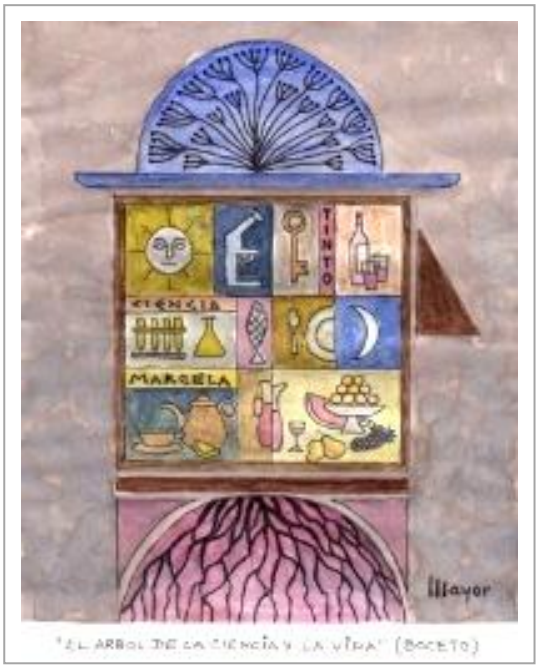
PEDECIBA-Biología

Sub-área Neurociencias

Tesis de Doctorado



Inducción por flavonoides de defensas antioxidantes endógenas a través de la vía Nrf2/ARE: implicancia en la neuroprotección

Lic. Florencia Arredondo

Director de tesis: Dr. Federico Dajas

Departamento de Neuroquímica

Instituto de Investigaciones Biológicas Clemente Estable

Montevideo, 2015

AGRADECIMIENTOS

En primer lugar, quisiera agradecer a los miembros del tribunal de tesis, las Dras. Silvia Olivera, Mónica Brauer y Patricia Lagos, por su buena disposición en evaluarme la tesis en brevísimo tiempo; por sus aportes y sus recomendaciones.

A mi director de Tesis, el Dr. Federico Dajas, gracias por permitirme integrarme al laboratorio. Gracias por su confianza y por su apoyo durante todo este tiempo.

A todos mis compañeros del Departamento de Neuroquímica de hoy y de siempre. A Fer por abrirme las puertas del cuarto de cultivo, y a Laura que junto con Fer me introdujeron en el mundo de las células. A Caroli, por tantos años compartiendo pecera y trabajo juntas. A Caroli, Rosina, Diego, Fer, Andrés y Giselle que colaboraron en la realización de parte de los ensayos presentados en esta tesis. A Giselle, Caroli y Andrés por haberse tomado el trabajo de leer y corregir esta tesis. A Costa por tantos años de laboratorio, comiditas y sobremesa juntos. A Jess por todas las experiencias personales y profesionales compartidas, padecidas y disfrutadas. A Felicia, por su cariño y apoyo emocional. A Estelita por mimarme tanto.

A Andrés y a Marcela Martinez por su asistencia con los ensayos que implicaron el uso de la plataforma analítica del IIBCE.

A Marcela Díaz y Andrés Di Paolo por su asistencia con el microscopio confocal.

A Héctor, Martín y Carmen del Bioterio. Por su buena disposición siempre.

Al Dr. Pablo Zunino, jefe del Depto de Micobiología del IIBCE, por permitirme realizar los trabajos de Real time PCR y de preparación y purificación de plásmidos necesarios para esta tesis. En especial a mi amiga Karina Antúnez, cuya ayuda fue fundamental para el desarrollo de estas metodologías. Gracias por su amistad, su apoyo, su energía.

Al Dr. Rafael Cantera, jefe del Depto de Biología del Neurodesarrollo del IIBCE, por permitirme realizar una pasantía en su laboratorio e introducirme en el mundo de Drosophila. En especial a mi amigo Santiago Ruiz, por su ayuda con los experimentos, por su buena onda, por su apoyo. A Rafael, Santi, Majo, Flavio y Martín, gracias por hacerme sentir como "de la casa".

Al Dr. Dean P. Jones, de la Div. de Medicina Pulmonar, Universidad de Emory , Atlanta, USA. Por permitirme realizar una pasantía en su laboratorio. En especial a la Dra. Young-Mi Go, por enseñarme las metodologías, por su paciencia. Por su tiempo en la discusión de resultados y la preparación de los manuscritos.

A los compañeros de Biología Celular y del Laboratorio de Neurofarmacologia experimental, por su buena onda y por todos los mangazos. Especialmente a Cecilia Scorza que siempre está disponible, ayudando y apoyando.

A Silvia Olivera, Pablo Díaz, Fede Rosconi, Alejandra Kun, por su asistencia a mis consultas, por los mangazos.

A PEDECIBA Biología por permitirme desarrollar esta tesis y por haber contribuido parcialmente a su financiación a través de sus alícuotas y becas para asistir a congresos. A la ANII por haberme otorgado una beca de postgrado. A la CAP por haberme otorgado una beca para finalizar este postgrado. A IBRO-LARC, ISN-CAEN y la SNU, que financiaron parcialmente mi asistencia a congresos y pasantías en el exterior. A la OPCW, principal agente financiador de nuestra línea de trabajo.

A mis amigas, por su amistad, por sus palabras de aliento.

A mis familiares, por su apoyo incondicional, por el aguante. A mis hermanos por poder contar con ellos siempre. A mis padres, por la educación recibida. En especial a mi madre, por ser un pilar fundamental en mi vida, por estar siempre, por su amor incondicional.

A Andrés, por quererme. Gracias por creer en mí, por su paciencia, por su apoyo.

A Mati y Guille, por ser la sorpresa más linda, el desafío más grande, el mejor de los regalos.

A todos…. Gracias!

RESUMEN

Las enfermedades que involucran muerte neuronal focal o masiva en el cerebro, y donde el estrés oxidativo juega un papel preponderante, son una causa frecuente de muerte e incapacidad en Uruguay y en el mundo. Pese a ello, no existen en la actualidad terapéuticas efectivas para su tratamiento.

Entre los posibles candidatos para el tratamiento de estas enfermedades se encuentra un grupo de compuestos polifenólicos, los flavonoides, los cuales han despertado un creciente interés ya que, además de ser potentes antioxidantes *in vitro*, se les han atribuido propiedades citoprotectoras frente a diversos estímulos nocivos. Sin embargo, los mecanismos de acción involucrados en el efecto protector de estos compuestos no han sido completamente dilucidados.

La presente tesis se centró en profundizar en la comprensión de los mecanismos subyacentes a la capacidad neuroprotectora de los flavonoides. Dicho efecto se ha explicado tradicionalmente por las propiedades antioxidantes directas de estos compuestos, secuestrando radicales libres. Sin embargo, la evidencia actual sugiere que esta propiedad no es suficiente para explicar su capacidad protectora. En particular, el objetivo de esta tesis consistió en caracterizar la inducción de la vía dependiente del factor de transcripción *Nuclear factor-erythroid 2-related factor 2* (Nrf2) por quercetina y flavonoides estructuralmente relacionados, como posible mecanismo involucrado en la neuroprotección frente a estrés oxidativo. Cabe destacar que esta vía es un interesante blanco terapéutico para la prevención de la muerte celular involucrada en diversas neuropatologías, ya que activa la expresión de una batería de genes ARE (*Antioxidant Response Element*) que codifican para proteínas citoprotectoras.

Para cumplir con nuestro objetivo, desarrollamos un modelo de muerte neuronal oxidativa en un cultivo primario de neuronas granulares de cerebelo. Utilizando este modelo evaluamos las propiedades neurotóxicas y neuroprotectoras del flavonoide quercetina. Los resultados obtenidos mostraron que, una vez agregada a los cultivos, quercetina se internaliza en las neuronas, alcanzando rápidamente el núcleo. Pasadas las 24 h de incubación no se detectan niveles intracelulares de quercetina, sin embargo ésta es capaz de prevenir la muerte neuronal frente al daño oxidativo. Por otra parte, el análisis de los requerimientos estructurales que aseguran el efecto neuroprotector de quercetina y flavonoides relacionados en nuestro modelo, no coinciden con aquellas características estructurales que determinan sus propiedades atrapadoras de radicales libres. Además encontramos que quercetina es capaz de activar el factor de transcripción Nrf2 y aumentar los niveles de expresión de la enzima γ-glutamilcisteína ligasa (GCL), que cataliza el paso limitante en la síntesis de glutatión (GSH). En este sentido, también encontramos que quercetina aumenta los niveles celulares de GSH total en los cultivos neuronales primarios. Asimismo nuestros resultados mostraron que la inhibición de GCL atenúa el efecto protector de quercetina, sugiriendo que la vía Nrf2/ARE a través de la modulación del sistema antioxidante del GSH, estaría involucrada en dicho efecto neuroprotector. Finalmente, el análisis de los requerimientos estructurales involucrados en la activación de Nrf2 por quercetina y flavonoides relacionados, mostró que el anillo A no sería relevante para la activación de la vía Nrf2/ARE, mientras que la planaridad sería un componente estructural importante. Asimismo, los flavonoides que presentaron actividad inductora de Nrf2, también fueron capaces de proteger a cultivos celulares sometidos a un daño oxidativo. Esto sugiere que habría una correlación entre la capacidad protectora y la actividad inductora de la vía Nrf2/ARE por parte de los flavonoides estudiados, lo cual reafirma la relevancia de esta vía en el efecto protector de los flavonoides.

En suma, el desarrollo de esta tesis permitió establecer la vía Nrf2/ARE como parte de los mecanismos responsables de la neuroprotección inducida por flavonoides, más allá de su capacidad atrapadora de radicales libres. Asimismo, se determinaron los requerimientos estructurales de los flavonoides para activar esta vía. Esto constituye una base para el diseño de nuevas moléculas capaces de activar esta vía citoprotectora, y con potencial valor terapéutico para el tratamiento de enfermedades neurodegenerativas.

TABLA DE CONTENIDO

INTRODUCCIÓN

El aumento del O_2 atmosférico hace 2000 millones de años significó una fuerza impulsora de la evolución. En ese entonces, organismos unicelulares debieron desarrollar soluciones adaptativas para evitar el O_2, tolerarlo y maximizar su utilización. Nuevos mecanismos de detección, procesamiento y señalización redox brindaron ventajas selectivas a organismos complejos capaces de colonizar ambientes diversos (Halliwell y Gutteridge 1999; Jones 2015).

Así, los metazoarios contemporáneos utilizan procesos redox para mantener su demanda energético-metabólica, su organización estructural, protegerse del entorno y reproducirse. Esta red de procesos redox es necesaria para el mantenimiento de la salud de los organismos. Fallas en su funcionamiento acumuladas durante el envejecimiento o su disrupción por estrés oxidativo (EO) contribuyen a la aparición de enfermedades (Jones 2015).

A continuación presentaremos los conceptos de: "especies reactivas", "defensas antioxidantes" y "homeostasis redox". También expondremos la definición de "estrés oxidativo" y analizaremos su papel en la ocurrencia de enfermedades neurodegenerativas. Luego mostraremos evidencias sobre la influencia de la dieta en la aparición de estas neuropatologías. En particular, nos centraremos en los flavonoides, como polifenoles dietarios de interés por sus propiedades neuroprotectoras. Finalmente presentaremos a la vía dependiente del factor de transcripción *Nuclear factor-erythroid 2-related factor 2* (Nrf2), como posible blanco terapéutico para la prevención de la neurodegeneración.

1. Especies reactivas

El uso del O_2 en la producción de energía supuso un enorme beneficio para la evolución de la vida en la tierra. Aunque también implicó la generación de subproductos del metabolismo del O_2 denominadas especies reactivas del oxígeno (ERO). En condiciones fisiológicas éstas se forman principalmente a partir de la cadena de trasporte electrónico mitocondrial. El 95 % del O_2 consumido por los organismos aerobios es reducido completamente a H_2O durante la respiración mitocondrial. Sin embargo menos del 2% deriva en la formación de ERO que son compuestos parcialmente reducidos y potencialmente tóxicos (Halliwell y Gutteridge 1999).

Las ERO incluyen radicales libres y otras especies no radicalarias. Los radicales libres son especies químicas, capaces de existir independientemente, caracterizados por poseer uno o más electrones desapareados. Esta propiedad los hace altamente reactivos, de forma que pueden dañar componentes celulares, comprometiendo la función normal de los tejidos. A diferencia de los radicales libres, las especies reactivas no radicalarias no presentan electrones desapareados, pero también son potencialmente tóxicas (Halliwell y Gutteridge 1999). Además de la mitocondria existen otros compartimientos subcelulares como los peroxisomas o el retículo endoplasmático, donde se producen ERO a través de la actividad enzimática de oxidasas, peroxidasas y lipooxigenasas, así como también de la citocromo P450. Cabe destacar que las ERO no sólo se producen como subproductos. También existen sistemas enzimáticos cuya función primaria es la formación de ERO. Tal es el caso de las NADPH oxidasas (Nox) de los fagocitos, que al generar ERO contribuyen a la destrucción microbiana. Por otra parte, existen fuentes exógenas de ERO, tales como la radiación ionizante, toxinas ambientales, rayos UV, agentes quimioterapéuticos, etc (Halliwell y Gutteridge 1999).

De forma análoga a las ERO también existen especies reactivas del nitrógeno (ERN), del cloro, del carbono, entre otros. A continuación en la tabla 1 se ejemplifican ERO y ERN, que son los principales grupos de especies reactivas implicados en funciones redox a nivel celular (Halliwell y Gutteridge 1999).

Tabla 1: Ejemplos de radicales libres y otras especies reactivas del oxígeno y del nitrógeno producidos en nuestro organismo.

Radical	Especie no radicalaria
Superóxido, $O_2^{\cdot -}$	Oxígeno singulete, $^1O_2$
Hidroxilo, $OH^{\cdot}$	Peróxido de hidrógeno, H_2O_2
Óxido nítrico, $NO^{\cdot}$	Peroxinitrito, $ONOO^-$

Entre éstos se destaca el peróxido de hidrógeno (H_2O_2) como uno de los principales ERO involucrados en la señalización redox en eucariotas. H_2O_2 es uno de los ERO más estables, con una vida media a nivel celular de 1 ms. Además, es una molécula no polar capaz de difundir a través de las membranas, aunque también su pasaje es facilitado por acuaporinas (peroxiporinas). Asimismo, está involucrado en procesos celulares tales como proliferación, diferenciación, reparación de tejidos, entre otros (Sies 2014).

En este sentido y de forma general podemos decir que las especies reactivas presentan un papel dual, pudiendo resultar tanto beneficiosas como perjudiciales para los organismos. En condiciones fisiológicas, participan como moduladores de señales intracelulares en procesos como la división celular, la diferenciación o la expresión génica, actuando sobre blancos moleculares redox-sensibles (enzimas, factores de transcripción, etc.) (Trachootham et al. 2008; Finkel 2011). Por otra parte, la formación constante de estas especies reactivas constituye una potencial amenaza para las células, ya que éstas son capaces de dañar proteínas, lípidos y ácidos nucleicos (Halliwell y Gutteridge 1999).

2. Defensas antioxidantes

Con el fin de mantener los niveles de las especies reactivas en proporciones no perjudiciales, los organismos aerobios desarrollaron múltiples mecanismos de defensa antioxidante. Gran parte de estas defensas actúan previniendo la formación de especies reactivas, neutralizándolas o convirtiéndolas en sustancias menos reactivas (Halliwell y Gutteridge 1999).

Podemos clasificar estas defensas antioxidantes en enzimáticas y no enzimáticas. Las defensas antioxidantes enzimáticas son proteínas endógenas que operan:

- evitando la generación de radicales libres a partir de otras moléculas, como es el caso de la catalasa y la glutatión peroxidasa;
- convirtiendo los radicales existentes en moléculas menos reactivas, antes de que éstos puedan afectar moléculas cercanas, como es el caso de la superóxido dismutasa.

Por otro lado, se encuentran los antioxidantes no enzimáticos, donde encontramos:

- proteínas endógenas que controlan los niveles de pro-oxidantes, como en el caso de la ferritina y transferrina con los niveles de iones hierro;
- antioxidantes de bajo peso molecular capaces de neutralizar/atrapar especies reactivas, evitando que éstas reaccionen y causen daños dentro de la célula. A su vez, pueden ser endógenos (como el glutatión, el urato, o el ubiquinol) o pueden provenir de la dieta, como las vitaminas C y E, y diversos compuestos polifenólicos (Halliwell y Gutteridge 1999). Entre estos polifenoles dietarios se destacan los flavonoides, sobre los cuales profundizaremos más adelante en este capítulo.

Asimismo, existen sistemas de reparación (proteasas, lipasas, y enzimas reparadoras de ADN) que complementan las acciones antioxidantes previamente descritas. Estos sistemas están encargados de recuperar la función de las biomoléculas modificadas, reparar el daño establecido, o

degradar las biomoléculas en caso de estar comprometida su función celular (Davies 2000).

Finalmente, otra estrategia desarrollada por los organismos aerobios implica la activación de sistemas de respuesta adaptativa frente a cambios en el estado oxidativo celular. Estos sistemas detectan alteraciones redox y desencadenan vías transitorias que causan la modulación de la expresión de genes que codifican enzimas citoprotectoras (Baird y Dinkova-Kostova 2011; Calabrese et al. 2012). Tal es el caso de la vía dependiente del factor de transcripción Nrf2, sobre la cual nos focalizaremos más adelante.

Una forma alternativa de clasificar a los antioxidantes de bajo peso molecular, es por su acción directa o indirecta (Figura 1).

Los antioxidantes directos son moléculas pequeñas con propiedades redox intrínsecas, que les permiten atrapar especies reactivas. En esa acción se consumen, de forma que luego de su interacción con las especies reactivas tienen que ser regenerados. Presentan una vida media corta y pueden causar efectos pro-oxidantes. Algunos de estos compuestos son endógenos, como el caso del glutatión, mientras que otros provienen de la dieta, como las vitaminas C y E (Dinkova-Kostova y Talalay 2008).

Los antioxidantes indirectos pueden o no tener propiedades redox intrínsecas. Ejercen su efecto biológico a través de la activación de vías de respuesta adaptativa como la dependiente de Nrf2, induciendo genes citoprotectores. Las proteínas efectoras actúan de forma catalítica, tienen vida media más larga y no causan efectos pro-oxidantes. Por lo tanto los efectos de los antioxidantes indirectos son más duraderos que en el caso de los directos. Podemos encontrar este tipo de antioxidante en la dieta, como es el caso de los isotiocianatos (Dinkova-Kostova y Talalay 2008).

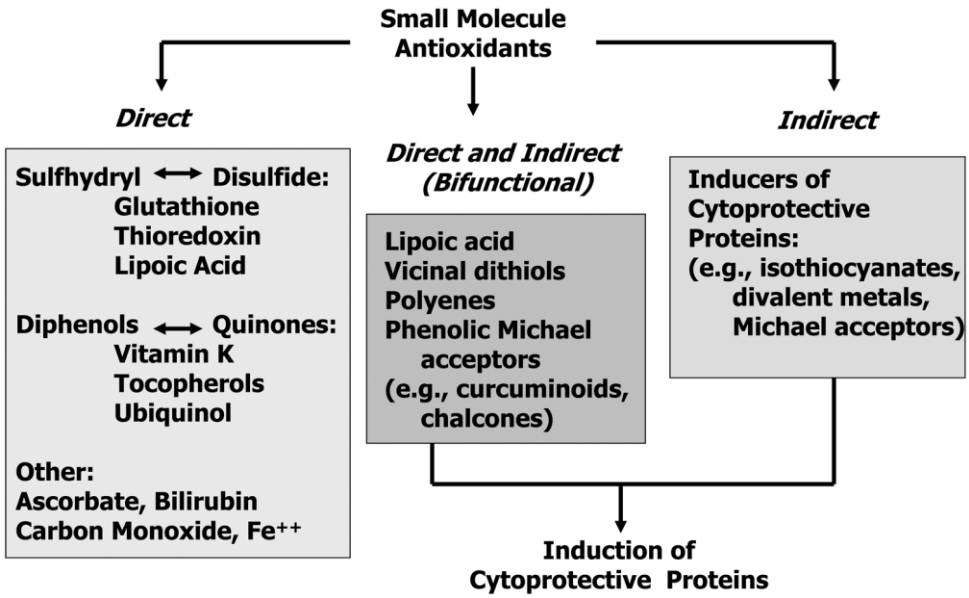


Figura 1: Antioxidantes directos, indirectos y bifuncionales (extraído de Dinkova-Kostova y Talalay 2008).

Por último, también están los antioxidantes que pueden actuar de ambas formas. A éstos los llamamos antioxidantes bifuncionales (Dinkova-Kostova y Talalay 2008). En ese grupo se encuentran los aceptores de Michael fenólicos como es el caso de los flavonoides. Sobre estas propiedades de los flavonoides también profundizaremos más adelante.

3. Homeostasis redox

Como expusimos previamente, el aumento del O_2 atmosférico impulsó el desarrollo de mecanismos de detección, procesamiento y señalización redox. Gran parte de estos procesos se acoplaron a la capacidad de oxidación reversible de residuos de aminoácidos como la cisteína (Cys) (Miseta y Csutora 2000). En este sentido, los diversos estados de oxidación del átomo de azufre de las Cys, componen un mecanismo de regulación dinámica de la estructura y función proteicas. Así, estos elementos redox-sensibles participan en procesos fisiológicos de señalización, regulación y tráfico macromolecular, mediante reacciones de óxido-reducción. Al mismo

tiempo, estas reacciones constituyen un mecanismo por el cual las especies reactivas pueden desencadenar y modular respuestas celulares (Jones 2015).

El estado de óxido-reducción de la célula está determinado por el balance entre los distintos componentes redox presentes en ella (Jones 2002). Definimos "homeostasis redox" al mantenimiento del estado redox celular dentro de un rango óptimo, que asegura el correcto funcionamiento celular.

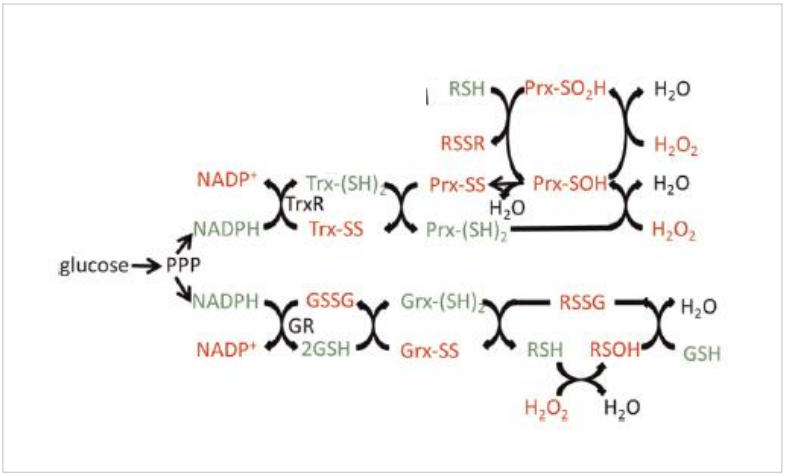


Figura 2: Red de pares redox que encontramos en sistemas biológicos y mantenimiento del estado redox celular (adaptado de McBean et al. 2015).

3.1. Los sistemas del glutatión y tiorredoxinas y su papel en el mantenimiento de la homeostasis redox

Uno de los actores principales en el mantenimiento de la homeostasis redox celular es el glutatión. Este tripéptido compuesto por L-glutamato, L-cisteína y glicina, es el más importante de los tioles no proteicos en las células de mamífero, donde alcanza concentraciones del orden milimolar. Su síntesis consta de 2 reacciones enzimáticas. Primero, la enzima γ-glutamilcisteína ligasa (GCL, también conocida como γ-glutamilcisteína

sintetasa) produce la γ-glutamilcisteína a partir del L-glutamato y la L-cisteína. Este es el paso limitante en la síntesis de glutatión. Posteriormente, la enzima glutatión sintetasa une la glicina al C-terminal de la γ-glutamilcisteína. El glutatión puede existir tanto en su estado reducido (GSH) como oxidado (glutatión disulfuro, GSSG), aunque la forma reducida es la preponderante a nivel celular (proporción de 100:1, aproximadamente). GSH y GSSG son intercambiables por la acción de dos enzimas: la glutatión peroxidasa (GPx) y la glutatión reductasa (GR) (Figura 2) (Bains y Shaw 1997). Una de las funciones principales del glutatión es atrapar radicales libres y en particular al radical $OH^{\cdot}$. Durante ese tipo de reacciones GSH se oxida formando GSSG. El GSSG es reducido por la GR para regenerar el GSH y para ello es necesaria la presencia del NADPH como donador de hidrógeno (Figura 2) (Bains y Shaw 1997). El GSH también participa en el proceso de detoxificación a cargo de la enzima glutatión-S- transferasa (GST). Esta cataliza la conjugación de GSH a xenobióticos, facilitando su eliminación. Cabe destacar que tanto GCL, como GR, GPx y GST son enzimas citoprotectoras (antioxidantes y detoxificantes) cuya expresión génica es modulada coordinadamente por la vía de respuesta adaptativa dependiente de Nrf2, al igual que otras proteínas de fase II (Baird y Dinkova-Kostova 2011). GSH también actúa de forma sinérgica con antioxidantes exógenos como la vitamina C. En este sentido, participa en el reciclado del ascorbato a partir de la reducción directa o enzimática del dehidroascorbato, contribuyendo de esa forma al restablecimiento de la homeostasis redox. Asimismo, el glutatión opera dentro de una red de pares redox formado por NAD+/NADH, NADP+/NADPH y GSH/GSSG que trabajan de forma coordinada con GSH/GR, glutarredoxinas (Grx), tiorredoxinas, tiorredoxina reductasa (TrxR) y peroxirredoxinas (Prx) con el fin de mantener la homeostasis redox (Figura 2) (McBean et al. 2015). Por las funciones que desempeña y por su elevada concentración a nivel celular, el potencial

redox GSH/GSSG (E_hGSSG) sirve como indicador del estado redox celular (Jones 2002).

Otro gran sistema tiol-dependiente involucrado en funciones de modulación redox es la familia de las tiorredoxinas (Trx). De forma complementaria al sistema de GSH, las Trx participan en la regulación de proteínas redox sensibles implicadas en señales de transducción y expresión génica. Se trata de proteínas redox pequeñas que mediante la oxidación reversible a nivel de sus residuos Cys, reducen los disulfuros de sus sustratos proteicos modulando su actividad. Las Trx actúan a su vez como antioxidantes directos, atrapando radicales libres. Asimismo, actúan como donadores de hidrógeno de las Prx durante el proceso de reducción de peróxidos. Las Trx oxidadas vuelven a su estado reducido por acción de la enzima TrxR, utilizando NADPH como fuente reductora (Figura 2) (Powis y Montfort 2001).

3.2. Alteraciones en la homeostasis redox y respuesta celular

Los sistemas de control y señalización redox están organizados funcionalmente en circuitos con distintos potenciales de óxido-reducción según cada compartimiento subcelular. Como expusimos previamente, el mantenimiento del balance redox en estos circuitos depende de los sistemas del GSH y Trx (Figura 2). Esta organización en circuitos redox restringidos física y cinéticamente entre sí, les permite a los distintos procesos de control y señalización activarse y funcionar de forma independiente. Sin embargo, esta disposición también hace vulnerables a las células. La disrupción en la organización redox puede llevar al mal funcionamiento celular y por ende a la aparición de patologías, como detallaremos más adelante (Jones y Go 2010).

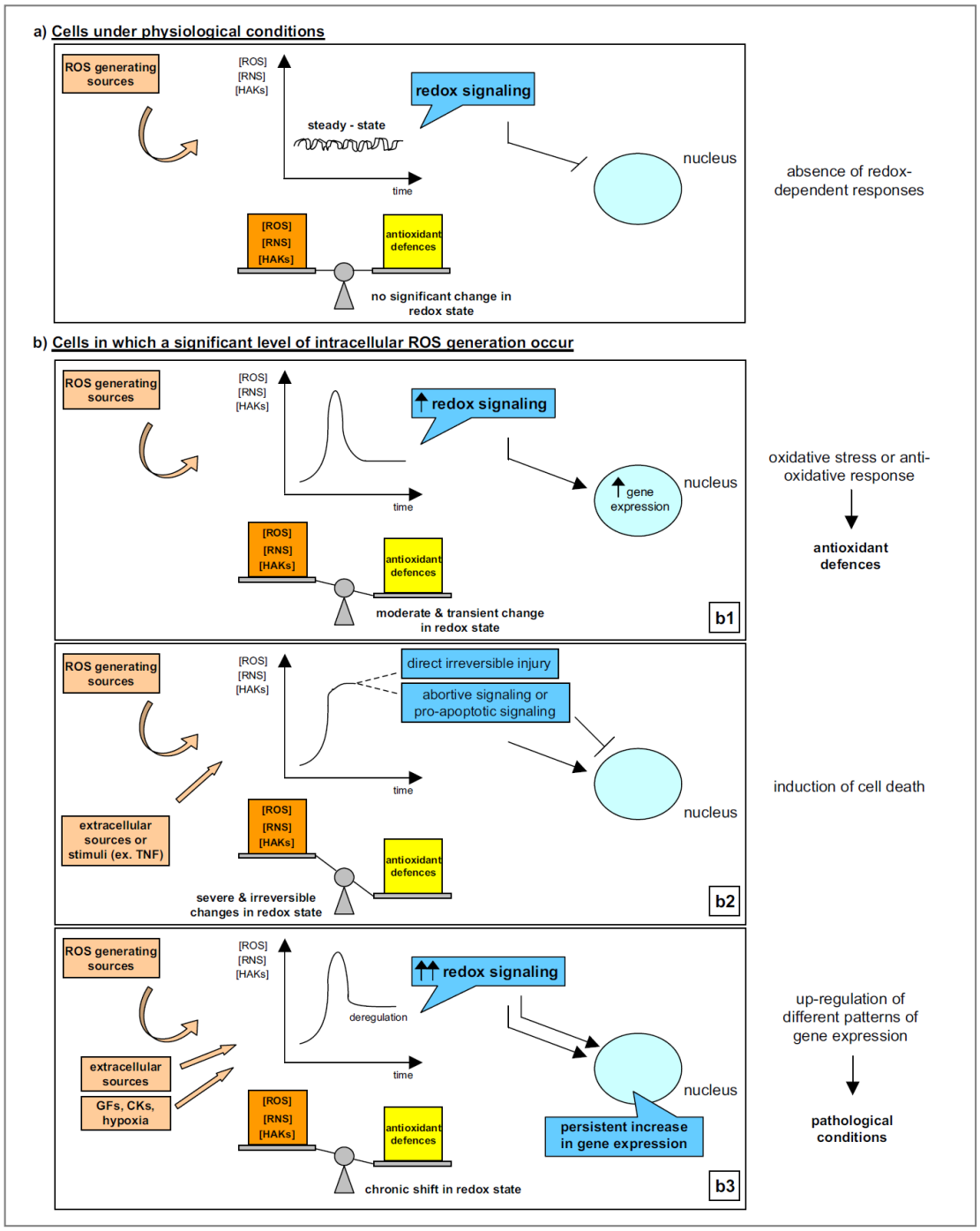


Figura 3: Homeostasis redox, señalización redox y respuesta celular: a) condiciones fisiológicas; b1) EO moderado y transitorio; b2) EO severo que lleva a la muerte celular; b3) EO severo y crónico que lleva a la adaptación redox (extraído de Novo y Parola 2008).

En condiciones fisiológicas, las especies reactivas se encuentran en niveles celulares controlados, debido al balance dinámico entre su

formación y eliminación. Fluctuaciones del estado redox celular por acción de especies reactivas dentro del rango tolerado, influyen sobre diversas funciones mediante vías de señalización redox-dependientes. Por otra parte, alteraciones en el estado redox celular más allá de los límites tolerables comprometen sus funciones biológicas. Así, un descenso en los niveles basales de especies reactivas puede interrumpir el papel fisiológico que éstas tienen, por ejemplo en la proliferación celular. Alternativamente, un exceso en los niveles de especies reactivas puede dañar biomoléculas, desencadenar respuestas adaptativas redox-sensibles e incluso causar muerte celular (Figura 3) (Sies 2015). El destino celular final dependerá del grado de desbalance oxidativo sobre el cual nos centraremos a continuación.

4. Estrés oxidativo

El estrés oxidativo (EO) se define como una situación de desbalance entre la generación celular de pro-oxidantes y la actividad de los sistemas antioxidantes que lleva al daño de componentes celulares (ácidos nucleicos, proteínas y lípidos) y a la disrupción del control y la señalización redox celular (Jones 2006; Sies y Jones 2007).

En otras palabras, el concepto de EO implica un desbalance más allá de los límites tolerables en el estado redox celular fisiológico, hacia un estado más oxidativo. Los efectos del EO a nivel celular dependen del tipo y entorno celular donde ocurra, de la magnitud del desbalance y de su duración. Así, un EO moderado y breve puede desencadenar vías transitorias redox-sensibles que modulen la expresión génica de proteínas capaces de restablecer la homeostasis redox (D'Autréaux y Toledano 2007). Sin embargo, frente a situaciones de EO severo, este tipo de respuestas adaptativas pueden ser insuficientes para contrarrestar el desbalance redox. Tal magnitud de EO puede llegar a causar daño en macromoléculas,

alteración de la estructura y función celular, e incluso puede llevar a la muerte celular. Asimismo, en algunas situaciones de estrés crónico, la célula puede alcanzar un nuevo estado de equilibrio redox celular más oxidativo. En esta situación, la célula es capaz de tolerar niveles elevados de especies reactivas. Además, puede activar vías de señalización que inducen la expresión de genes que contribuyen a la aparición de enfermedades crónicas (Figura 3) (Novo y Parola 2008).

En este sentido, el EO juega un papel clave en el envejecimiento así como en patologías cardiovasculares, neurodegenerativas y cáncer. Precisamente, estos procesos están asociados a cambios en el estado redox y a una disrupción del control y la señalización redox celular (Langley y Ratan 2004).

5. Sistema Nervioso Central, estrés oxidativo y su implicancia en las patologías neurodegenerativas

El Sistema Nervioso Central (SNC) es particularmente vulnerable al EO debido a su elevada demanda metabólica. El cerebro utiliza 20% del O_2 consumido por el organismo, aunque apenas representa el 2% de la masa corporal total (Halliwell y Gutteridge 1999). Asimismo, representa un entorno con elevada generación de especies reactivas, ya que en las neuronas éstas pueden producirse por diversos procesos celulares como:

- la auto-oxidación de neurotransmisores como la dopamina;
- la sobre-estimulación neuronal a partir de corrientes de Ca^{2+} por acción de aminoácidos excitatorios (excitotoxicidad);
- la deprivación de factores tróficos, entre otros.

Además, se trata de un tejido cuyas células presentan una baja o moderada tasa de recambio en comparación con otro tipo de tejidos. Asimismo, en relación a las células gliales y vasculares, las neuronas poseen bajos niveles de algunas enzimas antioxidantes como la catalasa. Por otra

parte, presentan un alto contenido de hierro y sustratos fácilmente oxidables, como los ácidos grasos poliinsaturados de sus membranas lipídicas (Langley y Ratan 2004).

El EO se ha relacionado tanto con procesos agudos como los accidentes cerebrovaculares (infartos cerebrales), como con patologías neurodegenerativas crónicas (Enfermedad de Alzheimer, Enfermedad de Parkinson, Esclerosis Lateral Amiotrófica, etc.) (Simonian y Coyle 1996; Gilgun-Sherki et al. 2001; Andersen 2004; Langley y Ratan 2004). Estas neuropatologías presentan diversas sintomatologías secundarias como producto de la muerte de poblaciones neuronales vulnerables específicas. Las características clínicas de los procesos neurodegenerativos incluyen desde desórdenes motores (como en las enfermedades de Huntington y Parkinson) hasta demencias severas (como en el Alzheimer) (Olesen et al. 2006).

La esperanza de vida a nivel mundial va en aumento. Así, entre los años 2000 y 2050 se prevé que la población por encima de 60 años duplique su cifra (World Health Organization 2008). A medida que las poblaciones envejecen, la incidencia de las neuropatologías aumenta y esto trae aparejado importantes secuelas socioeconómicas. Por ejemplo, la Enfermedad de Alzheimer es la enfermedad neurodegenerativa más común con 26 millones de enfermos en el mundo. También es la causa más frecuente de demencia y se estima que para el año 2050, 1 de cada 85 personas padecerá esta enfermedad (Chen y Lin 2015). Actualmente no existen herramientas terapéuticas efectivas en cuanto a la prevención y el tratamiento de las enfermedades neurodegenerativas. Esto se debe en parte a que son patologías complejas que involucran diversos factores.

Aunque estos desórdenes presentan distintas características clínicas y neuropatológicas, comparten ciertas alteraciones que contribuyen a la disfunción y muerte neuronal. Entre estas alteraciones encontramos: defectos en el sistema proteosomal y agregación proteica, disrupción de la

homeostasis del calcio, inflamación, excitotoxicidad, alteración de los procesos metabólicos y de la función mitocondrial, y aumento de daño oxidativo (Alexi et al. 2000). Aún no se ha podido establecer si el EO es un componente patológico primario o si surge como consecuencia del proceso degenerativo. Sin embargo, actualmente es considerado uno de los eventos celulares primordiales que llevan a la muerte neuronal asociada a las enfermedades neurodegenerativas (Ischiropoulos y Beckman 2003; Andersen 2004; Wang y Michaelis 2010).

6. Terapia antioxidante y sus limitaciones: compuestos secuestradores de radicales libres para el tratamiento de enfermedades asociadas al EO

Durante décadas se estudió ampliamente la aplicación de antioxidantes exógenos con propiedades secuestradores de radicales libres para el tratamiento de enfermedades asociadas al EO. Durante ese período se pensaba que compuestos capaces de atrapar radicales libres *in vitro* serían aptos para prevenir o enlentecer la aparición de estas enfermedades. Sin embargo, este tipo de terapias han sido controversiales y mayoritariamente inefectivas tanto a nivel pre-clínico como clínico (Moosmann y Behl 2002; Bouayed y Bohn 2010). Una posible explicación para dicho fracaso se basa en que existen limitantes cinéticas en la acción secuestradora de especies reactivas. El radical libre más reactivo reaccionará tanto con un antioxidante de bajo peso molecular como con una biomolécula (ácido nucleico, proteína, lípido), con cinéticas parecidas. Se necesitan elevadas concentraciones de antioxidantes para atrapar efectivamente las especies reactivas, pero sin embargo su concentración *in vivo* es limitada. Asimismo se necesita que el compuesto logre acceder al tejido donde hay una sobreproducción de especies reactivas, siendo este otro factor limitante para su eficiencia *in vivo* (Forman et al. 2014).

En dicho contexto, resulta de interés el desarrollo de nuevas estrategias terapéuticas, más abarcativas, capaces de modular las defensas antioxidantes endógenas, con el fin de mantener la homeostasis redox celular, previniendo o retrasando la aparición de los procesos neurodegenerativos.

7. Influencia de la dieta en la incidencia de neuropatologías

Existen evidencias clínicas y epidemiológicas sobre el efecto de la dieta y el estilo de vida en la incidencia de enfermedades crónicas (Limón-Pacheco y Gonsebatt 2009). En las últimas décadas, tradiciones culturales que involucran dietas saludables como las dietas mediterránea y oriental, han generado especial interés. En particular, diversos estudios clínicos y epidemiológicos han demostrado que este tipo de dietas ricas en frutas y verduras pueden reducir la incidencia de enfermedades asociadas al EO tales como cáncer, enfermedades cardiovasculares y neuropatologías (Checkoway et al. 2002; Tan et al. 2003; Kuriyama et al. 2006; Nurk et al. 2009; Li et al. 2012; Sääksjärvi et al. 2013). A su vez, estudios *in vivo* e *in vitro* sugieren que los fitoquímicos presentes en los alimentos de origen vegetal (frutas, vegetales, legumbres, semillas, hierbas, especias) serían los responsables de los efectos beneficiosos de estas dietas. Actualmente se sugiere que a pesar de no ser nutrientes esenciales, una ingesta pobre en fitoquímicos aumentaría el riesgo de aparición de estas enfermedades (Forman et al. 2014).

8. Los flavonoides como agentes neuroprotectores

Entre los distintos tipos de fitoquímicos presentes en la dieta, encontramos a la familia de los flavonoides. Desde el punto de vista estructural, estos polifenoles dietarios presentan un esqueleto de

difenilpropanos (C6-C3-C6), compuesto por dos anillos bencénicos (A y B) unidos por una cadena de tres átomos de carbono, que habitualmente da lugar a un heterociclo oxigenado (pirano, anillo C). Existen diversas subfamilias de flavonoides: flavanoles, flavanonas, flavonas, isoflavonas, flavonoles y antocianinas. Estas se diferencian entre sí según los grupos funcionales y el grado de oxidación del anillo C (Figura 4 y Tabla 2) (Crozier et al. 2009). Dentro de cada subfamilia, los distintos compuestos se diferencian por el número y localización de los grupos hidroxilo, metoxilo u otros sustituyentes en sus ciclos bencénicos (anillos A y B). En la naturaleza, la mayoría se encuentra conjugados a azúcares (glicósidos), aunque algunos pueden encontrarse en forma libre (agliconas) y otros están frecuentemente polimerizados (Middleton Jr. et al. 2000).

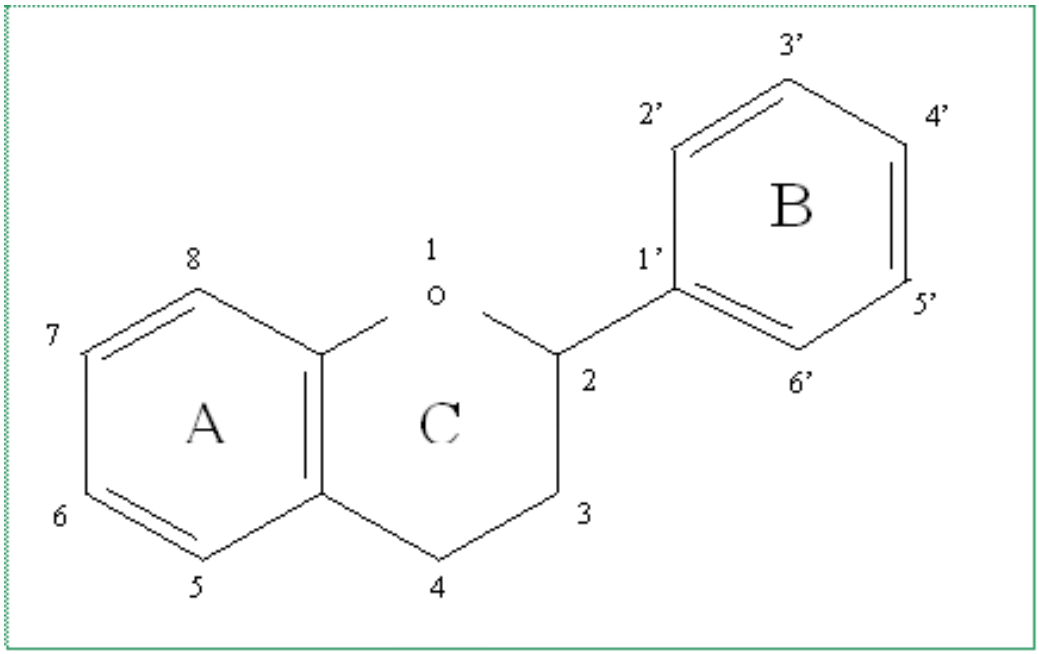


Figura 4: Estructura genérica de los flavonoides.

Tabla 2: Clasificación de los flavonoides según sus características estructurales.

Subfamilia	Insaturación en anillo C	Grupo funcional en anillo C
Flavanoles	-	3-hidroxi
Flavanonas	-	4-oxo
Flavonas	En posición 2-3	4-oxo
isoflavonas*	En posición 2-3	4-oxo
Flavonoles	En posición 2-3	3-hidroxi y 4-oxo
Antocianinas	En posición 1-2 y en 3-4	3-hidroxi

* La posición del anillo B respecto al anillo C es 2 en todos los casos, a excepción de las isoflavonas, que es 3.

Los flavonoides son productos del metabolismo secundario de las plantas donde participan en diversas funciones como la protección frente a las ERO producidas por radiaciones UV, los mecanismos de polinización, la tolerancia al frio, entre otras. Asimismo, son en parte responsables del color, sabor y texturas de vegetales y de productos derivados como el té, el vino, etc. La composición y concentración de flavonoides en las plantas es muy diversa; varían según su etapa en el desarrollo y en respuesta al ambiente (Middleton Jr. et al. 2000). Al ser compuestos sintetizados fundamentalmente en plantas, su presencia en tejidos animales está directamente relacionada al consumo de alimentos de origen vegetal (Scalbert y Williamson 2000). Por lo tanto, las acciones biológicas de estos compuestos en animales van a depender de su biodisponibilidad. En este sentido, una vez ingeridos, los flavonoides en el organismo se someten a procesos de absorción, distribución, metabolismo y excreción, que determinan el destino de la aglicona y sus metabolitos. La biodisponibilidad de los flavonoides dietarios varía ampliamente según el tipo de matriz del alimento en la cual se ingieren

y el tipo de glicosilación que posean. Normalmente, las concentraciones totales de los flavonoides en plasma humano están en el rango nanomolar. Sin embargo, tras la administración de suplementos o dietas ricas en flavonoides, los niveles de metabolitos pueden alcanzar el orden micromolar (Hollman et al. 1996).

8.1. Acciones biológicas de los flavonoides y mecanismos de acción propuestos

Desde hace décadas los flavonoides han adquirido interés por sus diversas acciones biológicas. Se les ha adjudicado numerosos efectos: antiinflamatorio, antiaterosclerótico antiviral, hepatoprotector, citoprotector, anticarcinogénico, citotóxico y mutagénico (Bjeldanes y Chang 1977; Kaul et al. 1985; Torel et al. 1986; Afanas'ev et al. 1989; Hertog et al. 1993a; Hertog et al. 1993b; Gaspar et al. 1994).

En los últimos años, dietas ricas en flavonoides así como flavonoides aislados han recibido especial atención por su potencial efecto en la prevención de enfermedades cardiovasculares, neurodegenerativas y cáncer (Knekt et al. 2002; Macready et al. 2009; Spencer 2010; Gao et al. 2012; Kennedy 2014).

En particular, respecto al potencial neuroprotector de los flavonoides, se les han atribuido propiedades citoprotectoras frente a diversos estímulos nocivos, incluyendo el EO (Joyeux et al. 1995; Harborne y Williams 2000; Ishige et al. 2001; Schroeter et al. 2002). Estos efectos han sido tradicionalmente explicados por sus reconocidos mecanismos de acción antioxidantes secuestrando radicales libres, quelando iones metálicos y modulando la actividad de enzimas como la cicloxigenasa, xantinoxidasa, 5-lipoxigenasa, etc. (Ratty et al. 1988; Cushman et al. 1991; Laughton et al. 1991; Chang et al. 1993; Gil et al. 1994; Rice-Evans 2001). Sin embargo, en los últimos años diversos estudios han sugerido que los flavonoides son capaces interactuar

con receptores y quinasas, modulando vías de señalización neuronal y la expresión de genes involucrados en procesos de plasticidad sináptica. Se ha demostrado que tales mecanismos previenen la muerte neuronal y/o promueven una mejora en los procesos de aprendizaje y memoria tanto en animales como en humanos (Macready et al. 2009; Williams y Spencer 2012; Rodriguez-Mateos et al. 2014). Asimismo, trabajos recientes aportaron importantes evidencias sobre la capacidad de algunos flavonoides de estimular vías de respuesta adaptativa frente a situaciones de estrés celular, como el oxidativo, metabólico, inflamatorio, etc (Figura 5)(Mandel et al. 2008; Spencer 2009; Forman et al. 2014). Como adelantáramos al comienzo, esta tesis se centró en este tipo de propiedad de los flavonoides, y en particular en la modulación de la vía dependiente del factor de transcripción Nrf2, como posible blanco terapéutico para la prevención de la neurodegeneración. La misma se detallará más adelante.

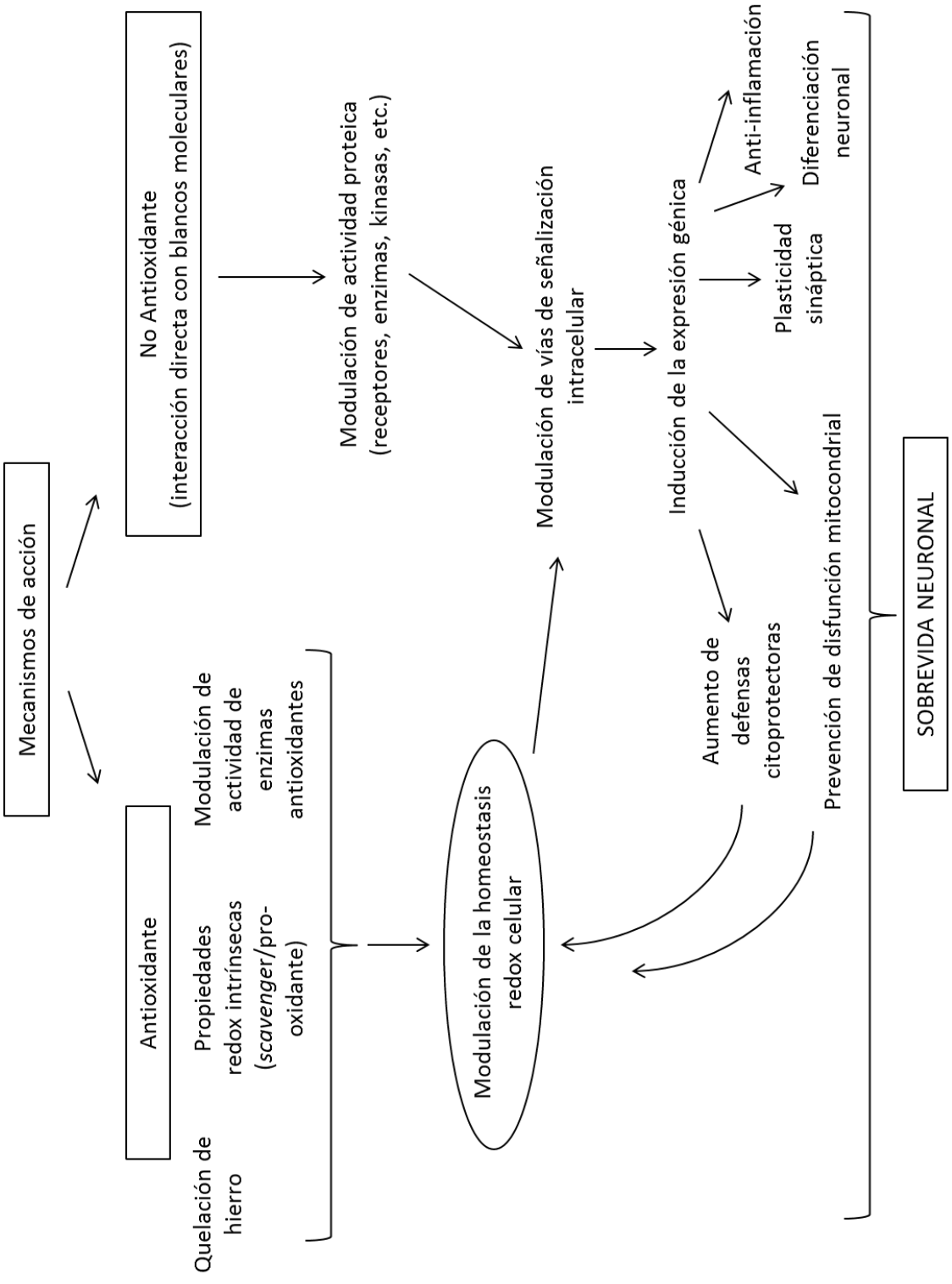


Figura 5: Esquema sobre los mecanismos de acción involucrados en la sobrevida neuronal propuestos para los flavonoides.

8.2. Acciones biológicas de los flavonoides: relación estructura actividad

Al día de hoy se han identificado más de 10.000 flavonoides (Pollastri y Tattini 2011). La gran diversidad estructural de estos compuestos ha permitido estudiar los requerimientos estructurales para ejercer algunas de las actividades biológicas descritas. El caso mejor estudiado y donde actualmente existe un consenso, es el relacionado a la actividad secuestradora de radicales libres. Estos estudios estructura-actividad mostraron que el grupo catecol en posiciones 3' y 4' del anillo B, la presencia de una insaturación en los carbonos 2 y 3, un grupo hidroxilo libre en la posición 3 y un grupo carbonilo en la posición 4, aseguran una elevada potencia antioxidante. Estas características estructurales les confieren la capacidad de donar hidrógeno/electrones a los radicales libres y de deslocalizar el radical resultante dentro de su estructura (Rice-Evans et al. 1996).

8.3. Quercetina

Uno de los flavonoides dietarios más abundantes y más estudiados es la quercetina (3,3´,4´,5,7-pentahidroxiflavona). Entre los alimentos y bebidas con mayores niveles de quercetina encontramos a la cebolla, la manzana, al té y al vino (Hertog et al. 1995). El consumo promedio de este flavonoide en una dieta occidental normal se sitúa entre 5 y 40mg/día. Sin embargo, en dietas ricas en frutas y verduras el consumo diario de quercetina puede situarse entre 200 y 500mg (Hertog et al. 1995; Rimm et al. 1996; Knekt et al. 1997; Kimira et al. 1998; Johannot y Somerset 2006; Lin et al. 2006).

Se han descrito un amplio espectro de efectos beneficiosos de la quercetina, tales como anti-cancerígenos, anti-inflamatorios,

cardioprotectores, entre otros (Casagrande y Darbon 2001; Egert et al. 2009; Chuang et al. 2010).

En particular, numerosos estudios *in vitro* e *in vivo* mostraron efectos neuroprotectores de este flavonoide. Estos incluyen desde modelos de muerte neuronal en líneas celulares y cultivos primarios frente a diversos agentes tóxicos (H_2O_2, tert-butil hidroperóxido, 6-hidroxi-dopamina, etc), hasta modelos experimentales de Parkinson, Alzheimer, hipoxia/isquemia, trauma, etc (Dajas et al. 2003a; Mercer et al. 2005; Pu et al. 2007; Sharma et al. 2007; Haleagrahara et al. 2011). Así, este cuerpo de evidencias sugieren que quercetina tendría potencial como herramienta terapéutica para el tratamiento o prevención de la neurodegeneración.

Sin embargo, los mecanismos de acción subyacentes a estos efectos no están completamente dilucidados. En este sentido, y como explicamos previamente para los flavonoides en general, tradicionalmente se propuso que la capacidad secuestradora de radicales libres de quercetina era la responsable de sus efectos beneficiosos (Formica y Regelson 1995). Quercetina presenta todas las características estructurales que expusimos previamente, necesarias para que ser un efectivo secuestrador de radicales libres (Silva et al. 2002). Sin embargo, como también dijimos, existen limitantes cinéticas en la acción secuestradora de especies reactivas; se necesitan elevadas concentraciones del compuesto para atrapar efectivamente las especies reactivas, mientras que su concentración *in vivo* es limitada (Forman et al. 2014). Por otra parte, las propiedades redox intrínsecas de este flavonoide hacen que luego de reaccionar con radicales libres, se convierta en un producto reactivo, con propiedades pro-oxidantes (Metodiewa et al. 1999; Boots et al. 2008). Se postula que la actividad pro-oxidante de quercetina se debe fundamentalmente a la presencia del grupo catecol en posiciones 3' y 4' del anillo B. Esta sustitución orto-di-hidroxi es susceptible a auto-oxidarse y convertirse en orto-semiquinonas y orto-quinonas/ metilen

quinonas, causando además la producción de ERO (Metodiewa et al. 1999; Boots et al. 2003; Chang et al. 2008; Walle 2009).

Así se propone que estos mecanismos pro-oxidantes serían los responsables de los efectos tóxicos y mutagénicos de dosis elevadas de quercetina (Rietjens et al. 2005). No obstante las evidencias indican que quercetina es mutagénico/carcinogénico *in vitro*, pero no *in vivo* (Harwood et al. 2007). Existen diversos mecanismos *in vivo* que limitan las acciones pro-oxidantes y tóxicas de quercetina. Consumida como parte de la dieta normal, los niveles sistémicos de quercetina libre son bajos, producto de la baja absorción y rápida metabolización del flavonoide. Por ejemplo, se observó en humanos que los niveles de quercetina libre en plasma luego de la administración oral de una dosis alta de quercetina de 1500 mg/día, alcanza los 8 ng/mL (Wang et al. 2005). Esta baja biodisponibilidad de quercetina limita sus efectos adversos descritos *in vitro* (Harwood et al. 2007). Asimismo, quercetina no actúa de forma aislada en el organismo, sino que forma parte de una red de interacción entre antioxidantes dietarios y endógenos. Esta red actúa de forma coordinada para regenerar los antioxidantes a partir de los productos oxidados (Boots et al. 2003). Actualmente se postula que luego de atrapar radicales libres, las formas pro-oxidantes de quercetina serían recicladas a quercetina o reaccionarían con GSH, formando conjugados no reactivos GS-quercetina: 6-glutationil-quercetina (6-GSQ) y 8-glutationil-quercetina (8-GSQ) (Boots et al. 2003; Ferraresi et al. 2005).

Por otra parte, en concentraciones bajas, las acciones pro-oxidantes de quercetina pueden alterar transitoriamente el estado redox celular a través de la generación de niveles moderados de ERO, o mediante la depleción parcial de GSH. Estos eventos desencadenan una situación de EO moderado capaz de modular vías intracelulares redox-sensibles, permitiendo la expresión de genes citoprotectores. Actualmente se sugiere que los flavonoides como la quercetina, con elevado potencial redox intrínseco que

les permitan generar ERO, serían potentes activadores de la vía dependiente de Nrf2 (Lee-Hilz et al. 2006). Esta propiedad resulta de especial interés en esta tesis, como profundizaremos al final del capítulo.

Tal como expusimos previamente, al igual que otros flavonoides, quercetina es capaz de modular otros factores de transcripción como el *Nuclear factor kappa-light-chain-enhancer of activated B* cells (NF-κB), *Activator protein 1* (AP-1) (Ishikawa et al. 1999; Kim et al. 2013). Asimismo quercetina es capaz de interactuar directamente con proteínas, enzimas y receptores. En particular se destaca la capacidad de modular una amplia gama de kinasas, como *phosphoinositide 3-kinase* (PI3-kinase), *protein kinase B/Akt* (PKB/Akt), *protein kinase C* (PKC), *mitogen-activated protein kinases* (MAPK), etc (Ferriola et al. 1989; Nagasaka y Nakamura 1998; Walker et al. 2000). De esa forma, el flavonoide es capaz de modular diversas funciones celulares a través de vías de señalización intracelular dependientes de kinasas. Además, puede inducir y activar la sirtuina 1 (SIRT1) (Davis et al. 2009). Esta desacetilasa regula diversos procesos relacionados al estrés, por ejemplo a través de la desacetilación de blancos proteicos como: histonas, *tumor protein p53* (p53), factores de transcripción *Forkhead box O* (FoxO), entre otros. Estas evidencias indican que quercetina es capaz de ejercer efectos pleiotrópicos mediante diversos mecanismos moleculares, más allá de sus clásicas propiedades antioxidantes directas.

Cabe destacar que quercetina es capaz de acumularse en la mitocondria, modulando el estado redox mitocondrial, la permeabilidad del poro de transición mitocondrial y favoreciendo la biogénesis mitocondrial (Davis et al. 2009; Fiorani et al. 2010). Estos mecanismos a nivel de la mitocondria resultan de interés si pensamos en el papel clave que juega este organelo en los procesos neurodegenerativos. Evidencias sugieren que el retículo plasmático sería otro organelo diana de la quercetina (Suganya et al. 2014). El estrés del retículo es otro tipo de respuesta celular al estrés y se lo ha involucrado con la muerte celular en diversas patologías

neurodegenerativas, cardíacas y diabetes. Estudios han indicado que quercetina sería capaz de controlar las vías que se activan durante el estrés del retículo. Esta acción moduladora podría ser un mecanismo adicional a través del cual quercetina previene el proceso neurodegenerativo.

9. La vía Nrf2 como blanco de estudio en la búsqueda de neuroprotección

Como expusimos previamente, durante la evolución los organismos desarrollaron sistemas de defensas endógenas inducibles en situaciones de estrés. Su activación por agentes exógenos o endógenos, promueve la expresión de genes que codifican para enzimas citoprotectoras. Existen diversos factores de transcripción involucrados en estos procesos, tales como NF-κB, AP-1 y Nrf2. NF-κB está implicado en la respuesta celular frente a estímulos de estrés como las citoquinas y juega un papel clave en la regulación de la respuesta inmune (Lawrence 2009). AP-1 es un factor de transcripción que es activado por factores de crecimiento, citoquinas y oncoproteínas e induce la transcripción de genes relacionados con proliferación, supervivencia, diferenciación y transformación celular (Hess et al. 2004). Por su parte, Nrf2 regula la expresión inducible de genes de fase II que incluyen enzimas detoxificantes y antioxidantes, entre otros (Moi et al. 1994; Itoh et al. 1997). La vía dependiente del factor de transcripción Nrf2 es una vía citoprotectora interesante como blanco de estudio para la prevención de la muerte celular por EO. En particular, diversos estudios de activación, sobreexpresión, y/o atenuación de Nrf2, sugieren que esta vía presenta un papel importante en la protección del sistema nervioso (Lee et al. 2003; Calkins et al. 2005; Vargas et al. 2008).

La vía dependiente de Nrf2 involucra 3 componentes moleculares principales con el fin de regular la expresión de proteínas citoprotectoras:

1. La proteína *Kelch-like ECH-associated protein 1* (Keap1), que actúa como sensor y blanco molecular de los inductores de la vía (Itoh et al. 1999);

2. El factor de transcripción Nrf2, responsable de la expresión basal e inducible de genes citoprotectores (Moi et al. 1994);

3. La secuencia de ADN *Antioxidant response element* (ARE), también conocida como *Electrophile response element* (EpRE), que es una secuencia específica localizada en la región promotora de los genes de fase II (Itoh et al. 1997).

Brevemente, esta vía inducible funciona de la siguiente manera. Nrf2 se encuentra constitutivamente inactivado por su unión a la proteína citoplasmática Keap1 y al citoesqueleto, por lo que el control primario de su función radica principalmente en su distribución subcelular, más que en la síntesis *de novo*. Cuando esta vía es activada, Nrf2 se disocia de Keap1 y se transloca al núcleo formando heterodímeros con proteínas *small Musculoaponeurotic fibrosarcoma* (sMaf), lo cual facilita su interacción con las regiones ARE y la consecuente transcripción de cientos de genes citoprotectores (Jaiswal 2004; Kensler et al. 2007).

9.1. Nrf2

Nrf2 fue descubierto hace poco más de 20 años (Moi et al. 1994). Se trata de una proteína de 66-kDa que presenta en su región C-terminal un dominio *cap'n'collar* (CNC, región homóloga a la proteína homónima de *Drosophila*) y una región básica con cierre (*zipper*) de leucinas (bZIP). Por tal motivo se la clasificó dentro de la familia de proteínas CNC-bZIP, que incluye además a los factores Nrf1, Nrf3 y p45 NF-E2. En roedores está conformada por 597 aminoácidos, mientras que en humanos comprende 605 aminoácidos. En lo que refiere a su estructura, presenta 7 dominios Neh

(*Nrf2–ECH-homology*) (Figura 6) (Tebay et al. 2015). Cada uno de estos dominios presenta funciones diferentes:

– Neh1 comprende la región CNC-bZIP que está conservada en todos los miembros de esta familia de factores de transcripción. Es necesaria para la unión del factor de transcripción al ADN y para formar heterodímeros con otras proteínas bZIP.

– La región N-terminal de Neh2 es necesaria para la asociación de Nrf2 con Keap1 y por lo tanto para su retención. En esta región se encuentran las secuencias peptídicas conservadas conocidas como ETGE y DLG, con las cuales Keap1 se une con afinidades alta y baja, respectivamente.

– La región C-terminal de Neh3 está involucrada en la activación transcripcional de Nrf2.

– Neh4 y Neh5 son dominios de transactivación de Nrf2. Actúan de forma cooperativa en la unión al co-activador *CREB binding protein* y de esa forma aumentan la tasa de transcripción génica.

– Neh6 es responsable de la regulación de Nrf2 independiente de Keap1. Contiene 2 secuencias peptídicas conservadas conocidas como DSGIS y DSAPGS, que son reconocidas por la proteína *β-transducin repeat-containing protein* (β-TrCP). El motivo DSGIS contiene el sitio de fosforilación de la enzima *glycogen synthase kinase-3* (GSK-3). La fosforilación de Nrf2 por GSK-3 potencia la regulación negativa de Nrf2 a través de β-TrCP.

– Neh7 está involucrado en la interacción directa entre Nrf2 y el dominio de unión al ADN del receptor *retinoid X receptor a* (RXRa). Esta interacción es inhibitoria ya que previene la unión de los co-activadores en los dominios Neh4 y Neh5 (Tebay et al. 2015).

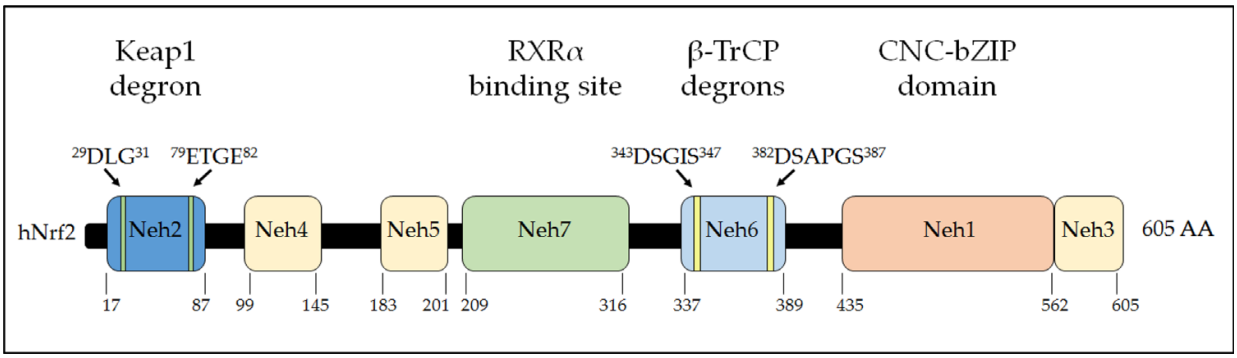


Figura 6: Estructura de Nrf2 humano (extraído de Tebay et al. 2015).

Como ya dijimos, para unirse al ADN, Nrf2 tiene que formar primero un heterodímero con las proteínas sMaf. Los niveles de estas pequeñas proteínas están finamente regulados: niveles muy bajos limitan la activación de Nrf2, mientras que niveles demasiado elevados causan la formación de homodímeros y por lo tanto quedan menos sMafs libres para la dimerización con Nrf2 (Katsuoka et al. 2005).

9.2. La región ARE

ARE es la región del ADN donde se unen los heterodímeros Nrf2-sMaf. Es una región amplificadora o "enhancer" tipo *cis*, descubierta a principios de los años ´90 y con una secuencia consenso definida como: 5'-A/GTGAC/GNNNGCA/G-3' ("N" representa residuos redundantes) (Nguyen et al. 2003). Su nombre *Antioxidant response element* deriva del hecho que se encontró por primera vez en la región promotora de genes inducibles por antioxidantes sintéticos fenólicos, como la tert-butil hidroquinona (tBHQ). Asimismo se evidenció que la secuencia ARE era imprescindible para la regulación de la expresión de genes de fase II detoxificantes y antioxidantes (Rushmore et al. 1991). Posteriormente, y con el descubrimiento de Nrf2, se

relacionó el papel de este factor de transcripción en la expresión génica dependiente de ARE (Itoh et al. 1997; Nguyen et al. 2000; Zhang 2006).

Actualmente se postula que Nrf2 se une a un amplio espectro de secuencias ARE. Su afinidad de unión dependerá del grado de similitud que tenga cada secuencia con respecto a la secuencia ARE consenso. Por otra parte Nrf2 se une a otras regiones que no presentan ARE; en estos casos se cree que se tratan de uniones indirectas o inespecíficas del factor (Lacher et al. 2015).

9.3 Regulación molecular de Nrf2

Nrf2 está sometido a mecanismos de regulación complejos, tanto a nivel transcripcional como post-transcripcional.

A nivel transcripcional, el gen que codifica para Nrf2, NFE2L2, presenta en su región promotora 2 secuencias tipo XRE (x*enobiotic response element*), que permiten la inducción génica a través del *aryl hydrocarbon receptor* (AhR) activado por hidrocarburos aromáticos policíclicos (PAHs, del inglés *polycyclic aromatic hydrocarbons*). Asimismo, evidencias indican que Nrf2 modularía la vía dependiente de AhR. Esto sugiere que existe un entrecruzamiento (*cross-talk)* entre estas dos vías adaptativas responsables de la inducción génica como respuesta a xenobióticos (Hayes et al. 2009). Además, la región promotora de NFE2L2 murino presenta elementos de respuesta a 12-O-tetradecanoilforbol-13-acetato (TPA) que permite su activación transcripcional por el oncogén $Kras^{G12D}$ a través de la via c-Jun y c-Fos. Además, NFE2L2 contiene un sitio de unión al factor NF-κB, que le permite ser inducible frente a estímulos inflamatorios (Rushworth et al. 2012). Por último, cabe destacar que la región promotora de NFE2L2 también presenta 2 secuencias ARE, que le permiten a Nrf2 inducir sus propios niveles de expresión (Kwak et al. 2002).

Por ser un factor de transcripción que se activa en respuesta a estrés, Nrf2 presenta una importante regulación a nivel post-transcripcional. La proteína es constantemente marcada por diversas E3 ubiquitin ligasas que forman complejos proteicos tales como: CRL (formado por: *Cullin 3* (Cul3)*, RING-box1* (RBX1) y *E3 ubiquitin ligase*) y SCF (formado por *S-phase kinase-associated protein 1* (Skp1), *cullin- 1* (Cul1) y *F-box protein E3 ubiquitin-ligase*). Esto asegura una constante degradación de Nrf2 a través del proteosoma 26S. Este mecanismo permite una dinámica activación en respuesta a estrés, ya que la inhibición de su degradación asegura que la proteína quede rápidamente disponible para traslocar al núcleo y actuar (Villeneuve et al. 2010).

9.3.1. Regulación de Nrf2 a través de Keap1

El regulador negativo de Nrf2 mejor estudiado es Keap1 (Itoh et al. 1999). Pertenece a la familia de las proteínas *broad complex,tram-track,bric-à-brac – Kelch* (BTB-Kelch). Es una proteína adaptadora de sustrato que en condiciones basales retiene a Nrf2 para su ubiquitinación a través del complejo proteico CRL^{Keap1} para su posterior degradación proteosomal. Presenta 5 dominios que detallamos a continuación:

- Una región N-terminal (NTR)
- Un dominio de interacción proteína-proteína BTB
- Una región intermedia (del inglés *intervening region*, IVR)
- Un dominio *double-glicine repeat* (DGR); también llamado *Kelch-repeat*, que consta de 6 subdominios con distintos motivos Gly-Gly cada uno
- Una región C-terminal (CTR).

El dominio BTB se une a Cul3, mientras que los dominios DGR y CTR se disponen formando una estructura β-hélice que interactúa con el dominio Neh2 de Nrf2 (Figura 7) (Tebay et al. 2015).

Keap1 se encuentra formando un homodímero a nivel del citoplasma y es de esta forma que se une a Nrf2. Uno de los modelos más aceptados de interacción Keap1-Nrf2 es el llamado *hinge and latch* (pestillo y bisagra). El mismo postula que uno de los Keap1 que conforman el homodímero se une a Nrf2 a la altura del motivo de alta afinidad ETGE del dominio Neh2, mientras que el motivo de baja afinidad DLG de Neh2 interacciona con la β-hélice del Keap1 adyacente. En condiciones de estrés oxidativo o ataque nucleofílico, las modificaciones de sus Cys le impiden a Keap1 mantener a Nrf2 para que sea ubiquitinado y luego degradado. De esa forma Nrf2 se acumula y puede traslocar al núcleo y actuar (Figura 8) (Baird y Dinkova-Kostova 2011).

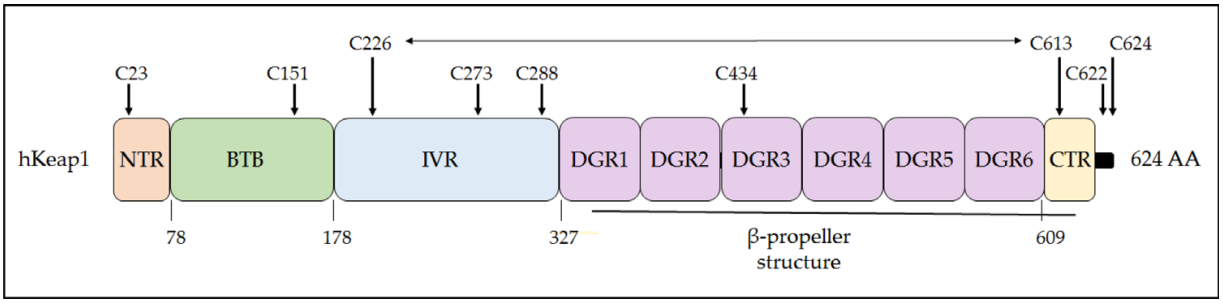


Figura 7: Estructura de Keap1 humano (extraído de Tebay et al. 2015).

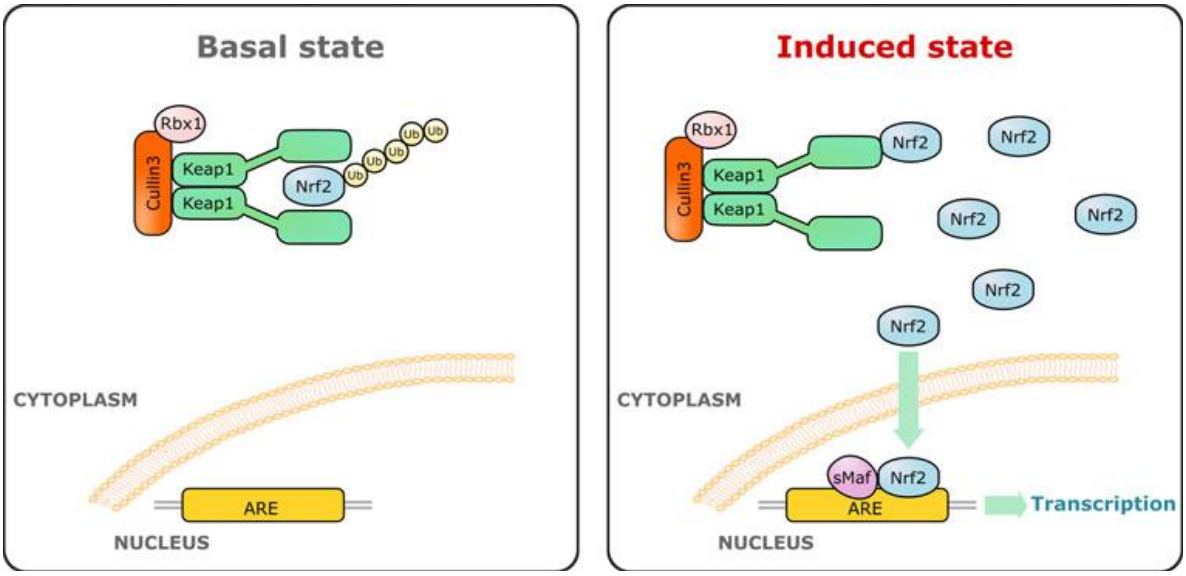


Figura 8: Modelo de interacción Keap1-Nrf2 *hinge and latch* (extraído de Baird y Dinkova-Kostova 2011).

Keap1 actúa de sensor de estrés celular. Su elevada sensibilidad redox se debe a que es una proteína rica en residuos de Cys. Estos residuos son susceptibles a la oxidación o a la modificación covalente por electrófilos. Cabe resaltar que la inactivación de Keap1 puede desencadenarse por un amplio espectro de compuestos. Se propone que existe un "código de cisteínas", que son modificadas de distintos modos según la naturaleza química del electrófilo. En este sentido, se han descrito diversos residuos sensores de estrés a lo largo de la proteína: Cys151, Cys273/Cys288, y Cys226/Cys434/Cys613, entre otras (Figura 7) (Baird y Dinkova-Kostova 2011).

9.3.2. Regulación de Nrf2 independiente de Keap1

Recientemente se descubrió otro tipo de regulación negativa de Nrf2 independiente de Keap1. Se trata de un mecanismo que depende de la proteína β-TrCP y de la kinasa GSK3 (Salazar et al. 2006; Rada et al. 2011). GSK3 fosforila serinas específicas del dominio Neh6 de Nrf2, convirtiéndolo en un domino de degradación que luego es reconocido por β-TrCP. A su vez, β-

TrCP actúa como adaptador de ubiquitin ligasa, y por lo tanto favorece el marcaje de Nrf2 por el complejo Cullin1/Rbx1, para su posterior degradación proteosomal (Figura 9).

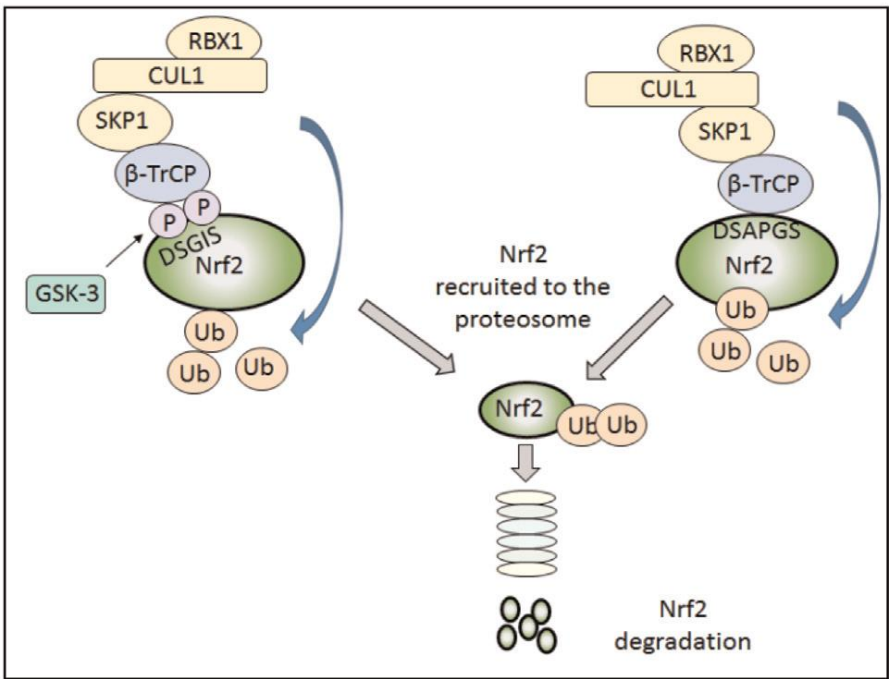


Figura 9: Modelo de regulación negativa vía GSK3/β-TrCP (extraído de Tebay et al. 2015).

GSK3 está involucrado en una gama muy diversa de vías que convergen en la activación de la fosfatidil inositol 3-fosfato kinasa (PI3K)/AKT y que participan en numerosos procesos metabólicos, de diferenciación, desarrollo, proliferación, etc.

El descubrimiento de Nrf2 como un nuevo blanco de esta kinasa, implica un nuevo grado de complejidad de las funciones de Nrf2. Asimismo, el eje β-TrCP/GSK3 aparece como una nueva estrategia terapéutica para la modulación de la actividad de Nrf2 (Figura 10) (Cuadrado 2015).

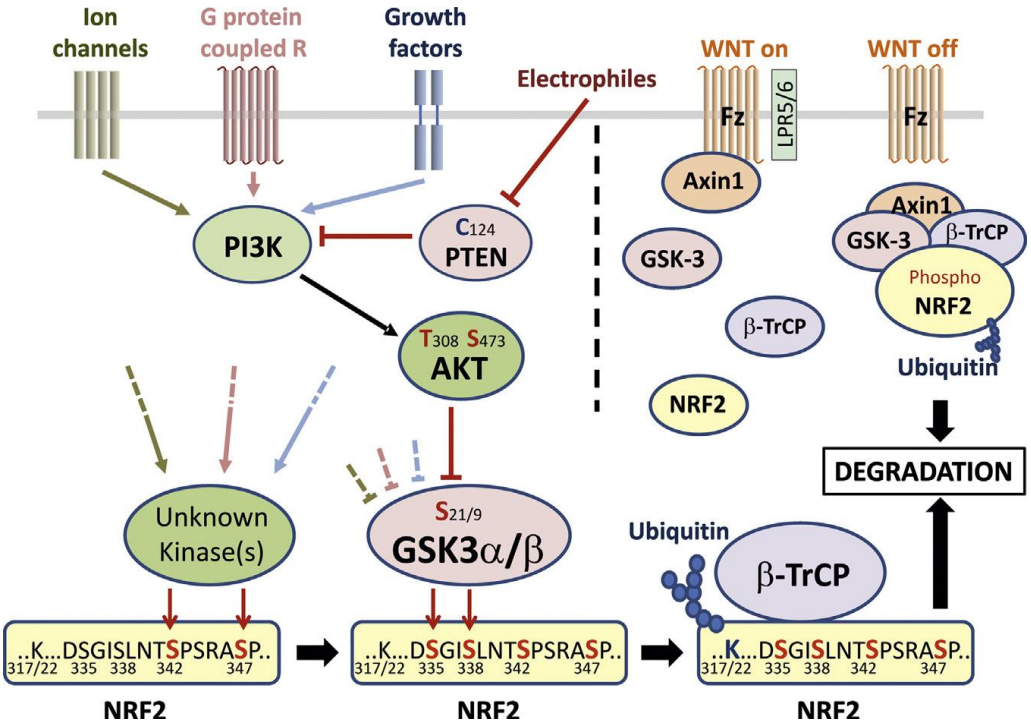


Figura 10: Vías de señalización intracelular que convergen y modulan el eje GSK3/βTrCP/NRF2 (extraído de Cuadrado 2015).

9.3.3. Inactivación de la vía Nrf2

Una vez activada la vía dependiente de Nrf2, también se desencadenan mecanismos endógenos de inactivación de la misma, para evitar su activación excesiva, y volver a la homeostasis celular. Entre estos mecanismos de retroalimentación negativa, se destacan:

– Algunos prooxidantes terminan promoviendo la activación de GSK3β por fosforilación de su tirosina 216, que a su vez fosforila a la proteína kinasa Fyn (p-Fyn). Esta se acumula en el núcleo y fosforila a Nrf2 en su tirosina 568. Una vez fosforilado, Nrf2 interactúa con las proteínas Crm1 o exportin 1 y de esa forma es exportado fuera del núcleo (Jain y Jaiswal 2006; Jain y Jaiswal 2007).

– La importación del complejo Rbx1-Cul3-Keap1 a través de la unión de Keap1 a la proteína *prothymosin a* (ProTa). Una vez en el núcleo, el

complejo se libera de ProTa y se une a Nrf2, promoviendo la ubiquitinación y degradación del Nrf2 nuclear (Niture y Jaiswal 2009).

– La "rama autorreguladora" de la vía Nrf2/ARE: Se trata de la presencia de secuencias ARE en las regiones promotoras de los genes que codifican para Cul3, Rbx1 y Keap1. De esta forma, cuando Nrf2 se activa, también induce la expresión del complejo Rbx1-Cul3-Keap1 que terminará reteniendo al factor de transcripción y mediando su degradación (Kaspar y Jaiswal 2010).

9.4. Tipos de inductores de la vía Nrf2

En la literatura se han propuesto diversos tipos de clasificaciones de los inductores de Nrf2, según su origen, su estructura química, su efecto a nivel de la vía, etc. Zhang y cols., propusieron una clasificación alternativa que se basa en la acción de los inductores sobre Keap1 o sobre la unión Keap1-Nrf2. Si bien debería complementarse con un listado de compuestos capaces de modular la vía a través del nuevo eje regulador β-TrCP/GSK3, esta clasificación permite evidenciar la gran diversidad de inductores de la vía. La detallamos a continuación:

– Inductores que actúan sobre IVR: Éste es un dominio con elevado contenido en Cys. Entre todas ellas, se destacan Cys273 y Cys288 que son claves para la actividad de Keap1. Su oxidación modifica la estructura de Keap1, reduce su afinidad por Nrf2 y lo disocia de Cul3. Los inductores de este grupo son en su mayoría aceptores de Michael, de tipo endógeno derivados de ácidos grasos poliinsaturados, como las prostaglandinas y los alquenales.

– Inductores que actúan sobre DGR: Este dominio se une al dominio Neh2 de Nrf2 y a la actina del citoesqueleto celular. Los inductores de este grupo interrumpen la unión Keap1-Nrf2. Se trata de proteínas endógenas como la *Nucleoporin p62* (p62), que se une al dominio DGR de

Keap1 causando su separación de Nrf2. También se caracterizó a *Cyclin-dependent kinase inhibitor 1A* ($p21^{Cip1}$) que compite con Keap1 por la unión directa a los motivos DLG y ETGE de Nrf2. Asimismo diversas kinasas pertenecen a este grupo, que mediante la fosforilación de residuos de Nrf2 les cambian su conformación y evitan su unión a Keap1. Entre ellas encontramos a MAPK, PI3K, y PKC.

– Inductores que actúan sobre múltiples dominios: En este grupo se encuentran los metales pesados como el Hg2+, Cd2+, Zn2+, etc. Se postula que los censores en este caso serían los residuos His225/Cys226 del dominio IVR y Cys613 del dominio DGR. Los mecanismos de acción de estos inductores no están esclarecidos.

– Inductores que actúan sobre el dominio BTB: BTB cumple 2 funciones relevantes, como dominio de dimerización de Keap1 y como sitio de unión a Cul3. En este dominio el residuo nucleofílico Cys 151 es fundamental. Ésta cumple un papel de sensor de estrés y es sensible a numerosos inductores mayoritariamente exógenos y de estructuras químicas muy diversas. Entre ellos encontramos a la tBHQ, aceptores de Michael como el ebseleno, fenoles oxidables, e isotiocianatos como el SFN, y también especies reactivas como el H_2O_2, $NO\cdot$, etc (Zhang et al. 2013).

9.5. Efectores ARE de la vía Nrf2

La vía Nrf2 controla la expresión de un conjunto muy amplio de genes con una enorme diversidad funcional y de esa forma le confiere a la célula múltiples niveles de protección.

A continuación enumeramos las distintas características funcionales de los genes diana de la vía:

1. Proteínas involucradas en el metabolismo y transporte de endo y xenobióticos. En este grupo encontramos a la NAD(P)H quinona deshidrogenasa 1 (NQO1), responsable de catalizar la reducción de las

quinonas a partir de NADH o NADPH. De esa forma, previene la susceptibilidad celular al EO ya que reduce la formación de ERO por parte de las quinonas, así como la depleción de los *pools* intracelulares de sulfhidrilos. Asimismo encontramos a las enzimas aldo-ceto-reductadas (AKRs), sulfotransferasas (SULTs), las Uridina difosfo-glucuronosil-transferasas (UGTs), y las GSTs que se encargan de detoxificar/activar, glucuronizar, sulfatar y glutationilar, respectivamente, facilitando la hidrofilicidad para la posterior excreción de endo y exoxenobióticos. Así, los productos de las reacciones de conjugación de estas enzimas son exportados de la célula a través de la proteínas de la familia *multidrug resistance-associated protein* (MRP), que también son efectores ARE (Chorley et al. 2012; Hirotsu et al. 2012).

2. Proteínas con función antioxidante. En este grupo encontramos a diversas proteínas involucradas en el sistema GSH: la subunidad χ-CT del transportador de membrana cistina/glutamato (que incorpora cistina, la cual es rápidamente reducida y sirve para la síntesis de GSH), a las ya presentadas GPx, GR y las subunidades catalítica y regulatoria de GCL. Además, encontramos diversas proteínas relacionadas con el sistema de Trx: por ejemplo las TrxR que son efectores ARE al igual que las Trx, que a su vez cumplen un papel clave en la catálisis de las Prxs, también efectores ARE. Por último, dentro de este grupo también encontramos a la hemoxigenasa 1 (HO-1), la cual genera bilirrubina, que juega un importante papel como antioxidante. La región promotora del gen de HO-1 cuenta con múltiples secuencias ARE, lo que lo hacen rápidamente inducible (Banning et al. 2005; Chorley et al. 2012; Hirotsu et al. 2012).

3. Enzimas que sintetizan poder reductor. En este grupo encontramos a la glucosa 6-fosfato deshidrogenasa (que provee de NADPH a GR) y a la enzima málica y la fosfogluconato deshidrogenasa (éstas forman NADPH que luego es usado por las TrxR) (Hirotsu et al. 2012).

4. Proteínas no enzimáticas, pero que cumplen un papel fundamental en la citoprotección. Por ejemplo, la ferritina, que almacena eficientemente el hierro libre y de esa forma no solo evita su toxicidad, sino que permite que éste sea utilizado cuando la célula lo requiera (Huang et al. 2013).

5. Proteínas que participan en los sistemas de reparación o degradación de proteínas dañadas, como es el caso de las subunidades del proteosoma 26S (Kwak y Kensler 2006).

Como se puede observar, el alcance regulatorio de Nrf2 se extiende más allá de genes antioxidantes. Incluso alcanza a inducir genes del metabolismo, genes proteostáticos y genes involucrados en vías de autorregulación. Asimismo, recientemente se observó que Nrf2 puede inducir proteínas anti-apoptóticas como *B-cell lymphoma 2* (Bcl-2), proteínas autofágicas como p62, anti-inflamatorias como *interleukin 10* (IL-10), factores de crecimiento como *brain derived neurotrophic factor* (BDNF) y co-factores de transcripción como Nrf1 y *peroxisome proliferator-activated receptor* (PPAR) *gamma coactivator 1-alpha* (PGC-1α) (Komatsu et al. 2010; Piantadosi et al. 2011; Sakata et al. 2012; Niture y Jaiswal 2012).

Actualmente se postula que debido a la gran diversidad de genes ARE, probablemente Nrf2 utilice también diversas estrategias regulatorias en respuesta al estrés. Si bien los genes antioxidantes canónicos de la vía parecen ser regulados de un modo "encendido-apagado" (*switch-like)*, probablemente otros genes ARE exhiban un tipo de respuesta más gradual frente a la activación de Nrf2. En este sentido, distintas combinaciones entre las secuencias amplificadoras, los complejos reguladores de la cromatina, e incluso el entrecruzamiento (*crosstalk)* con otras vías (AhR, NF-kB, p53, etc.), permitirían la modulación de la expresión génica por parte de Nrf2 de una forma muy versátil (Wakabayashi et al. 2010).

9.6. Efectores ARE de la vía Nrf2 conservados desde la mosca al hombre

Los factores de transcripción Cnc son una familia de proteínas conservada en gusanos, insectos, peces, aves y mamíferos, pero ausente en plantas y hongos. Los ortólogos funcionales a Nrf2 de *Caenorhabditis elegans* y *Drosophila melanogaster* son los factores transcripcionales *Skinhead family member 1* (Skn1) y la isoforma C de la proteína Cnc (CncC), respectivamente (Figura 11). Ambos se activan en respuesta al estrés, e inducen la expresión de genes antioxidantes (Sykiotis y Bohmann 2010). En el caso de la mosca, además del factor transcripcional, las proteínas Keap1 y sMaf también están conservadas funcional y estructuralmente.

En un trabajo recientemente realizado por Lacher y colaboradores, se estudió la homología entre las redes regulatorias de Nrf2 en Drosophila y humanos. De esa forma, se pudieron observar qué tipos de genes diana de Nrf2 estaban conservados entre ambas especies. El solapamiento entre grupos funcionales de genes diana de Nrf2 de estas dos especies distanciadas evolutivamente, sugiere que varias de las funciones regulatorias de este factor transcripcional fueron fuertemente conservadas. Entre los grupos de genes conservados se destacan: los genes antioxidantes "canónicos" de la vía, genes involucrados en mecanismos proteosomales y metabolismo glucídico, y genes implicados en procesos autorregulatorios. Esto sugiere por un lado, que las funciones de regulación del metabolismo xenobiótico, metabolismo glucídico y de la proteostasis fueron funciones centrales de la vía Nrf2 desde su formación. Por otro lado, se destaca la conservación de la "rama autorreguladora" de la vía Nrf2. Al igual que en humanos, en moscas el factor transcripcional induce la expresión del represor ortólogo de Keap1. Cabe destacar que si bien en *Caenorhabditis elegans* no existe una proteína ortóloga de Keap1, Skn1 también es

sometido a un proceso de autorregulación ya que induce la expresión de su represor. El mantenimiento de la función autorreguladora de Nrf2 a lo largo de la escala evolutiva sugiere que ésta es una de las funciones primordiales de la vía (Lacher et al. 2015).

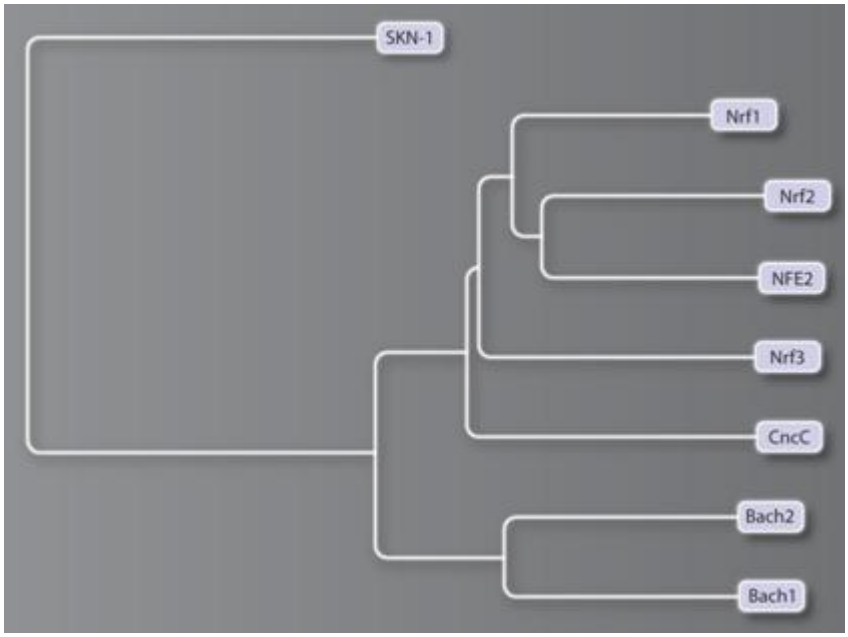


Figura 11: Árbol filogenético de los factores de transcripción Cnc y Bach (extraído de Sykiotis y Bohmann 2010).

9.7. El papel neuroprotector de la vía Nrf2

Nrf2 está ampliamente expresado en todo el SNC y se activa en respuesta a diferentes situaciones patológicas incluyendo tanto desórdenes neurodegenerativos agudos como crónicos (Zhang et al. 2013). Además de su papel como inductor de defensas antioxidantes endógenas, también participa como regulador de los procesos inflamatorios en el cerebro (Sandberg et al. 2014).

Asimismo se ha observado la alteración de esta vía en diversas neuropatologías como los procesos isquémicos, trauma, Enfermedad de

Parkinson, Enfermedad de Alzheimer y la Esclerosis Lateral Amiotrófica (Sarlette et al. 2008; von Otter et al. 2010; Mota et al. 2015).

Por último, diversos estudios de activación, sobreexpresión, y/o atenuación de Nrf2, sugieren que esta vía presenta un papel importante en la protección del sistema nervioso (Lee et al. 2003; Lee et al. 2005; Calkins et al. 2005; Johnson y Johnson 2015).

Por lo tanto Nrf2 resulta un interesante blanco terapéutico, donde moléculas capaces de activar esta vía tendrían un potencial valor para el tratamiento de enfermedades neurodegenerativas.

9.8. Los flavonoides como inductores de la vía Nrf2

Entre los fenoles oxidables capaces de inducir la vía Nrf2 que enumeramos en el punto 9.4, se encuentran los flavonoides. Como expusimos para el caso de quercetina, tradicionalmente las propiedades pro-oxidantes de los flavonoides han sido consideradas como efectos negativos, por ser responsables de su toxicidad. Sin embargo, desde hace algunos años se empezó a asociar la pro-oxidación de estos compuestos con su capacidad de modular vías de señalización intracelular redox-sensibles (Suzuki et al. 1997; Metodiewa et al. 1999). Asimismo, recientemente en la literatura comenzó a proponerse una hipótesis "hormética" sobre el papel que cumplen los flavonoides y otros fitoquímicos provenientes de la dieta, en la salud y prevención de enfermedades crónicas. En esta teoría se postula que los fitoquímicos serían reconocidos a nivel celular como xenobióticos que a dosis elevadas son tóxicos. Sin embargo a dosis moderadas activan sistemas de respuesta adaptativa, haciendo más resistentes a las células frente a otros agentes estresantes y promotores de la aparición de enfermedades (Mattson y Cheng 2006). Actualmente se especula que los flavonoides con sus propiedades redox intrínsecas causarían una situación de estrés oxidativo moderado. De esa forma serían

capaces de activar vías como la Nrf2/ARE, promoviendo la expresión de defensas celulares endógenas (Procházková et al. 2011).

En los últimos años diversos estudios aportaron evidencias sobre la capacidad de algunos flavonoides de inducir la expresión de genes a través de la vía Nrf2 en líneas celulares no neuronales. Sin embargo, estos estudios se centran principalmente en las acciones anticancerígenas de estos compuestos a través de la vía Nrf2 (Chow et al. 2005; Tanigawa et al. 2007; Lee et al. 2008). Poco se ha estudiado sobre estos efectos en neuronas (Lavoie et al. 2009)).

Por lo tanto, la inducción de proteínas citoprotectoras a través de la vía Nrf2 en neuronas por acción de flavonoides, presentaría un potencial terapéutico en la prevención de la muerte neuronal por EO asociada a enfermedades neurodegenerativas.

ANTECEDENTES PARTICULARES

Como expusimos en la introducción, las enfermedades que involucran muerte neuronal focal o masiva en el cerebro, y donde el estrés oxidativo juega un papel preponderante, son una causa frecuente de muerte e incapacidad en Uruguay y en el mundo. Pese a ello, no existen en la actualidad terapias efectivas para su tratamiento. Esto ha promovido la búsqueda de nuevas terapias capaces de prevenir la muerte neuronal inducida por EO implicado en estas patologías.

En este contexto, en el Departamento de Neuroquímica estudiamos desde hace más de 15 años los efectos neuroprotectores de compuestos antioxidantes de origen natural. En particular, analizamos las propiedades neuroprotectoras de los flavonoides en diversos modelos de muerte neuronal en cultivos y en modelos de neurodegeneración *in vivo* (Dajas et al. 2003b; Arredondo et al. 2004; Echeverry et al. 2004; Dajas et al. 2005; Rivera et al. 2008; Blasina et al. 2009; Dajas et al. 2013; Echeverry et al. 2015; Blasina et al. 2015; Dajas et al. 2015). Por un lado, nos focalizamos en dilucidar los mecanismos involucrados en la neuroprotección de estos compuestos. Por otro, desarrollamos preparaciones que sirvan de vehículo y faciliten el acceso de estos compuestos al cerebro.

Previo al desarrollo de esta tesis, demostramos que la capacidad atrapadora de radicales libres de flavonoides estructuralmente relacionados entre sí, era una propiedad necesaria pero no suficiente para asegurar la citoprotección frente a un daño oxidativo (Dajas et al. 2003b; Arredondo et al. 2004). Esta observación fue apoyada por otros grupos de investigación (Wang et al. 2001; Kang et al. 2004).

Planteamos así que el efecto neuroprotector de los flavonoides involucraría la modulación del estado redox celular mediante mecanismos más allá de sus propiedades antioxidantes directas. Ese mantenimiento de la homeostasis redox celular evitaría la muerte celular por EO, y jugaría un

papel crítico en la prevención y evolución de enfermedades neurodegenerativas.

En este sentido, el estado del conocimiento sobre los mecanismos de acción de los flavonoides cuando planificamos esta tesis, era el siguiente:

- Evidencias sugerían sobre la capacidad inductora de los niveles de GSH por parte de los flavonoides, a través de la regulación génica vía ARE de la enzima GCL (Myhrstad et al. 2002; Moskaug et al. 2005).

- Otros trabajos habían reportado acciones moduladoras de los flavonoides sobre la actividad de las enzimas implicadas en el reciclado del GSH, como la GR y la GPx (Minato et al. 2003; Hong y Liu 2004).

- Los estudios sobre los mecanismos de neuroprotección por quercetina se centraban principalmente en su capacidad atrapadora de radicales libres, o en su interacción con cascadas intracelulares dependientes de kinasas (Schroeter et al. 2002; Mandel et al. 2005; Shimizu y Weinstein 2005). Por otra parte, los pocos estudios centrados en la capacidad moduladora de los sistemas redox tiol-dependientes por parte de la quercetina, se habían realizado en células no neuronales (Myhrstad et al. 2002).

Asimismo, diversos estudios de activación, sobreexpresión, y/o atenuación de Nrf2, sugerían que esta vía presentaba un papel importante en la protección del sistema nervioso (Lee et al. 2003; Lee et al. 2005; Calkins et al. 2005).

HIPÓTESIS DE TRABAJO

A partir de estos antecedentes, la hipótesis de trabajo en la que nos apoyamos durante esta tesis fue la siguiente:

El efecto neuroprotector de quercetina frente a estrés oxidativo involucra la inducción de defensas antioxidantes endógenas a través de la vía Nrf2. Este mecanismo es común a otros flavonoides estructuralmente relacionados con quercetina.

OBJETIVOS

En base a esta hipótesis, nos planteamos el siguiente objetivo general:

Estudiar la inducción de la vía Nrf2 por quercetina y flavonoides estructuralmente relacionados, como posible mecanismo involucrado en la neuroprotección frente a estrés oxidativo.

En esencia, en esta tesis intentamos responder a 3 preguntas:

1. ¿Quercetina es capaz de prevenir la muerte neuronal por EO mediante mecanismos antioxidantes indirectos?

2. ¿Qué características estructurales de quercetina y flavonoides relacionados determinan este efecto neuroprotector?

3. ¿El efecto neuroprotector de la quercetina involucra la activación de la vía Nrf2/ARE?

Para responder a estas preguntas, nos trazamos los siguientes objetivos específicos:

a) Desarrollar un modelo *in vitro* de muerte neuronal por estrés oxidativo en un cultivo neuronal primario.

b) Evaluar la capacidad neuroprotectora de quercetina en el modelo *in vitro* de muerte neuronal por estrés oxidativo.

c) Estudiar la cinética de la biodistribución celular y el destino subcelular de quercetina en los cultivos neuronales primarios.

d) Realizar un análisis de estructura-actividad de la capacidad neuroprotectora de flavonoides estructuralmente relacionados a quercetina, en el modelo *in vitro* de muerte neuronal por estrés oxidativo.

e) Estudiar la activación de la vía Nrf2/ARE por quercetina en el modelo de cultivo neuronal primario.

f) Realizar un análisis de estructura-actividad de la activación de la vía Nrf2/ARE por flavonoides estructuralmente relacionados a quercetina.

g) Estudiar el papel de la vía Nrf2/ARE en el efecto neuroprotector de quercetina.

METODOLOGÍA Y RESULTADOS

1. After cellular internalization, quercetin causes Nrf2 nuclear translocation, increases glutathione levels and prevents neuronal death against an oxidative insult. F. Arredondo; C. Echeverry; JA Abin-Carriquiry; F. Blasina; K. Antúnez; DP Jones; YM Go; YL Liang; F. Dajas. Free Radical Biology and Medicine, v.: 49 5, p.: 738 - 747, 2010

En el artículo que presentamos a continuación nos focalizamos en los objetivos específicos a, b y c, con el fin de contestar la primera pregunta planteada en esta tesis: *¿Quercetina es capaz de prevenir la muerte neuronal por EO mediante mecanismos antioxidantes indirectos?*

Para ello, desarrollamos un modelo de muerte neuronal oxidativa en un cultivo primario de neuronas granulares de cerebelo sometido a un daño con H_2O_2 durante 24 h. Utilizando ese modelo evaluamos las propiedades neurotóxicas y neuroprotectoras de quercetina al ser agregada 24 h antes del insulto con H_2O_2 (protocolo de pre-tratamiento con respecto al daño oxidativo). Además, analizamos la cinética de la biodistribución celular de quercetina en los cultivos neuronales así como su destino subcelular, durante las 24 h de incubación. También evaluamos los efectos del pre-tratamiento con quercetina sobre los niveles de los tioles GSH y Trx2 en los cultivos neuronales. Finalmente estudiamos los efectos del pre-tratamiento con quercetina sobre la translocación nuclear de Nrf2 en los cultivos neuronales.

Los resultados obtenidos mostraron que quercetina se internaliza rápidamente en las neuronas, alcanzando el núcleo, y sus niveles intracelulares van disminuyendo durante el tiempo de incubación hasta que se hacen indetectables luego de las 24 h de tratamiento. Quercetina es capaz de prevenir la muerte neuronal aún cuando no está presente al momento del daño oxidativo. Asimismo, es capaz de traslocar el factor de transcripción Nrf2 al núcleo y aumentar los niveles de GSH.

Estos hallazgos abrieron la puerta para el estudio de la activación de la vía Nrf2 como un posible mecanismo involucrado en la prevención de la muerte neuronal por EO por parte de los flavonoides.

Free Radical Biology & Medicine 49 (2010) 738–747

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

After cellular internalization, quercetin causes Nrf2 nuclear translocation, increases glutathione levels, and prevents neuronal death against an oxidative insult

Florencia Arredondo [a,*], Carolina Echeverry [a], Juan A. Abin-Carriquiry [a], Fernanda Blasina [a], Karina Antúnez [b], Dean P. Jones [c], Young-Mi Go [c], Yong-Liang Liang [c], Federico Dajas [a]

[a] Departamento de Neuroquímica, Instituto de Investigaciones Biológicas Clemente Estable, 11600 Montevideo, Uruguay
[b] Departamento de Microbiología, Instituto de Investigaciones Biológicas Clemente Estable, 11600 Montevideo, Uruguay
[c] Division of Pulmonary Medicine, Department of Medicine, Emory University, Atlanta, GA 30322, USA

ARTICLE INFO

Article history:
Received 3 November 2009
Revised 18 May 2010
Accepted 24 May 2010
Available online 8 June 2010

Keywords:
Neuroprotection
Oxidative stress
Quercetin
Bioavailability
Intrinsic fluorescence
Nrf2
γ-Glutamate–cysteine ligase
Glutathione
Thioredoxin 2
Free radicals

ABSTRACT

In this work we describe the protective effects of quercetin against H_2O_2 in 24-h-pretreated neuronal cultures. We explored quercetin availability and subcellular fate through the use of HPLC-Diode Array Detection (DAD), epifluorescence, and confocal microscopy. We focused on quercetin modulation of thiol-redox systems by evaluating changes in mitochondrial thioredoxin Trx2, the levels of total glutathione (GSH), and the expression of the γ-glutamate–cysteine ligase catalytic subunit (GCLC), the rate-limiting enzyme of GSH synthesis, by the use of Western blot, HPLC, and real-time PCR techniques, respectively. We further explored the activation of the protective NF-E2-related factor 2 (Nrf2)-dependent signaling pathway by quercetin using immunocytochemistry techniques. Our results showed rapid quercetin internalization into neurons, reaching the nucleus after its addition to the culture. Quercetin pretreatment increased total GSH levels, but did not increase Trx2. Interestingly it caused Nrf2 nuclear translocation and significantly increased GCLC gene expression. At the moment of H_2O_2 addition, intracellular quercetin or related metabolites were undetectable in the cultures although quercetin pretreatment prevented neuronal death from the oxidant exposure. Our findings suggest alternative mechanisms of quercetin neuroprotection beyond its long-established ROS scavenging properties, involving Nrf2-dependent modulation of the GSH redox system.

Introduction

Reactive oxygen and nitrogen species (ROS/RNS) are recognized for playing a dual role as both deleterious and beneficial molecules. Under physiologic conditions, the balance between the generation and the elimination of ROS/RNS maintains a redox homeostasis to ensure the correct function of redox-sensitive signaling proteins. However, when it is disturbed, oxidative processes take place, damaging biomolecules, and aberrant redox cell signaling may lead to cell death, contributing to disease onset [1–4]. This recognized "two-faced" character of ROS/RNS has contributed to the establishment of a redefinition of oxidative stress from the classical "imbalance of pro-oxidants and antioxidants" to a more contemporary concept regarding a "disruption of redox signaling and control" [5,6].

The maintenance of cellular redox homeostasis appears as a clue to the correct functioning of redox signaling and control and the prevention of oxidative stress-related diseases. In this sense, cells have developed diverse mechanisms to maintain such homeostasis and to deal with ROS/RNS produced in excess during oxidative stress [2].

A main regulator of the intracellular redox status is glutathione, a cysteine-containing tripeptide with reducing and nucleophilic properties. It exists either as a reduced form (GSH) or as an oxidized form (GSSG). This thiol is a key modulator of cell functions including antioxidant defense, redox regulation of protein thiols, and maintenance of redox homeostasis [7–11].

The other major thiol-dependent system underlying redox modulation functions is the family of thioredoxins (Trx). Trx isozymes play an important role in redox regulation of protein thiols involved in signal transduction and gene regulation [12]. Particularly, the mitochondrial isoform Trx2 is abundantly and widely distributed in rat brain. It is essential for cell survival, and evidence suggests it has protective effects against oxidative stress [12–15].

An important number of studies on the central nervous system (CNS) support the idea that the disturbance of redox homeostasis, often associated with deficits in mitochondrial function, leads to oxidative stress events and neuronal death, thereby contributing to the development of neurodegenerative disorders such as Alzheimer and Parkinson disease [16–18]. Interestingly, several studies associate a shift in GSH levels with such pathologies [19–23]. In addition, a rapidly growing number of studies suggest that Trx proteins perform

* Corresponding author. Fax: +598 2 4872603.
E-mail address: flor@iibce.edu.uy (F. Arredondo).

0891-5849/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2010.05.020

important functions in the CNS, including neuroprotective actions [12].

In this context, pharmacological interventions that regulate redox homeostasis could be an important strategy to promote cell survival and treat or prevent neurodegeneration [11,19–23].

There is a growing interest in the neuroprotective potential of flavonoids although the exact mechanisms by which these compounds exert their benefits are not fully understood [24–27]. Though these favorable effects have been largely related to their classical hydrogen-donating antioxidant activity [28], in the past 5–10 years, evidence from cell studies suggests that flavonoids can influence cellular fate by other mechanisms of action involving protein and lipid interactions leading to enzymatic modulation, interaction with several receptors, modulation of intracellular signaling cascades, and modulation of gene expression [24,25,27,29–31]. In particular, flavonoids have been proposed to induce the expression of protective genes, including those encoding enzymes involved in GSH synthesis, through the activation of the NF-E2-related factor-2 (Nrf2) transcription factor pathway [32]. In addition, many of these multitarget mechanisms are redox sensitive, suggesting that the basis of the potential therapeutic capacity of flavonoids may be related to properties beyond scavenging and metal-chelating antioxidant activities.

Among the high number of naturally occurring flavonoids, quercetin is the most frequent flavonoid in the Western diet [33–35]. Studies of quercetin's neuroprotective mechanisms have been mainly focused either on ROS scavenging and metal-chelating properties or on interactions with kinase signaling cascades [29,30,36–38]. Less work has been done on the modulation of thiol-redox systems by quercetin and these studies have been mainly performed in nonneuronal cells [39]. Accordingly, in the present work we focused on the modulation of thiol-redox systems as a complementary antioxidant mechanism of quercetin neuroprotection. To this aim we developed a model of oxidative stress-induced neuronal death in primary cerebellar granule neurons, in which we studied neuroprotective and neurotoxic profiles of quercetin in 24-h pretreated cultures. We evaluated quercetin bioavailability in cultures during the 24 h of incubation previous to oxidative injury, and we investigated its subcellular fate in neurons by the use of its fluorogenic properties. Furthermore we studied changes in mitochondrial thioredoxin Trx2 and in cellular levels of GSH and changes in the expression of γ-glutamate–cysteine ligase catalytic subunit (GCLC), the rate-limiting enzyme of GSH synthesis, and we explored the activation of the protective Nrf2-dependent signaling pathway by quercetin.

Methods

Reagents

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless stated otherwise. Hydrogen peroxide (H_2O_2) was dissolved in distilled water and subsequently filtered before being added to the cultures at a final concentration of 60 μM. Quercetin (Great Dragon Biochemicals Co., Ltd., China; 98% purity; CAS No. 6151-25-3) was dissolved in 0.5% dimethyl sulfoxide (DMSO) and applied to the neuronal cultures at a final concentration range from 5 to 100 μM.

Neuronal culture

Primary cerebellar granule neurons (CGN) were obtained from 6- to 8-day-old Sprague–Dawley rats [40] and seeded in poly-L-ornithine precoated 24-well plates at a density of 3.1×10^5 cells/cm^2 and kept in basal medium Eagle supplemented with fetal bovine serum (PAA Laboratories, Pashing, Austria; 10%), 20 mM KCl, and 25 mM glucose, in a humidified chamber at 37 °C in a 5% CO_2 atmosphere. Glial growth was inhibited with cytosine arabinoside (10 μM). Cultures were maintained for 8 days in vitro until the experimental treatments.

Experimental treatments

For the evaluation of the protective capacity of quercetin, H_2O_2 was added at the 9th day in vitro (DIV9) for 24 h. Quercetin was applied 24 h before the H_2O_2 insult (pretreatment).

For the evaluation of the neurotoxicity of quercetin, at DIV8 cultures were exposed to quercetin for 24 h.

Assessment of cell viability: examination of morphology and metabolic activity of cells

Cell viability was evaluated by direct examination of the cultures under inverted light microscopy. Briefly, after experimental treatment cells were examined by phase-contrast microscopy (Axiovert 25; Zeiss, Oberkochen, Germany), to analyze morphological features that are indicators of cellular integrity and cellular death (brightness, shrinking, cellular fragmentation, neurite integrity).

Additionally, cell survival was quantified by analysis of the metabolic activity of cultures using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [41]. This test is based on the enzymatic reduction of MTT salts into formazan inside the cells [41]. As MTT lacks permeativity through the plasma membrane, it enters into cells by endocytosis, an ATP-dependent process characteristic of living cells [42]. Thus, this assay reflects the integrity of the metabolic machinery in viable neurons. In brief, after experimental treatment, cells were incubated for 45 min at 37 °C with MTT (0.1 mg/ml final concentration), and metabolically active cells reduced the dye to purple formazan. Formazan crystals were dissolved in DMSO, and the absorbance was measured on an MRX microplate reader (Dynex Technologies, Chantilly, VA, USA), using a reference wavelength of 630 nm and a test wavelength of 570 nm. Results were presented as the percentage of MTT reduction, assuming that absorbance of control cells was 100%.

HPLC analysis of quercetin availability in cell cultures

To evaluate the intracellular/cell-associated levels of quercetin, at DIV8 cultures were incubated with 25 μM quercetin for up to 24 h, quickly washed with phosphate-buffered saline (PBS) three times, and lysed (50% aqueous methanol in 0.1 M HCl). For measurement of extracellular levels, after quercetin incubation, samples from culture medium were diluted in methanol (1:1).

Freeze-thawed samples were microfuged at 10,000 rpm for 5 min at 4 °C, and supernatants were injected onto the HPLC system. Separation of constituents was achieved by reverse-phase HPLC using a C18 column (Phenomenex, Torrance, CA, USA) with 5-μm particle size. A binary HPLC pump (Waters 1525; Milford, MA, USA) with a 717 Plus autosampler (Waters) and a photodiode array detector (Waters 2998) linked to Empower 2 (Waters) chromatography data software was utilized. The temperature of the column was set at 30 °C. The mobile phases used were (A) 100% MeOH and (B) 0.5% H_3PO_4, pH 2, 5% MeOH, at 0.7 ml/min. The gradient system consisted of (min/%B) 0/80, 40/0, 41/80, and 47/80. The eluant was monitored by photodiode array detection at 375 nm and spectra of products obtained between 210 and 600 nm.

For the analysis of putative quercetin metabolites, new peaks with spectral characteristics similar to those of flavonoids were analyzed as quercetin equivalents.

Epifluorescence and confocal microscopy

CGN cultures were seeded in poly-L-ornithine-coated coverslips in 24 wells (3.1×10^5 cells/cm^2) and maintained as described above. To

evaluate the intracellular presence of quercetin, at DIV8 cultures were incubated with 25 μM quercetin for up to 24 h, quickly washed with PBS, and fixed with 4% formaldehyde for 5 min [43]. Nuclei were stained with Hoechst 33258 or TOPRO-3 (Molecular Probes, Eugene, OR, USA) dye. After that, coverslips were put upside down on the glass slides, with a drop of 30% glycerol.

To evaluate the Nrf2 nuclear translocation, at DIV8 cultures were incubated with 25 μM quercetin or with 20 μM *tert*-butylhydroquinone (tBHQ; positive modulator of Nrf2) for 3 h, quickly washed with PBS, and fixed with 4% paraformaldehyde for 10 min. Cells were left in permeabilization solution (0.1% Triton X-100 in PBS) for 15 min, and then in antibody blocking solution (2% bovine albumin, 2% horse serum in permeabilization solution) for 1 h, followed by an overnight incubation with primary antibody anti-Nrf2 (rabbit, 1:400; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) diluted in the same buffer at 4 °C. After being washed with PBS, the cells were incubated with Alexa 568-conjugated secondary antibody (goat anti-rabbit IgG–568; 1:400; Chemicon, Temecula, CA, USA) for 1 h at room temperature and then washed with PBS. Nuclei were stained with Hoechst 33258. Finally, coverslips were put upside down on the glass slides, with a drop of 30% glycerol.

Samples were first observed with epifluorescence (Olympus DP70; Center Valley, PA, USA), using 20× and 40× objectives and DPController software (2002; Olympus). Subsequently, samples were observed with a confocal laser scanning microscopy system (Olympus BX61), using 20×, 40×, and 60× objectives and software options (FluoView FV300; 4.3a version). Green fluorescence from quercetin was monitored at 488 nm ex/500–540 nm em; Hoechst 33258 and TOPRO-3 nuclear stains were detected at $\lambda_{ex} = 405$ nm and $\lambda_{ex} = 633$ nm, respectively; and finally Alexa 568-conjugated secondary antibody used for Nrf2 localization was detected at $\lambda_{ex} = 543$ nm.

Analysis of GCLC gene expression by real-time PCR

To assess whether 25 μM quercetin treatment caused GCLC induction, at DIV8 cultures were treated with the flavonoid or with vehicle (control) for 6 h, before total RNA isolation.

Total RNA was extracted from cell cultures using the QIAamp viral RNA mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Total RNA recovered was immediately reverse-transcribed to generate first-strand cDNAs using the QuantiTec reverse transcription kit (Qiagen), according to the manufacturer´s instructions.

GCLC transcript levels were analyzed by real-time PCR using the primers GCLC forward, 5′-ATGAAAGTGGCACAGGAGCGAG-3′, and GCLC reverse, 5′-AAACAGGCCTTCCTTCCCATT-3′ [44]. Transcript levels of 18 S RNA were also analyzed and used as reference gene data for normalization, by using the primers 18 S RNA forward, 5′-GTAACCCGTTGAACCCCATT-3′, and 18 S reverse, 5′-CCATCCAATCGG-TAGTAGCG-3′ [45].

Real-time PCRs were carried out using the QuantiTec SYBR green real-time PCR kit (Qiagen) and primers (Integrated DNA Technologies, Coralville, IA, USA) described above. Reaction mixes contained 25 μl of 2× QuantiTec SYBR green PCR master mix (Qiagen), 0.25 μM each primer, and 5 μl of 1:10 diluted cDNA in a final volume of 50 μl.

PCRs were carried out using a Corbett Rotor Gene 6000 cycler and the cycling program consisted in an initial preincubation step of 50 °C for 2 min and 95 °C for 15 min and 35 cycles of 94 °C for 15 s, 53 °C for 30 s, and 72 °C for 30 s. Fluorescence was measured in the elongation step and negative controls (without cDNA) were included in each reaction run. The specificity of the reaction was checked by analyzing the melting curve of the final amplified products, which was obtained through continuous reading over increasing temperatures from 65 to

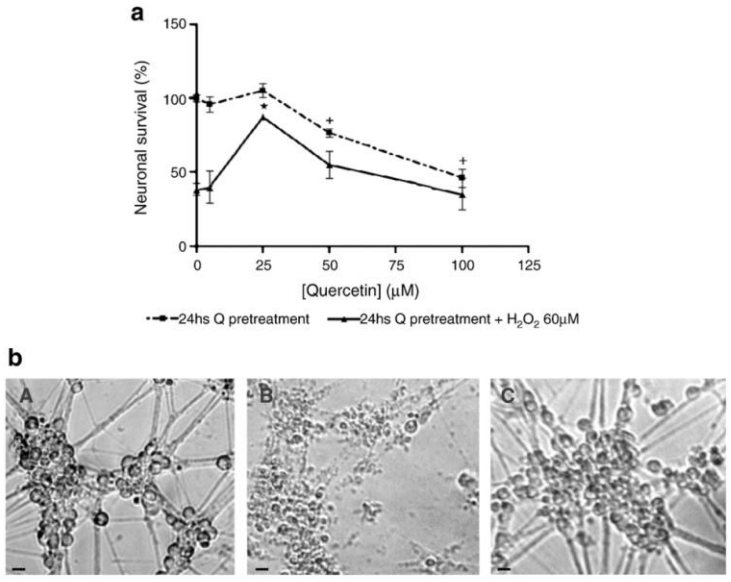


Fig. 1. Quercetin toxicity per se and protective effects against H_2O_2 on cerebellar granule neuronal cultures after 24 h preincubation. (a) Biphasic dose–response effect of quercetin on neuronal cells. For the evaluation of quercetin protective effects, neuronal cells were pretreated for 24 h with quercetin (0–100 μM) and then were exposed to 60 μM H_2O_2 for 24 h. For the analysis of quercetin toxicity, neuronal cells were exposed for 24 h to increasing concentrations of quercetin alone (0–100 μM). Data were normalized to control vehicle cells (neuronal cultures treated with vehicle alone for 24 h) and presented as means of % ± SEM of at least three experiments. $^{+}p<0.01$, significantly different from control vehicle cells; $^{*}p<0.001$, significantly different from control vehicle cells + H_2O_2. (b) Phase-contrast photomicrographs showing the morphological preservation of 25 μM quercetin pretreated cultures after 24 h of H_2O_2 injury: image A, control vehicle cells; image B, control vehicle cells + H_2O_2; image C, 25 μM quercetin-pretreated cells + H_2O_2. Scale bar, 10 μm.

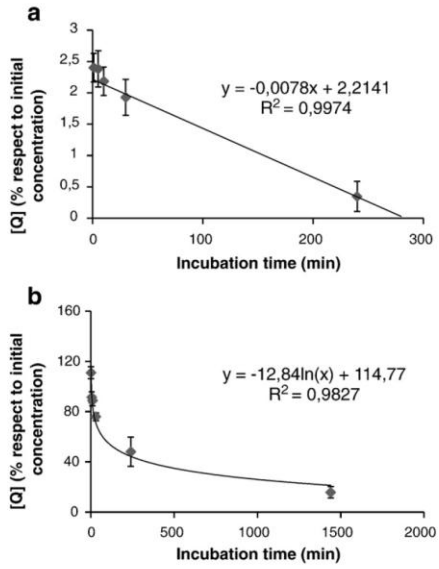


Fig. 2. Temporal course of (a) intracellular/cell-associated and (b) extracellular levels of quercetin in neuronal cultures during 24 h incubation. Neuronal cells were exposed to 25 μM quercetin for 1 min, 5 min, 10 min, 30 min, 4 h, and 24 h. Cell extracts and medium samples were analyzed by HPLC-Diode Array Detection (DAD) for quantification of quercetin intracellular/cell-associated and extracellular levels, respectively. Data are presented as means ± SEM of at least three experiments.

95 °C. Four 10-fold serial dilutions (1, 0.1, 0.01, and 0.001) of total cDNA samples were included in real-time PCR analysis to confirm that the amplification efficiencies of GCLC and of 18 S RNA were similar.

The amplification results were expressed as the threshold cycle number (C_T), which represents the number of cycles needed to generate a fluorescent signal greater than a predefined threshold. To perform data normalization, C_T for 18 S RNA was subtracted from C_T for GCLC (ΔC_T). The comparative $\Delta\Delta C_T$ method was used for the relative mRNA quantification as described previously [46].

Total glutathione analysis

After experimental treatment, GSH and GSSG concentrations of cultures were measured by HPLC with fluorescence detection as *S*-carboxymethyl-*N*-dansyl derivatives and using γ-glutamylglutamate as internal standard, as previously described [9].

Western blotting analysis

After experimental treatments, protein samples (40 μg) were resolved on 15% sodium dodecyl sulfate–polyacrylamide gel and transferred to nitrocellulose membrane (Hybond C; Amersham Biosciences, Buckinghamshire, UK). Membranes were blocked for 1 h in PBS containing 0.05% Tween 20 and 5% nonfat dry milk, followed by an overnight incubation with primary antibodies anti-Trx2 (rabbit serum; 1:500; Covance, Denver, PA, USA) and anti-α-tubulin (mouse; 1:1500) diluted in the same buffer at 4 °C. After being washed with 0.05% Tween 20 in PBS, the membrane was incubated with peroxidase-conjugated secondary antibodies (ECL donkey anti-rabbit IgG–HRP and ECL sheep anti-mouse IgG–HRP, respectively; GE Healthcare, Chalfont St. Giles, UK) for 1 h and then washed and developed using ECL Western blotting detection reagents (Amersham, GE Healthcare).

Statistical analysis

Experiments were done three times independent, and data are presented as means ± SEM. The results of cell viability versus quercetin concentration were statistically evaluated for significance with one-way ANOVA followed by a Tukey post hoc test. Results obtained from total GSH levels of different treatments related to different time points were statistically evaluated for significance with two-way ANOVA followed by a Bonferroni post hoc test. Real-time PCR experiments were done two times independently, and the variation in GCLC gene transcript levels (ΔC_T) between quercetin-treated and control groups was evaluated using a Student *t* test.

Results

Quercetin toxicity and protective effects against H_2O_2 on cerebellar granule neuronal cultures: 24 h pretreatment resulted in a biphasic dose–response effect

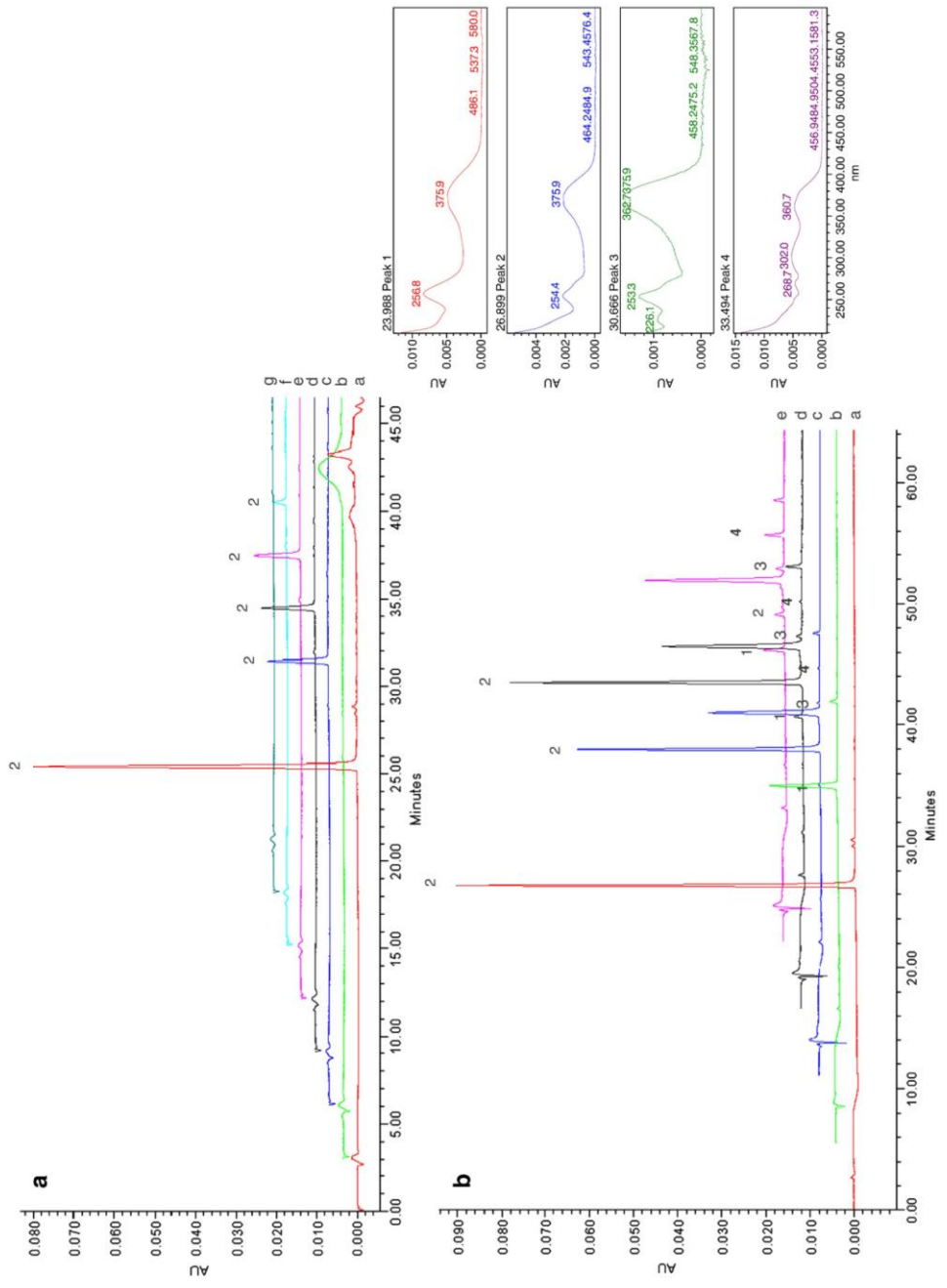
For the assessment of quercetin protection, we incubated primary neuronal cultures with quercetin 24 h before 60 μM H_2O_2 treatment. We also studied the toxic effect per se of 24-h quercetin pretreatment in these neuronal cultures. Fig. 1a presents toxic and protective profiles of quercetin (5–100 μM). We observed a biphasic dose–effect curve, in that concentrations lower than 25 μM did not prevent oxidant-induced neuronal death, and doses higher than 25 μM were toxic per se. As a result, from the concentration range studied only 25 μM quercetin showed protective effects (Fig. 1a). Indeed, whereas the addition of 60 μM H_2O_2 caused a shift in neuronal survival that attained 38 ± 4% after 24 h, 25 μM quercetin pretreatment significantly prevented oxidant-induced neuronal death after 24 h, resulting in an 88 ± 2% neuronal survival (Fig. 1a).

Phase-contrast micrographs in Fig. 1b show morphological features of vehicle-treated or 25 μM quercetin-treated cultures subsequently treated with H_2O_2 for 24 h. Control (vehicle-treated only) cells (image A) showed bright round somata, with a complex neurite network. In contrast, vehicle-treated cells injured with H_2O_2 for 24 h (image B) looked mostly shrunk and less bright, and the neurite network had become thinner and fragmented. However, and in agreement with MTT determinations, there were no evident morphological alterations in 25 μM quercetin-treated cultures after 24 h of H_2O_2 injury (image C), compared with control, indicating that pretreatment with 25 μM quercetin preserved neuronal integrity after 24 h of the oxidative insult.

Finally, to verify that this protective effect was not due to an external reaction between the compound and the oxidant, we repeated the experiment adding a washing step between quercetin pretreatment and H_2O_2 addition to cultures. The results showed that the neuroprotection was due to the effects of quercetin on the cells and not to a direct reaction with H_2O_2 (data not shown).

Temporal changes in quercetin availability in neuronal cultures during 24 h of pretreatment

We determined quercetin availability in neuronal cultures at various times before oxidative injury, by measuring changes in quercetin intracellular/cell-associated and extracellular levels in treated cultures up to 24 h (Figs. 2 and 3). Extracellular levels of quercetin decreased with time, and after 24 h of incubation, quercetin was still detectable in medium cultures (16 ± 5% of initial quercetin concentration added to cultures; (Figs. 2b and 3b). Intracellular/cell-associated quercetin levels were detected after 1 min of being added to cultures (2.4 ± 0.2% of total amount of quercetin added to cultures)



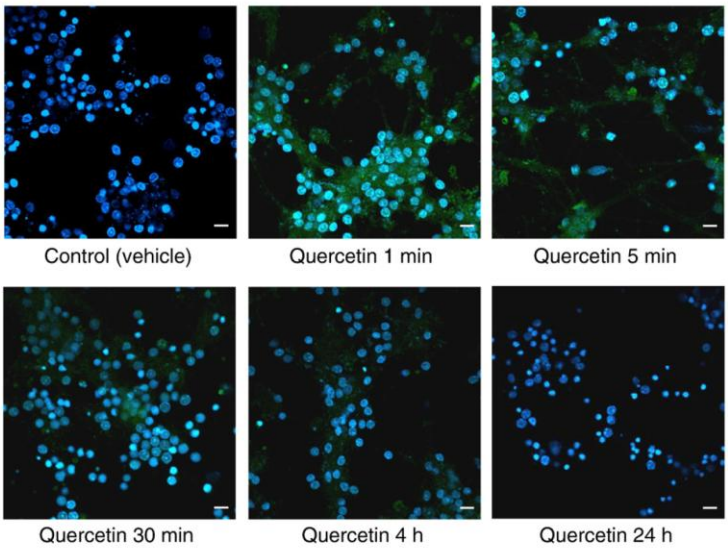


Fig. 4. Quercetin intracellular presence was confirmed by its fluorogenic properties. Confocal photomicrographs of vehicle-treated neuronal cultures (control) and cells incubated with 25 μM quercetin for 1 min, 5 min, 30 min, 4 h, and 24 h are shown. The merged images of the six treatments show nuclei stained with Hoechst 33258 dye (blue fluorescence) and quercetin green fluorescence monitored at 488 nm ex/500–540 nm em. Quercetin green fluorescence is present in the neurons immediately after 1 min of incubation, but it disappears after 24 h. Scale bar, 15 μm.

and they subsequently decreased along the 24-h period of treatment. By 24 h, when H_2O_2 was added, intracellular/cell-associated quercetin was undetectable (Figs. 2a and 3a). The data obtained from these analyses were fitted to kinetic rate equations of zero and first orders (Fig. 2a and b, respectively).

Furthermore, three new peaks, which had spectral characteristics similar to those of quercetin, were detected in extracellular samples (Fig. 3b). They increased with time and reached their maximum after 24 h. At this time, the most abundant of the three metabolites represented less than 5% of the initial quercetin concentration added to the cultures. None of these three peaks were detectable in intracellular/cell-associated samples by 24 h (Fig. 3a).

Analysis of internalization and subcellular fate of quercetin in neuronal cultures

Previous work in nonneuronal cells reported that quercetin cellular internalization is coupled to a transient binding of the molecule to protein targets, and this interaction can be visualized at specific fluorescence settings [43]. So, by using the fluorogenic properties of quercetin, we intended to find subcellular targets of the flavonoid in our neuronal cultures. We used epifluorescence and confocal imaging to see whether quercetin was associated with neurons at both extracellular and intracellular levels.

We found that as soon as 1 min after quercetin addition to cultures, neurons were fully stained with a strong green fluorescence. Quercetin fluorescence persisted with time, although the signal strength was progressively weakened, until it completely disappeared after 24 h of incubation (Fig. 4).

Furthermore, confocal imaging showed that quercetin particularly attained the nuclear level, although simultaneous analysis of nucleic acid stain and quercetin fluorescence showed different nuclear localizations (Fig. 5).

Effects of 25 μM quercetin pretreatment on total GSH concentration and Trx2 expression in neuronal cultures

We focused on modulation of intracellular levels of two major regulators of cellular redox status, glutathione and mitochondrial thioredoxin. We found that 24 h quercetin pretreatment resulted in an increase in the cellular levels of total GSH ($146.3 \pm 12\%$ with respect to control. Fig. 6a), although there were no differences in protein levels of Trx2 between control and quercetin-pretreated cultures (Fig. 6b).

Quercetin pretreatment caused nuclear accumulation of Nrf2 in neuronal cultures

The Nrf2-dependent cytoprotective pathway has been shown to induce gene expression of enzymes involved in GSH synthesis [32]. We therefore examined whether quercetin could activate the Nrf2 signaling pathway in our neuronal culture model. Upon activation,

Fig. 3. Chromatographic analysis of quercetin levels in neuronal cultures incubated with 25 μM flavonoid for up to 24 h. (a) Chromatograms showing the time course of intracellular/cell-associated levels of quercetin: (trace a) quercetin standard, (trace b) control (vehicle-treated cells), (trace c) 1 min of quercetin incubation, (trace d) 5 min of quercetin incubation, (trace e) 10 min of quercetin incubation, (trace f) 4 h of quercetin exposure, and (trace g) 24 h of quercetin exposure. Quercetin (peak 2, RT 26,899 min) was detected after 1 min of being added to cultures and subsequently decreased along the 24-h period of treatment. By 24 h, at the moment of the oxidant exposure, neither quercetin nor the other peaks with similar spectral characteristics were detectable. (b) Chromatograms showing the time course of extracellular levels of quercetin: (trace a) quercetin standard, (trace b) control (vehicle-treated cells), (trace c) 30 min of quercetin incubation, (trace d) 4 h of quercetin incubation, (trace e) 24 h of quercetin incubation. Quercetin (peak 2, RT 26,899 min) decreased with time, and after 24 h of incubation, it was still detectable in medium cultures. Furthermore, three new peaks, which had spectral characteristics similar to those of quercetin, were detected (peak 1, RT 23,988 min; peak 3, RT 30,666 min; peak 4, RT 33,494 min). They increased with time and reached their maxima after 24 h.

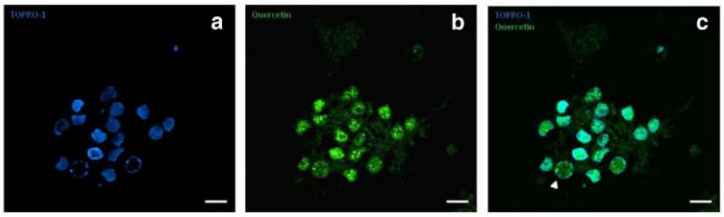


Fig. 5. Quercetin attained both cytoplasmic and nuclear compartments. Confocal fluorescence micrographs of cells incubated with 25 μM quercetin for 30 min. (Image a) Nuclei were stained with TOPRO-1 (blue fluorescence). (Image b) Green fluorescence from quercetin (monitored at 488 nm ex/500–540 nm em). (Image c) Merged images. Quercetin green fluorescence was present at both the nuclear and the cytoplasmic level. Dense nuclear green staining pattern suggests the presence of quercetin binding proteins in the nuclei (arrowhead). Bar, 10 μm.

Nrf2 accumulates in the nucleus. Thus, by the use of immunocytochemistry, we examined whether quercetin could cause nuclear translocation of Nrf2 in neurons.

Fig. 7 presents neuronal cultures treated with vehicle alone, 25 μM quercetin, or 20 μM tBHQ. Cultures treated with vehicle showed diffuse Nrf2 staining with low levels of Nrf2 immunoreactivity in the cytoplasm and nucleus. On the other hand, tBHQ positive control cultures showed a strong signal for Nrf2, which was particularly striking in the region of the nucleus. Finally, quercetin-treated cultures presented a major Nrf2 signal in both cytoplasm and nucleus. This signal, which was particularly remarkable in the nucleus, was even more prominent than that observed with tBHQ. Thus, quercetin caused nuclear translocation of Nrf2 in neuronal cultures.

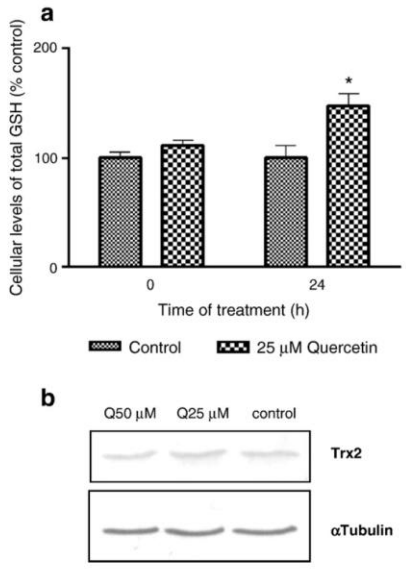


Fig. 6. Twenty-five micromolar quercetin caused an increase in cellular levels of total GSH, but not of Trx2. (a) Effects of 25 μM quercetin incubation for 24 h on total GSH concentration in neuronal cultures. Control, vehicle-treated cells; 25 μM quercetin, cells treated with quercetin 25 μM for 24 h. Results are expressed as means ± SEM of three experiments. *$p<0.01$, significantly different from control. (b) Effects of 25 μM quercetin incubation for 24 h on Trx2 expression. Immunoblotting with anti-α-tubulin is shown as a loading control. Control, vehicle-treated control cells; Q25 μM, cells treated with 25 μM quercetin for 24 h; Q50 μM, cells treated with 50 μM quercetin for 24 h. Similar results were obtained from three experiments.

Quercetin pretreatment induced GCLC expression in neuronal cultures

To see if the GSH increase observed could be a result of quercetin induction in GCLC expression through Nrf2 pathway activation, cultures were treated with the flavonoid or with vehicle (control) for 6 h, and gene expression of GCLC and 18 S RNA was determined by real-time PCR as described above. Gene-specific amplification was confirmed for the two primer pairs as single peaks in the melting curve analysis and through the T_m values.

Amplification efficiencies for GCLC and 18 S RNA genes were approximately equal (data not shown), validating the use of the $\Delta\Delta C_T$ method for relative quantification.

Quercetin induced significant gene expression of the GCLC subunit in cultures, which was increased by 2.7-fold after 6 h of incubation (Fig. 8).

Discussion

The results presented above showed a clear-cut protection by quercetin of cerebellar granule neurons in culture against an oxidative injury. Protection by quercetin of different cells in culture against a variety of insults has been previously described [36,38,47–51]. The novelty of the present data lies in that they showed protective effects in a pretreatment experimental model in neuronal cells, in which: (a) a limited amount of quercetin was quickly internalized into the neurons, reaching the nucleus level; (b) the Nrf2 cytoprotective signaling pathway was activated; (c) quercetin treatment resulted in an increase in GCLC expression and total GSH levels; and finally (d) neuronal death was prevented although intracellular/cell-associated quercetin or metabolites were not detectable at the moment of the oxidant exposure.

As an initial approach, the analysis of quercetin availability in neuronal cultures and its biotransformation could throw light on the mechanisms involved in quercetin protective effects. In this sense, our results showed a rapid internalization of the flavonoid into the neuronal cells that was limited to 2.4 ± 0.2% of total added quercetin (Fig. 2a). In addition, our chromatographic analysis showed that neither intracellular/cell-associated quercetin nor its metabolites were detectable after 24 h of quercetin exposure (Fig. 3a). The intracellular biotransformation of quercetin by the neurons was also evidenced by the gradual disappearance of its intrinsic fluorescence by 24 h incubation (Fig. 4). According to the low/undetectable intracellular availability of free quercetin or related metabolites at the moment of the oxidative insult, direct ROS scavenging or metal-chelating activities may have been limited under these circumstances. Nevertheless, we should take into account that a rapid intracellular autoxidation of internalized quercetin [52], and a subsequent reaction between these autoxidized forms and cellular macromolecules, could have led to the formation of adducts not detectable under our HPLC or microscopy conditions.

Furthermore, although quercetin and extracellular metabolites were detected after 24 h of quercetin exposure (Fig. 3b), the

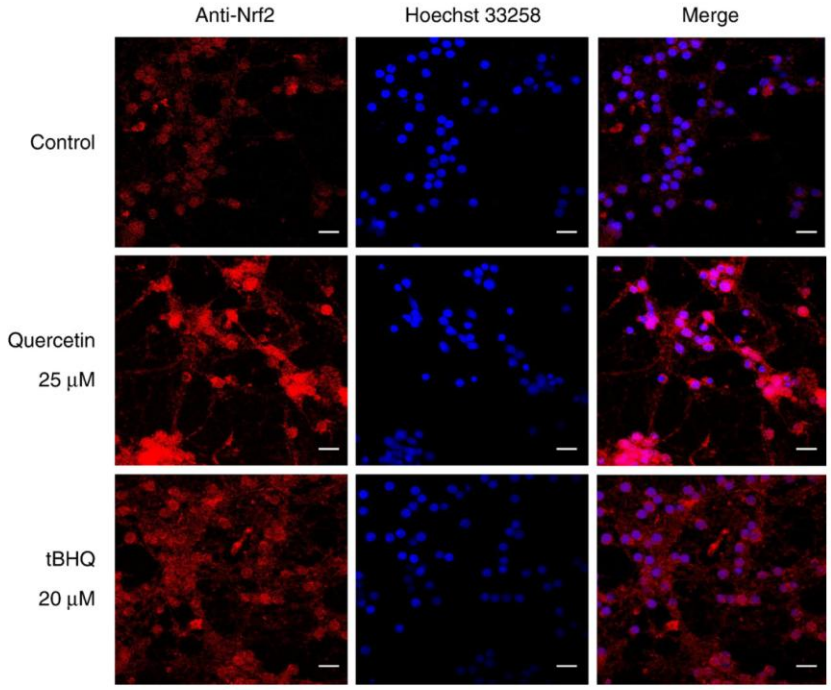


Fig. 7. Twenty-five micromolar quercetin caused Nrf2 nuclear translocation in neuronal cultures. Confocal photomicrographs show neuronal cultures treated with vehicle (control), 25 µM quercetin, or 20 µM tBHQ for 3 h. Shown are immunoreactivity for Nrf2 (red), nuclear staining with Hoechst 33258 (blue), and merged images of each of the three treatments. Cultures treated with quercetin presented a major Nrf2 signal, which was particularly dense at neuronal nuclei. Scale bar, 15 µm.

protective effects were not due to an antioxidant external reaction between the remnant extracellular quercetin or metabolites and the oxidant. These results strengthen the hypothesis that other intracellular mechanisms, probably involving protein interactions and/or induction of survival pathways [30,31], could explain the quercetin protective effects observed here.

In this sense, a remarkable finding was that after quercetin exposure, its fluorescence attained the nuclear level (Fig. 5). Simultaneous confocal analysis of nuclear stain and quercetin fluorescence showed different nuclear localizations (Fig. 5), which would give a basis for the interpretation of quercetin binding to nuclear proteins. Indeed, our findings are in agreement with previous research done in *Drosophila* follicles in which quercetin accumulated in the nuclei,

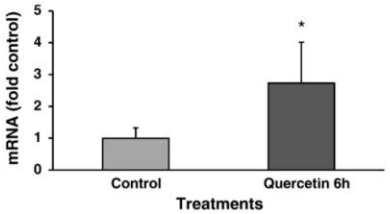


Fig. 8. Twenty-five micromolar quercetin induced GCLC expression in neuronal cultures. mRNA levels of GCLC after exposure to quercetin are shown. Cells were exposed to vehicle alone (control) or 25 µM quercetin for 6 h. GCLC mRNA levels from two experiments ($n=4$) were measured with the real-time PCR method. $^{*}p<0.05$, significantly different from control.

interacting with proteins associated with ring canals [53]. Quercetin was shown to interact with actin, which is particularly interesting because this protein has been suggested to be an essential component of the transcriptional machinery [54]. Further studies after isolation of molecular targets could provide additional information about the availability and mechanism of action of quercetin in neurons.

A last interesting finding related to quercetin uptake and biotransformation results from the kinetic rate equations of zero and first orders fitted by the temporal course of quercetin intracellular/cell-associated and extracellular levels, respectively (Fig. 2a and b). These results provide evidence that different chemical or enzymatic processes were involved in the disappearance or biotransformation of intracellular/cell-associated and extracellular quercetin. A rate equation of zero order corresponding to changes in intracellular/cell-associated levels suggests a mechanism independent of quercetin concentration. This would be the case, for example, of a saturated enzymatic reaction. On the other hand, a first-order rate equation corresponding to changes in extracellular levels of quercetin implies a mechanism that depends on quercetin concentration. This would be the case, for example, of an autoxidation reaction. Further analytical studies could provide additional information about the mechanisms of biotransformation of quercetin in neurons.

Focusing on the modulation of thiol-redox systems by quercetin, the analysis of protein levels of Trx2 did not show any difference between control and quercetin-pretreated neuronal cultures (Fig. 6b), indicating that quercetin protection was not accompanied by mitochondrial thioredoxin induction. Because previous studies reported cytoplasmic thioredoxin Trx1 regulation by quercetin in astrocytes [55], and quercetin modulation of mammalian thioredoxin

reductase [56], we cannot discard that other members of the Trx family could be modulated by quercetin in this model.

In contrast, quercetin pretreatment increased total GSH levels in neuronal cultures (Fig. 6a). Precisely, the high levels of GSH in neuronal cells might be able to cope with the toxicity of H_2O_2 and thus could be part of the mechanisms of neuroprotection involved in our model. Previously, quercetin has been shown to activate the Nrf2 transcription factor pathway [32,57,58]. This cytoprotective pathway is of special interest with regard to oxidative stress, because Nrf2 is responsible for the induction of a battery of genes encoding protective antioxidant proteins, and thus it promotes the regulation of the intracellular redox environment [59,60]. In this sense, our results also showed that quercetin caused Nrf2 nuclear translocation in neuronal cultures, evidencing the activation of such cytoprotective pathway (Fig. 7). Furthermore, our data showed that quercetin caused an increase in the expression of GCLC, the rate-limiting enzyme in GSH synthesis, whose gene is known to be up-regulated through Nrf2 activation. Our data are in accordance with previous work in cell lines, although little has been done to characterize the effect of quercetin on this cytoprotective pathway in neurons [32,39,58,61–63].

Finally, emerging data suggest that other dietary phytochemicals, such as benzyl isothiocyanate, sulforaphane, curcumin, and resveratrol, present biphasic dose–response effects similar to that found with quercetin (Fig. 1a): although they are toxic at high doses, at lower doses they can activate adaptive cellular stress responses pathways [64–67]. In this sense, findings from our work suggest that the cellular and molecular mechanisms underlying quercetin protection may involve the induction of antioxidant enzyme gene expression, as evidenced by the increase in GCLC expression and the rise in total GSH levels, through the activation of the Nrf2 cytoprotective signaling pathway. Although great care should be taken when extrapolating data obtained from cell cultures to dietary in vivo situations [68], it is undeniable that flavonoid bioactivities enumerated here are meaningful in the perspective of their beneficial effects in humans and could be taken into account for potential therapeutic uses in neuroprotection.

Acknowledgments

This work was supported by Organisation for the Prohibition of Chemical Weapons Grant L/ICA/ICB/139636 (The Hague, Netherlands), the Programa de Desarrollo de las Ciencias Básicas, the International Brain Research Organization–Latin American Regional Committee, the International Society for Neurochemistry, and Gautier Laboratories, Uruguay. We especially thank Dr. Octavio Garcia for his helpful suggestions to improve our work with the primary cerebellar granule neurons. We also thank Lic. Marcela Díaz from the microscopy service and Lic. Marcela Martínez from the HPLC service of the Instituto de Investigaciones Biológicas Clemente Estable for their technical support. Finally, we thank Estela Baglietto and Rosina Goenaga for their support with cell culture techniques.

References

[1] Finkel, T. Oxygen radicals and signaling. *Curr. Opin. Cell Biol.* **10:**248–253; 1998.
[2] Halliwell, B.; Gutteridge, J. M. C. eds. Free Radicals in Biology and Medicine. Oxford, Oxford University Press; 2007.
[3] Suzuki, Y. J.; Forman, H. J.; Sevanian, A. Oxidants as stimulators of signal transduction. *Free Radic. Biol. Med.* **22:**269–285; 1997.
[4] Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M. T.; Mazur, M.; Telser, J. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* **39:**44–84; 2007.
[5] Sies, H.; Jones, D. P. Oxidative Stress. In: Fink G. ed. Encyclopedia of Stress, 2nd edition. San Diego: Academic Press; pp. 45–48, 2007.
[6] Jones, D. P. Redefining oxidative stress. *Antioxid. Redox Signaling* **8:**1865–1879; 2006.
[7] Castagne, V.; Gautschi, M.; Lefevre, K.; Posada, A.; Clarke, P. G. Relationships between neuronal death and the cellular redox status: focus on the developing nervous system. *Prog. Neurobiol.* **59:**397–423; 1999.
[8] Dalton, T. P.; Chen, Y.; Schneider, S. N.; Nebert, D. W.; Shertzer, H. G. Genetically altered mice to evaluate glutathione homeostasis in health and disease. *Free Radic. Biol. Med.* **37:**1511–1526; 2004.
[9] Jones, D. P. Redox potential of GSH/GSSG couple: assay and biological significance. *Methods Enzymol.* **348:**93–112; 2002.
[10] Schafer, F. Q.; Buettner, G. R. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic. Biol. Med.* **30:**1191–1212; 2001.
[11] Pastore, A.; Federici, G.; Bertini, E.; Piemonte, F. Analysis of glutathione: implication in redox and detoxification. *Clin. Chim. Acta* **333:**19–39; 2003.
[12] Patenaude, A.; Murthy, M. R.; Mirault, M. E. Emerging roles of thioredoxin cycle enzymes in the central nervous system. *Cell. Mol. Life Sci.* **62:**1063–1080; 2005.
[13] Chen, Y.; Cai, J.; Murphy, T. J.; Jones, D. P. Overexpressed human mitochondrial thioredoxin confers resistance to oxidant-induced apoptosis in human osteosarcoma cells. *J. Biol. Chem.* **277:**33242–33248; 2002.
[14] Damdimopoulos, A. E.; Miranda-Vizuete, A.; Pelto-Huikko, M.; Gustafsson, J. A.; Spyrou, G. Human mitochondrial thioredoxin: involvement in mitochondrial membrane potential and cell death. *J. Biol. Chem.* **277:**33249–33257; 2002.
[15] Tanaka, T.; Hosoi, F.; Yamaguchi-Iwai, Y.; Nakamura, H.; Masutani, H.; Ueda, S.; Nishiyama, A.; Takeda, S.; Wada, H.; Spyrou, G.; Yodoi, J. Thioredoxin-2 (TRX-2) is an essential gene regulating mitochondria-dependent apoptosis. *EMBO J.* **21:** 1695–1703; 2002.
[16] Wallace, D. C. Mitochondrial genetics: a paradigm for aging and degenerative diseases? *Science* **256:**628–632; 1992.
[17] Wallace, D. C. Mitochondrial diseases in man and mouse. *Science* **283:**1482–1488; 1999.
[18] Zhou, C.; Huang, Y.; Przedborski, S. Oxidative stress in Parkinson's disease: a mechanism of pathogenic and therapeutic significance. *Ann. NY Acad. Sci.* **1147:** 93–104; 2008.
[19] Bains, J. S.; Shaw, C. A. Neurodegenerative disorders in humans: the role of glutathione in oxidative stress-mediated neuronal death. *Brain Res. Brain Res. Rev.* **25:**335–358; 1997.
[20] Ballatori, N.; Krance, S. M.; Notenboom, S.; Shi, S.; Tieu, K.; Hammond, C. L. Glutathione dysregulation and the etiology and progression of human diseases. *Biol. Chem.* **390:**191–214; 2009.
[21] Ceccon, M.; Giusti, P.; Facci, L.; Borin, G.; Imbesi, M.; Floreani, M.; Skaper, S. D. Intracellular glutathione levels determine cerebellar granule neuron sensitivity to excitotoxic injury by kainic acid. *Brain Res.* **862:**83–89; 2000.
[22] Schulz, J. B.; Lindenau, J.; Seyfried, J.; Dichgans, J. Glutathione, oxidative stress and neurodegeneration. *Eur. J. Biochem.* **267:**4904–4911; 2000.
[23] Townsend, D. M.; Tew, K. D.; Tapiero, H. The importance of glutathione in human disease. *Biomed. Pharmacother.* **57:**145–155; 2003.
[24] Mandel, S. A.; Amit, T.; Weinreb, O.; Reznichenko, L.; Youdim, M. B. Simultaneous manipulation of multiple brain targets by green tea catechins: a potential neuroprotective strategy for Alzheimer and Parkinson diseases. *CNS Neurosci. Ther.* **14:**352–365; 2008.
[25] Spencer, J. P. The impact of flavonoids on memory: physiological and molecular considerations. *Chem. Soc. Rev.* **38:**1152–1161; 2009.
[26] Zhao, B. Natural antioxidants protect neurons in Alzheimer's disease and Parkinson's disease. *Neurochem. Res.* **34:**630–638; 2009.
[27] Spencer, J. P. Flavonoids and brain health: multiple effects underpinned by common mechanisms. *Genes Nutr.* **4:**243–250; 2009.
[28] Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure–antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic. Biol. Med.* **20:**933–956; 1996.
[29] Nijveldt, R. J.; van Nood, E.; van Hoorn, D. E.; Boelens, P. G.; van Norren, K.; van Leeuwen, P. A. Flavonoids: a review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.* **74:**418–425; 2001.
[30] Williams, R. J.; Spencer, J. P.; Rice-Evans, C. Flavonoids: antioxidants or signalling molecules? *Free Radic. Biol. Med.* **36:**838–849; 2004.
[31] Fraga, C. G. Plant polyphenols: how to translate their in vitro antioxidant actions to in vivo conditions. *IUBMB Life* **59:**308–315; 2007.
[32] Myhrstad, M. C.; Carlsen, H.; Nordstrom, O.; Blomhoff, R.; Moskaug, J. O. Flavonoids increase the intracellular glutathione level by transactivation of the γ-glutamylcysteine synthetase catalytical subunit promoter. *Free Radic. Biol. Med.* **32:**386–393; 2002.
[33] de Vries, J. H.; Janssen, P. L.; Hollman, P. C.; van Staveren, W. A.; Katan, M. B. Consumption of quercetin and kaempferol in free-living subjects eating a variety of diets. *Cancer Lett.* **114:**141–144; 1997.
[34] Hertog, M. G.; Feskens, E. J.; Hollman, P. C.; Katan, M. B.; Kromhout, D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet* **342:**1007–1011; 1993.
[35] Hertog, M. G.; Hollman, P. C.; Katan, M. B.; Kromhout, D. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. *Nutr. Cancer* **20:**21–29; 1993.
[36] Ansari, M. A.; Abdul, H. M.; Joshi, G.; Opii, W. O.; Butterfield, D. A. Protective effect of quercetin in primary neurons against Abeta(1–42): relevance to Alzheimer's disease. *J. Nutr. Biochem.* **20:**269–275; 2009.
[37] Boots, A. W.; Haenen, G. R.; Bast, A. Health effects of quercetin: from antioxidant to nutraceutical. *Eur. J. Pharmacol.* **585:**325–337; 2008.
[38] Silva, B.; Oliveira, P. J.; Dias, A.; Malva, J. O. Quercetin, kaempferol and biapigenin from Hypericum perforatum are neuroprotective against excitotoxic insults. *Neurotox. Res.* **13:**265–279; 2008.
[39] Lavoie, S.; Chen, Y.; Dalton, T. P.; Gysin, R.; Cuenod, M.; Steullet, P.; Do, K. Q. Curcumin, quercetin, and tBHQ modulate glutathione levels in astrocytes and neurons: importance of the glutamate cysteine ligase modifier subunit. *J. Neurochem.* **108:** 1410–1422; 2009.

[40] Garcia, O.; Massieu, L. Strategies for neuroprotection against L-trans-2, 4-pyrrolidine dicarboxylate-induced neuronal damage during energy impairment in vitro. *J. Neurosci. Res.* **64:**418–428; 2001.

[41] Denizot, F.; Lang, R. Rapid colorimetric assay for cell growth and survival: modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Meth.* **89:**271–277; 1986.

[42] Liu, Y.; Peterson, D. A.; Kimura, H.; Schubert, D. Mechanism of cellular 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction. *J. Neurochem.* **69:**581–593; 1997.

[43] Nifli, A. P.; Theodoropoulos, P. A.; Munier, S.; Castagnino, C.; Roussakis, E.; Katerinopoulos, H. E.; Vercauteren, J.; Castanas, E. Quercetin exhibits a specific fluorescence in cellular milieu: a valuable tool for the study of its intracellular distribution. *J. Agric. Food Chem.* **55:**2873–2878; 2007.

[44] Vargas, M. R.; Pehar, M.; Cassina, P.; Martinez-Palma, L.; Thompson, J. A.; Beckman, J. S.; Barbeito, L. Fibroblast growth factor-1 induces heme oxygenase-1 via nuclear factor erythroid 2-related factor 2 (Nrf2) in spinal cord astrocytes: consequences for motor neuron survival. *J. Biol. Chem.* **280:**25571–25579; 2005.

[45] Mastrogiannaki, M.; Matak, P.; Keith, B.; Simon, M. C.; Vaulont, S.; Peyssonnaux, C. HIF-2alpha, but not HIF-1alpha, promotes iron absorption in mice. *J. Clin. Invest.* **119:**1159–1166; 2009.

[46] Bookout, A. L.; Mangelsdorf, D. J. Quantitative real-time PCR protocol for analysis of nuclear receptor signaling pathways. *Nucl. Recept. Signal.* **1:**e012; 2003.

[47] Angeloni, C.; Spencer, J. P.; Leoncini, E.; Biagi, P. L.; Hrelia, S. Role of quercetin and its in vivo metabolites in protecting H9c2 cells against oxidative stress. *Biochimie* **89:**73–82; 2007.

[48] de Groot, H.; Rauen, U. Tissue injury by reactive oxygen species and the protective effects of flavonoids. *Fundam. Clin. Pharmacol.* **12:**249–255; 1998.

[49] Hanneken, A.; Lin, F. F.; Johnson, J.; Maher, P. Flavonoids protect human retinal pigment epithelial cells from oxidative-stress-induced death. *Invest. Ophthalmol. Visual Sci.* **47:**3164–3177; 2006.

[50] Lin, R.; Liu, J.; Gan, W.; Ding, C. Protective effect of quercetin on the homocysteine-injured human umbilical vein vascular endothelial cell line (ECV304). *Basic Clin. Pharmacol. Toxicol.* **101:**197–202; 2007.

[51] Vauzour, D.; Ravaioli, G.; Vafeiadou, K.; Rodriguez-Mateos, A.; Angeloni, C.; Spencer, J. P. Peroxynitrite induced formation of the neurotoxins 5-*S*-cysteinyl-dopamine and DHBT-1: implications for Parkinson's disease and protection by polyphenols. *Arch. Biochem. Biophys.* **476:**145–151; 2008.

[52] Spencer, J. P.; Abd-el-Mohsen, M. M.; Rice-Evans, C. Cellular uptake and metabolism of flavonoids and their metabolites: implications for their bioactivity. *Arch. Biochem. Biophys.* **423:**148–161; 2004.

[53] Gutzeit, H. O.; Henker, Y.; Kind, B.; Franz, A. Specific interactions of quercetin and other flavonoids with target proteins are revealed by elicited fluorescence. *Biochem. Biophys. Res. Commun.* **318:**490–495; 2004.

[54] Bohl, M.; Czupalla, C.; Tokalov, S. V.; Hoflack, B.; Gutzeit, H. O. Identification of actin as quercetin-binding protein: an approach to identify target molecules for specific ligands. *Anal. Biochem.* **346:**295–299; 2005.

[55] Sharma, V.; Mishra, M.; Ghosh, S.; Tewari, R.; Basu, A.; Seth, P.; Sen, E. Modulation of interleukin-1beta mediated inflammatory response in human astrocytes by flavonoids: implications in neuroprotection. *Brain Res. Bull.* **73:** 55–63; 2007.

[56] Lu, J.; Papp, L. V.; Fang, J.; Rodriguez-Nieto, S.; Zhivotovsky, B.; Holmgren, A. Inhibition of mammalian thioredoxin reductase by some flavonoids: implications for myricetin and quercetin anticancer activity. *Cancer Res.* **66:**4410–4418; 2006.

[57] Bok, S. H.; Park, S. Y.; Park, Y. B.; Lee, M. K.; Jeon, S. M.; Jeong, T. S.; Choi, M. S. Quercetin dihydrate and gallate supplements lower plasma and hepatic lipids and change activities of hepatic antioxidant enzymes in high cholesterol-fed rats. *Int. J. Vitam. Nutr. Res.* **72:**161–169; 2002.

[58] Alia, M.; Mateos, R.; Ramos, S.; Lecumberri, E.; Bravo, L.; Goya, L. Influence of quercetin and rutin on growth and antioxidant defense system of a human hepatoma cell line (HepG2). *Eur. J. Nutr.* **45:**19–28; 2006.

[59] Friling, R. S.; Bensimon, A.; Tichauer, Y.; Daniel, V. Xenobiotic-inducible expression of murine glutathione S-transferase Ya subunit gene is controlled by an electrophile-responsive element. *Proc. Natl Acad. Sci. USA* **87:**6258–6262; 1990.

[60] Rushmore, T. H.; Morton, M. R.; Pickett, C. B. The antioxidant responsive element: activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J. Biol. Chem.* **266:**11632–11639; 1991.

[61] Rodgers, E. H.; Grant, M. H. The effect of the flavonoids, quercetin, myricetin and epicatechin on the growth and enzyme activities of MCF7 human breast cancer cells. *Chem. Biol. Interact.* **116:**213–228; 1998.

[62] Ishige, K.; Schubert, D.; Sagara, Y. Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. *Free Radic. Biol. Med.* **30:**433–446; 2001.

[63] Tanigawa, S.; Fujii, M.; Hou, D. X. Action of Nrf2 and Keap1 in ARE-mediated NQO1 expression by quercetin. *Free Radic. Biol. Med.* **42:**1690–1703; 2007.

[64] Kim, B. R.; Hu, R.; Keum, Y. S.; Hebbar, V.; Shen, G.; Nair, S. S.; Kong, A. N. Effects of glutathione on antioxidant response element-mediated gene expression and apoptosis elicited by sulforaphane. *Cancer Res.* **63:**7520–7525; 2003.

[65] Kirlin, W. G.; Cai, J.; DeLong, M. J.; Patten, E. J.; Jones, D. P. Dietary compounds that induce cancer preventive phase 2 enzymes activate apoptosis at comparable doses in HT29 colon carcinoma cells. *J. Nutr.* **129:**1827–1835; 1999.

[66] Moriarty-Craige, S. E.; Jones, D. P. Extracellular thiols and thiol/disulfide redox in metabolism. *Annu. Rev. Nutr.* **24:**481–509; 2004.

[67] Nair, S.; Li, W.; Kong, A. N. Natural dietary anti-cancer chemopreventive compounds: redox-mediated differential signaling mechanisms in cytoprotection of normal cells versus cytotoxicity in tumor cells. *Acta Pharmacol. Sin.* **28:**459–472; 2007.

[68] Crozier, A.; Jaganath, I. B.; Clifford, M. N. Dietary phenolics: chemistry, bioavailability and effects on health. *Nat. Prod. Rep.* **26:**1001–1043; 2009.

2. Pretreatment with natural flavones and neuronal cell survival after oxidative stress: a structure-activity relationship study. C. Echeverry; F Arredondo; Juan Andres Abin-Carriquiry; J Midiwo; C Ochieng; L. Kerubo; F. Dajas. Journal of Agricultural and Food Chemistry, v.: 58 4, p.: 2111 - 2115, 2010

En el artículo que presentamos a continuación nos focalizamos en el objetivo específico d, con el fin de contestar la segunda pregunta planteada en la tesis: *¿ Qué características estructurales de quercetina y flavonoides relacionados determinan este efecto neuroprotector?*

Para ello, utilizando el modelo de muerte neuronal oxidativa desarrollado previamente, evaluamos la capacidad neuroprotectora de 13 flavonas naturales estructuralmente similares a la quercetina. De este modo nos propusimos identificar los requerimientos estructurales necesarios para la neuroprotección por flavonoides. Los resultados obtenidos muestran que además de quercetina sólo 3 del conjunto de flavonas estructuralmente relacionadas fueron efectivamente protectoras. Además, las características estructurales que aseguran su efecto neuroprotector, no coinciden con aquellas características necesarias para la actividad atrapadora de radicales libres de estos compuestos.

Esto reafirma nuestra hipótesis de que en este modelo las flavonas neuroprotectoras estarían interactuando con vías de señalización intracelular, más allá de su capacidad atrapadora de radicales libres.

JFood | 3b2 | ver.9 | 19/1/010 | 22:22 | Msc: jf-2009-02951v | TEID: emr00 | BATID: 00000 | Pages: 4.82

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY ARTICLE

J. Agric. Food Chem. **XXXX,** *XXX,* 000–000 A
DOI:10.1021/jf902951v

Pretreatment with Natural Flavones and Neuronal Cell Survival after Oxidative Stress: A Structure−Activity Relationship Study

Carolina Echeverry,*,† Florencia Arredondo,† Juan Andres Abin-Carriquiry,† Jacob O. Ogweno Midiwo,§ Charles Ochieng,§ Leonidah Kerubo,§ and Federico Dajas†

†Departmento de Neuroquímica, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay, and §Department of Chemistry, University of Nairobi, Nairobi, Kenya

Quercetin shows structural features that have been related to the antioxidant potency of flavonoids and also shows neuroprotection in different models of oxidative death. Because only a few studies have focused on the flavonoid structural requirements for neuroprotection, this work evaluated the protective capacity of 13 flavones structurally related to quercetin, isolated from Kenyan plants, to rescue primary cerebellar granule neurons from death induced by a treatment with 24 h of hydrogen peroxide (150 μM). Each flavone (0−100 μM) was applied 24 h prior to the oxidative insult, and neuronal viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results suggest that the *o*-dihydroxy substitution in the B-ring is not necessary to afford neuroprotection and could be partly responsible for neurotoxic effects. Furthermore, the hydroxy substitutions in the positions C3 (C-ring) in C5 and C7 (A-ring) would be important for neuroprotection in this model.

KEYWORDS: Flavones; neuronal protection; oxidative stress; SAR

INTRODUCTION

The lack of effective therapies against neuronal death involved in neuropathologies such as Parkinson's and Alzheimer's diseases has led to the search for new therapeutic strategies mainly related to the events of oxidative stress implicated in these pathologies. In this sense, epidemiological studies have shown a lower incidence of such illnesses in people consuming flavonoid-rich diets (*1*, *2*), although the exact mechanisms by which these compounds exert their beneficial effects are not fully understood. Beyond their classical hydrogen-donating antioxidant activity, in the past 5−10 years, some evidence has emerged from cell studies suggesting that flavonoids could influence cellular fate by other mechanisms of action: enzymatic modulation, interaction with several receptors, and modulation of intracellular signaling cascades that control cell survival, death, differentiation, and gene expression (*3*−*6*). This capacity to modify multiple cellular targets is likely the basis of the therapeutic potential of flavonoids and also their toxic effects.

Flavonoids represent the most common group of polyphenolic compounds in the human diet and are widely found in plants. They consist of two aromatic carbon rings, benzopyran (A- and C-rings) and a benzene (B-ring), and can be divided into six groups depending on the degree of oxidation of the C-ring, the hydroxylation pattern, and the substitution at the C3-position. Among these, the flavones that are based on the backbone of 2-phenylchromen-4-one (2-phenyl-1-benzopyran-4-one) are the most commonly encountered, being ubiquitous within the families and genera of the higher plants (**Figure 1**). The structural diversity of these natural compounds offers the possibility to identify the molecular substitutions required for different biological actions. In this sense, there is an agreement in the literature on some of the molecular features that determine particular activities of flavonoids. Thus, their antioxidant potency has been linked to the *o*-dihydroxy substitution in the B-ring and the presence of 2,3-unsaturation and a 4-carbonyl in the C-ring (**Figure 1**) (*7*, *8*). Their activity as metal ion chelators appears to be defined by the presence of the *o*-dihydroxy substitution in the B-ring, the 4-carbonyl in the C-ring, and a 5-hydroxy substitution in the A-ring (*9*), whereas the prooxidant properties of flavonoids would be associated with the number of hydroxyl groups present in the molecule (*10*). In particular, the cytoprotective capacity of flavonoids against different insults has been mainly attributed to their antioxidant potency (*11*, *12*). However, in a previous work (*13*), assessing the cytoprotective potency of several structurally related flavonoids against oxidative stress, we found that cytoprotection did not correlate with the antioxidation potency. Nevertheless, the antioxidant structural features described above were common to all of the cytoprotective flavonoids studied, as shown by other authors (*14*, *15*). In this context, the search for particular structural features that afford protection would be significant for the development of new neuroprotective molecules

*Address correspondence to this author at the Departamento de Neuroquímica, Instituto de Investigaciones Biológicas Clemente Estable, Avda. Italia 3318, CP 11600, Montevideo, Uruguay (telephone/fax +598 2 4872603; e-mail echever@iibce.edu.uy).

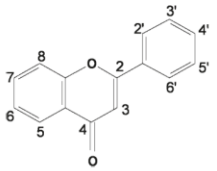


Figure 1. Molecular structure of the flavone backbone.

and for the understanding of the mechanisms of action of flavonoids.

To this aim we performed a structure−activity relationship (SAR) study focused on the protective effects of flavonoids against oxidative stress in neuronal cultures. We assessed the protective capacity of 13 natural flavones in an experimental paradigm in which an oxidative insult that leads to cell death is applied to cultured neurons after previous exposure to the flavones. The flavones were mainly isolated from Kenyan plants, and they are structurally related to quercetin, a prototypic antioxidant molecule and a potent neuroprotective compound in different experimental paradigms (*13*, *16*). To assess more accurately the potential value of the protective molecules as leading compounds to design molecules of therapeutic use, we also assessed the toxicity of the selected flavones on the cultured neurons.

The main results showed the structural requirements for neuronal cell protection, which are different from those that afford antioxidant capacity.

MATERIALS AND METHODS

General Chemicals. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Hydrogen peroxide (H_2O_2) was dissolved in distilled water and subsequently filtered before addition to the cultures

Flavones Utilized. Quercetin (3,5,7,3′,4′-pentahydroxyflavone) was acquired from Xi'an Sino-Dragon I/E Co., Ltd., China. Apigenin (5,7,4′-trihydroxyflavone), luteolin (5,7,3′,4′-tetrahydroxyflavone), and fisetin (3,7,3′,4′-tetrahydroxyflavone) were obtained from Sigma-Aldrich. The rest of the flavones studied were isolated by Prof. J. Midiwo's research group at the Department of Chemistry, University of Nairobi, from the following plants: *Dodonaea angustifolia*, *Psiadia punctulata*, *Gardenia ternifolia*, *Senecio roseiflorus*, and *Hypericum lanceolatum*. The following compounds were obtained from these plants (**Table 1**): compound **1** (5,7-dihydroxy-3,6,4′-trimethoxyflavone), compound **2** (5-hydroxy-3,7,4′-trimethoxyflavone), compound **3** (5,7-dihydroxy-3,4′-dimethoxyflavone), compound **4** (3,5-dihydroxy-7,4′-dimethoxyflavone), compound **5** (5,6,7-trihydroxy-3,4′-dimethoxyflavone), compound **6** (3,5,7-trihydroxy-4′-methoxyflavone), compound **7** (5,7,4′-trihydroxy-3-methoxyflavone), compound **8** (3,5,7,3′-tetrahydroxy-4′methoxyflavone, tamarixetin), and compound **9** (3,5,7,4′-tetrahydroxy-3′methoxyflavone, isorhamnetin).

Each molecule was dissolved in dimethyl sulfoxide (DMSO) 30%, prior to bioassay experiments.

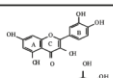
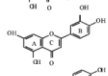
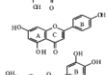
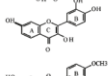
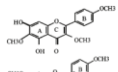
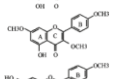
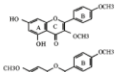
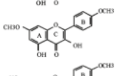
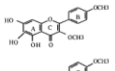
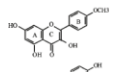
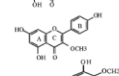
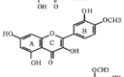
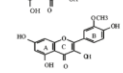
Plant Materials. The plants used and voucher specimens have been described previously (*17*, *18*).

The leaf and flowers of the plants mentioned were collected from their geographical location and handled according to established procedures.

Extraction. Extraction was performed as previously described for exudates from aerial parts (*17*, *18*); the aerial branches and leaves were dipped into organic solvent and shaken for not more than 15 s to avoid extraction of internal tissue substances indicated by the appearance of the green of chlorophyll. This wash was dried in vacuo using a rotary evaporator to give a solid gum. Other parts of the plants were dried before the powder was extracted at ambient temperature with various organic solvents, dichloromethane/methanol (1:1) followed by methanol, and solvent was removed to yield the gummy solid.

Analysis was done using analytical TLC: Merck precoated silica gel 60 F254 plates with standard previously characterized compounds to locate desired compounds in the extracts, CC on silica gel 60 (70−230 mesh).

Table 1. Lethal Concentration 50 (LC_{50}) and Effective Concentration 50 (EC_{50}) of the Flavones Studied

Compound	Structure	LC_{50} (μM)	EC_{50} (μM)
Quercetin		80.3	5.7
Luteolin		79.3	-
Apigenin		76.2	-
Fisetin		63.5	-
1		29.7	-
2		26.0	-
3		25.1	-
4		41.3	-
5		24.0	-
6		72.1	2.6
7		14.4	-
8		> 100	2.9
9		85.6	5.6

EIMS: direct inlet, 70 eV, ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) run on DRX-500 (Bruker), ARX 300 (Bruker), or Varian-Mercury spectrometers using solvent resonances to calibrate the spectra.

Neuronal Culture. Primary cerebellar granule neurons (CGN) were obtained from PN6−8 Sprague−Dawley rats (*19*) and seeded in poly-L-ornithine precoated 96-well plates at a density of 200,000 cells/well. They were kept in basal medium Eagle supplemented with fetal bovine serum (PAA Laboratories, Austria) (10%), 20 mM KCl, and 25 mM glucose in a humidified chamber at 37 °C in a 5% CO_2 atmosphere. Glial growth was inhibited by addition of cytosine arabinoside (10 μM).

Experimental Treatments. For flavone neuroprotective capacity evaluation, neuronal death was induced at seven days in culture (DIC7) by adding 150 μM hydrogen peroxide (H_2O_2) for 24 h. Flavones (0−100 μM in 1.5% DMSO, final concentration) were applied 24 h before the H_2O_2 insult. Neurotoxicity assessment was done by exposing granule cell cultures to 0−100 μM concentrations of each flavone during 24 h.

Cell Viability Measurements. Neuronal mitochondrial activity, as an indicator of cell viability, was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (*20*).

Briefly, after experimental treatments, cells were incubated for 90 min at 37 °C with MTT (0.1 mg/mL final concentration) that was reduced by metabolically active cells to purple formazan. Formazan crystals were dissolved with DMSO, and the absorbance was measured on an MRX microplate reader (Dynex Technologies), using a reference wavelength of 630 nm and a test wavelength of 570 nm.

Results are presented as percentage of MTT reduction, assuming that absorbance of control cells was 100%.

Neuronal viability was also examined qualitatively by phase-contrast microscopy (Axiovert 25, Zeiss, Germany).

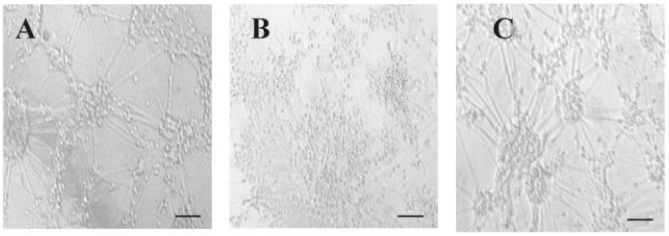


Figure 2. Phase contrast photomicrographs of neurons in culture showing neuroprotection by pretreatment with 10 μM compound **9**: (**A**) control vehicle neurons (DMSO 1.5%); (**B**) H_2O_2 vehicle neurons (DMSO 1.5% + H_2O_2 150 μM 24 h); (**C**) H_2O_2−compound **9** neurons (10 μM compound **9** 24 h + H_2O_2 150 μM 24 h). Scale bar: 35 mm.

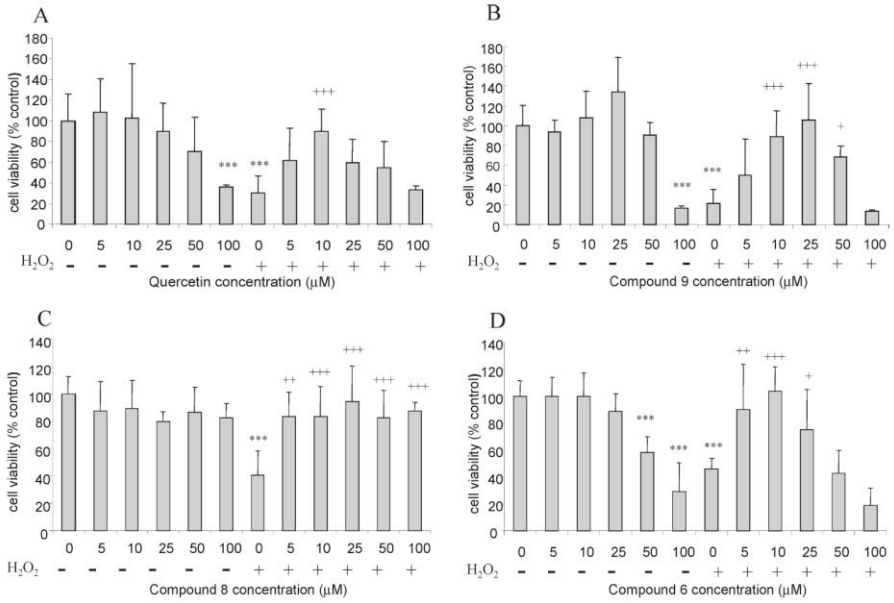


Figure 3. Flavone CGN toxicity and protection against oxidative insult: (**A**) quercetin (3,5,7,3′,4′-pentahydroxyflavone) profile; (**B**) compound **9** (3,5,7,4′-tetrahydroxy-3′-methoxyflavone, isorhamnetin) profile; (**C**) compound **8** (5,7,3′-trihydroxy-3,4′-dimethoxyflavone, tamarixetin) profile; (**D**) compound **6** (3,5,7-trihydroxy-4′-methoxyflavone) profile. Data are presented as mean of cell viability ± SD (***, $p < 0.001$ as compared to CV; +++, $p < 0.001$ as compared to PV; +2, $p < 0.05$ as compared to PV; +, $p < 0.01$ as compared to PV, by ANOVA−Tukey multiple-comparison test). CV, control vehicle neurons; PV, peroxide vehicle neurons.

Statistical Analysis. Cell viability data are presented as mean ± SD and were analyzed by ANOVA−Tukey multiple-comparison tests.

RESULTS

From the molecules studied, compound **8** (5,7,3′-trihydroxy-3,4′-dimethoxyflavone, tamarixetin), compound **9** (3,5,7,4′-tetrahydroxy-3′-methoxyflavone, isorhamnetin), compound **6** (3,5,7-trihydroxy-4′-methoxyflavone), and quercetin (3,5,7,3′,4′-pentahydroxyflavone) prevented the H_2O_2-induced cell death, evidenced by phase contrast microscopy and MTT assays, indicating that they are neuroprotective.

A representative set of phase contrast photomicrographs of neurons in culture that were treated with 10 μM compound **9** and 24 h later exposed to the oxidative insult are shown in **Figure 2**. Treatment with H_2O_2 caused marked neuronal death (**Figure 2B**). In contrast, neurons pretreated with 10 μM compound **9** and subsequently exposed to the same oxidative insult (**Figure 2C**) appeared to be morphologically similar to control cells (**Figure 2A**).

Effective half maximal concentrations for toxicity (LC_{50}) and for protection (EC_{50}) were calculated from the dose−response curves (**Table 1**).

The toxicity profile of each neuroprotective flavone was different (**Figure 3**). Only compound **8** did not show toxicity in the concentrations tested (**Table 1** and **Figure 3**). Although the correlation between the number of OH and LC_{50} was positive, it was low ($r^2 = 0.537$, **Figure 4**). The compounds with minor LC_{50} values (compounds **7**, **1**, **2**, **3**, and **5**) show methoxy substitution in C3 (C-ring).

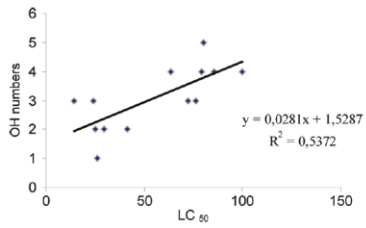


Figure 4. Correlation between OH numbers of flavones and LC_{50}.

The protective compounds **8**, **9**, **6**, and quercetin showed an EC_{50} = 2.9, 5.6, 2.6, and 5.7 μM, respectively (**Table 1**; **Figure 3**). Interestingly, apart from quercetin, the rest of the neuroprotective molecules do not present the *o*-dihydroxy substitution in the B-ring, although they have similar potencies.

In this model fisetin did not show neuroprotection (**Table 1**), although its only structural difference from quercetin is the absence of OH substitution at C5 of the A-ring. On the other hand, compound **4**, that does not show protection, possesses an OH in position C5, but has an OMe group at C7 of the A-ring (**Table 1**).

Of the other nonprotective compounds, apigenin and luteolin both have the OH substitution at C5 and C7 in the A-ring, although they lack the OH substitution at C3 (C-ring); compounds **7** and **3**, which also have an OH group in positions C5 and C7 (A-ring), present an OMe group at C3 (C-ring) and, finally, compounds **1**, **2**, and **5** have an OMe group at C3 (C-ring) and present diverse substitutions in the A-ring (**Table 1**).

in summary, the common structural characteristics of the four neuroprotective molecules are an OH substitution at the C5- and C7-positions of the A-ring, an OH substitution at the C3 position of the C-ring, and variable B-ring substitutions.

DISCUSSION

The results described above showed that only 4 of the 13 flavones studied exhibited neuronal protective activity, strongly indicating that specific structural features are related to this action. Results suggest that the hydroxy substitutions in the A-ring (C5 and C7) and at position C3 (C-ring) of the flavones would be necessary to afford neuronal cell protection. The OH substitution at position C3 (C-ring) has been previously shown as important in a wide range of biological activities such as scavenger activity and anti-inflammatory effects (*21*, *22*). This structural feature appears to be significant for the cationic divalent chelating properties (*21*), and it appears to be responsible for the increase in the molecular coplanarity that has been implicated in these biological activities (*21*, *23*).

Our results are in agreement with Kang and co-workers, who studied the neuroprotective capacity of four structurally related flavones, wogonin, chrysin, apigenin, and luteolin, by testing whether these flavones protect SH-SY5Y human neuroblastoma cells against oxidative stress-induced cell death. They found that one hydroxyl group at C3 is essential to exert protection (*14*).

Additionally, a few studies have demonstrated that both the 5-OH and 7-OH groups of the A-ring are significant for flavonoid bioactivities. Lee and co-workers have suggested that OH substitutions at C5 and/or C7 might significantly affect the anti-apoptotic properties of flavonoids (*24*). Furthermore, Van Hoorn et al. (*25*) showed the importance of these substitutions on xanthine oxidase inhibition by flavones. Because free radicals are produced by xanthine oxidase activity, its inhibition has an indirect antioxidant effect that could contribute to neuroprotection. In this case, the authors sustained the notion that 3′- and 4′-hydroxyl moieties (B-ring) strengthen this activity.

When we compare the activity profiles of the four protective molecules with the nonprotective fisetin, it can be concluded that the *o*-dihydroxy substitution in the B-ring is not needed for protection in our model. This is an important and novel contribution of our work because several studies have shown the importance of this structural feature in diverse bioactivities of flavonoids (*26*−*29*), particularly for their scavenger capacity. Thus, it is generally accepted that the positions and numbers of OH in the B-ring give the scavenger capacity of these compounds, whereas OH groups on the A-ring do not appear to be significant for this activity (*21*). Indeed, it has been suggested that OH groups on the A-ring have poor reactivity toward peroxyl radicals (*30*, *31*). Accordingly, our results showed that structural features implicated in neuronal protection are different from those that provide the free radical scavenging capacity of flavonoids. It has to be borne in mind that in the model utilized in this work, flavones are added to cells in culture 24 h before they are submitted to the oxidative aggression. This is an important difference with most utilized models that place the flavonoids and the oxidative insult in close relationship (*14*, *32*). In our model, the flavonoid molecule could interact with intracellular targets before the oxidative insult is applied, likely inducing the expression of cell signaling molecules. Further research now in progress in our laboratory aims to detect the intracellular presence of the flavones during the preteatment period, a fact already demonstrated for some flavonoids (*33*, *34*). Metabolomic studies are also being performed to identify putative active metabolites as it has been claimed in previous studies (*33*).

The toxicity studies of the flavones evidenced a positive correlation between the number of OH substitutions and the LC_{50} values. This result indicates that in neurons in culture, toxicity may be inversely related to hydrophilicity. The low correlation coefficient would suggest that besides the number of OH, their positions would also be important for toxicity. Although some studies have shown the involvement of OH and OMe groups in cytotoxicity (*35*), none has implicated the OMe substitution, particularly at C3 (C-ring), with this action. Our work shows for the first time that the OMe substitution at C3 (C-ring) would increase the neurotoxicity. This feature should be studied further to identify possible mechanisms implicated in the toxicity of flavones.

Structure−acivity studies like those reported here are a contribution to the knowledge of the pharmacophoric structures (**Figure 1**: OH substitutions at C5, C7, and C3; OH, OCH_3, or H substitutions at C3′; and OH or OCH_3 substitutions at C4′) that afford neuronal cell protection and could be meaningful for the design of new molecules for the treatment of brain pathologies that involve massive neuronal cell death.

LITERATURE CITED

(1) Fraser, M. L.; Mok, G. S.; Lee, A. H. Green tea and stroke prevention: emerging evidence. *Complement. Ther. Med.* **2007**, *15*, 46–53.

(2) Lemeshow, S.; Letenneur, L.; Dartigues, J. F.; Lafont, S.; Orgogozo, J. M.; Commenges, D. Illustration of analysis taking into account complex survey considerations: the association between wine consumption and dementia in the PAQUID study. *Am. J. Epidemiol.* **1998**, *148*, 298–306.

(3) Dajas, F.; Arredondo, F.; Echeverry, C.; Ferreira, M.; Morquio, A.; Rivera, F. Flavonoids and the brain: evidences and putative mechanisms for a protective capacity. *Curr. Neuropharmacol.* **2005**, *3*, 193–206.

(4) Rice-Evans, C. Flavonoid antioxidants. *Curr. Med. Chem.* **2001**, *8*, 797–807.

(5) Schroeter, H.; Boyd, C.; Spencer, J. P.; Williams, R. J.; Cadenas, E.; Rice-Evans, C. MAPK signaling in neurodegeneration: influences of flavonoids and of nitric oxide. *Neurobiol. Aging* **2002**, *23*, 861–880.

(6) Mandel, S. A.; Amit, T.; Kalfon, L.; Reznichenko, L.; Youdim, M. B. Targeting multiple neurodegenerative diseases etiologies with multimodal-acting green tea catechins. *J. Nutr.* **2008**, *138*, 1578S–1583S.

(7) Bors, W.; Heller, W.; Michel, C.; Saran, M. Radical chemistry of flavonoid antioxidants. *Adv. Exp. Med. Biol.* **1990**, *264*, 165–170.

(8) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure−antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933–056.

(9) Mira, L.; Fernández, M. T.; Santos, M.; Rocha, R.; Florencio, M. H.; Jennings, K. R. Interactions of flavonoids with iron and copper ions: a mechanism for their antioxidant activity. *Free Radical Res.* **2002**, *36*, 1199–1208.

(10) Heim, K. E.; Tagliaferro, A. R.; Bobilya, D. J. Flavonoid antioxidants: chemistry, metabolism and structure−activity relationships. *J. Nutr. Biochem.* **2002**, *13*, 572–584.

(11) Cainelli, G.; Angeloni, C.; Cervellati, R.; Galletti, P.; Giacomini, D.; Hrelia, S.; Sinisi, R. New polyphenolic β-lactams with antioxidant activity. *Chem. Biodivers.* **2008**, *5*, 811–829.

(12) Zhang, J.; Stanley, R. A.; Adaim, A.; Melton, L. D.; Skinner, M. A. Free radical scavenging and cytoprotective activities of phenolic antioxidants. *Mol. Nutr. Food Res.* **2006**, *50*, 996–1005.

(13) Dajas, F.; Rivera, F.; Blasina, F.; Arredondo, F.; Echeverry, C.; Lafón, L.; Morquio, A.; Heinzen, H. Cell culture protection and in vivo neuroprotective capacity of flavonoids. *Neurotox. Res.* **2003**, *5*, 377–384.

(14) Kang, S. S.; Lee, J. Y.; Choi, Y. K.; Kim, G. S.; Han, B. H. Neuroprotective effects of flavones on hydrogen peroxide-induced apoptosis in SH-SY5Y neuroblostoma cells. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2261–2264.

(15) Wang, C. N.; Chi, C. W.; Lin, Y. L.; Chen, C. F.; Shiao, Y. J. The neuroprotective effects of phytoestrogens on amyloid β protein-induced toxicity are mediated by abrogating the activation of caspase cascade in rat cortical neurons. *J. Biol. Chem.* **2001**, *276*, 5287–5295.

(16) Dajas, F.; Rivera, F.; Blasina, F.; Arredondo, F.; Abín, A.; Costa, G.; Echeverry, C.; Lafón, L.; Heinzen, H.; Ferreira, M.; Morquio, A. Neuroprotection by flavonoids. *Braz. J. Med. Biol. Res.* **2003**, *36*, 1613–1620.

(17) Yenesew, A.; Irungu, B.; Derese, S.; Midiwo, J. O.; Heydenreich, M.; Peter, M. G. Two prenylated flavonoids from the stem bark of *Erythrina burttii*. *Phytochemistry* **2003**, *63*, 445–448.

(18) Juma, B. F.; Yenese, A.; Midiwo, J. O.; Waterman, P. G. Flavones and phenylpropenoids in the surface exudate of *Psiadia punctulata*. *Phytochemistry* **2001**, *57*, 571–574.

(19) García, O.; Massieu, L. Strategies for neuroprotection against L-*trans*-2,4-pyrrolidine dicarboxylate-induced neuronal damage during energy impairment in vitro. *J. Neurosci. Res.* **2001**, *64*, 418–428.

(20) Denizot, F.; Lang, R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* **1986**, *89*, 271–277.

(21) Arora, A.; Nair, M. G.; Strasburg, G. M. Structure−activity relationships for antioxidant activities of a series of flavonoids in a liposomal system. *Free Radical Biol. Med.* **1998**, *24*, 1355–1363.

(22) Theoharides, T. C.; Alexandrakis, M.; Kempuraj, D.; Lytinas, M. Anti-inflammatory actions of flavonoids and structural requirements for new design. *Int. J. Immunopathol. Pharmacol.* **2001**, *14*, 119–127.

(23) Guo, M.; Perez, C.; Wei, Y.; Rapoza, E.; Su, G.; Bou-Abdallah, F.; Chasteen, N. D. Iron-binding properties of plant phenolics and cranberry's bio-effects. *Dalton Trans.* **2007**, *43*, 4951–4961.

(24) Lee, E. R.; Kang, Y. J.; Kim, J. H.; Lee, H. T.; Cho, S. G. Modulation of apoptosis in HaCaT keratinocytes via differential regulation of ERK signaling pathway by flavonoids. *J. Biol. Chem.* **2005**, *280*, 31498–31507.

(25) Van Hoorn, D.; Nijveldt, R. J.; Van Leeuwen, P. A. M.; Hofman, Z.; Rabet, L. M.; De Bont, D. B. A.; Van Norren, K. Acurate prediction of xanthine oxidase ihibition based on the structure of flavonoids. *Eur. J. Pharmacol.* **2002**, *451*, 111–118.

(26) Rice-Evans, C. A.; Miller, N. J.; Bolwell, P. G.; Bramley, P. M.; Pridham, J. B. The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Res.* **1995**, *22*, 375–383.

(27) Odontuya, G.; Hoult, J. R.; Houghton, P. J. Structure−activity relationship for antiinflammatory effect of luteolin and its derived glycosides. *Phytother. Res.* **2005**, *19*, 782–786.

(28) Kaneko, T.; Baba, N.; Matsuo, M. Structure−activity relationship of antioxidants for inhibitors of linoleic acid hydroperoxide-induced toxicity in cultured human umbilical vein endothelial cells. *Cytotechnology* **2001**, *35*, 43–55.

(29) Melidou, M.; Riganakos, K.; Galaris, D. Protection against nuclear DNA damage offered by flavonoids in cells exposed to hydrogen peroxide: the role of iron chelation. *Free Radical Biol. Med.* **2005**, *15*, 1591–1600.

(30) van Acker, S. A.; van den Berg, D. J.; Tromp, M. N.; Griffioen, D. H.; van Bennekom, W. P.; van der Vijgh, W. J.; Bast, A. Structural aspects of antioxidant activity of flavonoids. *Free Radical Biol. Med.* **1996**, *20*, 331–342.

(31) Roginsky, V. A.; Barsukova, T. K.; Remosova, A. A.; Bors, W. Moderate antioxidant efficiencies of flavonoids during peroxidation of methyl linoleate in homogenous and micellar solutions. *J. Am. Oil Chem. Soc.* **1996**, *73*, 777–786.

(32) Kim, S. R.; Park, M. J.; Lee, M. K.; Sung, S. H.; Park, E. J.; Kim, J.; Kim, S. Y.; Oh, T. H.; Markelonis, G. J.; Kim, Y. C. Flavonoids of Inula britannica protect cultured cortical cells from necrotic cell death induced by glutamate. *Free Radical Biol. Med.* **2002**, *32*, 596–604.

(33) Spencer, J. P. E.; Kuhnle, G. G. C.; Williams, R. J.; Rice-Evans, C. Intracellular metabolism and bioactivity of quercetin and its *in vivo* metabolites. *Biochem. J.* **2003**, *372*, 173–181.

(34) Angeloni, C.; Spencer, J. P. E.; Leoncini, E.; Biagi, P. L.; Hrelia, S. Role of quercetin and its in vivo metabolites in protecting H9c2 cells against oxidative stress. *Biochimie* **2007**, *89*, 73–82.

(35) Jeong, J. M.; Choi, C. H.; Kang, S. K.; Lee, I. H.; Lee, J. Y.; Jung, H. Antioxidant and chemosensitizing effects of flavonoids with hydroxy and/or methoxy groups and structure−activity relationship. *J. Pharm. Pharm. Sci.* **2007**, *10*, 537–546.

Received for review August 27, 2009. Revised manuscript received January 8, 2010. Accepted January 11, 2010. This work was supported by Grant L/ICA/139636 from the International Cooperation and Assistance Office of the OPCW (Organization for the Prohibition of Chemical Weapons), The Hague, The Netherlands.

3. Involvement of Nrf2-dependent up-regulation of GSH biosynthetic enzymes in quercetin neuronal protection against an oxidative insult. F. Arredondo, C. Echeverry, D. Carvalho, JA. Abin-Carriquiry, G. Prunell, K. Antúnez, DP Jones, YM Go, F. Dajas. Neurotoxicity Research (submitted, 2015).

En este artículo nos centramos en los objetivos específicos e, f y g, con el fin de contestar la última pregunta planteada en la tesis: *¿El efecto neuroprotector de la quercetina involucra la activación de la vía ARE/Nrf2?*

En este sentido, utilizando el mismo modelo de cultivo neuronal, estudiamos la inducción de genes ARE a través de la vía Nrf2 en cultivos tratados con quercetina. Luego utilizamos inhibidores farmacológicos específicos de las proteínas cuya expresión había sido inducida por activación de la vía Nrf2: GCL y HO-1. Aplicamos los inhibidores a los cultivos neuronales, previo al tratamiento con quercetina, luego sometimos a los cultivos al daño oxidativo y finalmente evaluamos la viabilidad neuronal. Los resultados mostraron que el efecto neuroprotector de la quercetina se ve atenuado por la inhibición de la enzima GCL y no por la inhibición de HO-1.

Esto sugiere que el efecto protector de quercetina involucraría la activación de la vía Nrf2 y que el sistema antioxidante del GSH sería relevante en este efecto.

Por otra parte, realizamos un estudio estructura-actividad para determinar los requerimientos estructurales necesarios para la activación de Nrf2 por flavonoides. Para ello, transfectamos células de la línea PC12 con un plásmido ARE-Luc reportero de la expresión del gen ARE GCL. Utilizamos estas células debido a la baja eficiencia de transfección obtenidos en los cultivos primarios neuronales. Los resultados mostraron que el anillo A no sería un componente estructural relevante para la activación de la vía Nrf2, mientras que la planaridad sería un componente estructural importante. Asimismo, quercetina y los flavonoides relacionados que presentaron

actividad inductora de Nrf2, también fueron capaces de proteger a los cultivos sometidos a un daño oxidativo.

Esto sugiere que habría una correlación entre la capacidad citoprotectora y la actividad inductora de la vía Nrf2 por parte de los flavonoides estudiados, lo cual reforzaría nuestra hipótesis sobre la relevancia de la vía Nrf2 en el efecto protector de los flavonoides.

Involvement of Nrf2-dependent up-regulation of GSH biosynthetic enzymes in quercetin neuronal protection against an oxidative insult

F. Arredondo[1*], C. Echeverry[1], D. Carvalho[1], JA. Abin-Carriquiry[1], G. Prunell[1], K. Antúnez[2], D. P. Jones[3], Y.-M. Go[3], F. Dajas[1].

[1]Departamento de Neuroquímica, Instituto de Investigaciones Biológicas "Clemente Estable", Montevideo, Uruguay. [2]Depto. Microbiología, Instituto de Investigaciones Biológicas "Clemente Estable", Montevideo, Uruguay. [3]Division of Pulmonary Medicine, Department of Medicine, Emory University, Atlanta, USA.

Correspondence to: Florencia Arredondo
Departamento de Neuroquímica, Instituto de Investigaciones Biológicas Clemente Estable,
Avda Italia 3318, 11600, Montevideo, Uruguay
Tel/Fax: 598 2487 2603
Email: florarredondou@gmail.com

1. Introduction

Neurodegenerative diseases share several common pathological features, including protein misfolding, mitochondrial dysfunction, inflammation and alterations in cellular redox status (Halliwell 2006). In particular, numerous studies support the idea that disturbance of redox balance leads to oxidative stress events and neuronal death underlying the onset or progression of such disorders (Ischiropoulos and Beckman 2003; Barnham et al. 2004). In this context, pharmacological interventions that regulate cellular redox homeostasis could be an important strategy to promote cell survival and treat or prevent neurodegeneration.

An interesting approach for restoring redox homeostasis involves activation of the transcription factor Nuclear factor erythroid 2-related factor 2 (Nrf2). This member of the Cap'n'Collar family of basic leucine zipper transcription factors plays a central role in the regulation of cellular redox status through the modulation of a wide range of adaptive responses to intrinsic and extrinsic cellular stresses (Moi et al. 1994; Kensler et al. 2007). Nrf2 activates the transcription of more than 200 genes that are involved in the metabolism of drugs, cytoprotection and cellular integrity. In particular it induces the expression of many protective antioxidant and phase II enzymes, such as NAD(P)H-quinone oxidoreductase 1 (NQO1), γ-glutamylcysteine ligase (GCL), heme oxygenase 1 (HO-1), among others, promoting detoxification and restoration of redox status (Jaiswal 2004; Kensler et al. 2007). Nrf2-inducible genes share a common promoter element called the antioxidant response element (ARE), also known as electrophile-responsive element (EpRE). Under basal conditions, Nrf2 is retained in the cytoplasm by its negative regulator Kelch-like erythroid-cell-derived protein with CNC homology-associating protein 1 (Keap1), which links it to the cytoskeleton and promotes its degradation. Upon Nrf2 activation, Nrf2 escapes from Keap1-mediated proteasomal degradation and translocates into the nucleus, where it binds ARE/EpRE and activates gene transcription (Wasserman and Fahl 1997; Itoh et al. 1999).

The Nrf2 system is widely expressed in the Central Nervous System (CNS) and it is activated in response to different pathological conditions like acute cerebral injuries and neurodegenerative diseases (reviewed in Zhang et al., 2013). Beyond its main regulatory role in endogenous cellular defense systems, Nrf2 is also an important regulator of inflammation in the brain (Sandberg et al. 2014). Interestingly, alteration of this pathway has been related to diverse brain pathologies (reviewed in Zhang et al., 2013).

Furthermore, numerous studies reveal that activation of Nrf2 pathway, either by genetic or chemical methodologies, confers neuroprotection in diverse *in vitro* and *in vivo* models of CNS pathologies, through the overexpression of phase II genes (Zhang et al., 2013).

Thus, thinking of Nrf2 as a pharmacological target for therapeutic purposes, there is an increasing clinical interest in using Nrf2 activators for the treatment of CNS diseases (de Vries et al. 2008; Son et al. 2008). In this sense, many dietary phytochemicals have been shown to activate adaptive cellular stress response pathways, including Nrf2 pathway (Lee et al. 2014). Among these compounds, flavonoids have gained further interest because of their neuroprotective potential, although the exact mechanisms by which they exert their benefits are not fully understood (Spencer 2009).

Quercetin is the most frequent flavonoid in the Western diet (Hertog et al. 1993; De Vries et al. 1997). It has been widely studied by *in vitro* and *in vivo* neuronal models. Certainly, diverse *in vitro* studies have shown that treatment with quercetin can increase the survival of neurons or neuronal-derived cell lines against oxidative insults, among others injuries (Dajas et al. 2003; Dajas et al. 2005; Jakubowicz-Gil et al. 2008; Silva et al. 2008; Ansari et al. 2009; Ossola et al. 2009). Preclinical *in vivo* studies of quercetin preventive effects on neurodegeneration have mostly focused on cognitive impairments, ischemia, and traumatic injury (Kelsey et al. 2010; Dajas et al. 2015). Hence, quercetin has the potential to be developed into a novel therapy for neurodegeneration. The beneficial effects of quercetin have been classically attributed to its potent direct antioxidant capacity (Rice-Evans et al. 1996). Nevertheless, previous studies in our laboratory have demonstrated that direct antioxidation is a necessary but not sufficient property for the neuroprotective activity of quercetin (Echeverry et al. 2010). Attention was more recently directed to the wide interaction of flavonoids, including quercetin, with intracellular targets and its capacity to modulate signaling cascades and transcription factors, regulating the expression of critical genes (Williams et al. 2004; Spencer et al. 2012; Williams and Spencer 2012; Rendeiro et al. 2015). Accordingly, a promising strategy for CNS disease prevention would be the induction of Nrf2 pathway activation. In this sense, we have previously described the protective effects of quercetin against H_2O_2 in 24-h-pre-treated cerebellar granule neuronal cultures. Quercetin rapidly internalized into neurons, reaching the nucleus after its addition to the culture. It prevented neuronal death from H_2O_2 exposure although at the moment of the oxidant addition intracellular quercetin or related metabolites were undetectable in the cultures.

Quercetin pre-treatment caused Nrf2 nuclear translocation and increased the gene expression of the catalytic subunit of GCL enzyme (GCL_C) and total glutathione (GSH) levels. Our findings suggest complementary mechanisms of quercetin neuroprotection, possibly involving Nrf2-dependent pathway modulation (Arredondo et al. 2010).

An open question of the previous work was whether up-regulation of GCL and/or other Nrf2-dependent cytoprotective proteins were effectively involved in quercetin neuroprotective effect. To this aim, we utilized the previous experimental paradigm of flavonoid 24h pre-treatment prior to the oxidative insult. As far as quercetin was undetectable at the moment of H_20_2 insult, we would be able to assess its mechanisms of action beyond the classical direct antioxidant effect. Considering the importance of Nrf2 pathway modulation, we also examined flavonoid structural requirements for such bioactivity, and its correlation with the protective capacity. This would throw light on lead structures for the synthesis of new therapeutic neuroprotective compounds.

2. Methodology

2.1. Reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

2.2. Plasmid constructs

The plasmids used in this study are as follows: pT81 luciferase vector (ATCC 37584), pARE4-Luc (comprising the ARE4 region of GCL_C gene inserted into a pT81 luciferase vector) (Mulcahy et al. 1997), and pEGFP-N1 (Clontech 6085-1). The pARE4-Luc reporter and pT81-Luc plasmids were kindly provided by Drs. Go and Jones (Go et al. 2004). *Escherichia coli* XL-1 Blue was used for maintenance of the plasmids (Bullock et al. 1987). It was grown in Luria-Bertani (LB) medium at 37° C, and supplemented with ampicillin (100 μg/ml) when necessary. Plasmids were purified using QIAprep Spin Miniprep kit following the manufacturer's instructions (Qiagen). The pEGFP-N1 plasmid was provided ready-to-use by Prof. Pablo Diaz-Amarilla and Prof. Federico Rosconi (Instituto de Investigaciones Biológicas "Clemente Estable", Uruguay).

2.3. Cell culture

2.3.1. Neuronal Primary culture

Primary cerebellar granule neurons (CGN) were obtained from 6-to 8-day-old Sprague–Dawley rats as previously described (Echeverry et al. 2010; Arredondo et al. 2010). Cells were seeded in poly-L-ornithine pre-coated 24-well or 96-well plates at a density of 3.1×10^5 cells/cm^2 or 6.1×10^5 cells/cm^2, respectively.

All procedures involving animals and their care were approved by the ethics committee of the "Instituto de Investigaciones Biológicas Clemente Estable". All efforts were made to minimize the number of animals used and their suffering.

2.3.2. PC12 cell culture

PC12 cells were grown and maintained, as previously described (Greene and Tischler 1976; Arredondo et al. 2004). Cells were seeded in collagen pre-coated 96-well plates or in poly-L-ornithine pre-coated 6-well plates at a density of 1.2×10^5 cells/cm^2.

2.4. Cell culture treatments

Flavonoids used in this study are as follows: Quercetin (Great Dragon Biochemicals Co., Ltd., China; 98% purity; CAS No. 6151-25-3), isorhamnetin, fisetin, apigenin, taxifolin, and catechin (Fig. 1). All of them were dissolved in dimethyl sulfoxide (DMSO) (1,5% and 0,3% final concentrations for primary CGN and PC12 cells cultures, respectively).

Primary CGN neuronal cultures were used at 8-10 days in vitro (DIV) for this study. To test if quercetin up-regulation of the GSH biosynthetic enzymes underlies its neuroprotective effect, we examined the effect of L-Buthionine-[S,R]-sulfoximine (BSO) on quercetin neuronal protection against H_2O_2 insult. BSO is an irreversible inhibitor of GCL, and concentrations ranging from 5 to 10 µM are sufficient to provoke a decrease of glutathione content in both astrocytes and neurons (Zerarka et al. 2001; Saito et al. 2007). We chose 9 µM BSO concentration since it did not influence on control neuronal survival. After 8 days plating, cultures were incubated with 25 µM quercetin in the presence or absence of 9 µM BSO for 24 h. Cultures were exposed to 180 µM H_2O_2 for 24 h at 9 DIV. Cell viability was determined after treatments at 10 DIV. To investigate the involvement of HO-1 in quercetin neuroprotective effects, we examined the effect of zinc-protoporphyrin IX (ZNPP IX, a competitive HO-1 inhibitor) on quercetin neuronal protection against H_2O_2 insult. We chose 1 µM ZNPP IX concentration in accordance with previous studies and since it was the highest inhibitor concentration that did not influence on control neuronal survival (Li et al. 2006). After 8

days plating, cultures were pre-treated for 24h with 25 µM quercetin in the presence or absence of 1 µM ZNPP IX and then exposed to H_2O_2 oxidative insult at 9 DIV. Cell viability was determined after treatments at 10 DIV.

For the cytoprotective activity assays in PC12 cells, at 5 DIV cells were pre-treated for 24 h with each flavonoid (0–150 µM) and then exposed to 500 µM H_2O_2 for 24 h. Cell viability was determined after treatments at 7 DIV.

2.5. Assessment of cell viability

Cell survival was quantified by analysis of the metabolic activity of cultures using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Applichem, Germany), which reflects the integrity of the metabolic machinery in viable neurons (Denizot and Lang 1986). In brief, after experimental treatment, cells were incubated for 45 min at 37 °C with MTT (0.1 mg/ml final concentration), and metabolically active cells reduced the dye to purple formazan. Formazan crystals were dissolved in DMSO, and the absorbance was measured on a Varioskan Flash Multimode Reader (Thermo Scientific™, Massachusetts, USA), using a reference wavelength of 630 nm and a test wavelength of 570 nm. Results were presented as the percentage of formazan absorbance, assuming that absorbance of vehicle-treated cells (control) was 100%.

2.6. Quantitative real time-PCR

To assess whether treatment with 25 µM quercetin caused ARE/EpRE genes induction, at 8 DIV primary CGN neuronal cultures were treated with the flavonoid or with vehicle (control) for 6 h, before total RNA isolation.

Total RNA was extracted from cell cultures using the RNeasy Plus Mini kit (Qiagen), according to the manufacturer's instructions. Total RNA recovered was immediately reverse-transcribed to generate first-strand cDNAs using the QuantiTec reverse transcription kit (Qiagen), according to the manufacturer´s instructions. Transcript levels were analyzed by quantitative real-time PCR (qRT-PCR) using the following primers: HO-1 forward: 5´-TGCTCGCATGAACACTCTG-3´ and reverse: 5´-TCCTCTGTCAGCAGTGCCT-3 (123pb) (Scapagnini et al. 2002); NQO1 forward: 5´-CTGGAAGGGTGGAAGAAGCGTC-3´ and reverse: 5´-TCTGGTTGTCGGCTGGAATGG-3´ (196pb); glutathione peroxidase 1 (GPx1) forward: 5´-TCAGTTCGGACATCAGGAGAATGG-3´ and reverse: 5´-

GGAAGGTAAAGAGCGGGTGAGC-3´ (150pb); modifying subunit of GCL (GCL_m) forward: 5´-ATCTTGCCTCCTGCTGTGTGATG-3´ and reverse: 5´-GGCTTCAATGTCAGGGATGCTTTC-3´ (153pb); GCL_c forward: 5´-ATGAAAGTGGCACAGGAGCGAG-3´ and reverse: 5´-AAACACGCCTTCCTTCCCATTG-3´ (186pb); Nrf2 forward: 5´-TTCCTCTGCTGCCATTAGTCAGTC-3´ and reverse: 5´-GCTCTTCCATTTCCGAGTCACTG-3´ (242pb) (Vargas et al. 2005). Transcript levels of 18 S RNA were also analyzed and used as reference gene data for normalization, by using the primers 18 S RNA forward: 5′- GTAACCCGTTGAACCCCATT-3′ and reverse: 5′-CCATCCAATCGGTAGTAGCG- 3′ (Mastrogiannaki et al. 2009).

Real-time PCRs were carried out using the QuantiTec SYBR green real-time PCR kit (Qiagen) and primers (Integrated DNA Technologies, Coralville, IA, USA) described above, according to the manufacturer´s instructions.

PCRs were carried out using a Corbett Rotor Gene 6000 cycler and the cycling program consisted in an initial preincubation step of 50 °C for 2 min and 95 °C for 15 min and 35 cycles of 94 °C for 15 s, 53 °C for 30 s, and 72 °C for 30 s, as previously described (Arredondo et al. 2010). Fluorescence was measured in the elongation step and negative controls (without cDNA) were included in each reaction run. The specificity of the reaction was checked by analyzing the melting curve of the final amplified products, which was obtained through continuous reading over increasing temperatures from 65 to 95 °C. Four 10-fold serial dilutions (1, 0.1, 0.01, and 0.001) of total cDNA samples were included in real-time PCR analysis to obtain amplification efficiencies of each gene.

The amplification results were expressed as the threshold cycle number (CT), and Pfaffl comparative method was used for mRNA relative quantification (Pfaffl 2001).

2.7. Western Blot Analysis

After experimental treatments, protein samples (40 µg) were resolved on 12% sodium dodecyl sulfate–polyacrylamide gel and transferred to PVDF membrane (Amersham, GE Healthcare). Membranes were blocked for 1 h in PBS containing 0.05% Tween 20 and 5% bovine serum albumin, followed by an overnight incubation with primary antibodies anti-Nrf2 (mouse; 1:1000; AbCam) and anti-α- tubulin (mouse; 1:2000) diluted in the same buffer at 4 °C. After being washed with 0.05% Tween 20 in PBS, the membrane was incubated with a fluorophore-labeled secondary antibody (Alexa Fluor®

647 goat anti-mouse; 1:15000; Thermo Fisher) for 1 h and then washed. Bands fluorescence was quantified using a Next Generation Multi-purpose Image Scanner (FLA900, FUJIFILM).

2.8. Transfection and luciferase reporter gene assay

For transient transfections, we used PC12 cell line. These cells share their embryological origin with neuroblastic cells, and are suitable hosts for DNA transfection (Gordon et al. 2013). PC12 cells were grown overnight and co-transfected at 1 DIV with pARE4-Luc or pT81 (empty vector) (Go et al. 2004) and a pEGFP vector (as efficiency control) using Lipofectamine 2000 (Life Technologies), according to the manufacturer´s instructions. At 5 DIV cells were treated for 16h with the vehicle (control) or with the different flavonoids at 25 µM final concentration (Fig. 1). Luciferase activity was measured according to the Luciferase Reporter Assay manufacturer's instruction (Promega). Transfection efficiency was controlled by co-expressing pEGFP vector. Luminescence measured was expressed in relation to the basal activity of cells transfected with empty vectors, after normalization to EGFP fluorescence. Data were expressed as fold of induction respect to vehicle-treated (control) cells.

2.9. Statistical analysis

Experiments were done at least three independent times, and data were presented as mean ± SEM. All statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Differences among different experimental groups were tested for significance using Student´s t test, or one-way analysis of variance (ANOVA) followed by Tukey's or Dunnet´s *post hoc* tests. A difference was considered significant at $p < 0.05$.

3. Results

3.1. Quercetin increased Nrf2 levels in CGN primary cultures

In the present study, to examine the effect of quercetin on Nrf2 protein levels we analyzed Nrf2 protein expression in neuronal extracts at various times after 25 µM quercetin exposure. The result shows that Nrf2 protein levels were increased with time, and significantly higher after 6 h and 24 h of quercetin incubation (1.7- fold and 1.6-

fold, respectively, compared to control) (Fig. 2a).

To examine if quercetin regulates Nrf2 expression at the transcriptional level in CGN cultures, we treated the cells with 25 µM quercetin for 6 h and determined Nrf2 mRNA levels. We chose expression time in accordance with previous work (Tanigawa et al. 2007). As shown in Fig. 2b, Nrf2 expression was increased in response to quercetin treatment when comparing with control (Fig. 2b), but differences were not statistically significant (p= 0.0594).

3.2. Quercetin upregulated Nrf2-inducible genes in CGN primary cultures

Subsequently, we investigated whether quercetin-elevated Nrf2 activation stimulates expression of cellular detoxifying, antioxidant and anti-inflammatory genes such as NQO-1, GPX1, GCL, and HO-1 in neuronal cells by qRT-PCR. The results show that quercetin at 25 µM for 6 h elevated HO-1 expression significantly (3.9-fold, Fig 3a). This result was consistent with an increase in HO-1 protein levels in CGN cultures after 15 h of quercetin treatment (data not shown). Quercetin also increased expression of catalytic subunit of GCL (GCL_c) significantly, (2.6-fold, Fig. 3b), consistent with our previous analysis of GCL_c expression in quercetin-treated CGN cultures (Arredondo et al. 2010). Furthermore, GCL_m expression increased in response to quercetin treatment (2.8-fold) when comparing with control (Fig. 3c), but differences were not statistically significant (p=0.0762). Both GCL_c and GCL_m expression results were in accordance with our previous findings showing an increase in in total GSH, levels in CGN cultures after 24 h of quercetin pre-treatment (Arredondo et al. 2010). No significant changes were observed in NQO1 and GPx1 gene transcript levels after 6h quercetin treatment (Fig.3d and 3e, respectively).

3.3. Involvement of glutathione antioxidant system and HO-1 in neuroprotection by quercetin against oxidative stress

Quercetin stimulated elevation of HO-1, GCL_c and in GCL_m genes (Fig.3b and c) led us to examine if neuronal protective effect of quercetin was dependent on GCL and/or HO-1 using pharmacological inhibitors for GCL and HO-1. Inhibition of GCL by 9 µM BSO significantly suppressed protective effects of quercetin on CGN survival against H_2O_2 insult, suggesting an involvement of GSH antioxidant system in quercetin neuronal protection against oxidative stress (Fig.4a).

On the other hand, the data shows that HO-1 inhibition by 1 μM ZnPP IX did not alter the protective effects of quercetin against H_2O_2-induced neuronal death (Fig.4b), suggesting that protective role of quercetin against oxidant-induced neuronal death was regulated by a mechanism involving Nrf2-elevated GCL activation but not HO-1 expression.

3.4. Flavonoid structure-activity relationship for ARE/EpRE mediated transcription activation

To delineate the structural molecular features of quercetin in association with Nrf2 and Nrf2- dependent antioxidant gene transcription, we further evaluated cellular sensitivity to various flavonoids, comparing ARE-luc reporter activity (Mulcahy et al. 1997) as a measure of Nrf2 transcriptional response to indicated flavonoids (Fig. 1). To measure ARE-Luc activity, PC12 cells transiently transfected with pARE4-Luc reporter were treated with different flavonoids at equimolar concentrations (25 μM).

From all the flavonoids tested, the result shows that fisetin and isorhamnetin stimulated ARE-Luc activation comparable to that of quercetin, with a ranking order of efficacy as follows: quercetin > fisetin > isorhamnetin (Fig. 5). On the other hand, apigenin, taxifolin and catechin did not show significant ARE-Luc activation at 25 μM (Fig. 5).

3.5. Flavonoids protective effects against oxidative stress correlated with ARE/EpRE mediated transcription activation

As shown the comparable significant effects of fisetin and isorhamnetin to quercetin on Nrf2-dependent ARE transcriptional activation in Fig 5, we further examined these flavonoids on cell protection against oxidative stress by dose-dependent manner (Fig. 6). Consistent with the data of Fig 5, fisetin and isorhamnetin had significant cytoprotective effects comparable to that of quercetin, with a ranking order of cytoprotective potency as follows: quercetin > fisetin > isorhamnetin, while isorhamnetin showed the most efficacious cytoprotective effect at 150 μM. On the other hand, apigenin, taxifolin and catechin did not show significant protective effects at any concentration (Fig. 6). Lethal concentration 50 (LC50) of these compounds in PC12 cell culture paradigm were as follows: LC50 quercetin: 79,1 μM; LC50 fisetin: 114,2 μM; LC50 isorhamnetin > 150 μM; LC50 apigenin: 78,3 μM; LC50 taxifolin > 150 μM; LC50 catechin > 150 μM.

4. Discussion

In a previous study we demonstrated that quercetin pre-treatment prevented neuronal death from H_2O_2 oxidative insult. As well, we indirectly suggested that quercetin underlying mechanisms of action may involve Nrf2 pathway beyond its classical direct antioxidant effect (Arredondo et al. 2010).

In the present work we directly demonstrated for the first time that mechanisms underlying quercetin neuroprotective effects involve Nrf2-dependent modulation of endogenous defense systems, since ARE/EpRE-driven glutathione biosynthetic enzymes were necessary for neuroprotection against oxidative stress in CGN neuronal culture. As further evidence, ARE/EpRE-driven transcription was directly correlated with the protective effect, since only those flavonoids with ARE/EpRE-driven transcription activation properties showed protective effects against oxidative stress in PC12 cells.

The increased total Nrf2 protein levels and mRNA expression in response to quercetin treatment (Fig. 2a and b) are in agreement with previous work from Tanigawa and co-workers in HepG2 cells, where quercetin enhances Nrf2 through up-regulating transcription and stabilizing protein at post transcription (Tanigawa et al. 2007). Since multiple molecular mechanisms of Nrf2 induction operate with cell-type and inducer-specific variations (Lee et al. 2005), further studies should be made in order to determine the molecular mechanisms by which quercetin influences Nrf2 activation in neuronal cells.

Of the ARE/EpRE-driven enzymes analyzed, only HO-1 and GCL_c gene expression levels were significantly increased (Fig.3). These results confirm and complement our previous studies of activation of Nrf2 by quercetin (Arredondo et al. 2010), and are in agreement with other studies that showed HO-1 and GCL activation with other polyphenols in neuronal cultures (Mizuno et al. 2011; González-Reyes et al. 2013; Lou et al. 2014). Not all Nrf2 target genes are induced at similar levels of expression, since they depend on different ARE/EpRE enhancers which respond differently to Nrf2 activation (Lacher et al. 2015). Moreover, a cell-specific ARE/EpRE gene expression has been previously described (Lee et al. 2005), which could explain in part the selective ARE/EpRE-driven gene expression observed after quercetin treatment in neuronal cultures.

Based on these gene expression results, and to explain the underlying mechanisms of quercetin neuroprotection, we first focused on HO-1. This enzyme catalyzes

degradation of heme to yield free iron, carbon monoxide and biliverdin, which is subsequently converted to bilirubin through biliverdin reductase (Mancuso et al. 2006). Interestingly HO-1 has protective effects against oxidative stress (Chen 2014). Nevertheless, our results showed that HO-1 inhibition did not alter the neuroprotective effects of quercetin against oxidative stress (Fig.4b). Neuroprotection is a complex phenomenon comprising diverse convergent mechanisms with different weight in the final protective outcome. In the case of HO-1, its up-regulation appears not to be relevant enough to afford quercetin neuroprotective effect in the experimental paradigm utilized. We next focused on the role of GCL, the rate limiting enzyme in glutathione biosynthesis, in quercetin neuroprotection. Glutathione is a key modulator of cell functions including antioxidant defense, redox regulation of protein thiols, and maintenance of redox homeostasis (Schafer and Buettner 2001; Jones 2002). Our results showed that GCL irreversible inhibition suppressed the protection of quercetin (Fig.4a), suggesting that Nrf2-driven induction of intracellular GSH levels plays a critical role in quercetin neuroprotective effect in the experimental paradigm utilized. Mizuno and co-workers reported similar results from the analysis of isothiocyanates neuroprotective effect against paraquat or H_2O_2 oxidative stress in primary striatal cultures (Mizuno et al. 2011).

To delineate the structural molecular features of quercetin involved in the observed stimulation of Nrf2-dependent gene transcription, we performed a flavonoid structure-activity relationship study for ARE/EpRE-mediated transcription activation (Fig.5). In parallel, we analyzed the cytoprotective capacity against an oxidative insult of the selected flavonoids (Fig.1) in the PC12 cell culture paradigm (Fig.6). Our results showed that although fisetin lacks the 5-hydroxyl group on the A ring compared to quercetin (Fig. 1), it showed significant luciferase induction, as well as cytoprotective effect (Fig. 5 and 6), suggesting that substitutions in the A ring would not be relevant for these properties. Likewise, isorhamnetin presents a 3´-methoxy substitution in B ring compared to quercetin (Fig. 1), and it still showed significant ARE/EpRE-mediated gene transcription activation and cytoprotective effect (Fig. 5 and 6). As a result, the catechol substitutions in positions 3', 4´ of the B ring would not be important for ARE/EpRE induction and protection either. Besides the absence of the catechol group of the B ring, apigenin lacks the 3-hydroxyl group on the C ring compared to quercetin (Fig.1). As this flavone reached neither a significant luciferase induction nor a cytoprotective effect (Fig. 5 and 6), we might consider that the 3-hydroxyl group on the

C ring would be a relevant structural feature for these properties. Similarly, neither taxifolin nor catechin reached a significant luciferase induction effect or showed any cytoprotective effect (Fig. 5 and 6). These two molecules possess the same hydroxylation pattern as quercetin, although they both lack the unsaturation at the C2-C3 bond in C ring resulting in a non-planar C-ring (Fig. 1). Thus, planar aromatic C-ring may be an important structural feature for efficient ARE/EpRE-mediated transcription activation and cytoprotection against oxidative stress by flavonoids. Our results are in accordance with previous studies from Boerboom and co-workers who reported that the unsaturation at the C2-C3 bond in C ring ensuring a planar C-ring of the flavonoid is essential for ARE/EpRE-mediated transcription activation. These authors suggested that there might be a specific upstream stereochemical interaction, between flavonoids and possibly Keap1, or other effector or receptor proteins (Boerboom et al. 2006). Finally, beyond structural requirements already described, it is noteworthy that flavonoid cytoprotection against oxidative stress correlated with ARE/EpRE mediated transcription activation, reinforcing the involvement of Nrf2 pathway in neuroprotection.

In summary, we demonstrated for the first time that quercetin neuroprotection against oxidative stress in CGN cultures involves Nrf2-dependent up-regulation of GSH biosynthetic enzymes. Additionally structure-activity relationship study for ARE/EpRE-mediated transcription activation outlined structural feature requirements for Nrf2 activation by flavonoids. This could be helpful for identifying new candidates with therapeutic potential against oxidative stress- mediated neuronal death. Together, our results contributed to the understanding of physiologically relevant mechanisms of action of flavonoids, as inducers of endogenous protective defense systems.

5. Acknowledgements

This work was supported by Grant L/ICA/139636 from the Organization for the Prohibition of Chemical Weapons (OPCW, The Hague, The Netherlands), and by Programa de Desarrollo de las Ciencias Básicas (PEDECIBA, Montevideo, Uruguay). It was also supported in part by grants from the National Institutes of Health (R21 ES025632 and R01 ES023485). We especially thank Prof. Pablo Diaz-Amarilla and Prof. Federico Rosconi (Instituto de Investigaciones Biológicas "Clemente Estable", Uruguay) for contributing with pEGFP plasmid. We also thank Professor Ramón

Bernabeu (UBA, Argentina) for providing us the PC12 cell line. Finally, we thank Estela Baglietto for her support with cell culture techniques.

6. Figure captions

Fig 1: Chemical structure of the flavonoids studied: Quercetin (3,3´,4´,5,7-pentahydroxyflavone), isorhamnetin (3,4´,5,7-tetrahydroxy-3´-methoxyflavone), fisetin (3,3´,4',7-tetrahydroxyflavone), apigenin (4´,5,7-trihydroxyflavone), taxifolin (3,3',4',5,7-pentahydroxyflavanone), catechin (3,3´,4´,5,7-pentahydroxyflavane). Basic chemical structure of flavonoids (bottom).

Fig 2: Analysis of Nrf2 levels in CGN primary cultures after quercetin treatment. Cells were exposed to vehicle alone (control) or 25 μM quercetin for 1.5, 3, 6, or 24 h. Nrf2 protein (a) and mRNA levels (b) were determined by Western blot and qPCR. Nrf2 protein and mRNA levels were corrected by α-tubulin levels or 18S RNA cDNA amplification, respectively and expressed as fold increase respect to control. Values are expressed as the mean ± SEM of at least three independent experiments. *$p<0.05$, significantly different from control.

Fig 3: Nrf2-inducible gene modulation by quercetin in CGN primary cells.
HO-1, GCLc, GCLm, NQO-1, and GPx1 gene expression levels were detected by qPCR in CGN cells after a 25 μM quercetin treatment for 6 h (a, b, c, d, and e, respectively). Data are shown as ratios of gene expression levels in treated cells to that in vehicle-treated controls after normalization based on the expression of the 18S RNA housekeeping gene. Values are expressed as the mean ± SEM of almost five independent experiments. *$p<0.05$, significantly different from control.

Fig 4: Glutathione antioxidant system influenced quercetin protective effect against oxidative stress, while HO-1 did not underlie this effect.
CGN cells were pre-treated with 25 μM quercetin for 24h in the presence of BSO (a) or ZnPP IX (b) and then exposed to H_2O_2 oxidative insult for additional 24h. Neuronal survival was determined by metabolic activity of cells (MTT assay). Values are expressed as mean ± SEM of percent of survival respect to control, of at least three

independent experiments. *p<0.05, significantly different from control.

Fig.5: Induction of luciferase activity in PC12 cells transiently transfected with pARE4-Luc reporter at equimolar concentrations of flavonoids structurally related to quercetin. At 1 DIV PC12 cells were co-transfected with pARE4-Luc or pT81 (empty vector) and a pEGFP vector (as efficiency control). At 5 DIV cells were treated for 16h with the different flavonoids at 25µM final concentration. Subsequently, GFP fluorescence was quantified and the luciferase assay was performed. Values are expressed as mean ± SEM of ratios of luciferase activity in treated cells to that in vehicle-treated controls after normalization based on pEGFP control fluorescence, of three independent experiments. *p<0.05, significantly different from control.

Fig.6: Analysis of protective effects against H2O2 on PC12 cells after 24 h pre-incubation of flavonoids structurally related to quercetin. At 5 DIV PC12 cells were pretreated for 24 h with flavonoids (0–150 µM) and then were exposed to 500 µM H_2O_2 for 24 h. Subsequently, cell survival was assessed and quantified by MTT assay. Data were normalized to control vehicle cells (cultures treated with vehicle alone for 24 h) and presented as means of %±SEM of at least three experiments. *p<0.05, significantly different from control vehicle cells treated with H_2O_2 (dotted line).

7. References

Ansari MA, Abdul HM, Joshi G, et al (2009) Protective effect of quercetin in primary neurons against Abeta(1-42): relevance to Alzheimer's disease. J Nutr Biochem 20:269–75. doi: 10.1016/j.jnutbio.2008.03.002

Arredondo F, Echeverry C, Abin-Carriquiry J a, et al (2010) After cellular internalization, quercetin causes Nrf2 nuclear translocation, increases glutathione levels, and prevents neuronal death against an oxidative insult. Free Radic Biol Med 49:738–47. doi: 10.1016/j.freeradbiomed.2010.05.020

Arredondo MF, Blasina F, Echeverry C, et al (2004) Cytoprotection by Achyrocline satureioides (Lam) D.C. and some of its main flavonoids against oxidative stress. J Ethnopharmacol 91:13–20. doi: 10.1016/j.jep.2003.11.012

Barnham KJ, Masters CL, Bush AI (2004) Neurodegenerative diseases and oxidative stress. Nat Rev Drug Discov 3:205–214. doi: 10.1038/nrd1330

Boerboom AMJF, Vermeulen M, van der Woude H, et al (2006) Newly constructed

stable reporter cell lines for mechanistic studies on electrophile-responsive element-mediated gene expression reveal a role for flavonoid planarity. Biochem Pharmacol 72:217–226. doi: 10.1016/j.bcp.2006.04.002

Bullock WO, Fernandez JM, Short JM (1987) XL1-Blue: A high efficiency plasmid transforming recA Escherichia coli strain with beta-galactosidase selection. Biotechniques 5:376–379.

Chen J (2014) Heme oxygenase in neuroprotection: From mechanisms to therapeutic implications. Rev Neurosci 25:269–280. doi: 10.1515/revneuro-2013-0046

Dajas F, Abin-Carriquiry JA, Arredondo F, et al (2015) Quercetin in brain diseases: Potential and limits. Neurochem Int 89:140–8. doi: 10.1016/j.neuint.2015.07.002

Dajas F, Arredondo F, Echeverry C, et al (2005) Flavonoids and the Brain: Evidences and Putative Mechanisms for a Protective Capacity. Curr. Neuropharmacol. 3:193–205.

Dajas F, Rivera-Megret F, Blasina F, et al (2003) Neuroprotection by flavonoids. Braz J Med Biol Res 36:1613–20.

de Vries HE, Witte M, Hondius D, et al (2008) Nrf2-induced antioxidant protection: A promising target to counteract ROS-mediated damage in neurodegenerative disease? Free Radic. Biol. Med. 45:1375–1383.

De Vries JHM, Janssen PLTMK, Hollman PCH, et al (1997) Consumption of quercetin and kaempferol in free-living subjects eating a variety of diets. In: Cancer Letters. pp 141–144

Denizot F, Lang R (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods 89:271–277. doi: 10.1016/0022-1759(86)90368-6

Echeverry C, Arredondo F, Abin-Carriquiry JA, et al (2010) Pretreatment with natural flavones and neuronal cell survival after oxidative stress: a structure-activity relationship study. J Agric Food Chem 58:2111–5. doi: 10.1021/jf902951v

Go Y-M, Gipp JJ, Mulcahy RT, Jones DP (2004) H2O2-dependent activation of GCLC-ARE4 reporter occurs by mitogen-activated protein kinase pathways without oxidation of cellular glutathione or thioredoxin-1. J Biol Chem 279:5837–45. doi: 10.1074/jbc.M307547200

González-Reyes S, Guzmán-Beltrán S, Medina-Campos ON, Pedraza-Chaverri J (2013) Curcumin pretreatment induces Nrf2 and an antioxidant response and prevents hemin-induced toxicity in primary cultures of cerebellar granule neurons of rats. Oxid Med Cell Longev. doi: 10.1155/2013/801418

Gordon J, Amini S, White M (2013) General Overview of Neuronal Cell Culture. In: Amini S, White MK (eds) Neuronal Cell Culture SE - 1. Humana Press, pp 1–8

Greene LA, Tischler AS (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci U S A 73:2424–2428. doi: 10.1073/pnas.73.7.2424

Halliwell B (2006) Oxidative stress and neurodegeneration: Where are we now? J. Neurochem. 97:1634–1658.

Hertog MG, Feskens EJ, Hollman PC, et al (1993) Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet 342:1007–1011. doi: 10.1016/0140-6736(93)92876-U

Ischiropoulos H, Beckman JS (2003) Oxidative stress and nitration in neurodegeneration: Cause, effect, or association? J. Clin. Invest. 111:163–169.

Itoh K, Wakabayashi N, Katoh Y, et al (1999) Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev 13:76–86. doi: 10.1101/gad.13.1.76

Jaiswal AK (2004) Nrf2 signaling in coordinated activation of antioxidant gene expression. Free Radic. Biol. Med. 36:1199–1207.

Jakubowicz-Gil J, Rzeski W, Zdzisinska B, et al (2008) Different sensitivity of neurons and neuroblastoma cells to quercetin treatment. Acta Neurobiol Exp (Wars) 68:463–476.

Jones DP (2002) Redox potential of GSH/GSSG couple: Assay and biological significance. Methods Enzymol 348:93–112. doi: 10.1016/S0076-6879(02)48630-2

Kelsey NA, Wilkins HM, Linseman DA (2010) Nutraceutical antioxidants as novel neuroprotective agents. Molecules 15:7792–7814.

Kensler TW, Wakabayashi N, Biswal S (2007) Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu Rev Pharmacol Toxicol 47:89–116. doi: 10.1146/annurev.pharmtox.46.120604.141046

Lacher SE, Lee JS, Wang X, et al (2015) Beyond antioxidant genes in the ancient NRF2 regulatory network. Free Radic Biol Med. doi: 10.1016/j.freeradbiomed.2015.06.044

Lee J, Jo D-G, Park D, et al (2014) Adaptive cellular stress pathways as therapeutic targets of dietary phytochemicals: focus on the nervous system. Pharmacol Rev 66:815–68. doi: 10.1124/pr.113.007757

Lee J-M, Li J, Johnson DA, et al (2005) Nrf2, a multi-organ protector? FASEB J 19:1061–1066. doi: 10.1096/fj.04-2591hyp

Li M-H, Cha Y-N, Surh Y-J (2006) Peroxynitrite induces HO-1 expression via PI3K/Akt-dependent activation of NF-E2-related factor 2 in PC12 cells. Free Radic Biol Med 41:1079–1091. doi:

http://dx.doi.org/10.1016/j.freeradbiomed.2006.06.010

Lou H, Jing X, Wei X, et al (2014) Naringenin protects against 6-OHDA-induced neurotoxicity via activation of the Nrf2/ARE signaling pathway. Neuropharmacology 79:380–388. doi: 10.1016/j.neuropharm.2013.11.026

Mancuso C, Pani G, Calabrese V (2006) Bilirubin: an endogenous scavenger of nitric oxide and reactive nitrogen species. Redox Rep 11:207–213. doi: 10.1179/135100006X154978

Mastrogiannaki M, Matak P, Keith B, et al (2009) HIF-2??, but not HIF-1??, promotes iron absorption in mice. J Clin Invest 119:1159–1166. doi: 10.1172/JCI38499

Mizuno K, Kume T, Muto C, et al (2011) Glutathione biosynthesis via activation of the nuclear factor E2-related factor 2 (Nrf2)--antioxidant-response element (ARE) pathway is essential for neuroprotective effects of sulforaphane and 6-(methylsulfinyl) hexyl isothiocyanate. J Pharmacol Sci 115:320–328. doi: 10.1254/jphs.10257FP

Moi P, Chan K, Asunis I, et al (1994) Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. Proc Natl Acad Sci U S A 91:9926–9930. doi: 10.1073/pnas.91.21.9926

Mulcahy RT, Wartman MA, Bailey HH, Gipp JJ (1997) Constitutive and beta-naphthoflavone-induced expression of the human gamma-glutamylcysteine synthetase heavy subunit gene is regulated by a distal antioxidant response element/TRE sequence. J Biol Chem 272:7445–7454.

Ossola B, Kääriäinen TM, Männistö PT (2009) The multiple faces of quercetin in neuroprotection. Expert Opin Drug Saf 8:397–409. doi: 10.1517/14740330903026944

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45. doi: 10.1093/nar/29.9.e45

Rendeiro C, Rhodes JS, Spencer JPE (2015) The mechanisms of action of flavonoids in the brain: Direct versus indirect effects. Neurochem Int 89:126–139. doi: 10.1016/j.neuint.2015.08.002

Rice-Evans CA, Miller NJ, Paganga G (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic. Biol. Med. 20:933–956.

Saito Y, Nishio K, Numakawa Y, et al (2007) Protective effects of 15-deoxy-Δ12,14-prostaglandin J2 against glutamate-induced cell death in primary cortical neuron cultures: induction of adaptive response and enhancement of cell tolerance primarily through up-regulation of cellular glutathione. J Neurochem 102:1625–1634. doi: 10.1111/j.1471-4159.2007.04701.x

Sandberg M, Patil J, D'Angelo B, et al (2014) NRF2-regulation in brain health and disease: Implication of cerebral inflammation. Neuropharmacology 79:298–306. doi: 10.1016/j.neuropharm.2013.11.004

Scapagnini G, D'Agata V, Calabrese V, et al (2002) Gene expression profiles of heme oxygenase isoforms in the rat brain. Brain Res 954:51–59. doi: 10.1016/S0006-8993(02)03338-3

Schafer FQ, Buettner GR (2001) Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic. Biol. Med. 30:1191–1212.

Silva B, Oliveira PJ, Dias A, Malva JO (2008) Quercetin, kaempferol and biapigenin from hypericum perforatum are neuroprotective against excitotoxic insults. Neurotox Res 13:265–279. doi: 10.1007/BF03033510

Son TG, Camandola S, Mattson MP (2008) Hormetic dietary phytochemicals. NeuroMolecular Med. 10:236–246.

Spencer JPE (2009) Flavonoids and brain health: Multiple effects underpinned by common mechanisms. Genes Nutr. 4:243–250.

Spencer JPE, Vafeiadou K, Williams RJ, Vauzour D (2012) Neuroinflammation: Modulation by flavonoids and mechanisms of action. In: Molecular Aspects of Medicine. pp 83–97

Tanigawa S, Fujii M, Hou DX (2007) Action of Nrf2 and Keap1 in ARE-mediated NQO1 expression by quercetin. Free Radic Biol Med 42:1690–1703. doi: 10.1016/j.freeradbiomed.2007.02.017

Vargas MR, Pehar M, Cassina P, et al (2005) Fibroblast growth factor-1 induces heme oxygenase-1 via nuclear factor erythroid 2-related factor 2 (Nrf2) in spinal cord astrocytes: Consequences for motor neuron survival. J Biol Chem 280:25571–25579. doi: 10.1074/jbc.M501920200

Wasserman WW, Fahl WE (1997) Functional antioxidant responsive elements. Proc Natl Acad Sci U S A 94:5361–5366. doi: 10.1073/pnas.94.10.5361

Williams RJ, Spencer JPE (2012) Flavonoids, cognition, and dementia: Actions, mechanisms, and potential therapeutic utility for Alzheimer disease. Free Radic. Biol. Med. 52:35–45.

Williams RJ, Spencer JPE, Rice-Evans C (2004) Flavonoids: Antioxidants or signalling molecules? Free Radic. Biol. Med. 36:838–849.

Zerarka S, Pellerin L, Slosman D, Magistretti PJ (2001) Astrocytes as a predominant cellular site of (99m)Tc-HMPAO retention. J Cereb Blood Flow Metab 21:456–468. doi: 10.1097/00004647-200104000-00014

Zhang M, An C, Gao Y, et al (2013) Emerging roles of Nrf2 and phase II antioxidant enzymes in neuroprotection. Prog. Neurobiol. 100:30–47.

Figure 1

Click here to download Figure Fig 1.tif

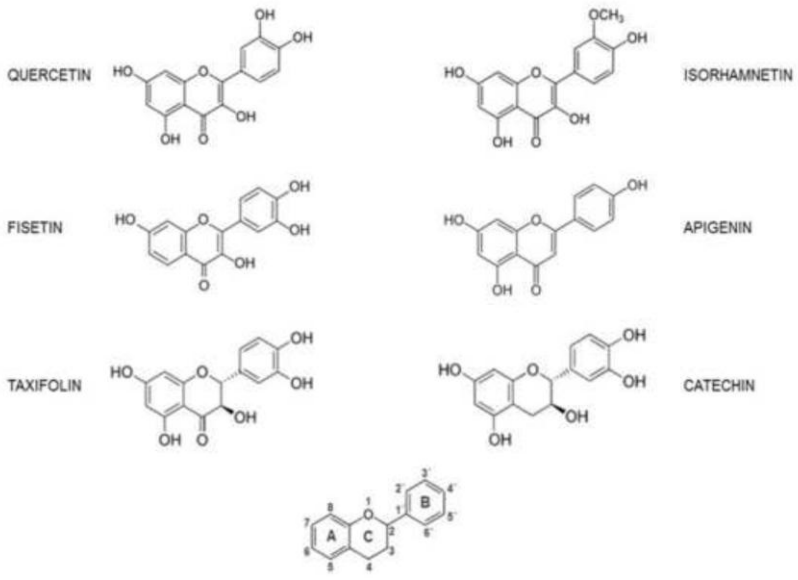


Figure 2

Click here to download Figure fig2.tif

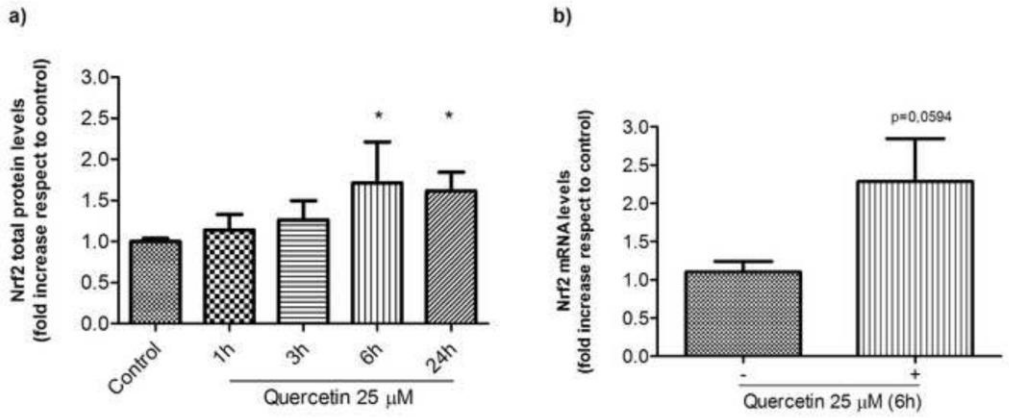


Figure 3

Click here to download Figure fig3.tif

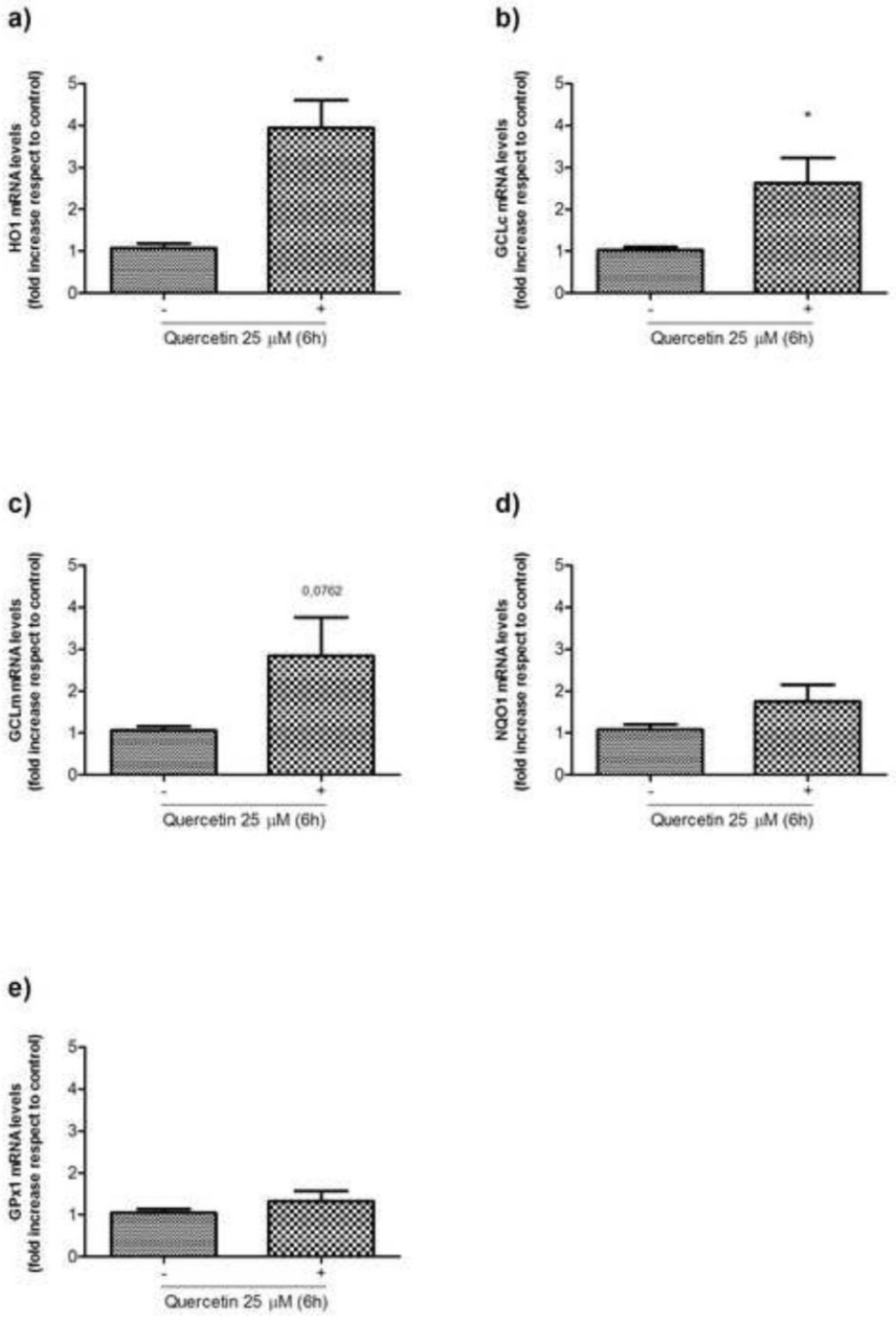


Figure 4

Click here to download Figure fig4.tif

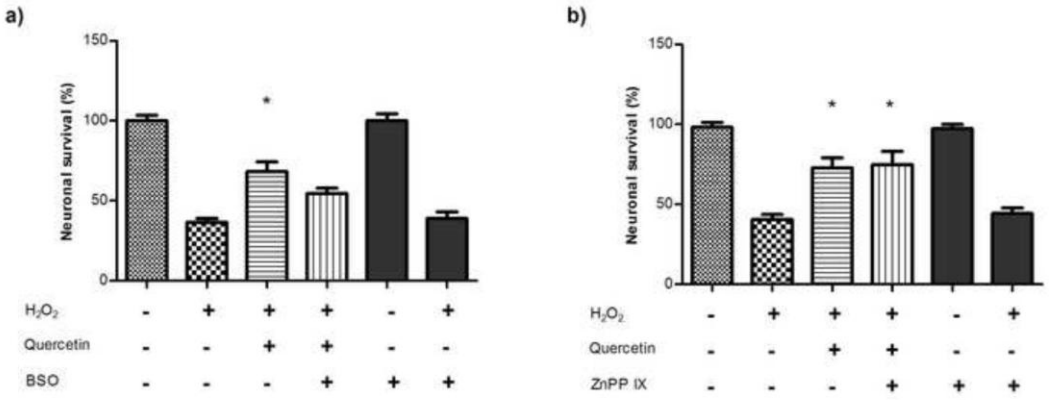


Figure 5

Click here to download Figure fig5.tif

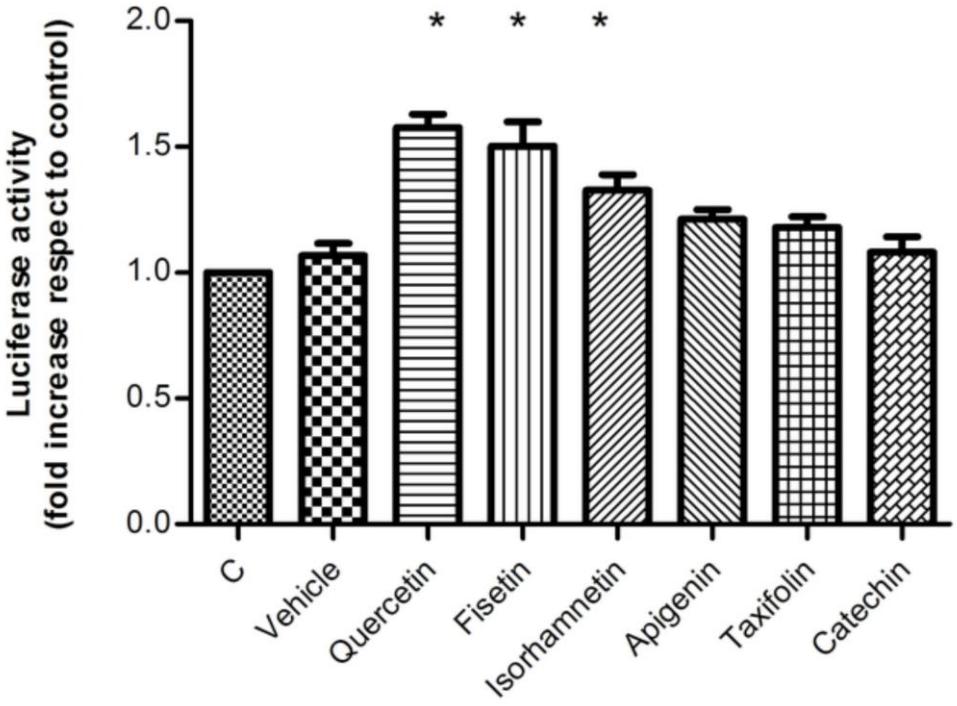
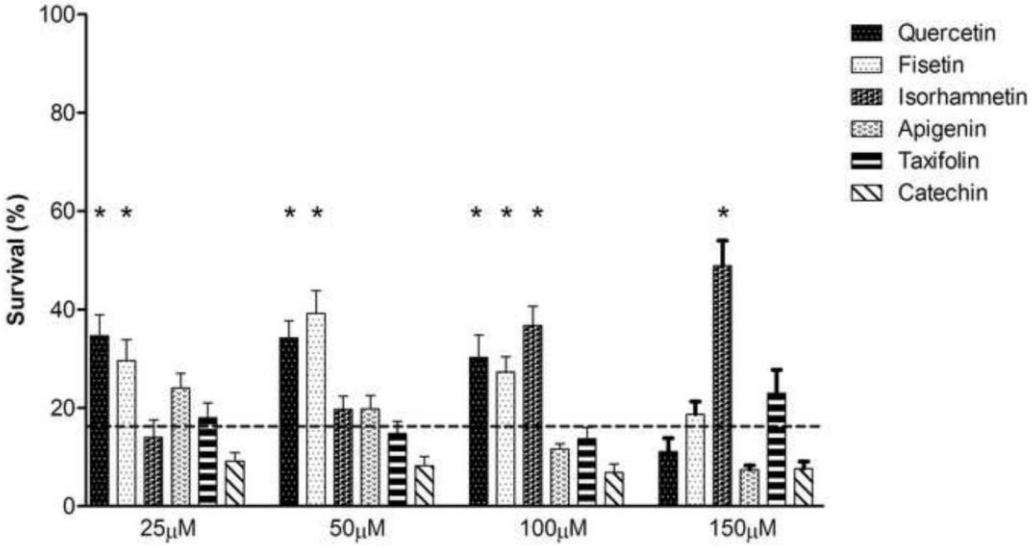


Figure 6

Click here to download Figure fig6.tif



DISCUSIÓN

En esta tesis profundizamos en el conocimiento de los mecanismos de acción subyacentes al efecto neuroprotector de quercetina y flavonoides estructuralmente relacionados. En particular, estudiamos el papel que cumple la vía dependiente de Nrf2.

Primero, cabe destacar el paradigma experimental desarrollado, donde cultivos primarios de neuronas granulares de cerebelo fueron pre-tratados con quercetina durante 24 horas previo al daño oxidativo. Esta fue una diferencia relevante respecto a diversos modelos experimentales utilizados previos a esta tesis, en donde generalmente la adición del flavonoide y la injuria estaban muy próximos en el tiempo (Kim et al. 2002; Kang et al. 2004). Los resultados obtenidos sobre la disponibilidad intracelular de la quercetina en los cultivos, muestran que en el protocolo de pre-tratamiento, no se detectan niveles de quercetina ni de metabolitos al momento del daño oxidativo. Esto permite sugerir que la quercetina podría estar actuando por mecanismos diferentes a la capacidad atrapadora de radicales o quelantes, ya que estos mecanismos antioxidantes directos se verían limitados por la ausencia del flavonoide al momento de la adición del oxidante. De forma complementaria, si bien se detectaron niveles bajos de quercetina y metabolitos a nivel extracelular, los efectos protectores no se debieron a una reacción externa directa entre estos compuestos y el oxidante. Esto refuerza nuestra hipótesis de que otros mecanismos intracelulares, probablemente a través de la inducción de vías de señalización intracelular, estarían involucrados en los efectos neuroprotectores de quercetina observados en este modelo.

Paralelamente, el pre-tratamiento de los cultivos con quercetina causó la translocación nuclear de Nrf2 y un aumento en sus niveles proteicos. Asimismo aumentó la expresión de los genes ARE de las enzimas GCL y HO-1en los cultivos. Esto a su vez se condice con el aumento de los niveles de

GSH total y de la proteína HO-1 observados. Por otra parte, el efecto neuroprotector de quercetina se atenuó por la inhibición de la enzima GCL. Por lo tanto, nuestros resultados sugieren que los mecanismos moleculares responsables del efecto neuroprotector de quercetina en este modelo involucran la inducción de defensas endógenas a través de la activación de la vía Nrf2. En particular, en este efecto neuroprotector estaría involucrado el sistema de defensa dependiente de GSH, donde niveles elevados de GSH en los cultivos serían capaces de afrontar la toxicidad por H_2O_2. Este aumento del GSH causado por quercetina coincide con resultados previos obtenidos en otros modelos en cultivo, predominantemente no neuronales (Rodgers y Grant 1998; Ishige et al. 2001; Myhrstad et al. 2002; Alía et al. 2006; Lavoie et al. 2009). De modo similar, trabajos recientes demostraron que quercetina modula la vía Nrf2, aunque existe poca información sobre esta acción en neuronas (Kang et al. 2009; Granado-Serrano et al. 2012; Hayashi et al. 2012).

De las características estructurales que evaluamos en el estudio de relación estructura-actividad, las más relevantes para la inducción de Nrf2 por quercetina y flavonoides relacionados fueron: la insaturación en posición 2,3, el carbonilo en posición 4, y las sustituciones en posición 3' y 4' del anillo B, donde al menos una de ellas sea un hidroxilo. Al comparar estas características con los requerimientos estructurales descritos para la actividad secuestradora de radicales libres de flavonoides (Rice-Evans et al. 1996), encontramos varias coincidencias. Observamos una asociación entre las propiedades redox intrínsecas de los flavonoides (que les confieren capacidades antioxidantes/pro-oxidantes) y la capacidad de inducir la vía Nrf2. Por otra parte, si comparamos estas características con los requerimientos estructurales necesarios para el efecto neuroprotector, aparece una clara diferencia en relación al anillo A de los flavonoides. Mientras que este anillo no parece ser relevante para secuestrar radicales libres ni para activar Nrf2, éste sí sería importante para su efecto

neuroprotector. Esta diferencia se puede explicar si pensamos en la neuroprotección como un efecto complejo que implica la convergencia de acciones positivas (antioxidación, activación de Nrf2, dependientes de los anillos B y C) y negativas (pro-oxidación, citotoxicidad, donde podría estar en parte relacionado el anillo A).

A partir de los resultados obtenidos en esta tesis, proponemos que quercetina y flavonoides estructuralmente relacionados son capaces de proteger neuronas frente a un daño oxidativo a través de la inducción de defensas celulares endógenas dependientes de la vía Nrf2/ARE. La activación de esta vía por parte de los flavonoides podría deberse a sus propiedades redox intrínsecas que les otorgarían actividad pro-oxidante. De esta forma, a partir de propiedades tradicionalmente consideradas tóxicas, los flavonoides podrían contribuir a la prevención de la neurodegeneración mediante la inducción de proteínas citoprotectoras. Esto va de la mano con una reciente propuesta realizada por Forman y cols (2014), sobre el mecanismo por el cual los fitoquímicos dietarios (entre ellos, los flavonoides) ejercen sus efectos beneficiosos en el organismo. Los autores proponen que estos compuestos no actúan como antioxidantes directos, secuestrando radicales libres, ya que estas propiedades no son cinéticamente factibles en el organismo. Proponen que los fitoquímicos dietarios actúan de forma sinérgica entre sí, como agentes electrofílicos capaces de activar sistemas de respuesta adaptativa (como la vía Nrf2/ARE) que desencadenan la inducción de proteínas citoprotectoras y un aumento en sustratos nucleofílicos como el GSH, tiorredoxinas y NADPH. Los autores proponen que los fitoquímicos dietarios serían los responsables de esta inducción y mantenimiento del "tono nucleofílico". Y definen a este "tono nucleofílico" como la capacidad de respuesta celular adaptativa frente a daños oxidativos causados por electrófilos (Forman et al. 2014).

Si bien esta tesis se centró en el potencial de los flavonoides como activadores de vías de respuesta celular adaptativa, cabe recordar que

estos compuestos son también capaces de interactuar directamente con diversos blancos moleculares como receptores y kinasas, y de esa forma modular el destino celular. Debido a las limitantes estequiométricas antes descritas para estos compuestos, es improbable que actúen como antioxidantes directos. Por lo tanto sería el conjunto de mecanismos de antioxidación indirecta y no antioxidantes que darían como resultado los efectos pleiotrópicos de los flavonoides asociados con la protección neuronal.

CONCLUSIONES

En el modelo de pre-tratamiento durante 24 h con quercetina en cultivos neuronales previo a la adición de H_2O_2, observamos que:

a) quercetina fue rápidamente internalizada a las neuronas, llegando hasta el núcleo;

b) el factor transcripcional Nrf2 se activó;

c) hubo un aumento en los niveles proteicos de Nrf2, y en la expresión de los genes ARE que codifican para las enzimas GCL y HO-1;

d) se observó un aumento de los niveles de GSH total y de la proteína HO-1 en los cultivos neuronales;

e) los niveles intracelulares de quercetina o metabolitos no fueron detectables al momento de la adición del daño oxidativo;

f) se previno la muerte neuronal frente al daño oxidativo;

g) el efecto neuroprotector de quercetina se atenuó por la inhibición de la enzima GCL y no por la inhibición de HO-1.

En el estudio estructura-actividad del efecto neuroprotector por flavonoides encontramos que las características estructurales que aseguran este efecto, no coinciden con aquellas características necesarias para la actividad atrapadora de radicales libres de los flavonoides.

En el estudio estructura-actividad de la activación de Nrf2 por flavonoides observamos que:

a) el anillo A no sería un componente estructural relevante para la activación de Nrf2;

b) la planaridad sería un componente estructural importante para la activación de Nrf2;

c) los flavonoides que presentaron actividad inductora de Nrf2, también fueron capaces de proteger a los cultivos sometidos a un daño oxidativo.

PERSPECTIVAS

El desarrollo de esta tesis nos ha conducido a plantearnos una serie de interrogantes, entre las que se destacan:

1. ¿Cuáles son los mecanismos "corriente arriba" (*upstream)* de activación de Nrf2 por parte de quercetina?

Nuestras evidencias así como las de otros autores sugieren que la actividad pro-oxidante de los flavonoides activaría la vía Nrf2/ARE (Lee-Hilz et al. 2006). Sin embargo falta confirmar si Keap1 sería el blanco de acción de los flavonoides, y en ese caso, mediante la modificación de qué Cys de la proteína estarían actuando. Una alternativa a este mecanismo sería la interacción con otros blancos proteicos, como por ejemplo kinasas que terminaran causando la activación de Nrf2. En este sentido, cabe destacar que estudios recientes de nuestro equipo de trabajo demostraron mediante métodos bioinformáticos de *docking* reverso, que uno de los blancos proteicos posibles de quercetina sería GSK3β (Carvalho et al. 2015, enviado, ver apéndice I). Este resultado nos permite direccionarnos en el análisis de la capacidad inhibitoria de flavonoides sobre GSK, y evaluar si estos compuestos son capaces de activar Nrf2 a través de la otra rama regulatoria independiente de Keap1.

2. ¿Sobre qué células estarían actuando los flavonoides? ¿Neuronas, astrocitos o ambos?

Reportes previos demostraron que la expresión de genes ARE puede tener lugar en cultivos primarios de neuronas corticales. Sin embargo argumentan que su respuesta a la activación de Nrf2 es moderada, debido a que tienen bajos niveles proteicos de Nrf2. Asimismo proponen que el aumento en los niveles de GSH mediado por Nrf2 en astrocitos sería necesario y suficiente para proteger a las neuronas corticales de daños

oxidativos (Shih et al. 2003). Por otra parte otros estudios han mostrado que quercetina es capaz de activar Nrf2 en astrocitos y microglia (Lavoie et al. 2009; Kang et al. 2013). En este sentido, es probable que las células gliales jueguen un papel en la neuroprotección mediada por quercetina.

En el caso particular de esta tesis, los cultivos primarios que usamos, se componen en más de un 95% de neuronas granulares de cerebelo. En el 5% restante encontramos otros tipos celulares, entre ellos los astrocitos. Si analizamos los ensayos de inmunofluorescencia de Nrf2, los resultados mostraron traslocación y aumento en la señal de Nrf2 en todos los elementos celulares del cultivo, en su mayoría neuronas granulares de cerebelo. Por lo tanto, quercetina activa la vía Nrf2 en neuronas. Resta confirmar en qué medida la activación de esta vía en los astrocitos circundantes, puede contribuir a la protección neuronal frente al daño oxidativo.

3. ¿Cuál es el efecto de los flavonoides sobre la vía Nrf2/ARE *in vivo*?

Al día de hoy, el efecto de los flavonoides sobre la vía Nrf2/ARE ha sido caracterizado en diversos modelos de cultivos celulares, en su mayoría líneas celulares y en relación a efectos anticancerígenos y hepatoprotectores (Qin et al. 2014; Saw et al. 2014; Ji et al. 2015). También encontramos algunos modelos de cultivos neuronales frente a injurias tales como glutamato, H_2O_2 y 6-OHDA (Lou et al. 2012; Lou et al. 2014; Lee et al. 2014). En el caso particular de quercetina, en su mayoría se tratan de estudios de hepatoprotección. En relación al sistema nervioso encontramos trabajos con quercetina en neuronas de la raíz dorsal y microglía (Kang et al. 2013; Shi et al. 2013)

En lo que refiere a modelos *in vivo*, recientemente se han reportado evidencias de inducción de la vía Nrf2 por parte de epicatequina, en modelos de isquemia cerebral experimental y luteolina en modelo de trauma (Han et al. 2014; Shah et al. 2014; Xu et al. 2014). Por lo tanto todavía

resta mucho por estudiar y comprender cómo estos compuestos alteran la vía Nrf2 en el SNC en modelos *in vivo*.

En este sentido, proponemos que el uso de *Drosophila melanogaster* como modelo de estudio de la activación por flavonoides de la vía CnCc (ortólogo de Nrf2 en moscas), puede resultar de gran utilidad. Como dijimos previamente, en mosca la vía Nrf2/Keap1/Smaf está conservada. Asimismo, se ha reportado que la inducción de CncC promueve el largo de vida en estos organismos (Sykiotis y Bohmann 2010). Así por ejemplo, se pueden realizar ensayos del largo de vida en mosca con el fin de evaluar la capacidad inductora de CncC de compuestos de interés, como en el caso de los flavonoides. Por su pequeño tamaño y su alta tasa de reproducción, los estudios en Drosophila son más fáciles de realizar que en vertebrados. Asimismo, la versatilidad genética de este modelo permitiría disecar el papel funcional de los principales componentes de la vía CncC. Por lo tanto, este modelo podría ser útil como una etapa de *screening* de compuestos previa a los estudios *in vivo* en roedores.

BIBLIOGRAFÍA

Afanas'ev IB, Dorozhko AI, Brodskii A V, et al (1989) Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. Biochem Pharmacol 38:1763-1769. doi: 10.1016/0006-2952(89)90410-3

Alexi T, Borlongan C V, Faull RLM, et al (2000) Neuroprotective strategies for basal ganglia degeneration: Parkinson's and Huntington's diseases. Prog Neurobiol 60:409-470. doi: 10.1016/S0301-0082(99)00032-5

Alía M, Mateos R, Ramos S, et al (2006) Influence of quercetin and rutin on growth and antioxidant defense system of a human hepatoma cell line (HepG2). Eur J Nutr 45:19-28. doi: 10.1007/s00394-005-0558-7

Andersen JK (2004) Oxidative stress in neurodegeneration: cause or consequence? Nat Med 10 Suppl:S18-S25. doi: 10.1038/nrn1434

Arredondo MF, Blasina F, Echeverry C, et al (2004) Cytoprotection by Achyrocline satureioides (Lam) D.C. and some of its main flavonoids against oxidative stress. J Ethnopharmacol 91:13-20. doi: 10.1016/j.jep.2003.11.012

Bains JS, Shaw C a. (1997) Neurodegenerative disorders in humans: The role of glutathione in oxidative stress-mediated neuronal death. Brain Res Rev 25:335-358. doi: 10.1016/S0165-0173(97)00045-3

Baird L, Dinkova-Kostova AT (2011) The cytoprotective role of the Keap1-Nrf2 pathway. Arch Toxicol 85:241-272. doi: 10.1007/s00204-011-0674-5

Banning A, Deubel S, Kluth D, et al (2005) The GI-GPx gene is a target for Nrf2. Mol Cell Biol 25:4914-4923. doi: 10.1128/MCB.25.12.4914-4923.2005

Bjeldanes LF, Chang GW (1977) Mutagenic activity of quercetin and related compounds. Science 197:577-578. doi: 10.1126/science.327550

Blasina F, Vaamonde L, Silvera F, et al (2015) Intravenous nanosomes of quercetin improve brain function and hemodynamic instability after severe hypoxia in newborn piglets. Neurochem Int 89:149-56. doi: 10.1016/j.neuint.2015.08.007

Blasina MF, Vaamonde L, Morquio A, et al (2009) Differentiation induced by Achyrocline satureioides (Lam) infusion in PC12 cells. Phytother Res 23:1263-9. doi: 10.1002/ptr.2607

Boots AW, Haenen GRMM, Bast A (2008) Health effects of quercetin: From antioxidant to nutraceutical. Eur. J. Pharmacol. 585:325-337.

Boots AW, Kubben N, Haenen GRMM, Bast A (2003) Oxidized quercetin reacts with thiols rather than with ascorbate: implication for quercetin supplementation. Biochem Biophys Res Commun 308:560-5.

Bouayed J, Bohn T (2010) Exogenous antioxidants - Double-edged swords in cellular redox state: Health beneficial effects at physiologic doses versus deleterious effects at high doses: Oxid Med Cell Longev 3:228-237. doi: 10.4161/oxim.3.4.12858

Calabrese V, Cornelius C, Dinkova-Kostova AT, et al (2012) Cellular stress responses, hormetic phytochemicals and vitagenes in aging and longevity. Biochim Biophys Acta - Mol Basis Dis 1822:753-783. doi: 10.1016/j.bbadis.2011.11.002

Calkins MJ, Jakel RJ, Johnson DA, et al (2005) Protection from mitochondrial complex II inhibition in vitro and in vivo by Nrf2-mediated transcription. Proc Natl Acad Sci United States Am 102 :244-249. doi: 10.1073/pnas.0408487101

Casagrande F, Darbon J-M (2001) Effects of structurally related flavonoids on cell cycle progression of human melanoma cells: regulation of cyclin-dependent kinases CDK2 and CDK1. Biochem Pharmacol 61:1205-1215. doi: 10.1016/S0006-2952(01)00583-4

Chang H, Mi M, Ling W, et al (2008) Structurally related cytotoxic effects of flavonoids on human cancer cells in vitro. Arch Pharm Res 31:1137-44. doi: 10.1007/s12272-001-1280-8

Chang WS, Lee YJ, Lu FJ, Chiang HC (1993) Inhibitory effects of flavonoids on xanthine oxidase. Anticancer Res 13:2165-2170.

Checkoway H, Powers K, Smith-Weller T, et al (2002) Parkinson's disease risks associated with cigarette smoking, alcohol consumption, and caffeine intake. Am J Epidemiol 155:732-8. doi: 10.1093/aje/155.8.732

Chen Z, Lin Z (2015) Tea and human health: biomedical functions of tea active components and current issues. J Zhejiang Univ Sci B 16:87-102. doi: 10.1631/jzus.B1500001

Chorley BN, Campbell MR, Wang X, et al (2012) Identification of novel NRF2-regulated genes by ChiP-Seq: Influence on retinoid X receptor alpha.

Nucleic Acids Res 40:7416-7429. doi: 10.1093/nar/gks409

Chow J-M, Shen S-C, Huan SK, et al (2005) Quercetin, but not rutin and quercitrin, prevention of H2O2-induced apoptosis via anti-oxidant activity and heme oxygenase 1 gene expression in macrophages. Biochem Pharmacol 69:1839-1851. doi: 10.1016/j.bcp.2005.03.017

Chuang C-C, Martinez K, Xie G, et al (2010) Quercetin is equally or more effective than resveratrol in attenuating tumor necrosis factor-{alpha}-mediated inflammation and insulin resistance in primary human adipocytes. Am J Clin Nutr 92:1511-1521. doi: 10.3945/ajcn.2010.29807

Crozier A, Jaganath IB, Clifford MN (2009) Dietary phenolics: chemistry, bioavailability and effects on health. Nat Prod Rep 26:1001-1043. doi: 10.1039/b802662a

Cuadrado A (2015) Structural and functional characterization of Nrf2 degradation by glycogen synthase kinase 3/β-TrCP. Free Radic Biol Med 88:147-57. doi: 10.1016/j.freeradbiomed.2015.04.029

Cushman M, Nagarathnam D, Burg DL, Geahlen RL (1991) Synthesis and protein-tyrosine kinase inhibitory activities of flavonoid analogues. J Med Chem 34:798-806. doi: 10.1021/jm00106a047

D'Autréaux B, Toledano MB (2007) ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. Nat Rev Mol Cell Biol 8:813-824. doi: 10.1038/nrm2256

Dajas F, Abin-Carriquiry JA, Arredondo F, et al (2015) Quercetin in brain diseases: Potential and limits. Neurochem Int 89:140-8. doi: 10.1016/j.neuint.2015.07.002

Dajas F, Andres A-CJ, Florencia A, et al (2013) Neuroprotective actions of flavones and flavonols: mechanisms and relationship to flavonoid structural features. Cent Nerv Syst Agents Med Chem 13:30-5.

Dajas F, Arredondo F, Echeverry C, et al (2005) Flavonoids and the Brain: Evidences and Putative Mechanisms for a Protective Capacity. Curr. Neuropharmacol. 3:193-205.

Dajas F, Rivera F, Blasina F, et al (2003a) Cell culture protection and in vivo neuroprotective capacity of flavonoids. Neurotox Res 5:425-32. doi: 10.1007/BF03033172

Dajas F, Rivera-Megret F, Blasina F, et al (2003b) Neuroprotection by flavonoids. Braz J Med Biol Res 36:1613-20.

Davies KJ (2000) Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. IUBMB Life 50:279-289. doi: 10.1080/713803728

Davis JM, Murphy EA, Carmichael MD, Davis B (2009) Quercetin increases brain and muscle mitochondrial biogenesis and exercise tolerance. Am J Physiol Regul Integr Comp Physiol 296:R1071-1077. doi: 10.1152/ajpregu.90925.2008

Dinkova-Kostova AT, Talalay P (2008) Direct and indirect antioxidant properties of inducers of cytoprotective proteins. Mol Nutr Food Res 52 Suppl 1:S128-38. doi: 10.1002/mnfr.200700195

Echeverry C, Arredondo F, Martínez M, et al (2015) Antioxidant Activity, Cellular Bioavailability, and Iron and Calcium Management of Neuroprotective and Nonneuroprotective Flavones. Neurotox Res 27:31-42. doi: 10.1007/s12640-014-9483-y

Echeverry C, Blasina F, Arredondo F, et al (2004) Cytoprotection by neutral fraction of tannat red wine against oxidative stress-induced cell death. J Agric Food Chem 52:7395-9. doi: 10.1021/jf040053q

Egert S, Bosy-Westphal A, Seiberl J, et al (2009) Quercetin reduces systolic blood pressure and plasma oxidised low-density lipoprotein concentrations in overweight subjects with a high-cardiovascular disease risk phenotype: a double-blinded, placebo-controlled cross-over study. Br J Nutr 102:1065-1074. doi: 10.1017/S0007114509359127

Ferraresi R, Troiano L, Roat E, et al (2005) Essential requirement of reduced glutathione (GSH) for the anti-oxidant effect of the flavonoid quercetin. Free Radic Res 39:1249-58. doi: 10.1080/10715760500306935

Ferriola PC, Cody V, Middleton EJ (1989) Protein kinase C inhibition by plant flavonoids. Kinetic mechanisms and structure-activity relationships. Biochem Pharmacol 38:1617-1624. doi: 10.1016/0006-2952(89)90309-2

Finkel T (2011) Signal transduction by reactive oxygen species. J Cell Biol 194:7-15. doi: 10.1083/jcb.201102095

Fiorani M, Guidarelli A, Blasa M, et al (2010) Mitochondria accumulate large amounts of quercetin: Prevention of mitochondrial damage and release

upon oxidation of the extramitochondrial fraction of the flavonoid. J Nutr Biochem 21:397-404. doi: 10.1016/j.jnutbio.2009.01.014

Forman HJ, Davies KJA, Ursini F (2014) How do nutritional antioxidants really work: nucleophilic tone and para-hormesis versus free radical scavenging in vivo. Free Radic Biol Med 66:24-35. doi: 10.1016/j.freeradbiomed.2013.05.045

Formica J V, Regelson W (1995) Review of the biology of Quercetin and related bioflavonoids. Food Chem Toxicol an Int J Publ Br Ind Biol Res Assoc 33:1061-1080.

Gao X, Cassidy a., Schwarzschild M a., et al (2012) Habitual intake of dietary flavonoids and risk of Parkinson disease. Neurology 78:1138-1145. doi: 10.1212/WNL.0b013e31824f7fc4

Gaspar J, Rodrigues a, Laires a, et al (1994) On the mechanisms of genotoxicity and metabolism of quercetin. Mutagenesis 9:445-9.

Gil B, Sanz MJ, Terencio MC, et al (1994) Effects of flavonoids on Naja naja and human recombinant synovial phospholipases A2 and inflammatory responses in mice. Life Sci 54:PL333-8.

Gilgun-Sherki Y, Melamed E, Offen D (2001) Oxidative stress induced-neurodegenerative diseases: The need for antioxidants that penetrate the blood brain barrier. Neuropharmacology 40:959-975. doi: 10.1016/S0028-3908(01)00019-3

Granado-Serrano AB, Martín MA, Bravo L, et al (2012) Quercetin modulates Nrf2 and glutathione-related defenses in HepG2 cells: Involvement of p38. Chem Biol Interact 195:154-164. doi: 10.1016/j.cbi.2011.12.005

Haleagrahara N, Siew CJ, Mitra NK, Kumari M (2011) Neuroprotective effect of bioflavonoid quercetin in 6-hydroxydopamine-induced oxidative stress biomarkers in the rat striatum. Neurosci Lett 500:139-43. doi: 10.1016/j.neulet.2011.06.021

Halliwell B, Gutteridge JM (1999) Free Radicals in Biology and Medicine.

Han J, Wang M, Jing X, et al (2014) (−)-Epigallocatechin Gallate Protects Against Cerebral Ischemia-Induced Oxidative Stress via Nrf2/ARE Signaling. Neurochem Res 39:1292-1299. doi: 10.1007/s11064-014-1311-5

Harborne JB, Williams C a. (2000) Advances in flavonoid research since 1992.

Phytochemistry 55:481-504. doi: 10.1016/S0031-9422(00)00235-1

Harwood M, Danielewska-Nikiel B, Borzelleca JF, et al (2007) A critical review of the data related to the safety of quercetin and lack of evidence of in vivo toxicity, including lack of genotoxic/carcinogenic properties. Food Chem Toxicol 45:2179-205. doi: 10.1016/j.fct.2007.05.015

Hayashi Y, Matsushima M, Nakamura T, et al (2012) Quercetin protects against pulmonary oxidant stress via heme oxygenase-1 induction in lung epithelial cells. Biochem Biophys Res Commun 417:169-174. doi: 10.1016/j.bbrc.2011.11.078

Hayes JD, Dinkova-Kostova AT, McMahon M (2009) Cross-talk between Transcription Factors AhR and Nrf2: Lessons for Cancer Chemoprevention from Dioxin. Toxicol Sci 111 :199-201. doi: 10.1093/toxsci/kfp168

Hertog MG, Feskens EJ, Hollman PC, et al (1993a) Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet 342:1007-1011. doi: 10.1016/0140-6736(93)92876-U

Hertog MG, Hollman PC, Katan MB, Kromhout D (1993b) Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. Nutr Cancer 20:21-9. doi: 10.1080/01635589309514267

Hertog MG, Kromhout D, Aravanis C, et al (1995) Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. Arch Intern Med 155:381-6.

Hess J, Angel P, Schorpp-Kistner M (2004) AP-1 subunits: quarrel and harmony among siblings. J Cell Sci 117:5965-5973.

Hirotsu Y, Katsuoka F, Funayama R, et al (2012) Nrf2-MafG heterodimers contribute globally to antioxidant and metabolic networks. Nucleic Acids Res 40:10228-39. doi: 10.1093/nar/gks827

Hollman PCH, Gaag MVD, Mengelers MJB, et al (1996) Absorption and disposition kinetics of the dietary antioxidant quercetin in man. Free Radic Biol Med 21:703-707. doi: 10.1016/0891-5849(96)00129-3

Hong H, Liu G-Q (2004) Scutellarin attenuates oxidative glutamate toxicity in PC12 cells. Planta Med 70:427-31. doi: 10.1055/s-2004-818970

Huang B-W, Ray PD, Iwasaki K, Tsuji Y (2013) Transcriptional regulation of the human ferritin gene by coordinated regulation of Nrf2 and protein

arginine methyltransferases PRMT1 and PRMT4. FASEB J 27:3763-74. doi: 10.1096/fj.12-226043

Ischiropoulos H, Beckman JS (2003) Oxidative stress and nitration in neurodegeneration: Cause, effect, or association? J. Clin. Invest. 111:163-169.

Ishige K, Schubert D, Sagara Y (2001) Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. Free Radic Biol Med 30:433-446. doi: 10.1016/S0891-5849(00)00498-6

Ishikawa Y, Sugiyama H, Stylianou E, Kitamura M (1999) Bioflavonoid quercetin inhibits interleukin-1-induced transcriptional expression of monocyte chemoattractant protein-1 in glomerular cells via suppression of nuclear factor-kappaB. J Am Soc Nephrol 10:2290-6.

Itoh K, Chiba T, Takahashi S, et al (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun 236:313-322. doi: 10.1006/bbrc.1997.6943

Itoh K, Wakabayashi N, Katoh Y, et al (1999) Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev 13:76-86. doi: 10.1101/gad.13.1.76

Jain AK, Jaiswal AK (2006) Phosphorylation of Tyrosine 568 Controls Nuclear Export of Nrf2. J Biol Chem 281 :12132-12142. doi: 10.1074/jbc.M511198200

Jain AK, Jaiswal AK (2007) GSK-3β Acts Upstream of Fyn Kinase in Regulation of Nuclear Export and Degradation of NF-E2 Related Factor 2. J Biol Chem 282 :16502-16510. doi: 10.1074/jbc.M611336200

Jaiswal AK (2004) Nrf2 signaling in coordinated activation of antioxidant gene expression. Free Radic. Biol. Med. 36:1199-1207.

Ji L-L, Sheng Y-C, Zheng Z-Y, et al (2015) The involvement of p62-Keap1-Nrf2 antioxidative signaling pathway and JNK in the protection of natural flavonoid quercetin against hepatotoxicity. Free Radic Biol Med 85:12-23. doi: 10.1016/j.freeradbiomed.2015.03.035

Johannot L, Somerset SM (2006) Age-related variations in flavonoid intake and sources in the Australian population. Public Health Nutr. doi:

10.1017/PHN2006971

Johnson DA, Johnson JA (2015) Nrf2-a therapeutic target for the treatment of neurodegenerative diseases. Free Radic Biol Med 88:253-267. doi: 10.1016/j.freeradbiomed.2015.07.147

Jones DP (2015) Redox theory of aging. Redox Biol 5:71-79. doi: 10.1016/j.redox.2015.03.004

Jones DP (2006) Redefining Oxidative Stress. Antioxid Redox Signal 8:1-16. doi: 10.1089/ars.2006.8.1865

Jones DP (2002) Redox potential of GSH/GSSG couple: Assay and biological significance. Methods Enzymol 348:93-112. doi: 10.1016/S0076-6879(02)48630-2

Jones DP, Go YM (2010) Redox compartmentalization and cellular stress. Diabetes Obes Metab 12 Suppl 2:116-125. doi: 10.1111/j.1463-1326.2010.01266.x

Joyeux M, Lobstein A, Anton R, Mortier F (1995) Comparative antilipoperoxidant, antinecrotic and scavenging properties of terpenes and biflavones from Ginkgo and some flavonoids. Planta Med 61:126-9. doi: 10.1055/s-2006-958030

Kang CH, Choi YH, Moon SK, et al (2013) Quercetin inhibits lipopolysaccharide-induced nitric oxide production in BV2 microglial cells by suppressing the NF-kappaB pathway and activating the Nrf2-dependent HO-1 pathway. Int Immunopharmacol 17:808-813. doi: 10.1016/j.intimp.2013.09.009

Kang JH, Chang SY, Jang HJ, et al (2009) Quercetin-induced upregulation of human GCLC gene is mediated by cis-regulatory element for early growth response protein-1 (EGR1) in INS-1 beta-cells. J Cell Biochem 108:1346-1355. doi: 10.1002/jcb.22365

Kang SS, Lee JY, Choi YK, et al (2004) Neuroprotective effects of flavones on hydrogen peroxide-induced apoptosis in SH-SY5Y neuroblostoma cells. Bioorg Med Chem Lett 14:2261-4. doi: 10.1016/j.bmcl.2004.02.003

Kaspar JW, Jaiswal AK (2010) An Autoregulatory Loop between Nrf2 and Cul3-Rbx1 Controls Their Cellular Abundance. J Biol Chem 285 :21349-21358. doi: 10.1074/jbc.M110.121863

Katsuoka F, Motohashi H, Ishii T, et al (2005) Genetic evidence that small maf proteins are essential for the activation of antioxidant response element-dependent genes. Mol Cell Biol 25:8044-51. doi: 10.1128/MCB.25.18.8044-8051.2005

Kaul TN, Middleton E, Ogra PL (1985) Antiviral effect of flavonoids on human viruses. J Med Virol 15:71-79. doi: 10.1002/jmv.1890150110

Kennedy DO (2014) Polyphenols and the human brain: plant «secondary metabolite» ecologic roles and endogenous signaling functions drive benefits. Adv Nutr 5:515-33.

Kensler TW, Wakabayashi N, Biswal S (2007) Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu Rev Pharmacol Toxicol 47:89-116. doi: 10.1146/annurev.pharmtox.46.120604.141046

Kim BH, Choi JS, Yi EH, et al (2013) Relative antioxidant activities of quercetin and its structurally related substances and their effects on NF-κB/CRE/AP-1 signaling in murine macrophages. Mol Cells 35:410-420. doi: 10.1007/s10059-013-0031-z

Kim SR, Park MJ, Lee MK, et al (2002) Flavonoids of Inula britannica protect cultured cortical cells from necrotic cell death induced by glutamate. Free Radic. Biol. Med. 32:596-604.

Kimira M, Arai Y, Shimoi K, Watanabe S (1998) Japanese intake of flavonoids and isoflavonoids from foods. J Epidemiol 8:168-175. doi: 10.2188/jea.8.168

Knekt P, Järvinen R, Seppänen R, et al (1997) Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. Am J Epidemiol 146:223-230. doi: 10.1093/oxfordjournals.aje.a009257

Knekt P, Kumpulainen J, Järvinen R, et al (2002) Flavonoid intake and risk of chronic diseases. Am J Clin Nutr 76:560-568.

Komatsu M, Kurokawa H, Waguri S, et al (2010) The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. Nat Cell Biol 12:213-223.

Kuriyama S, Shimazu T, Ohmori K, et al (2006) Green tea consumption and mortality due to cardiovascular disease, cancer, and all causes in Japan: the Ohsaki study. JAMA 296:1255-65. doi: 10.1001/jama.296.10.1255

Kwak M-K, Itoh K, Yamamoto M, Kensler TW (2002) Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: role of antioxidant response element-like sequences in the nrf2 promoter. Mol Cell Biol 22:2883-92. doi: 10.1128/MCB.22.9.2883-2892.2002

Kwak MK, Kensler TW (2006) Induction of 26S proteasome subunit PSMB5 by the bifunctional inducer 3-methylcholanthrene through the Nrf2-ARE, but not the AhR/Arnt-XRE, pathway. Biochem Biophys Res Commun 345:1350-1357. doi: 10.1016/j.bbrc.2006.05.043

Lacher SE, Lee JS, Wang X, et al (2015) Beyond antioxidant genes in the ancient NRF2 regulatory network. Free Radic Biol Med. doi: 10.1016/j.freeradbiomed.2015.06.044

Langley B, Ratan RR (2004) Oxidative stress-induced death in the nervous system: cell cycle dependent or independent? J Neurosci Res 77:621-9. doi: 10.1002/jnr.20210

Laughton MJ, Evans PJ, Moroney MA, et al (1991) Inhibition of mammalian 5-lipoxygenase and cyclo-oxygenase by flavonoids and phenolic dietary additives. Biochem Pharmacol 42:1673-1681. doi: 10.1016/0006-2952(91)90501-U

Lavoie S, Chen Y, Dalton TP, et al (2009) Curcumin, quercetin, and tBHQ modulate glutathione levels in astrocytes and neurons: Importance of the glutamate cysteine ligase modifier subunit. J Neurochem 108:1410-1422. doi: 10.1111/j.1471-4159.2009.05908.x

Lawrence T (2009) The Nuclear Factor NF-κB Pathway in Inflammation. Cold Spring Harb Perspect Biol 1:a001651. doi: 10.1101/cshperspect.a001651

Lee D-S, Ko W, Kim D-C, et al (2014) Cudarflavone B provides neuroprotection against glutamate-induced mouse hippocampal HT22 cell damage through the Nrf2 and PI3K/Akt signaling pathways. Molecules 19:10818-31. doi: 10.3390/molecules190810818

Lee J-M, Li J, Johnson DA, et al (2005) Nrf2, a multi-organ protector? FASEB J 19:1061-1066. doi: 10.1096/fj.04-2591hyp

Lee J-M, Shih AY, Murphy TH, Johnson JA (2003) NF-E2-related Factor-2 Mediates Neuroprotection against Mitochondrial Complex I Inhibitors and Increased Concentrations of Intracellular Calcium in Primary Cortical Neurons. J Biol Chem 278 :37948-37956. doi: 10.1074/jbc.M305204200

Lee KW, Kang NJ, Heo Y-S, et al (2008) Raf and MEK Protein Kinases Are Direct Molecular Targets for the Chemopreventive Effect of Quercetin, a Major Flavonol in Red Wine. Cancer Res 68 :946-955. doi: 10.1158/0008-5472.CAN-07-3140

Lee-Hilz YY, Boerboom a. MJF, Westphal AH, et al (2006) Pro-oxidant activity of flavonoids induces EpRE-mediated gene expression. Chem Res Toxicol 19:1499-1505. doi: 10.1021/tx060157q

Li F-J, Ji H-F, Shen L (2012) A meta-analysis of tea drinking and risk of Parkinson's disease. ScientificWorldJournal 2012:923464. doi: 10.1100/2012/923464

Limón-Pacheco J, Gonsebatt ME (2009) The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. Mutat Res - Genet Toxicol Environ Mutagen 674:137-147. doi: 10.1016/j.mrgentox.2008.09.015

Lin J, Zhang SM, Wu K, et al (2006) Flavonoid intake and colorectal cancer risk in men and women. Am J Epidemiol 164:644-51. doi: 10.1093/aje/kwj296

Lou H, Jing X, Ren D, et al (2012) Eriodictyol protects against H(2)O(2)-induced neuron-like PC12 cell death through activation of Nrf2/ARE signaling pathway. Neurochem Int 61:251-7. doi: 10.1016/j.neuint.2012.05.013

Lou H, Jing X, Wei X, et al (2014) Naringenin protects against 6-OHDA-induced neurotoxicity via activation of the Nrf2/ARE signaling pathway. Neuropharmacology 79:380-388. doi: 10.1016/j.neuropharm.2013.11.026

Macready AL, Kennedy OB, Ellis JA, et al (2009) Flavonoids and cognitive function: a review of human randomized controlled trial studies and recommendations for future studies. Genes Nutr 4:227-42. doi: 10.1007/s12263-009-0135-4

Mandel S a., Amit T, Weinreb O, et al (2008) Simultaneous manipulation of multiple brain targets by green tea catechins: A potential neuroprotective strategy for Alzheimer and Parkinson diseases. CNS Neurosci Ther 14:352-365. doi: 10.1111/j.1755-5949.2008.00060.x

Mandel S a., Avramovich-Tirosh Y, Reznichenko L, et al (2005) Multifunctional activities of green tea catechins in neuroprotection: Modulation of cell survival genes, iron-dependent oxidative stress and PKC signaling

pathway. NeuroSignals 14:46-60. doi: 10.1159/000085385

Mattson MP, Cheng A (2006) Neurohormetic phytochemicals: Low-dose toxins that induce adaptive neuronal stress responses. Trends Neurosci 29:632-9. doi: 10.1016/j.tins.2006.09.001

McBean GJ, Aslan M, Griffiths HR, Torrão RC (2015) Thiol redox homeostasis in neurodegenerative disease. Redox Biol 5:186-194. doi: 10.1016/j.redox.2015.04.004

Mercer LD, Kelly BL, Horne MK, Beart PM (2005) Dietary polyphenols protect dopamine neurons from oxidative insults and apoptosis: investigations in primary rat mesencephalic cultures. Biochem Pharmacol 69:339-45. doi: 10.1016/j.bcp.2004.09.018

Metodiewa D, Jaiswal AK, Cenas N, et al (1999) Quercetin may act as a cytotoxic prooxidant after its metabolic activation to semiquinone and quinoidal product. Free Radic Biol Med 26:107-16.

Middleton Jr. E, Kandaswami C, Theoharides TC (2000) The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev 52:673-751.

Minato KI, Miyake Y, Fukumoto S, et al (2003) Lemon flavonoid, eriocitrin, suppresses exercise-induced oxidative damage in rat liver. Life Sci 72:1609-1616. doi: 10.1016/S0024-3205(02)02443-8

Miseta a, Csutora P (2000) Relationship between the occurrence of cysteine in proteins and the complexity of organisms. Mol Biol Evol 17:1232-1239. doi: 10.1093/oxfordjournals.molbev.a026406

Moi P, Chan K, Asunis I, et al (1994) Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. Proc Natl Acad Sci U S A 91:9926-9930. doi: 10.1073/pnas.91.21.9926

Moosmann B, Behl C (2002) Antioxidants as treatment for neurodegenerative disorders. Expert Opin Investig Drugs 11:1407-1435. doi: 10.1517/13543784.11.10.1407

Moskaug JO, Carlsen H, Myhrstad MC, Blomhoff R (2005) Polyphenols and glutathione synthesis regulation. Am J Clin Nutr 81:277-283.

Mota SI, Costa RO, Ferreira IL, et al (2015) Oxidative stress involving changes

in Nrf2 and ER stress in early stages of Alzheimer's disease. Biochim Biophys Acta 1852:1428-41. doi: 10.1016/j.bbadis.2015.03.015

Myhrstad MCW, Carlsen H, Nordström O, et al (2002) Flavonoids increase the intracellular glutathione level by transactivation of the γ-glutamylcysteine synthetase catalytical subunit promoter. Free Radic Biol Med 32:386-393. doi: 10.1016/S0891-5849(01)00812-7

Nagasaka Y, Nakamura K (1998) Modulation of the heat-induced activation of mitogen-activated protein (MAP) kinase by quercetin. Biochem Pharmacol 56:1151-5.

Nguyen T, Huang HC, Pickett CB (2000) Transcriptional regulation of the antioxidant response element. Activation by Nrf2 and repression by MafK. J Biol Chem 275:15466-15473. doi: 10.1074/jbc.M000361200

Nguyen T, Sherratt PJ, Pickett CB (2003) Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. Annu Rev Pharmacol Toxicol 43:233-260. doi: 10.1146/annurev.pharmtox.43.100901.140229

Niture SK, Jaiswal AK (2009) Prothymosin-a Mediates Nuclear Import of the INrf2/Cul3·Rbx1 Complex to Degrade Nuclear Nrf2. J Biol Chem 284 :13856-13868.

Niture SK, Jaiswal AK (2012) Nrf2 Protein Up-regulates Antiapoptotic Protein Bcl-2 and Prevents Cellular Apoptosis. J Biol Chem 287 :9873-9886. doi: 10.1074/jbc.M111.312694

Novo E, Parola M (2008) Redox mechanisms in hepatic chronic wound healing and fibrogenesis. Fibrogenesis Tissue Repair 1:5. doi: 10.1186/1755-1536-1-5

Nurk E, Refsum H, Drevon CA, et al (2009) Intake of Flavonoid-Rich Wine , Tea , and Chocolate by Elderly Men and Women Is Associated with Better Cognitive Test Performance. J Nutr 139:120-127. doi: 10.3945/jn.108.095182

Olesen J, Baker MG, Freund T, et al (2006) Consensus document on European brain research. J Neurol Neurosurg Psychiatry 77:i1-i49.

Piantadosi CA, Withers CM, Bartz RR, et al (2011) Heme Oxygenase-1 Couples Activation of Mitochondrial Biogenesis to Anti-inflammatory Cytokine Expression. J Biol Chem 286 :16374-16385. doi: 10.1074/jbc.M110.207738

Pollastri S, Tattini M (2011) Flavonols: Old compounds for old roles. Ann Bot 108:1225-1233. doi: 10.1093/aob/mcr234

Powis G, Montfort WR (2001) Properties and biological activities of thioredoxins. Annu Rev Pharmacol Toxicol 41:261-95. doi: 10.1146/annurev.pharmtox.41.1.261

Procházková D, Boušová I, Wilhelmová N (2011) Antioxidant and prooxidant properties of flavonoids. Fitoterapia 82:513-523. doi: 10.1016/j.fitote.2011.01.018

Pu F, Mishima K, Irie K, et al (2007) Neuroprotective effects of quercetin and rutin on spatial memory impairment in an 8-arm radial maze task and neuronal death induced by repeated cerebral ischemia in rats. J Pharmacol Sci 104:329-334. doi: 10.1254/jphs.FP0070247

Qin S, Deng F, Wu W, et al (2014) Baicalein modulates Nrf2/Keap1 system in both Keap1-dependent and Keap1-independent mechanisms. Arch Biochem Biophys 559:1-9. doi: 10.1016/j.abb.2014.03.011

Rada P, Rojo AI, Chowdhry S, et al (2011) SCF/β-TrCP Promotes Glycogen Synthase Kinase 3-Dependent Degradation of the Nrf2 Transcription Factor in a Keap1-Independent Manner . Mol Cell Biol 31:1121-1133. doi: 10.1128/MCB.01204-10

Ratty AK, Sunamoto J, Das NP (1988) Interaction of flavonoids with 1,1-diphenyl-2-picrylhydrazyl free radical, liposomal membranes and soybean lipoxygenase-1. Biochem Pharmacol 37:989-995. doi: 10.1016/0006-2952(88)90499-6

Rice-Evans C (2001) Flavonoid antioxidants. Curr Med Chem 8:797-807. doi: 10.2174/0929867013373011

Rice-Evans CA, Miller NJ, Paganga G (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic. Biol. Med. 20:933-956.

Rietjens IMCM, Boersma MG, Van Der Woude H, et al (2005) Flavonoids and alkenylbenzenes: Mechanisms of mutagenic action and carcinogenic risk. Mutat Res - Fundam Mol Mech Mutagen 574:124-138. doi: 10.1016/j.mrfmmm.2005.01.028

Rimm EB, Katan MB, Ascherio A, et al (1996) Relation between Intake of Flavonoids and Risk for Coronary Heart Disease in Male Health

Professionals. Ann Intern Med 125:384-389. doi: 10.7326/0003-4819-125-5-199609010-00005

Rivera F, Costa G, Abin A, et al (2008) Reduction of ischemic brain damage and increase of glutathione by a liposomal preparation of quercetin in permanent focal ischemia in rats. Neurotox Res 13:105-14.

Rodgers EH, Grant MH (1998) The effect of the flavonoids, quercetin, myricetin and epicatechin on the growth and enzyme activities of MCF7 human breast cancer cells. Chem Biol Interact 116:213-228. doi: 10.1016/S0009-2797(98)00092-1

Rodriguez-Mateos A, Vauzour D, Krueger CG, et al (2014) Bioavailability, bioactivity and impact on health of dietary flavonoids and related compounds: an update. Arch Toxicol 88:1803-53. doi: 10.1007/s00204-014-1330-7

Rushmore TH, Morton MR, Pickett CB (1991) The antioxidant responsive element: Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. J Biol Chem 266:11632-11639.

Rushworth SA, Zaitseva L, Murray MY, et al (2012) The high Nrf2 expression in human acute myeloid leukemia is driven by NF-kappaB and underlies its chemo-resistance. Blood 120:5188-5198. doi: 10.1182/blood-2012-04-422121; 10.1182/blood-2012-04-422121

Sääksjärvi K, Knekt P, Lundqvist A, et al (2013) A cohort study on diet and the risk of Parkinson's disease: the role of food groups and diet quality. Br J Nutr 109:329-37. doi: 10.1017/S0007114512000955

Sakata H, Niizuma K, Yoshioka H, et al (2012) Minocycline-Preconditioned Neural Stem Cells Enhance Neuroprotection after Ischemic Stroke in Rats. J Neurosci 32 :3462-3473. doi: 10.1523/JNEUROSCI.5686-11.2012

Salazar M, Rojo AI, Velasco D, et al (2006) Glycogen Synthase Kinase-3β Inhibits the Xenobiotic and Antioxidant Cell Response by Direct Phosphorylation and Nuclear Exclusion of the Transcription Factor Nrf2. J Biol Chem 281 :14841-14851. doi: 10.1074/jbc.M513737200

Sandberg M, Patil J, D'Angelo B, et al (2014) NRF2-regulation in brain health and disease: Implication of cerebral inflammation. Neuropharmacology 79:298-306. doi: 10.1016/j.neuropharm.2013.11.004

Sarlette A, Krampfl K, Grothe C, et al (2008) Nuclear erythroid 2-related factor 2-antioxidative response element signaling pathway in motor cortex and spinal cord in amyotrophic lateral sclerosis. J Neuropathol Exp Neurol 67:1055-62. doi: 10.1097/NEN.0b013e31818b4906

Saw CLL, Guo Y, Yang AY, et al (2014) The berry constituents quercetin, kaempferol, and pterostilbene synergistically attenuate reactive oxygen species: involvement of the Nrf2-ARE signaling pathway. Food Chem Toxicol 72:303-11. doi: 10.1016/j.fct.2014.07.038

Scalbert A, Williamson G (2000) Dietary Intake and Bioavailability of Polyphenols. J Med Food 3:121-125. doi: 10.1089/109662000416311

Schroeter H, Boyd C, Spencer JPE, et al (2002) MAPK signaling in neurodegeneration: Influences of flavonoids and of nitric oxide. Neurobiol Aging 23:861-880. doi: 10.1016/S0197-4580(02)00075-1

Shah ZA, Li R, Ahmad AS, et al (2014) The flavanol (−)-epicatechin prevents stroke damage through the Nrf2/HO1 pathway. J Cereb Blood Flow Metab 34:735. doi: 10.1038/jcbfm.2014.10

Sharma V, Mishra M, Ghosh S, et al (2007) Modulation of interleukin-1beta mediated inflammatory response in human astrocytes by flavonoids: implications in neuroprotection. Brain Res Bull 73:55-63. doi: 10.1016/j.brainresbull.2007.01.016

Shi Y, Liang X, Zhang H, et al (2013) Quercetin protects rat dorsal root ganglion neurons against high glucose-induced injury in vitro through Nrf-2/HO-1 activation and NF-[kappa]B inhibition. Acta Pharmacol Sin 34:1140-1148.

Shih AY, Johnson D a, Wong G, et al (2003) Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress. J Neurosci 23:3394-3406. doi: 23/8/3394 [pii]

Shimizu M, Weinstein IB (2005) Modulation of signal transduction by tea catechins and related phytochemicals. Mutat Res 591:147-60. doi: 10.1016/j.mrfmmm.2005.04.010

Sies H (2014) Role of metabolic H2O2 generation: redox signaling and oxidative stress. J Biol Chem 289:8735-41. doi: 10.1074/jbc.R113.544635

Sies H (2015) Oxidative stress: a concept in redox biology and medicine.

Redox Biol 4C:180-183. doi: 10.1016/j.redox.2015.01.002

Sies H, Jones D (2007) Oxidative Stress.

Silva MM, Santos MR, Caroco G, et al (2002) Structure-antioxidant activity relationships of flavonoids: A re-examination. Free Radic. Res. 36:1219-1227.

Simonian N a, Coyle JT (1996) Oxidative stress in neurodegenerative diseases. Annu Rev Pharmacol Toxicol 36:83-106. doi: 10.1146/annurev.pa.36.040196.000503

Spencer JPE (2010) The impact of fruit flavonoids on memory and cognition. Br J Nutr 104 Suppl :S40-S47. doi: 10.1017/S0007114510003934

Spencer JPE (2009) Flavonoids and brain health: Multiple effects underpinned by common mechanisms. Genes Nutr. 4:243-250.

Suganya N, Bhakkiyalakshmi E, Suriyanarayanan S, et al (2014) Quercetin ameliorates tunicamycin-induced endoplasmic reticulum stress in endothelial cells. Cell Prolif 47:231-240. doi: 10.1111/cpr.12102

Suzuki YJ, Forman HJ, Sevanian A (1997) Oxidants as stimulators of signal transduction. Free Radic Biol Med 22:269-285. doi: Doi 10.1016/S0891-5849(96)00275-4

Sykiotis GP, Bohmann D (2010) Stress-activated cap'n'collar transcription factors in aging and human disease. Sci Signal 3:re3. doi: 10.1126/scisignal.3112re3

Tan EK, Tan C, Fook-Chong SMC, et al (2003) Dose-dependent protective effect of coffee, tea, and smoking in Parkinson's disease: A study in ethnic Chinese. J Neurol Sci 216:163-167. doi: 10.1016/j.jns.2003.07.006

Tanigawa S, Fujii M, Hou DX (2007) Action of Nrf2 and Keap1 in ARE-mediated NQO1 expression by quercetin. Free Radic Biol Med 42:1690-1703. doi: 10.1016/j.freeradbiomed.2007.02.017

Tebay LE, Robertson H, Durant ST, et al (2015) Mechanisms of activation of the transcription factor Nrf2 by redox stressors, nutrient cues and energy status, and pathways through which it attenuates degenerative disease. Free Radic Biol Med. doi: 10.1016/j.freeradbiomed.2015.06.021

Torel J, Cillard J, Cillard P (1986) Antioxidant activity of flavonoids and reactivity with peroxy radical. Phytochemistry 25:383-385. doi:

10.1016/S0031-9422(00)85485-0

Trachootham D, Lu W, Ogasawara M a, et al (2008) Redox regulation of cell survival. Antioxid Redox Signal 10:1343-1374. doi: 10.1089/ars.2007.1957

Vargas MR, Johnson DA, Sirkis DW, et al (2008) Nrf2 Activation in Astrocytes Protects against Neurodegeneration in Mouse Models of Familial Amyotrophic Lateral Sclerosis. J Neurosci 28 :13574-13581. doi: 10.1523/JNEUROSCI.4099-08.2008

Villeneuve NF, Lau A, Zhang DD (2010) Regulation of the Nrf2–Keap1 Antioxidant Response by the Ubiquitin Proteasome System: An Insight into Cullin-Ring Ubiquitin Ligases. Antioxid Redox Signal 13:1699-1712. doi: 10.1089/ars.2010.3211

von Otter M, Landgren S, Nilsson S, et al (2010) Association of Nrf2-encoding NFE2L2 haplotypes with Parkinson's disease. BMC Med Genet 11:36. doi: 10.1186/1471-2350-11-36

Wakabayashi N, Slocum SL, Skoko JJ, et al (2010) When NRF2 talks, who's listening? Antioxid Redox Signal 13:1649-1663. doi: 10.1089/ars.2010.3216

Walker EH, Pacold ME, Perisic O, et al (2000) Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporine. Mol Cell 6:909-19. doi: 10.1016/S1097-2765(05)00089-4

Walle T (2009) Methylation of dietary flavones increases their metabolic stability and chemopreventive effects. Int J Mol Sci 10:5002-19. doi: 10.3390/ijms10115002

Wang CN, Chi CW, Lin YL, et al (2001) The Neuroprotective Effects of Phytoestrogens on Amyloid β Protein-induced Toxicity Are Mediated by Abrogating the Activation of Caspase Cascade in Rat Cortical Neurons. J Biol Chem 276:5287-5295. doi: 10.1074/jbc.M006406200

Wang X, Michaelis EK (2010) Selective neuronal vulnerability to oxidative stress in the brain. Front Aging Neurosci 2:12. doi: 10.3389/fnagi.2010.00012

Wang X, Wolkoff AW, Morris ME (2005) Flavonoids as a novel class of human organic anion-transporting polypeptide OATP1B1 (OATP-C) modulators. Drug Metab Dispos 33:1666-1672. doi: 10.1124/dmd.105.005926

Williams RJ, Spencer JPE (2012) Flavonoids, cognition, and dementia: Actions,

mechanisms, and potential therapeutic utility for Alzheimer disease. Free Radic. Biol. Med. 52:35-45.

World Health Organization (2008) 2008-2013 Action Plan for the Global Strategy for the Prevention and Control of Noncommunicable Diseases.

Xu J, Wang H, Ding K, et al (2014) Luteolin provides neuroprotection in models of traumatic brain injury via the Nrf2-ARE pathway. Free Radic Biol Med 71:186-95. doi: 10.1016/j.freeradbiomed.2014.03.009

Zhang DD (2006) Mechanistic studies of the Nrf2-Keap1 signaling pathway. Drug Metab Rev 38:769-789. doi: 10.1080/03602530600971974

Zhang M, An C, Gao Y, et al (2013) Emerging roles of Nrf2 and phase II antioxidant enzymes in neuroprotection. Prog. Neurobiol. 100:30-47.

ANEXO I: PUBLICACIONES CIENTÍFICAS VINCULADAS A LA TESIS

-**Arredondo F**, Echeverry C, Blasina F, Vaamonde L, Díaz M, Rivera F, Martinez M, Abin-Carriquiry JA, Dajas F. Flavones and flavonols in brain and disease: facts and pitfalls. Libro: *Bioactive Nutraceuticals and Dietary Supplements in Neurological and Brain Disease: Prevention and therapy*, 2015, 25:229-23. Organizadores: Ronald Ross Watson and Victor Preedy (Publicación 1).

-Echeverry C, **Arredondo F**, Martinez M, Abin-Carriquiry JA, Midiwo F, Dajas F C. Antioxidant activity, cellular bioavailability, iron and calcium management of neuroprotective and non-neuroprotective flavones. *Neurotoxicity Research*, 2015, 27:31-42 (Publicación 2).

-Dajas F, Abin-Carriquiry JA, **Arredondo F**, Blasina F, Echeverry C, Martinez M, Rivera F, Vaamonde L. Quercetin in brain diseases: Potencial and limits. *Neurochemistry International*, 2015, 89: 140–148 (Publicación 3).

-Carvalho D, Paulino M, Polticelli F, **Arredondo F**, Abin-Carriquiry JA. Quercetin Protein Targets Identification by Reverse Virtual Screening. *Journal of Molecular Modeling,* 2015, enviado (Publicación 4)

-Dajas F, Abin-Carriquiry JA, **Arredondo F**, Echeverry E, Rivera F. Neuroprotective Actions of Flavones and Flavonols: Mechanisms and Relationship to Flavonoid Structural Features. *Current Medicinal Chemistry - Central Nervous System Agents*, 2013, 13:30-35 (Publicación 5).

-Dajas F, JA Abin-Carriquiry, **Arredondo F**, Blasina F, Diaz M, Echeverry E, Rivera F, Vaamonde L. The Neuroprotective Capacity of *Achyrocline*

satureioides (Lam) D.C. and its flavonoids. Mechanisms of action. Libro: *Herbal Medicines For Human Health: Development and Evaluation of Plant-Derived Medicines*, 2011, 19: 339 - Organizadores: Eds G. Bagetta, M.T. Corasaniti , M. Cosentino, S. Sakurada (Publicación 6).

ANEXO I: PUBLICACIONES CIENTÍFICAS VINCULADAS A LA TESIS

-**Arredondo F**, Echeverry C, Blasina F, Vaamonde L, Díaz M, Rivera F, Martinez M, Abin-Carriquiry JA, Dajas F. Flavones and flavonols in brain and disease: facts and pitfalls. Libro: *Bioactive Nutraceuticals and Dietary Supplements in Neurological and Brain Disease: Prevention and therapy*, 2015, 25:229-23. Organizadores: Ronald Ross Watson and Victor Preedy (Publicación 1).

-Echeverry C, **Arredondo F**, Martinez M, Abin-Carriquiry JA, Midiwo F, Dajas F C. Antioxidant activity, cellular bioavailability, iron and calcium management of neuroprotective and non-neuroprotective flavones. *Neurotoxicity Research*, 2015, 27:31-42 (Publicación 2).

-Dajas F, Abin-Carriquiry JA, **Arredondo F**, Blasina F, Echeverry C, Martinez M, Rivera F, Vaamonde L. Quercetin in brain diseases: Potencial and limits. *Neurochemistry International*, 2015, 89: 140–148 (Publicación 3).

-Carvalho D, Paulino M, Polticelli F, **Arredondo F**, Abin-Carriquiry JA. Quercetin Protein Targets Identification by Reverse Virtual Screening. *Journal of Molecular Modeling,* 2015, enviado (Publicación 4)

-Dajas F, Abin-Carriquiry JA, **Arredondo F**, Echeverry E, Rivera F. Neuroprotective Actions of Flavones and Flavonols: Mechanisms and Relationship to Flavonoid Structural Features. *Current Medicinal Chemistry - Central Nervous System Agents*, 2013, 13:30-35 (Publicación 5).

-Dajas F, JA Abin-Carriquiry, **Arredondo F**, Blasina F, Diaz M, Echeverry E, Rivera F, Vaamonde L. The Neuroprotective Capacity of *Achyrocline*

satureioides (Lam) D.C. and its flavonoids. Mechanisms of action. Libro: *Herbal Medicines For Human Health: Development and Evaluation of Plant-Derived Medicines*, 2011, 19: 339 - Organizadores: Eds G. Bagetta, M.T. Corasaniti , M. Cosentino, S. Sakurada (Publicación 6).

AUTHOR QUERY FORM

ELSEVIER	**Book: WATSON-9780124114623** **Chapter: 25**	**Please e-mail your responses and any corrections to:** **E-mail: Ca.Johnson@elsevier.com**

Dear Author,

Any queries or remarks that have arisen during the processing of your manuscript are listed below and are highlighted by flags in the proof. (AU indicates author queries; ED indicates editor queries; and TS/TY indicates typesetter queries.) Please check your proof carefully and answer all AU queries. Mark all corrections and query answers at the appropriate place in the proof (e.g., by using on-screen annotations in the PDF file http://www.elsevier.com/book-authors/science-and-technology-book-publishing/overview-of-the-publishing-process) or compile them in a separate list, and tick off below to indicate that you have answered the query.

Please return your input as instructed by the project manager.

Location in article	**Query / remark**	
AU1, page 3	Is this the correct word here?	☐
AU2, page 7	As few of references are not in alphabetical order, we have reordered the references. Please check and confirm if it is fine	☐
AU3, page 2	We have shortend the running footer. Kindly confirm.	☐

CHAPTER

25

c0025

Flavones and Flavonols in Brain and Disease

Facts and Pitfalls

Florencia Arredondo, Carolina Echeverry, Fernanda Blasina, Lucía Vaamonde, Marcela Díaz, Felicia Rivera, Marcela Martínez, Juan Andres Abin-Carriquiry, Federico Dajas

Department of Neurochemistry, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay

OUTLINE

s0010 p0010

INTRODUCTION

s0015

BRAIN PATHOLOGY: OXIDATIVE STRESS AND INFLAMMATION

p0015 Brain pathology is a leading cause of death all over the world, with an incidence of about 2 in 1000 and an 8% total death rate (Kolominsky-Rabas et al., 1998; Samsa et al., 1999). Acute brain injury results from the combined effects of cellular energy failure, acidosis, glutamate release, intracellular calcium accumulation, lipid peroxidation, and nitric oxide neurotoxicity that serve to disrupt essential components of the cell, resulting in death (Halliwell & Gutteridge, 1999; Bains & Shaw, 1997). The formation of reactive oxygen and nitrogen species (ROS and NOS) in brain tissues (Siesjo, 1992) can activate diverse downstream signaling pathways, including the transcription factor nuclear factor kappa B (NF-κB), regulating the expression of genes encoding a variety of proinflammatory proteins, such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS; Clemens, 2000; József & Filep, 2003). Oxidative stress and inflammatory processes may also explain the high incidence of degenerative (Parkinson's and Alzheimer's diseases) and acute (cerebrovascular attacks) neuropathology in countries with aging populations (Joseph et al., 1999).

p0020 If during adulthood there are diverse neurological pathologies related to ischemia and neurodegeneration, then the brain of the newborn is particularly vulnerable to oxygen deprivation and ischemia. Neonatal encephalopathy due to hypoxia-ischemia occurs in one to six per 1000 live term births in developed countries. Approximately 15–20% of affected newborns die in the postnatal period, and 25% of survivors develop severe childhood disabilities. Until recently, management of hypoxic ischemic encephalopathy (HIE) has been limited to supportive intensive care (Olsen et al.,

http://dx.doi.org/10.1016/B978-0-12-411462-3.00025-4

10025-WATSON-9780124114623

2013). Early interventions to minimize brain damage are critical, particularly to modify cell death resulting from secondary collapse of neuronal energy. Immediate resuscitation to restore oxygen supply and blood circulation aims to limit the extent of this damage. The mechanisms believed to be important in this process include oxidative stress and high intracellular calcium leading to apoptosis (McGuire, 2007).

p0025 Despite intensive research aimed at developing neuroprotective treatments for brain injury, no drugs have been proven successful in advanced clinical trials (Heim et al., 2002), although some studies demonstrate that a number of antioxidants can reduce ROS-mediated reactions and rescue neurons from cerebral ischemic damage in animal models (Clemens, 1991). It has also been reported that the use of anti-inflammatory compounds has great potential as a therapeutic strategy for neuroprotection following ischemic brain injury (Barone & Feuerstein, 1999).

p0030 Epidemiological evidence has called attention to natural compounds with antioxidant and anti-inflammatory properties as potential neuroprotective molecules for brain pathology. In this sense, polyphenols show a broad spectrum of biological functions, such as antioxidative, antibacterial, antitumoral, antiviral, anti-inflammatory, and cardiovascular protection activities (Higdon & Frei, 2003; Mukhtar & Ahmad, 2000), and they are a group of molecules that deserve to be studied in their neuroprotective potential.

s0020 FLAVONES AND FLAVONOLS: CHEMICAL AND STRUCTURAL FEACTURES

p0035 Flavonoids are diphenylpropanes commonly occurring in plants, vegetables, seeds, tea, wine, and various plant products that are frequently components of the human diet. Flavonoids are synthesized as secondary metabolic products of most plants. These compounds are partially responsible for imparting color, flavor, and texture to fruits, vegetables, tea, coffee, and wine (Middleton et al., 2000; Schroeter et al., 2002). They are also capable of regulating growth, differentiation, and/or cell death in bacteria, fungi, plants, and animals (Formica & Regelson, 1995; Harborne & Williams, 2000).

p0040 From a structural point of view, flavonoids have a common structure consisting of two benzene rings linked by a chain of three carbon atoms (C6C3C6) and are categorized according to their chemical structure into monomeric flavanols, flavanones, anthocyanidins, flavones, and flavonols (Rice-Evans et al., 1996). Individual differences within each group result from the variation in number and arrangement of the hydroxyl groups as well as from the nature and extent of alkylation and/or glycosylation. The preferred glycosylation sites on the structure of flavonoids are the three position and, less frequently, the seven position. Glucose is the most usual sugar residue, but others may include galactose, rhamnose, and xylose. The flavonols and to a lesser extent flavones are the most commonly encountered, being ubiquitous within the families and genera of the higher plants (Harborne & Mabry, 1975; Herrmann, 1989; Kühnau, 1976). The most commonly occurring flavones and flavonols are those with dihydroxylation in the 3′ and 4′ positions of the B ring and, to a lesser extent, those with a lone B ring-hydroxyl group in the 4′ position. Flavones are characterized by having a planar structure due to the double bond in the central aromatic ring.

BRAIN EFFECTS OF FLAVONES AND FLAVONOLS: *IN VIVO* EVIDENCE s0025

p0045 Currently there has been great interest in the potential of flavonoids, among other polyphenols, to modulate neuronal function and prevent neurodegeneration. The use of these compounds in humans and in animal dietary supplementation studies has shown improvements in cognitive function possibly by protecting vulnerable neurons or enhancing existing neuronal function (Youdim & Joseph, 2001).

p0050 Numerous experimental studies *in vivo* describe the beneficial effects of flavones (e.g., luteolin, apigenin, jaceosidin, and eupatilin) and flavonols (e.g., myricetin, kaempferol, and quercetin) in diseases such as brain ischemia, Parkinson's disease (PD), and Alzheimer's disease (AD; Dajas et al., 2003; Simonyi et al., 2005). Experimental evidence has shown that flavones such as luteolin reduce neuroinflammation (Nam et al., 2013) and provide protection in the transient global ischemia model in mice (Cai et al., 2012). Kaempferol is one of the most studied flavonoid molecules, and preclinical studies have shown that this flavonol and some of its glucosides have a wide range of pharmacological activities, including antioxidant, anti-inflammatory, and neuroprotection in cerebral ischemia (Calderón-Montaño et al., 2011; López-Sánchez et al., 2007), PD (Li & Pu, 2011), and AD (Kim et al., 2010) experimental models. Kaempferol could prevent the loss of dopaminergic neurons induced by the neurotoxin 1-methyl1-4-phenyl1-1, 2, 3, 6-tetrahydropyridine (MPTP) in a mouse model of PD (Li & Pu 2011). Another well-known flavone, baicalein, has been shown to reduce the production of oxygen peroxide (H2O2), preventing 6-Hydroxydopamine (6-OHDA)-induced dopaminergic dysfunction in *in vitro* and *in vivo* experimental models of PD (Yin et al., 2011). This flavone also exerts neuroprotective

actions against MPTP-induced damage in mice (Yu et al., 2012).

p0055 Quercetin has been undoubtedly the most extensively studied flavonol for its neuroprotective potential *in vivo*, and recent experimental evidence confirms the neuroprotective effects of quercetin against cerebral ischemia (Ahmad et al., 2011). Analytical studies have shown that quercetin metabolites accumulated in several tissues in pigs and rats after oral administration (Halliwell & Gutteridge, 1999; Bains & Shaw, 1997), making unclear whether biological activity is directly due to quercetin itself. In particular, there is controversy over the activity of flavonoids in the brain, because the blood–brain barrier (BBB) makes it difficult for them to enter the brain. Therefore, strategies developed to improve brain bioavailability of flavonoids include the delivery through liposomal particles that facilitate the crossing of the BBB (Dajas et al., 2013; Rivera et al., 2008). In this regard, the time course of the effects of liposomal quercetin (LQ) has been followed in experimental focal ischemia in rats. Using a tetrazolium salt and a spectrophotometric method, it was shown that when the LQ preparation was administered 30 minutes, one hour, and four hours after ischemia the optical density of the formazan increased in the striatum as an indication of the metabolic tissue recovery only at the earlier times (30 min and 1 hr). In the same direction, spectrophotometric values obtained in the cortex after LQ treatments increased only 30 minutes after focal ischemia. Four hours after the ischemic insult, the LQ treatment did not change the protective parameters analyzed in the experimental conditions (Rivera et al., 2008). Similar results were obtained with behavioral scores. According to these results, the LQ treatment showed a reduced therapeutic window in agreement with other experimental therapeutic approaches. It is likely that the highly oxidative and inflammatory brain microenvironment generated one to four hours post-ischemia consuming available antioxidants breaks the continuity of antioxidative chains, triggering the pro-oxidant effects of quercetin.

p0060 In the case of PD experimental models, the results obtained with quercetin in relation to increased neuronal survival against oxidative insult have been contradictory (Kääriäinen et al., 2008), and there are some differences between the results obtained using *in vitro* and *in vivo* models. Thus studies in PC12 cells and primary cultures of rat cortical neurons showed neuroprotective effects after oxidative damage by H2O2 or 6-OHDA (Arredondo et al., 2004; Dok-Go et al., 2003). Otherwise, and in contrast with what is described in the case of ischemia, the results for experimental Parkinsonism related to quercetin are mainly negative regarding neuroprotection (Dajas et al., 2003; Ossola et al., 2009; Yokoyama et al., 2011). Quercetin negative effects have been explained by some toxicity to neurons (Ossola et al., 2008). This dual pharmacological profile of quercetin in cerebrovascular and neurodegenerative models is most likely the explanation for results recently obtained in a PD experimental model: Tissue levels of malondialdehyde in the substantia nigra (SN) increased significantly in the injured side 48 hours after a 6-OHDA lesion and were reverted by treatment with nanosomal quercetin one hour or 24 hours after injury. In contrast, a parenteral chronic treatment with quercetin did not increase the survival of TH positive neurons in the SN, which was 6.5% lower than in animals treated with an acute injection (Diaz, personal communication). These results could be an indication of the referred antioxidant or prooxidant profile of quercetin at different points of the pathological process, depending on the cellular microenvironment conditions, the concentration at the site of action, and the levels and duration of the oxidative process. In this sense, although the antioxidant effectiveness of the flavonoids *in vivo* has been extensively documented, some prooxidant actions have been described for these compounds (Yen et al., 2003). Therefore, further research is needed before they can be utilized as safe and effective antioxidant therapeutics.

p0065 In the case of the neonatal encephalopathy due to hypoxia-ischemia, in spite of the intensive research, the therapeutic arsenal available to help those infants at high risk of developing a neuropathological lesion is limited, and thus even induced hypothermia, a therapeutic tool widely utilized, shows limited beneficial outcomes in the long term (six or seven years; Shankaran et al., 2012). The brain is the most affected system, and neuropathological lesions can lead to squeal. Peripheral hemodynamic changes and multiorgan dysfunction (e.g., arterial blood pressure, heart rate, blood pH, etc.) can deepen the severity of the systemic pathology and sometimes become the cause of death during the acute period of the illness. Multiorgan failure, including hypotension, perfusion deficit, pulmonary hypertension, vasculopathic enterocolitis, renal failure, and thrombo-embolic complications, could give more complexity to the pathology (Armstrong et al., 2012). [AU1]

p0070 The beneficial effects of quercetin as antioxidant, anti-inflammatory, and modulator of survival proteins gene expression make it a potential alternative to treat HIE. Undoubtedly, the pathological brain process in neonatal asphyxia is a global and complex one in which appropriate bioavailability of pharmacological agents at the neuronal interface is critical. Drug nanosomal delivery systems for adequate and safe brain delivery are being increasingly utilized to

f0010

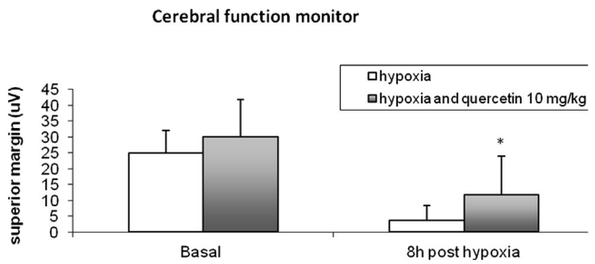


FIGURE 25.1 Superior margin of the monitorized amplitude integrated electroencephalography at the beginning and after eight hours of the experimental period. Compared with the hypoxic animals, those animals receiving one dose of quercetin liposomal preparation after the hypoxic episode improved the encephalographic voltage (*=p>0.05).

minimize this limitation (Mayank Gandhi, 2010), and it is known that frequently used glycolated preparations are not well tolerated by the cardiovascular system (Doenicke et al., 1994), specially during childhood (Zar et al., 2007).

p0075 In this context, a drug-delivery system for quercetin adequate for intravenous administration in the rat and piglet has been developed, improving several preparations already being studied (Priprem et al., 2008). The nanosomal preparation of quercetin stabilized key hemodynamic parameters altered during asphyxia in newborn piglets in the period following reanimation after a severe hypoxic episode. This is a key effect because the quality of survival could be related to initial improvement of these hemodynamic changes (Liu et al., 2007). More interestingly, the endovenous administration of one dose of 10 mg/kg of quercetin preparation induces an improvement in the brain voltage as evaluated by the monitor of cerebral function (amplitude-integrated EEG) eight hours after a severe hypoxic episode, as shown in Figure 25.1.

s0030

WORKING MECHANISMS UNDERLYING THE BRAIN EFFECTS OF FLAVONES AND FLAVONOLS: *IN VITRO* EVIDENCE

p0080 As described earlier, a substantial amount of evidence supports the diverse effects of flavones and flavonols within the brain, including the suppression of neuroinflammation, the promotion of neuronal differentiation, the protection of neurons against injury induced by neurotoxins, and the improvement in memory and learning functions. This wide range of effects appears to involve mechanisms that could be grouped into antioxidant and non-antioxidant properties. Both mechanisms may finally converge in the modulation of common intracellular-signaling pathways, leading to cell survival among other beneficial effects (Spencer, 2009; Dajas et al., 2013; Figure 25.2).

Antioxidant Properties s0035

p0085 Traditionally, the beneficial effects of these compounds have been attributed mainly to their antioxidant capacity, including their direct free radical scavenger and their metal-chelating activity properties. Additionally, they show broader indirect antioxidant effects through their capacity to interact and modulate antioxidant enzyme activities (Nijveldt et al., 2001). More recently, evidence from cell studies suggests that flavones and flavonols can influence antioxidant gene expression by modulation of redox-sensitive transcription factors, such as nuclear factor κB (NFκB) and NF-E2-related factor-2 (Nrf2). These mechanisms result in the induction of genes encoding for prosurvival, detoxifying, and antioxidant proteins (Seelinger et al., 2008; Arredondo et al., 2010). As an important result of antioxidant activities, flavones and flavonols would re-establish the redox regulation of proteins, transcription factors, and survival signaling cascades otherwise altered by oxidative-stress events.

p0090 Moreover, experimental work revealed that mitochondria are a plausible main target of redox properties of flavones, mediating, at least in part, their preventive actions against oxidative stress and mitochondrial dysfunction–associated pathologies (Lagoa et al., 2011).

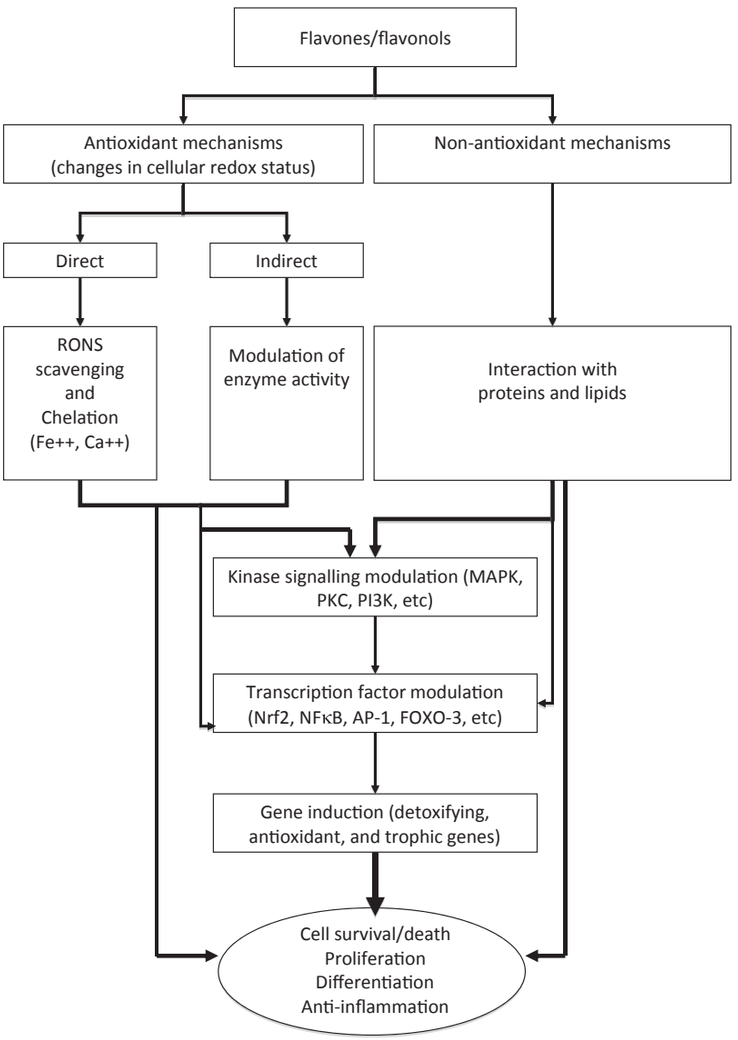
Non-Antioxidant Properties s0040

p0095 Besides their pharmacological role as potent antioxidants, flavones and flavonols show an important additional pharmacological activity represented by their capacity to directly interact with proteins. Some examples of these interactions include their potential to bind to the ATP-binding sites of a large number of proteins, their interactions with receptors such as GABA-A and adenosine receptors, or the modulation of mitochondrial-associated proapoptotic factors, such as DIABLO/smac (Johnson et al., 2011; Shimmyo et al., 2008; Spencer, 2009).

Modulation of Intracellular Signaling Pathways s0045

p0100 The enlisted properties of these compounds may finally converge in the modulation of a number of protein kinase and lipid kinase signaling cascades, such as the phosphatidylinositol 3-kinase (PI3K)/Akt, tyrosine kinase, protein kinase C (PKC), and mitogen-activated protein kinase (MAP kinase) signaling pathways. In this sense, attention is turned towards the

10025-WATSON-9780124114623



f0015

FIGURE 25.2 Working mechanisms of flavones/flavonols and their cellular effects.

modulation of these intracellular cascades as a therapeutic approach against neurodegeneration, because these signaling pathways have been involved in neuronal survival, neurogenesis, and cognitive performance through changes in synaptic plasticity. By their interaction with these intracellular signaling cascades, the beneficial cellular effects of flavones and flavonols may be mediated through changes in the phosphorylation state of target molecules, resulting in modulation of cellular function and gene expression and leading to cell survival (Spencer, 2007, 2009).

p0105 Nevertheless, as stated earlier in the discussion of *in vivo* evidence, the final effect within these signaling cascades may be beneficial or negative in the context of the oxidative state of the brain and the flavonoid concentration. Indeed, it should be important to take into account that their interactions with intracellular signaling pathways could have unpredictable consequences and will be dependent on the cell type (neuron or glia), the disease studied, and the insult applied.

Table 25.1 shows some *in vitro* experiments with p0110
the beneficial effects of several flavones and flavonols against diverse oxidative injuries and the suggested mechanisms of action. From the table, it can be concluded that the range of active concentrations is wide, from nM to μM, 100 μM being the highest. An important aspect is that flavones and flavonols are beneficial against very diverse oxidative aggressions, showing a multitarget action, although described mechanisms are mainly scavenging and antilipid peroxidation. The relationship of flavones and flavonols with intracellular signaling is not frequently addressed.

t0010 **TABLE 25.1** Evidence *In Vitro*, 2009–2013

Compound	Cell Culture	Concentration Range	Injury Model	Mechanisms Proposed	References
Apigenin	Human neuroblastoma SH-SY5Y cells overexpressing the Swedish mutant form of human APP	0.1, 1.0, and 10.0 μM	AB-mediated neurotoxicity induced by copper	Regulate redox imbalance, preserve mitochondrial function, inhibit MAPK, depress neuronal apoptosis	a
Fisetin	Hippocampal neuronal HT22 cells	60 μM	Oxidative stress caused by fluoride and dexamethasone.	Reduced enhanced ROS, increased NO level and apoptosis	b
Myricetin, Apigenin, kaempferol, luteolin, and quercetin	Rat C6 glioma cells	5–20 μM	Peroxide-induced cytotoxicity 24 hrs	Inhibit lipid peroxidation	c
Myricetin Quercetin	C6 glioma	Myricetin 100 pM, 1 nM, and 10 nM Quercetin 10μM and 25μM.	Oxygen-glucose deprivation (OGD)	Attenuated increase in ROS/RNS, and intracellular calcium Myricetin, attenuated MMP depolarization	d
Luteolin Quercetin	PC12	6 and 25 μMol/L	t-BHP and H2O2 and FeSO4	Increased GSH, reduced MDA levels Antilipoperoxidant activity	e
Luteolin	PC12	3.13–50 μM	6-OHDA	Suppression of Bax overexpression, inhibition of Bcl2 reduction, downregulation of p53	f
7,8-dihydroxyflavone	Rat retinal ganglion cells and retinal neuronal precursor RGC-5 cell line	20, 50, 100, 250, and 500 nM	Excitotoxic and oxidative stress-induced apoptosis and cell death	Upregulates TrkB, downstream AKT, and MAPK/ERK Promotes neuritogenesis	g
Kaempferol	SH-SY5Y cells and primary neurons	30 μM	Rotenone-induced acute toxicity	Enhancement of mitochondrial turnover by autophagy	h
Quercetin	Oligodendrocyte precursor cells (OPCs)		Oxygen/glucose deprivation (OGD)	Activation of the PI3K/Akt	i
Kaempferol	Microglial BV2 cells		LPS-induced inflammation	Downregulation of TLR4, NF-κB, p38 MAPK, JNK, and AKT	j
Quercetin	Primary rat mixed glial cells	75–100 μM	H2O2-induced toxicity		k
Baicalein	BV2 cells, PC12 cells and primary neuron cells	≅ 20uM	Oxygen-glucose deprivation (OGD) in vitro	Downregulates the expression of NOD2 and TNFα in both mRNA and protein levels	l
Quercetin and galangin	Isolated rat liver mitochondria	IC(50) = 1.23 +/- 0.27 and 2.39 +/- 0.79 M, respectively	Fe (2+)/citrate-mediated membrane lipid peroxidation (LPO)	Superoxide scavenging, decrease in membrane fluidity and/or mitochondrial uncoupling	m
Quercetin	Neuronal SH-SY5Y cells.		6-OHDA-induced toxicity	Inhibition of caspase activation	n

[a] *Zhao et al., 2012*
[b] *Inkielewicz-Stepniak, Radomski, & Wozniak, 2012*
[c] *Seibert et al., 2011*
[d] *Panickar & Anderson, 2011*
[e] *Pavlica & Gebhardt, 2010*
[f] *Guo et al., 2013*
[g] *Gupta et al., 2013*
[h] *Filomeni et al., 2012*
[i] *Wang et al., 2011*
[j] *Park et al., 2011*
[k] *Kabadere et al., 2011*
[l] *Li & Pu, 2011*
[m] *Dorta et al., 2008*
[n] *Ossola, Kääriäinen, & Männistö, 2009*

s0050

CONCLUSION

p0115 Given the major role attributed to oxidative processes in cardiovascular and brain pathology, great promise was given to the potent antioxidant capacity of flavonoids (particularly flavones and flavonols), ubiquitous molecules that constitute an important part of some diets. Diverse epidemiological studies apparently confirmed the assumption.

p0120 Preliminary experimental evidence *in vitro* and *in vivo*, summarized earlier, further contributed to confirm the protective hypothesis. Nevertheless, from the *in vitro* studies, the range of protective concentrations of flavones and flavonols is already narrow and toxicity is observed regularly over 100 μM. Although positive epidemiological results have been observed after chronic nutritional intake, the *in vivo* experimental studies utilize chronic or acute pharmacological doses that, even when protective, were much lower than those observed in *in vitro* studies. Preliminary observations of the liposomal carriers developed to improve this situation are showing that the increased brain bioavailability producing higher concentrations of flavones and flavonols, in a highly oxidative environment of brain pathology, can trigger the prooxidant potential of these molecules, resulting in further damage.

p0125 Clearly, actions other than antioxidants are active in the brain *in vivo*. Wide interaction with enzymes and proteins, interfering with intracellular phosphorylation/dephosphorylation and redox processes modulating signaling cascades, could give a major unpredictable final effect.

p0130 Although the positive protective results are promising for clinical pathological conditions, globally considering the previously described situation should lead to great caution being undertaken when designing clinical studies, mainly regarding dosage, particularly if pharmacological carriers are utilized. Whether or not nutritional low and chronic administration would have a firm perspective for prevention, acute pharmacological approaches still need further research. The utilization of the flavone/flavonol structure as a lead to generate molecules with less pro-oxidant potential could be a promising alternative.

s0055

References

[AU2] Ahmad, A., et al., 2011. Quercetin protects against oxidative stress associated damages in a rat model of transient focal cerebral ischemia and reperfusion. Neurochem. Res. 36 (8), 1360–1371.

Armstrong, K., et al., 2012. Cardiovascular dysfunction in infants with neonatal encephalopathy. Arch. Dis. Child. 97, 372–375.

Arredondo, M.F., et al., 2004. Cytoprotection by achyrocline satureioides (Lam) D.C. and some of its main flavonoids against oxidative stress. J. Ethnopharmacol. 91 (1), 13–20.

Arredondo, F., et al., 2010. After cellular internalization, quercetin causes Nrf2 nuclear translocation, increases glutathione levels, and prevents neuronal death against an oxidative insult. Free Radic. Biol. Med. 49, 738–747.

Bains, J.S., Shaw, C.A., 1997. Neurodegenerative disorders in humans: the role of glutathione in oxidative stress-mediated neuronal death. Brain Res. Brain Res. Rev. 25 (3), 335–358.

Barone, F.C., Feuerstein, G.Z., 1999. Inflammatory mediators and stroke: new opportunities for novel therapeutics. J. Cereb. Blood Flow. Metab. 19 (8), 819–834.

Cai, M., et al., 2012. The neuroprotective effect of eupatilin against ischemia/reperfusion-induced delayed neuronal damage in mice. Eur. J. Pharmacol. 689 (1–3), 104–110.

Calderón-Montaño, J.M., et al., 2011. A review on the dietary flavonoid kaempferol. Mini. Rev. Med. Chem. 11 (4), 298–344.

Clemens, M.R., 1991. Free radicals in chemical carcinogenesis. Klin. Wochenschr. 69 (21–23), 1123–1134.

Clemens, J.A., 2000. Cerebral ischemia: gene activation, neuronal injury, and the protective role of antioxidants. Free Radic. Biol. Med. 28 (10), 1526–1531.

Dajas, F., et al., 2003. Cell culture protection and in vivo neuroprotective capacity of flavonoids. Neurotox. Res. 5 (6), 425–432.

Dajas, F., et al., 2013. Neuroprotective actions of flavones and flavonols: mechanisms and relationship to flavonoid structural features. Central nervous system agents in Med. Chem. 13 (1), 30–35.

Doenicke, A., et al., 1994. A comparison of two formulations for etomidate, 2-hydroxypropyl-beta-cyclodextrin (HPCD) and propylene glycol.

Dok-Go, H., et al., 2003. Neuroprotective effects of antioxidative flavonoids, quercetin, (+)-dihydroquercetin and quercetin 3-methyl ether, isolated from opuntia ficus-indica var. saboten. Brain research 965 (1–2), 130–136.

Dorta, D.J., et al., 2008. Antioxidant activity of flavonoids in isolated mitochondria. Phytother. Res. 22 (9), 1213–1218.

Filomeni, G., et al., 2012. Neuroprotection of kaempferol by autophagy in models of rotenone-mediated acute toxicity: possible implications for parkinson's disease. Neurobiol of aging 33 (4), 767–785.

Formica, J.V., Regelson, W., 1995. Review of the biology of quercetin and related bioflavonoids. Food Chem. Toxicol. 33 (12), 1061–1080.

Guo, D.-J., et al., 2013. Neuroprotective effects of luteolin against apoptosis induced by 6-hydroxydopamine on rat pheochromocytoma PC12 cells. Pharmaceutical biology 51 (2), 190–196.

Gupta, V.K., et al., 2013. Protective effects of 7,8-dihydroxyflavone on retinal ganglion and RGC-5 cells against excitotoxic and oxidative stress. J. Mol. Neurosci. 49 (1), 96–104.

Halliwell, B., Gutteridge, J.M.C., 1999. Free Radicals in Biology and Medicine. In: Gutteridge, J.M.C. (Ed.). Oxford University Press.

Harborne, J.B., Mabry, H., 1975. The Flavonoids. Academic Press.

Harborne, J.B., Williams, C.A., 2000. Advances in flavonoid research since 1992. Phytochemistry 55 (6), 481–504.

Heim, K.E., Tagliaferro, A.R., Bobilya, D.J., 2002. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. J. Nutr. Biochem. 13 (10), 572–584.

Herrmann, K., 1989. Occurrence and content of hydroxycinnamic and hydroxybenzoic acid compounds in foods. Crit. Rev. Food Sci. Nutr. 28, 315–347.

Higdon, J.V., Frei, B., 2003. Tea Catechins and Polyphenols: Health Effects, Metabolism, and Antioxidant Functions. Crit. Rev. Food. Sci. Nutr. 43 (1), 89–143.

Inkielewicz-Stepniak, I., Radomski, M.W., Wozniak, M., 2012. Fisetin prevents fluoride- and dexamethasone-induced oxidative damage in osteoblast and hippocampal cells. Food Chem. Toxicol. 50 (3–4), 583–589.

Johnson, J.L., et al., 2011. Citrus flavonoids luteolin, apigenin, and quercetin inhibit glycogen synthase kinase-3β enzymatic activity by lowering the interaction energy within the binding cavity. J. Med. Food 14, 325–333.

Joseph, J.A., et al., 1999. Reversals of age-related declines in neuronal signal transduction, cognitive, and motor behavioral deficits with blueberry, spinach, or strawberry dietary supplementation. J. Neurosci. 19 (18), 8114–8121.

József, L., Filep, J.G., 2003. Selenium-containing compounds attenuate peroxynitrite-mediated NF-kappaB and AP-1 activation and interleukin-8 gene and protein expression in human leukocytes. Free Radic. Biol. Med. 35 (9), 1018–1027.

Kääriäinen, T.M., et al., 2008. Lack of robust protective effect of quercetin in two types of 6-hydroxydopamine-induced parkinsonian models in rats and dopaminergic cell cultures. Brain Res. 1203, 149–159.

Kabadere, S., et al., 2011. Quercetin both partially attenuates hydrogen peroxide-induced toxicity and decreases viability of rat glial cells. Acta. biologica. Hungarica. 62 (3), 221–227.

Kim, J.K., et al., 2010. Protective effects of kaempferol (3,4′,5,7-tetrahydroxyflavone) against amyloid beta peptide (Abeta)-induced neurotoxicity in ICR mice. Biosci. Biotechnol. Biochem. 74 (2), 397–401.

Kolominsky-Rabas, P.L., et al., 1998. A prospective community-based study of stroke in Germany--the Erlangen Stroke Project (ESPro): incidence and case fatality at 1, 3, and 12 months. Stroke: A Journal of Cerebral Circulation 29 (12), 2501–2506.

Kühnau, J., 1976. The flavonoids. A class of semi-essential food components: their role in human nutrition. World Rev. Nutr. Diet. 24, 117–191.

Lagoa, R., et al., 2011. Complex I and cytochrome c are molecular targets of flavonoids that inhibit hydrogen peroxide production by mitochondria. Biochimica. et. Biophysica. Acta. 1807, 1562–1572.

Li, S., Pu, X.-P., 2011. Neuroprotective effect of kaempferol against a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced mouse model of Parkinson's disease. Biological pharmaceutical bulletin 34 (8), 1291–1296.

Liu, J., Li, J., Gu, M., 2007. The correlation between myocardial function and cerebral hemodynamics in term infants with hypoxic-ischemic encephalopathy. J. Trop. Pediatr. 53, 44–48.

López-Sánchez, C., et al., 2007. Blood micromolar concentrations of kaempferol afford protection against ischemia/reperfusion-induced damage in rat brain. Brain Res. 1182, 123–137.

Mayank Gandhi, H.B., 2010. Nanotechnology in Blood Brain Barrier: A Review. International Journal of Pharmaceutical & Biological 1, 37–43.

McGuire, W., 2007. Perinatal asphyxia. Clinical Evid. 2007.

Middleton, E.J., Kandaswami, C., Theoharides, T.C., 2000. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol. Rev. 52 (4), 673–751.

Mukhtar, H., Ahmad, N., 2000. Tea polyphenols: prevention of cancer and optimizing health. Am. J. Clin. Nutr. 71(6 Suppl), p.1698S–702S; discussion 1703S–4S.

Nam, Y., et al., 2013. Natural flavone jaceosidin is a neuroinflammation inhibitor. Phytotherapy research: PTR 27 (3), 404–411.

Nijveldt, R.J., et al., 2001. a review of probable mechanisms of action and potential applications. Am. J. Clin. Nutr. 74, 418–425. Flavonoids.

Olsen, S.L., et al., 2013. Optimizing therapeutic hypothermia for neonatal encephalopathy. Pediatrics 131 (2), e591–e603.

Ossola, B., et al., 2008. Time-dependent protective and harmful effects of quercetin on 6-OHDA-induced toxicity in neuronal SH-SY5Y cells. Toxicology 250 (1), 1–8.

Ossola, B., Kääriäinen, T.M., Männistö, P.T., 2009. The multiple faces of quercetin in neuroprotection. Expert Opin. Drug. Saf. 8 (4), 397–409.

Panickar, K.S., Anderson, R.A., 2011. Mechanisms underlying the protective effects of myricetin and quercetin following oxygen-glucose deprivation-induced cell swelling and the reduction in glutamate uptake in glial cells. Neuroscience 183, 1–14.

Park, S.E., et al., 2011. Kaempferol acts through mitogen-activated protein kinases and protein kinase B/AKT to elicit protection in a model of neuroinflammation in BV2 microglial cells. Br. J. Pharmacol. 164 (3), 1008–1025.

Pavlica, S., Gebhardt, R., 2010. Protective effects of flavonoids and two metabolites against oxidative stress in neuronal PC12 cells. Life sciences 86 (3–4), 79–86.

Priprem, A., et al., 2008. Anxiety and cognitive effects of quercetin liposomes in rats. Nanomedicine : nanotechnology, biology, and medicine 4 (1), 70–78.

Rice-Evans, C.A., Miller, N.J., Paganga, G., 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free. Radic. Biol. Med. 20, 933–956.

Rivera, F., et al., 2008. Reduction of ischemic brain damage and increase of glutathione by a liposomal preparation of quercetin in permanent focal ischemia in rats. Neurotox. Res. 13 (2), 105–114.

Samsa, G.P., et al., 1999. Epidemiology of recurrent cerebral infarction: a medicare claims-based comparison of first and recurrent strokes on 2-year survival and cost. Stroke. 30 (2), 338–349.

Schroeter, H., et al., 2002. MAPK signaling in neurodegeneration: influences of flavonoids and of nitric oxide. Neurobiology of aging 23 (5), 861–880.

Seelinger, G., Merfort, I., Schempp, C.M., 2008. Anti-oxidant, anti-inflammatory and anti-allergic activities of luteolin. Planta. Med. 74, 1667–1677.

Seibert, H., et al., 2011. Cytoprotective activity against peroxide-induced oxidative damage and cytotoxicity of flavonoids in C6 rat glioma cells. Food Chem. Toxicol. 49 (9), 2398–2407.

Shankaran, S., et al., 2012. Childhood outcomes after hypothermia for neonatal encephalopathy. N. Engl. J. Med. 366, 2085–2092.

Shimmyo, Y., et al., 2008. Flavonols and flavones as BACE-1 inhibitors: structure-activity relationship in cell-free, cell-based and in silico studies reveal novel pharmacophore features. Biochimica. et. Biophysica. Acta. 1780, 819–825.

Siesjo, B.K.B., 1992. Pathophysiology and treatment of focal cerebral ischemia. Part I: pathophysiology. J. Neurosurg.. 77
(2
), pp.169–184.

Simonyi, A., et al., 2005. Polyphenols in cerebral ischemia: novel targets for neuroprotection. Mol. Neurobiol. 31 (1–3), 135–147.

Spencer, J.P.E., 2007. The interactions of flavonoids within neuronal signaling pathways. Genes nutrition 2, 257–273.

Spencer, J.P.E., 2009. Flavonoids and brain health: multiple effects underpinned by common mechanisms. Genes nutrition 4, 243–250.

Wang, X.-Q., et al., 2011. Quercetin protects oligodendrocyte precursor cells from oxygen/glucose deprivation injury in vitro via the activation of the PI3K/Akt signaling pathway. Brain research bulletin 86 (3–4), 277–284.

Yen, G.-C., et al., 2003. Pro-oxidative properties of flavonoids in human lymphocytes. Biosci. Biotechnol. Biochem. 67 (6), 1215–1222.

Yin, F., et al., 2011. Baicalin prevents the production of hydrogen peroxide and oxidative stress induced by Aβ aggregation in SH-SY5Y cells. Neuroscience letters 492 (2), 76–79.

Yokoyama, H., et al., 2011. Role of glial cells in neurotoxin-induced animal models of Parkinson's disease. Neurol. Sci. 32 (1), 1–7.

Youdim, K.A., Joseph, J.A., 2001. A possible emerging role of phytochemicals in improving age-related neurological dysfunctions: a multiplicity of effects. Free. Radic. Biol. Med. 30 (6), 583–594.

Yu, X., et al., 2012. Assessment of the treatment effect of baicalein on a model of Parkinsonian tremor and elucidation of the mechanism. Life sciences 91 (1–2), 5–13.

Zar, T., Graeber, C., Perazella, M.A., 2007. Recognition, treatment, and prevention of propylene glycol toxicity. Semin. Dial. 20, 217–219.

Zhao, L., et al., 2012. Apigenin attenuates copper-mediated β-amyloid neurotoxicity through antioxidation, mitochondrion protection and MAPK signal inactivation in an AD cell model. Brain Res. null.

WATSON: 25

Non-Print Items

Abstract
Flavones and flavonols are molecules present in most plants that are an important component of some human diets. Epidemiological evidence shows the beneficial effects of these molecules in cardiovascular and neuropathological diseases. Experimental evidence *in vitro* and *in vivo* has confirmed the neuroprotective effects in neurons in culture against oxidative insults and in models of focal ischemia and experimental Parkinsonism. Nevertheless, the active concentration range *in vitro* is very narrow, and effects on brain pathology have been shown mostly after chronic administration. Although the preventive effects of flavones and flavonols in brain pathology could be considered mostly substantiated, the positive neuroprotective activity after acute administration still deserves more research.

Keywords: flavonoids; flavones; flavonols; neuroprotection; brain pathology.

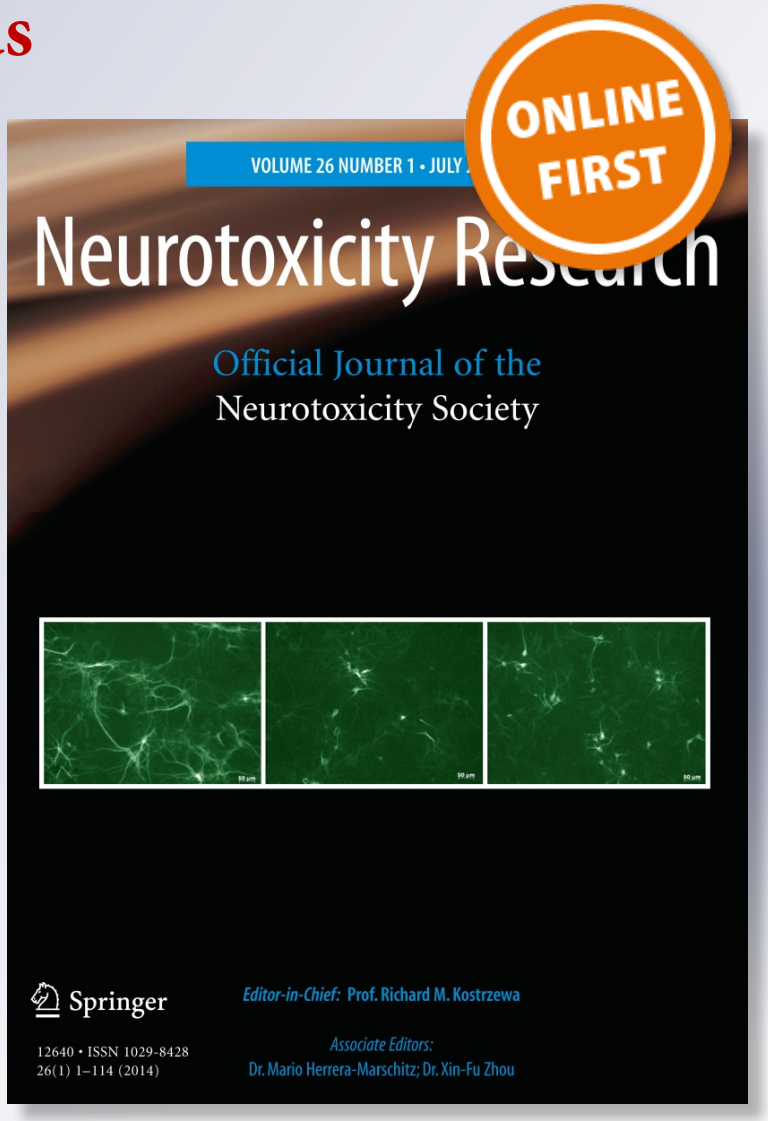
Antioxidant Activity, Cellular Bioavailability, and Iron and Calcium Management of Neuroprotective and Nonneuroprotective Flavones

Carolina Echeverry, Florencia Arredondo, Marcela Martínez, Juan Andrés Abin-Carriquiry, Jacob Midiwo & Federico Dajas

Neurotoxicity Research
Neurodegeneration, Neuroregeneration, Neurotrophic Action, and Neuroprotection

ISSN 1029-8428

Neurotox Res
DOI 10.1007/s12640-014-9483-y



Springer

Neurotox Res
DOI 10.1007/s12640-014-9483-y

Antioxidant Activity, Cellular Bioavailability, and Iron and Calcium Management of Neuroprotective and Nonneuroprotective Flavones

Carolina Echeverry · Florencia Arredondo · Marcela Martínez · Juan Andrés Abin-Carriquiry · Jacob Midiwo · Federico Dajas

Received: 26 March 2014 / Revised: 5 June 2014 / Accepted: 10 June 2014

Abstract Few studies have been undertaken on the relationship of the structure of flavones and neuroprotection. Previously, we described the structural determinants of the neuroprotective activity of some natural flavones in cerebellar granule neurons in culture against an oxidative insult (H_2O_2). In the present work, we analyzed anti-oxidant activity, cellular iron, and Ca^{2+} levels and cellular bioavailability of neuroprotective and nonneuroprotective flavones in the same experimental paradigm. Oxidative cellular damage produced by H_2O_2 was prevented by all of the studied flavones with rather similar potency for all of them. Labile Iron Pool was neither affected by protective nor nonprotective flavones. Intracellular Ca^{2+} homeostasis was not affected by protective flavones either. Nonetheless, fisetin, the nonprotective flavone, decreased Ca^{2+} levels modifying Ca^{2+} homeostasis. Methylation of the catechol group, although weakens anti-oxidant capacity, keeps the neuroprotective capacity with less degradation and lower toxicity, constituting promising structural alternatives as leads for the design of neuroprotective molecules.

Keywords Flavonoids · Flavones · Neuroprotection · Anti-oxidation

C. Echeverry (✉) · F. Arredondo · M. Martínez · J. A. Abin-Carriquiry · F. Dajas
UNESCO CHAIR: "Neuroprotective Natural Compounds", Department of Neurochemistry, Instituto de Investigaciones Biológicas Clemente Estable, Av. Italia 3318, 11600 Montevideo, Uruguay
e-mail: caroliecheverry@gmail.com

J. Midiwo
Department of Chemistry, University of Nairobi, Nairobi, Kenya

Introduction

Flavonoids, a family of the polyphenol compounds that give colour and taste to plants and flowers, ubiquitous in vegetables, plants, and beverages like tea and wine, are structurally characterized by having two benzene rings on either side of a 3-carbon ring (Clifford 2001). Flavonoids differ in the arrangements of hydroxyl, methoxy, and glycosidic side groups as well as in the conjugation between the A- and B- rings. The term flavonoid encompasses flavones, flavonols, flavan-3-ols, isoflavones, flavanones, and the anthocyanins. Among these, the flavones, which are based on the backbone of 2-phenylchromen-4-one (2-phenyl-1-benzopyran-4-one), are the most commonly encountered, being abundant within the families and genera of the higher plants (Andersen and Markham 2010). The flavones are characterized by a planar structure because of the double bond with the keto group in the central aromatic ring. One of the best-described flavonoids, quercetin, is a member of this group (Nijveldt et al. 2001). The structural difference in each flavonoid family results from the variation in the number and substitution pattern of the hydroxyl groups and the extent of glycosylation of these groups (Harborne 1994).

Most of the biological actions of flavones have been attributed to their anti-oxidant, hydrogen-donating capacity (Nijveldt et al. 2001; Rice-Evans 2001). Beyond the powerful direct scavenger action, flavones exert an important indirect antioxidant activity contributing to the homeostasis of calcium, metal chelation, stabilization of membranes through anti-lipid peroxidation, and enzymatic activity modulation (Nijveldt et al. 2001; Williams et al. 2004; Middleton et al. 2000).

The structural diversity of these natural compounds offers the possibility to identify the molecular substitutions

Published online: 28 June 2014

required for their different biological actions. Thus, important research has generated evidence supporting the role of specific structural components as requisites for radical scavenging, chelation, and oxidant activity (Bors et al. 1990; Rice-Evans et al. 1996; Heim et al. 2002). Their activity as transition metal ions chelators is defined by the presence of the *ortho*-dihydroxy substitution in B-ring, the 4-carbonyl in C-ring, and a 5-hydroxy substitution in A-ring (Mira et al. 2002). Early work already demonstrated that the structural requirement considered to be essential for effective radical scavenging is the electron donating capacity given by the presence of a catechol structure in the B-ring. The 3-OH moiety in combination with the C2–C3 double bond conjugated with a 4-keto group, which is responsible for electron delocalization from the B-ring, further enhances the radical-scavenging capacity (Bors et al. 1990; Rice-Evans et al. 1996). The B-ring hydroxyl configuration is the most significant determinant of scavenging and its fundamental, although not exclusive, role in antioxidation has been also demonstrated e.g., in the scavenging of peroxynitrite, a lethal oxidative molecule generated by oxidative stress in neurons (Heijnen et al. 2001).

It is important to mention that the direct and indirect antioxidant activity domains of flavones can neutralize several steps in the cascade of oxidative events occurring in the neuron after ischemia or in neurodegenerative diseases (Simonyi et al. 2005; Li and Pu 2011; Spencer 2009). Since numerous in vitro and in vivo experimental evidences have demonstrated a neuroprotective capacity of several flavonoids, for example, quercetin, fisetin, luteolin, and catechins (Dajas et al. 2003; Ossola et al. 2009; Dajas 2012), one accepted hypothesis is that the efficacy of flavones and flavonols as neuroprotective molecules is closely correlated with their antioxidant activity (Pietta 2000; Almajano et al. 2011).

Studying in cerebellar granule neurons the structural features that characterize the neuroprotective capacity of 14 natural flavones against an oxidative insult, we found that the catechol in B-ring is not necessary to afford neuroprotection (Echeverry et al. 2010) against an oxidative insult provoked by hydrogen peroxide. Furthermore, the hydroxyl substitutions in the position C3 (C-ring) and in C5 (A-ring) would be important for neuroprotection that, accordingly, appears to be linked to specific structural motifs, beyond those involved in anti-oxidation (Echeverry et al. 2010). These results would be in apparent discrepancy with the prevalent hypothesis about the role of antioxidation in neuroprotection.

In this context, this contribution was a natural continuation of these previous studies, aimed at exploring key mechanisms of neuroprotection, as would be the antioxidation activity of the four already identified neuroprotective flavones (Fig. 1a),[1] their management of intracellular scavenging of reactive oxygen species (ROS), and their anti-lipid peroxidation effects, when compared with treatment with a non neuroprotective flavone (Fig. 1b). The effects of protective and nonprotective flavones on iron and calcium homeostasis were also studied given their key importance in neuronal death. Moreover, cellular bioavailability was also studied and compared, since access to intracellular molecules is a key aspect of neuroprotection. We utilized the pre-treatment experimental paradigm with application of hydrogen peroxide after 24 h of flavone treatment, as it gives an assessment of the effects of the flavones on the modulation of survival signaling, independently of interaction with generated free radicals at the moment of the oxidative insult. Thus, results after pre-treatment would indicate the strengthening by flavones of the neuron condition to manage oxidative insults.

Materials and Methods

General Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. Hydrogen peroxide (H_2O_2) was dissolved in distilled water and subsequently filtered before added to the cultures.

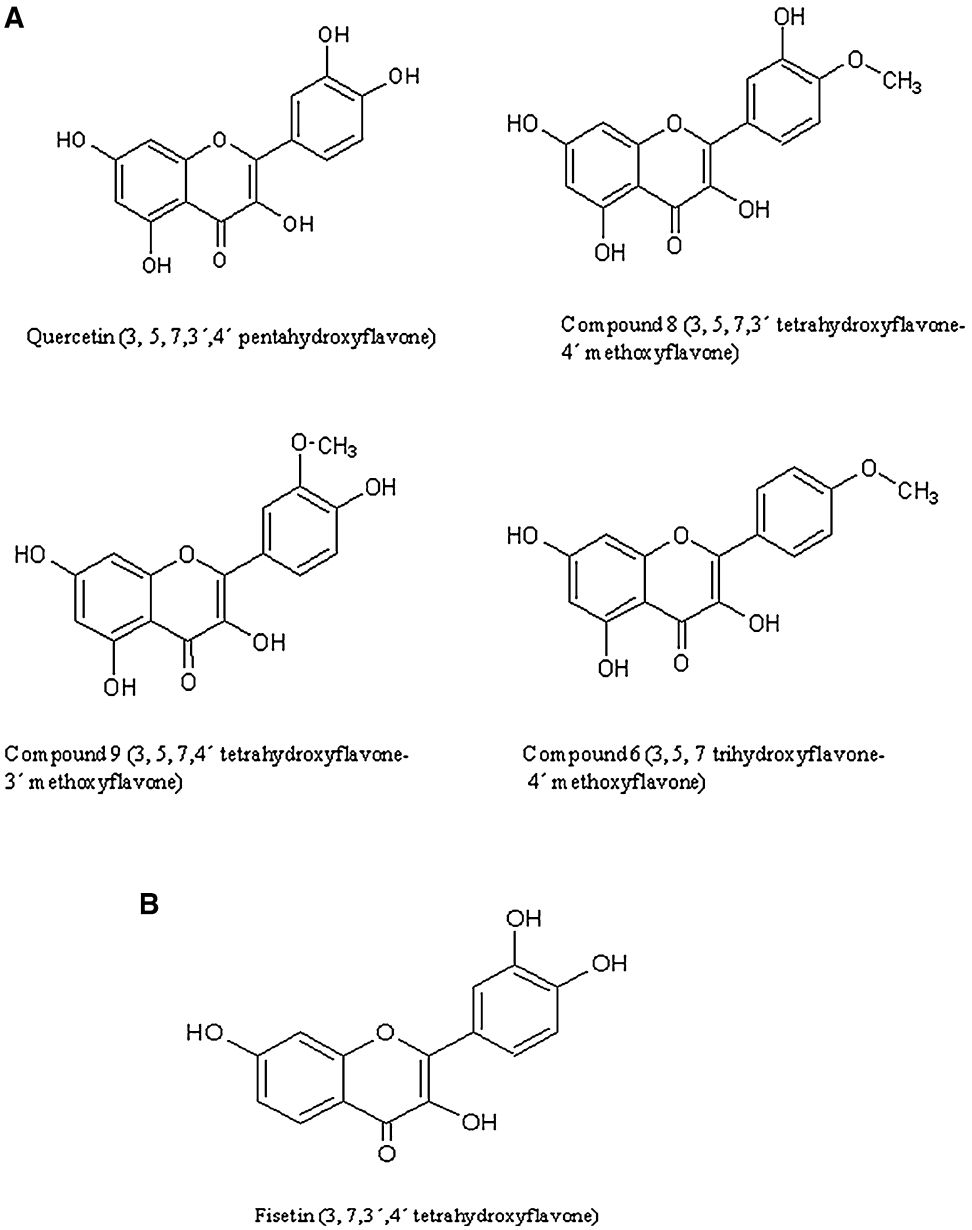
Flavones Utilized

Quercetin (3,5,7,3′,4′-pentahydroxyflavone) was acquired from Xi'an Sino-Dragon I/E Co., Ltd., China, and Fisetin (3,7,3′,4′tetrahydroxyflavone) was obtained from Sigma-Aldrich (St. Louis, MO, USA). The rest of the flavones studied were isolated by Prof. J. Midiwo's research group at the Department of Chemistry, University of Nairobi from the following plants: *Dodonaea angustifolia, Psiadia punctulata, Gardenia ternifolia, Senecio roseiflorus, and Hypericum lanceolatum.* The following compounds were obtained from these plants: compound **6** (3,5,7-trihydroxy-4′-methoxyflavone), compound **8** (3,5,7,3′-tetrahydroxy-4′-methoxyflavone, tamarixetin), compound **9** (3,5,7,4′-tetrahydroxy-3′-methoxyflavone, isorhamnetin).

Each molecule was dissolved in dimethyl sulfoxide (DMSO) 30 %, prior to bioassay experiments.

[1] Because they all share the basic backbone of 2-phenylchromen-4-one (2-phenyl-1-benzopyran-4-one), the molecules studied can be primarily considered as flavones and as such are taken in this work, knowing that as hydroxyl flavones they can also be considered as flavonols.

Fig. 1 **a** Protective flavones; **b** nonprotective flavones



Plant Materials

The plants used and its vouchers have been described previously (Yenesew et al. 2003; Juma et al. 2001).

The leaf and flowers of the plants mentioned were collected from their geographical location and handled by established procedures.

Extraction

Extraction was performed as previously described for exudates from aerial parts (Yenesew et al. 2003; Juma et al. 2001); the aerial branches and leaves were dipped into organic solvent and shaken for not more than 15 s to avoid extraction of internal tissue substances indicated by the appearance of the green of chlorophyll. This wash was dried *in vacuo* using a rotary evaporator to give a solid gum. Other parts of the plants were dried before extracting the powder at ambient temperature with varying organic solvents dichloromethane: methanol (1:1) followed by methanol and solvent removed to yield the gummy solid.

Analysis was done using analytical TLC: Merck precoated silica gel 60 F254 plates with standard previously characterized compounds to locate desired compounds in the extracts, CC on silica gel 60 (70–230 mesh). EIMS: direct inlet, 70 eV, ^{1}H NMR (500 MHz), and ^{13}C NMR (125 MHz) run on a DRX-500 (Bruker), ARX 300

(Bruker), or Varian-Mercury spectrometers using solvent resonances to calibrate the spectra.

The compounds used were at least 99.99 % pure. The purity was established by analytical techniques—thin layer chromatography, crystallization to constant melting point—and spectroscopic techniques—proton and C-13 NMR, Mass spec data.

Measurement of Free Radical Scavenger Capacity

The free radical scavenger capacity in vitro of each flavone was evaluated by studying its ability to scavenge the $ABTS^{\bullet+}$ radical (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) (Miller et al. 1993).

$ABTS^{\bullet+}$ was produced by the reaction of ABTS (7 mM) with ammonium persulfate (140 mM) for 12–16 h, in the dark and at room temperature. Prior to use, the stock solution was filtered and diluted in PBS (pH 7.4) so as to achieve an absorbance at 734 nm of 0.70 ± 0.02. One milliliter of diluted $ABTS^{\bullet+}$ was mixed with 10 μL of each flavone at different concentrations (0–20 μM final concentration), and the percentage decrease of absorbance at 734 nm was calculated. The IC50 (μM) was determined for each flavone.

Neuronal Culture

Primary cerebellar granule neurons (CGN) were obtained from PN6-8 Sprague–Dawley rats (García and Massieu 2001) and seeded in poly-L-ornithine pre-coated 96-wells plates at a density of 200,000 cells/well. They were kept in Basal Medium Eagle supplemented with foetal bovine serum (PAA Laboratories, Austria) (10 %), 20 mM KCl, and 25 mM glucose, in a humidified chamber at 37 °C in a 5 % CO_2 atmosphere. Glial growth was inhibited by addition of cytosine arabinoside (10 μM).

All procedures involving animals and their care were approved by the ethics committee of the "Instituto de Investigaciones Clemente Estable." All efforts were made to minimize the number of animals used and their suffering.

Experimental Paradigm

At 6 days in culture (DIC6), the flavones (in 1.5 % DMSO, final concentration) were preincubated during 24 h before the H_2O_2 insult. The neuronal death was induced at DIC7 by adding 180 μM hydrogen peroxide (H_2O_2) for 24 h.

Determination of Intracellular Formation of Reactive Oxygen Species (ROS)

ROS was assessed using oxidation sensitive dye, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), as the substrate (Okimotoa et al. 2000). CGN cells growing in 96-well plates were loaded with 100 μM DCFH-DA and incubated for 40 min at 37 °C. Nonfluorescent DCFH-DA dye that freely penetrates the cells, is hydrolyzed by intracellular esterases to 2′,7′-dichlorodihydrofluororescein (DCFH) and is trapped inside the cells. After washing the cells with PBS three times, cells were treated with 180 μM H_2O_2 at different times. The formation of 2′,7′-dichlorofluorescein (DCF) due to oxidation of DCFH in the presence of various ROS was read at the excitation wavelength (Ex) of 485 nm and the emission wavelength (Em) of 528 nm using a fluorescence micro plate reader (Varioskan Flash, Thermo Scientific).

Measurement of Lipid Peroxidation

Malondialdehyde (MDA) as an end product of lipid peroxidation was quantified by the thiobarbituric acid assay and subsequent HPLC analysis. This method is a global measurement of lipid peroxidation and was applied because of its sensitivity and simplicity (Draper et al. 1993). After washing the cells with PBS, 200 μL of 20 % (w/v) trichloroacetic acid containing 0.8 % (w/v) thiobarbituric acid was added to each culture dish. The cells were scratched off with a rubber policeman; the suspensions were transferred to glass centrifuge tubes and heated for 45 min at 90 °C.

After cooling to room temperature and centrifugation, the MDA–TBA complex in the supernatant was determined by HPLC (5u C18 100 A, 150 × 4.6 mm) with fluorescence detector (515 nm excitation, 553 nm emission). The mobile phase was a mixture of 5 mM water and methanol (50:50, v/v). The flow rate was maintained isocratically at 1 mL/min.

The amount of TBARS was expressed as nmol MDA equivalents formed per cultured dish.

Cellular Labile Iron Pool Evaluation

The cellular labile iron content was estimated by a fluorimetric assay using the metal-sensitive probe calcein (CA) (Breuer et al. 1995) and the strong membrane-permeant iron chelator SIH (salicylaldehyde isonicotinoyl hydrazone), generously provided by Prof. Prem Ponka (McGill University, Montreal, QC, Canada). Briefly, both control cells and cells preincubated with flavones were loaded with 0.25 μM calcein-AM for 20 min at 37 °C. CA-AM rapidly penetrates across the plasma membrane and is intracellularly hydrolysed to release free CA. After loading, the cultures were washed of excess CA-AM two times with PBS. Cellular CA fluorescence was recorded in a fluorescence micro plate reader (Varioskan Flash, Thermo Scientific) using an excitation wavelength of 485 nm and an

emission wavelength of 530 nm (slits 5 nm). Cell cultures without CA-AM were used as blank to correct nonspecific autofluorescence. Once hydrolyzed, calcein becomes trapped in the cytoplasm and emits intense green fluorescence. The calcein-loaded cells have a fluorescence component that is quenched by intracellular iron and can be revealed by addition of 200 μM SIH. The rise in fluorescence is equivalent to the change in calcein concentration or to the amount of cellular iron originally bound to CA. Thus, the changes in CA fluorescence intensity were directly proportional to the iron labile pool.

Cell viability was >95 % and unchanged during the assay.

Measurement of Intracellular Ca^{2+}

Intracellular Ca^{2+} was monitored using the fluorescent Ca^{2+}-sensitive dye, Fura 2-acetoxymethy ester (Fura 2-AM) (Liu and Wang 2001). The control cells and cells preincubated with flavones were preloaded with Fura 2-AM (5 μM) for 30 min at room temperature in the dark, then 30 min at 37 °C in a humidified incubator. Cells were then gently rinsed three times with PBS solution and incubated in the presence of 180 μM H_2O_2 for 15 min. After the fluorescence was measured at emission wavelength of 510 nm and excitation wavelengths of 340 and 380 nm using a fluorescence microplate reader (Varioskan Flash, Thermo Scientific), the fluorescence ratio (F340/F380) was calculated as an indicator of intracellular Ca^{2+}.

Each well was normalized to cell numbers using calcein cell viability assay (Mueller et al. 2004).

This series of experiments was performed with two protective molecules (quercetin and compound **9**) and fisetin (non protective).

Cell Viability Measurements of Cultures Pre-treated with Quercetin and Calcium Chelator (EGTA)

At DIC 6, the cells were preincubated with EGTA (1 mM) and quercetin during 24 h before the H_2O_2 insult.

The neuronal mitochondrial activity, as an indicator of cell viability, was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) assay (Denizot and Lang 1986).

Briefly, after experimental treatments, cells were incubated for 90 min at 37 °C with MTT (0.1 mg/mL final concentration) that was reduced by metabolically active cells to purple formazan. Formazan crystals were dissolved with DMSO, and the absorbance was measured on a MRX microplate reader (Dynex Technologies), using a reference wavelength of 630 nm and a test wavelength of 570 nm.

Results were presented as percentage of MTT reduction, assuming that absorbance of control cells was 100 %.

Neuronal viability was also examined qualitatively by phase-contrast microscopy (Axiovert 25, Zeiss, Germany).

HPLC Analysis of Flavones Availability in Cell Cultures

To evaluate the intracellular and extracellular levels of flavones, cultures were incubated with 25 μM of different flavones for 24 h, quickly washed with PBS 3 times and lysed (aqueous methanol 50 % in 0.1 M HCl). For measurement of extracellular levels, after flavones incubation, samples from culture medium were diluted in methanol (1:1).

Freeze-thawed samples were microfuged at 10,000 rpm for 5 min at 4 °C, and supernatants were injected onto the HPLC system. Separation of constituents was achieved by reverse-phase HPLC using a C18 column (Phenomenex, USA) with 5 μm particle size. A binary HPLC pump (Waters 1525) with a 717 plus auto sampler Waters and a photodiode array detector Waters 2998 linked to Empower 2 (Waters) chromatography data software was utilized. The temperature of the column was set at 30 °C. The mobile phase used was (A) MeOH 100 %, (B) H_3PO_4 0.5 % pH 2, MeOH 5 %, at 0.7 mL/min. The gradient system consisted of (min/%B): 0/80, 40/0, 41/80, and 47/80. The eluant was monitored by photodiode array detection at 375 nm and spectra of products obtained between 210 and 600 nm.

Statistical Analysis

The results were expressed as mean ± SEM. Differences among different experimental groups were tested for significance using one-or two-way analysis of variance (ANOVA) followed by Tukey's or Newman-Keuls multiple comparison tests, respectively, tacking $p < 0{,}05$ as significant

Results

Free Radical Scavenger Capacity

All the flavones showed marked scavenger capacity. Fisetin, the nonprotective flavone, has stronger scavenger capacity than isorhamnetin, a protective flavone, or stronger scavenger activity than compound **6** and 8, also protective flavones (Table 1). Accordingly, there is no direct relationship between the neuronal protection and the scavenger capacity.

Furthermore, in this experimental paradigm, the catechol motif appeared as important for anti-oxidation (quercetin and fisetin) and not for protection, as shown by results obtained with compounds **6**, **8**, and **9**.

Table 1 Free radical scavenger capacity of flavones evaluated by ABTS assay

Compound	IC_{50} (μM)
Quercetin	4,4 ± 1.3[a]
Comp **9** (isorhamnetin)	5,7 ± 1.1[a]
Comp **8** (tamarixetin)	9,5 ± 2.9
Compound **6**	12,4 ± 2.3
Fisetin	5,6 ± 1.5[a]

Each value is presented as mean ± SD ($n = 3$)

[a] $p < 0.5$ versus compound **6**

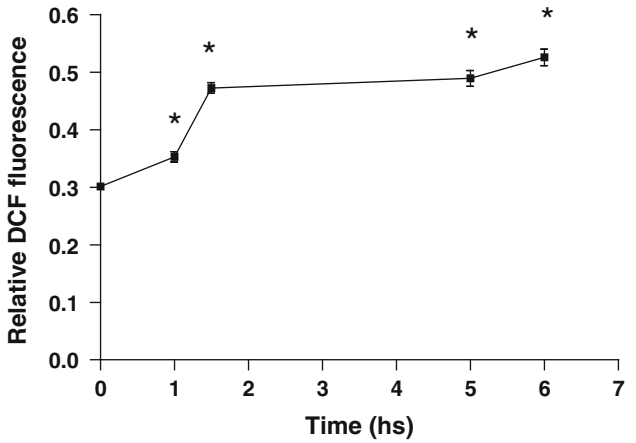


Fig. 2 ROS production at different times after H_2O_2 180 μM addition to neuronal cultures. Data presented as mean ± SEM of 3 separated experiments. *$p < 0.001$ versus vehicle-treated cells

Levels of Intracellular Reactive Oxygen Species

The addition of H_2O_2 to neuronal culture produced a significant increase in intra cellular ROS production, starting at 90 min, and maintained up to 7 h later (Fig. 2).

The pre-treatment of neuronal cultures with different concentrations of protective flavones, previously to the addition of H_2O_2, produced a significant decrease of intracellular ROS production with all concentrations (5–100 μm) in the case of quercetin and compound **9** (Fig. 3a). These were the flavones that had shown the most potent scavenger capacity in vitro. Only the smaller concentration of compound **8** decreased ROS production, while compound **6** had no effect at all (Fig. 3a). The latter had shown the least scavenger capacity in ABTS studies.

Pre-treatment with fisetin produced a significant decrease of intracellular ROS production at concentrations between 10 and 50 μM (Fig. 3b).

Lipid Peroxidation (Malondialdehyde Formation)

Four hours after the addition of H_2O_2 to neuronal cultures, there was a significant increase on MDA production (Fig. 4).

Twenty-four hours pre-treatment with 25 μM of each flavone (protective and non protective), before the oxidative insult, prevented the MDA increase after 4 h of addition of 180 μM H_2O_2 (Fig. 5).

Labile Iron Pool (LIP) Evaluation and Intracellular Free Ca^{2+} Concentrations

Pre-treatment of granule neurons with 25 μM of each flavone for 24 h did not affect LIP levels (Fig. 6). Regarding intracellular Ca^{2+} levels, the pre-incubation with protective molecules quercetin and compound **9** neither produced any change (Fig. 7a). Addition of H_2O_2 produced an increase in intracellular Ca^{2+} (Fig. 7) that was not reverted by quercetin or compound **9**. In contrast, pre-treatment with Fisetin produced a decrease of Ca^{2+} intracellular levels (Fig. 7b). Increased Ca^{2+} levels after H_2O_2 addition were not modified by Fisetin (Fig. 7b).

Since these contrasting results would be indicating that maintenance of intracellular Ca^{2+} levels is important for neuroprotection particularly in granule neurons, a Ca^{2+} chelating agent (EGTA) was added to CGN alone or together with quercetin, and survival assessment was performed. The experimental concentrations of EGTA utilized were those producing a decrease in Ca^{2+} levels similar to Fisetin after 24 h of treatment (data not shown).

While EGTA alone did not modify neuronal death provoked by H_2O_2 (Fig. 8), it blocked the protection obtained with quercetin pre-treatment in the concentrations utilized (Fig. 8).

Cell Availability of Flavones

After 24 h of flavone exposition, those with catechol group in B-ring (quercetin and fisetin) showed a higher degradation process shown by low extracellular concentrations and nondetectable intracellular levels (Fig. 9). In contrast, flavones that had at least one methylation in the B-ring (compound **8** and compound **9**) were more stable, showing high extracellular levels and detectable intracellular concentrations. Compound **8** was clearly the most stable of the group.

Discussion

As it was explained above, this work is a natural continuation of our previous discovery of four neuroprotective flavones, identified from a group of 13 natural flavones isolated from east Africa (Echeverry et al. 2010). For the first time, specific structural features of flavonoids were linked to the increased survival against a strong oxidative insult in neurons. The study of the mechanisms involved in

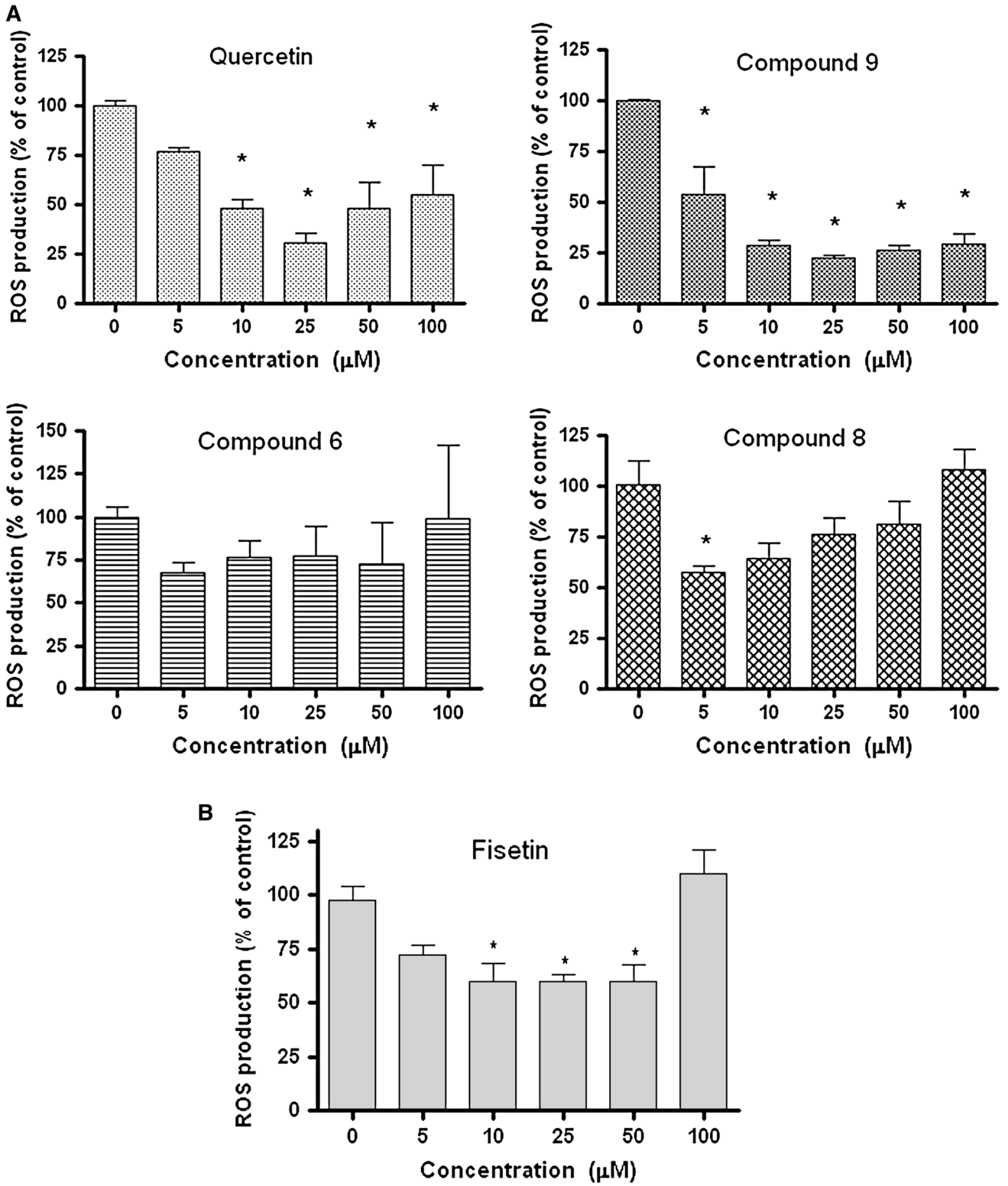


Fig. 3 Intracellular ROS production after H_2O_2 180 μM addition to neuronal cultures pre-treated for 24 h with different concentrations of the protective flavones (**a**) and fisetin, the nonprotective flavone (**b**) as indicated in each graph. Data presented as mean ± SEM of 3 separated experiments. *$p < 0.001$ versus vehicle-treated cells + H_2O_2 (ANOVA-Tukey multiple comparison test)

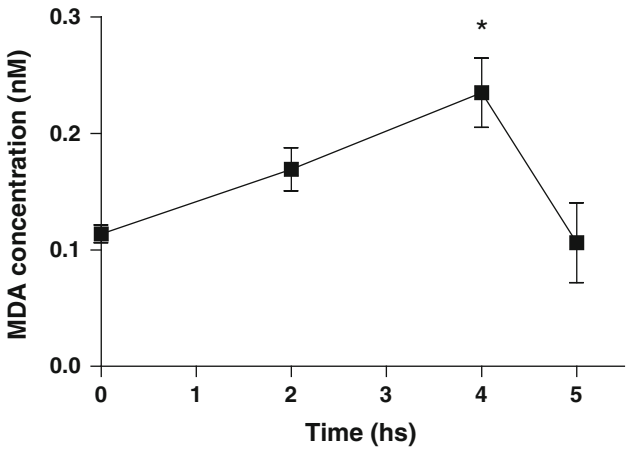


Fig. 4 MDA formation at different time after H_2O_2 180 μM addition to neuronal cultured. Data presented as mean ± SEM of 3 separated experiments. $^*p < 0.01$ versus vehicle-treated cells (two-way ANOVA-Newman Keuls test)

the protection appeared as an immediate endeavor to understand the neuroprotective process.

Before starting the analysis of results, a key point to stress is the specific characteristic of the experimental paradigm utilized: neurones in culture were *pre-treated* with the flavones for 24 h, before the addition of H_2O_2. At the moment of the insult, therefore, the intracellular concentration of flavones, as shown by HPLC, was very low, and the experimental paradigm can be primarily considered a model of a new metabolic condition that makes neurons more resistant to oxidative insults independently of the presence of the flavone at the moment of the insult. Nevertheless, with the methodology utilized, we cannot discard completely the presence of flavones metabolites.

Neuroprotection is likely related to increased expression of survival proteins. Results from our laboratory showed that quercetin pre-treatment modulates the GSH redox system (Arredondo et al. 2010). Quercetin caused Nrf2 nuclear translocation in neuronal cultures and significantly increased the γ-glutamate–cysteine ligase catalytic subunit gene expression, the rate limiting enzyme in GSH synthesis (Arredondo et al. 2010).

The study of cellular bioavailability at the moment of the insult showed that only compounds **8** and **9** were still present in significant intracellular concentrations. These molecules were precisely the ones that have at least one methylated change in positions 3́ or 4́ of the catechol group. After 24 h of flavone exposition, those with catechol group in B-ring showed a higher degradation process. These results can be considered an important contribution of the present study. Since previous experimental evidence has linked the presence of hydroxyls in the catechol group to an increased toxicity (Chang et al. 2008; Walle 2009), present results on bioavailability are important in the direction of obtaining molecules with less toxicity and better neuronal bioavailability. As further evidence, compound **8**, the more stable molecule, was precisely the only nontoxic flavone in the previous study (Echeverry et al. 2010).

Regarding antioxidation, results showed good scavenger activity for all the flavones, being those that have a catechol group the most potent. In this sense, the results agree with studies that show the importance of the *ortho*-dihydroxy substitution in B-ring for several bioactivities specially related to scavenger capacity (Bors et al. 1990; Rice-Evans et al. 1996). It is generally accepted that the position and number of OH in the B-ring greatly determine the scavenger capacity of these compounds. The presence of the *o*-dihydroxy structure on the B-ring confers a higher degree of stability to the phenoxy radicals by participating in electron delocalization.

On the other hand, regarding the intracellular markers of oxidative stress, particularly ROS production, all flavones except compound **6** reduced free radical levels at different concentrations. The only additional observation is that compound **8** reduced free radicals species at a lower and single concentration, while the other flavones decreased levels at a wider range of concentrations. The most active scavenger molecules were those that showed the higher capacity of ROS reduction. The increase of lipid peroxidation, the other marker of oxidative stress utilized, was also prevented by all flavones. From these experiments, we can conclude that, in the pre-treatment experimental conditions, there is no direct relationship between the neuronal protection and the antioxidant capacity of flavones. Fisetin, the nonprotective flavone, decreases oxidative markers in the same range as protective molecules. Our experimental data confirm now what we have advanced as a hypothesis in the previous work: antioxidant capacity appears as a necessary but not enough property for neuroprotection.

Regarding anti-lipid peroxidation experiments, Peng and Kuo (2003), studying anti-lipid peroxidation of 7 flavones, found that quercetin, myricetin, and luteolin inhibited lipid peroxidation in Caco-2 intestinal cells whereas kaempferol did not. They concluded that the most important structural criterion in protecting live cells from lipid peroxidation is the adjacent hydroxyl groups in the B-ring structure. According to our results, not only adjacent hydroxyl substitutions in B-ring are important for lipid peroxidation inhibition, since flavones with methylation in B-ring also decreased lipid peroxidation.

Iron is a very important element in living organisms, and iron-containing enzymes are key components of many essential biological reactions, like energy metabolism, oxygen transport, DNA synthesis and repair, detoxification of ROS, etc. (Kruszewski 2003). However, in some particular conditions, iron, via Fenton/Haber–Weiss reaction

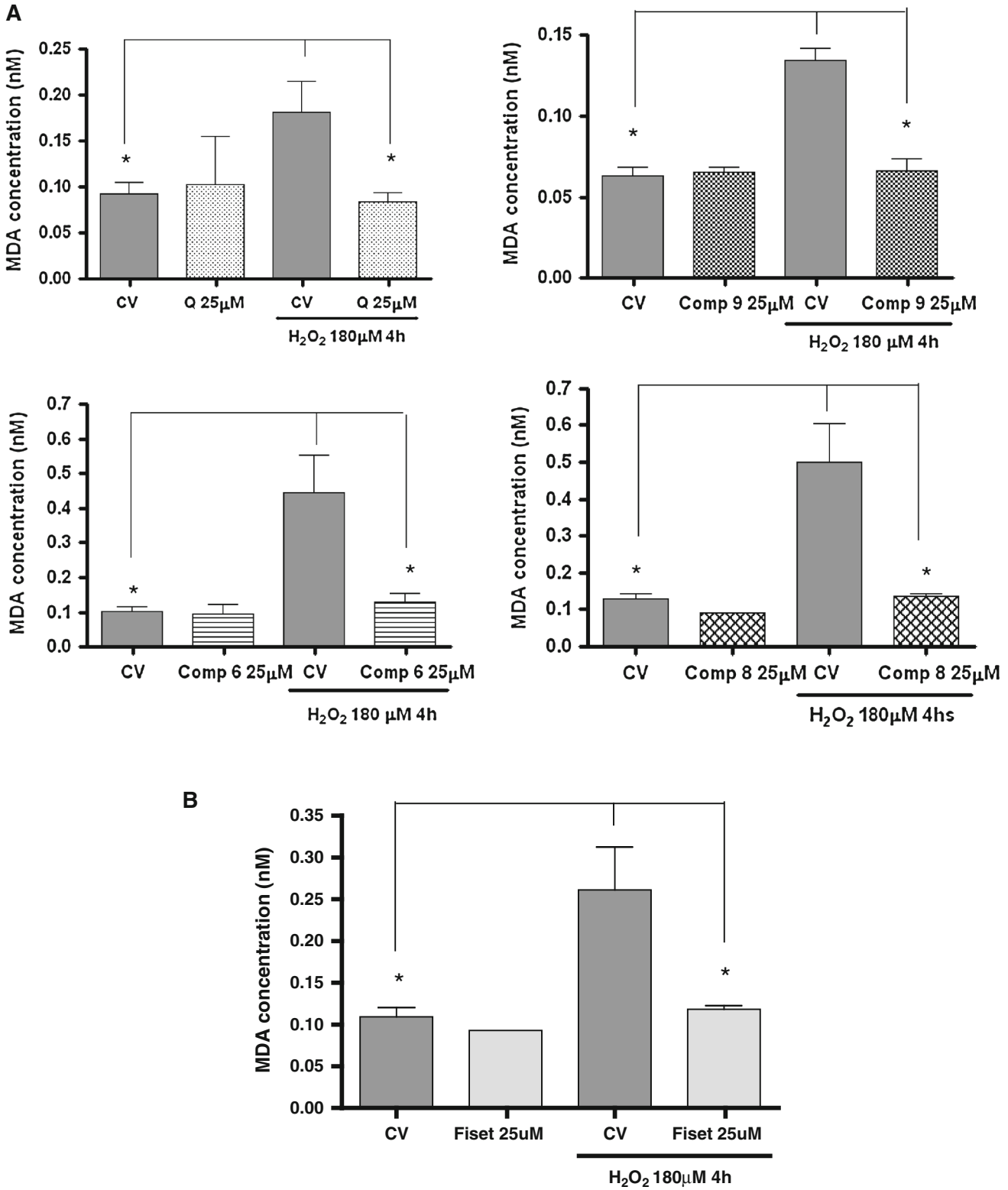


Fig. 5 Malondialdehyde formation after 24 h of pre-treatment with each flavone and addition of 180 μM H_2O_2 for 4 h. Data presented as mean ± SEM of 3 separated experiments. *$p < 0.01$ versus vehicle-treated cells and versus vehicle-treated cells + H_2O_2. **a** Protective flavones. **b** Nonprotective flavone

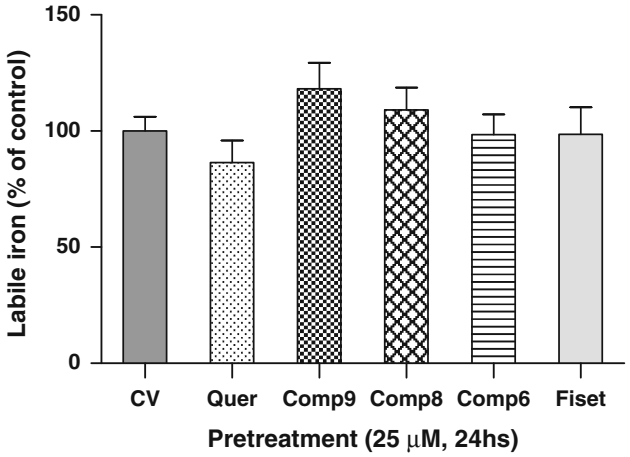


Fig. 6 LIP levels after 24 h pre-incubation with 25 μM of each flavone. Data presented as mean ± SEM of 3 separated experiments

cycle, can catalyze production of highly toxic hydroxyl radicals that can have deleterious effects for cell structures. These are the reasons why the cell keeps a tight control of iron homeostasis. Nevertheless, due to the high iron metabolism, there is always a pool of labile iron (LIP) that is utilized for proteins, enzymes, etc. Pre-treatment with all flavones did not change the levels of LIP, indicating that in the experimental paradigm utilized, protection would not involve a differential iron homeostasis as a key factor.

It is known that oxidative stress can induce neurotoxic insults by increasing intracellular Ca^{2+} and that hydrogen peroxide induces Ca^{2+} dysregulation as evidenced by an increase in Ca^{2+} baselines. Several studies have shown the importance of quercetin for Ca^{2+} homeostasis (Wang and Joseph 1999). In these experiments, where cells were incubated with quercetin before H_2O_2 application, quercetin was able to block the effects of H_2O_2, restoring Ca^{2+}

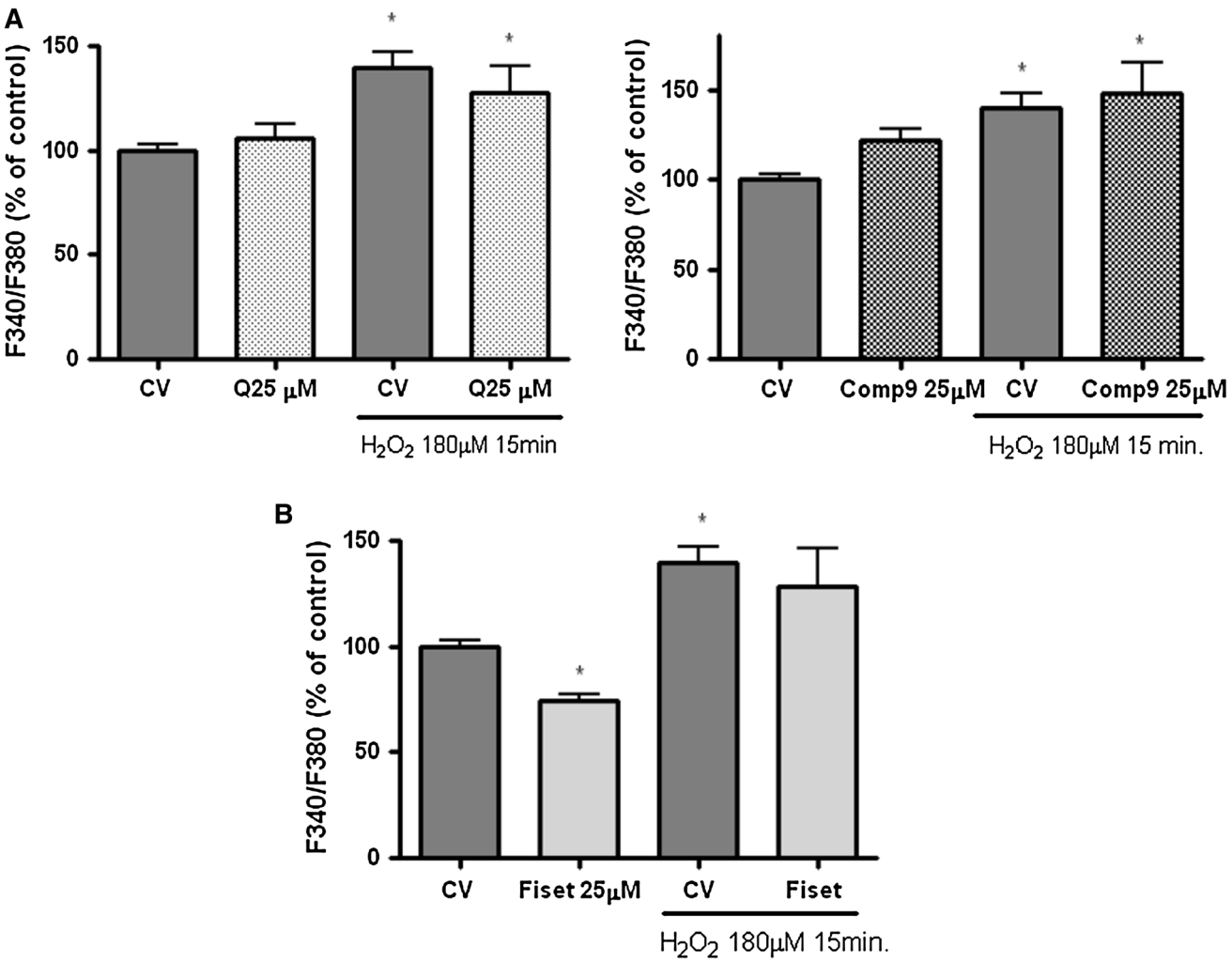


Fig. 7 Ca^{2+} levels after 24 h pre-incubation with 25 μM Quercetin, compound **9** (**a**) and Fisetin (**b**). Data presented as mean ± SEM of 3 separated experiments. *$p < 0.01$ versus vehicle-treated cells (two-way ANOVA-Newman Keuls test)

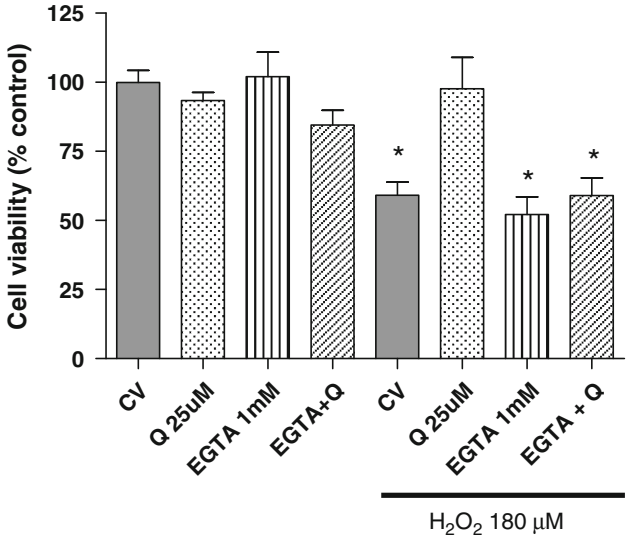


Fig. 8 Evaluation of cell viability in CGN after pre-treatment with quercetin and EGTA alone and subjected to H_2O_2 during 24 h. Data presented as mean of cell viability ± SD. *$p < 0.01$ versus vehicle-treated cells (two-way ANOVA-Newman Keuls test)

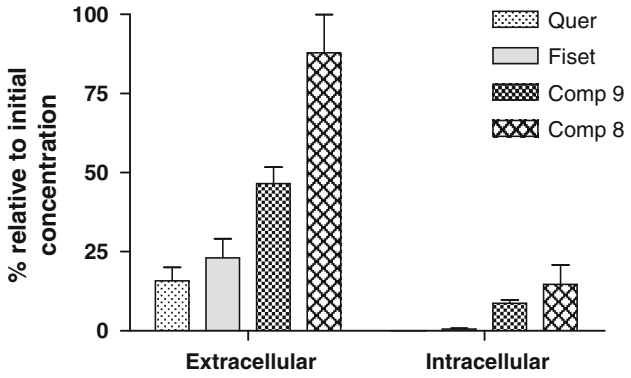


Fig. 9 Extracellular and intracellular concentrations of flavones expressed as percent of initial concentrations. Cell extracts and medium samples were analyzed by HPLC–DAD after 24 h of 25 µM flavones exposition. Data are presented as mean ± SEM of almost 3 separate experiments

homeostasis. A simultaneous structure–activity study showed the importance of the catechol group and the 2,3-double bond in conjugation with a 4-oxo group in the C-ring, along with the polyphenol structure for the protective effect against H_2O_2. Quercetin effects were stronger than catechin, cyanidin, and taxifolin (Wang and Joseph 1999). In our experiments, quercetin did not modify the H_2O_2-induced intracellular Ca^{2+} increase, a result likely reflecting the different experimental paradigms utilized. Results obtained with quercetin, and compound **9** would be showing that, globally, protective flavones do not appear to modify Ca^{2+} homeostasis. In contrast, nonprotective fisetin pre-treatment produced a decrease in Ca^{2+} intracellular concentrations, indicating an interference with homeostasis, likely contributing to the nonprotective profile. Previous work has shown that flavones without hydroxylation substitutions in A-ring present different effects on Ca^{2+} mechanisms regulating relaxation in rat-isolated thoracic aorta (Chan et al. 2000). If an equilibrated Ca^{2+} homeostasis is critically important for all cells, it is particularly important for the granule neurons. NMDA receptors are abundant in granule neurons, and low Ca^{2+} concentrations can lead these neurons to death (Franklin and Johnson 1992). Accordingly, decreased Ca^{2+} concentrations would not be compatible with survival and can explain the nonprotective results as shown by our results when EGTA blocks quercetin protection by decreasing Ca^{2+}.

In summary, oxidative cellular damage produced by H_2O_2 was prevented by all the studied flavones in a first antioxidant defense that was rather similar in potency for all of them, as shown by intracellular ROS and MDA production. Nonetheless, Fisetin, the only nonprotective flavone studied, modifying Ca^{2+} homeostasis, likely interferes with intracellular signaling in a way that could lead neurons to death.

In our previous work (Echeverry et al. 2010), we have shown that methylation of the catechol group in B-ring of flavones keeps the neuroprotective capacity with a minor toxicity, in spite of a lower antioxidant activity observed in the present work. Together with the less degradation observed in this work by the catechol changes, the results are showing that methylated flavones could be a lead for safer neuroprotective molecules of therapeutic potential.

Acknowledgments This work was supported by Grant L/ICA/139636 from the International Cooperation and Assistance Office of the OPCW (Organization for the Prohibition of Chemical Weapons), The Hague, The Netherlands. We thank Prof. Prem Ponka for the generous gift of the iron chelator SIH.

Conflict of interest The authors declare no conflicts of interest.

References

Almajano MP, Vila I, Gines S (2011) Neuroprotective effects of white tea against oxidative stress-induced toxicity in striatal cells. Neurotox Res 20:372–378

Andersen OM, Markham KR (2010) Flavonoids: chemistry, biochemistry and applications. CRC Press, Boca Raton

Arredondo F, Echeverry C, Abin-Carriquiry JA, Blasina F, Antúnez K, Jones DP, Go YM, Liang YL, Dajas F (2010) After cellular internalization, quercetin causes Nrf2 nuclear translocation, increases glutathione levels, and prevents neuronal death against an oxidative insult. Free Radic Biol Med 49:738–747

Bors W, Heller W, Michel C, Saran M (1990) Radical chemistry of flavonoid antioxidants. Adv Exp Med Biol 264:165–170

Breuer W, Epsztejn S, Millgram P, Cabantchik ZI (1995) Transport of iron and other transition metals into cells as revealed by a fluorescent probe. Am J Physiol 268:1354–1361

Chan EC, Pannangpetch P, Woodman OL (2000) Relaxation to flavones and flavonols in rat isolated thoracic aorta: mechanism of action and structure–activity relationships. Cardiovasc Pharmacol 35:326–333

Chang H, Mi M, Ling W, Zhu J, Zhang Q, Wei N, Zhou Y, Tang Y, Yuan J (2008) Structurally related cytotoxic effects of flavonoids on human cancer cells in vitro. Arch Pharm Res 31:1137–1144

Clifford M (2001) A nomenclature for phenols with special reference to tea. Crit Rev Food Sci Nutr 41:393–397

Dajas F (2012) Life or death: neuroprotective and anticancer effects of quercetin. J Ethnopharmacol 143:383–396

Dajas F, Rivera F, Blasina F, Arredondo F, Echeverry C, Lafon L, Morquio A, Heizen H (2003) Cell culture protection and in vivo neuroprotective capacity of flavonoids. Neurotox Res 5:425–432

Denizot F, Lang R (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods 89:271–277

Draper HH, Squires EJ, Mahmoodi H, Wu J, Agarwal S, Hadley M (1993) A comparative evaluation of thiobarbituric acid methods for the determination of malondialdehyde in biological materials. Free Radic Biol Med 15:353–363

Echeverry C, Arredondo F, Abin-Carriquiry JA, Midiwo JO, Ochieng C, Kerubo L, Dajas F (2010) Pretreatment with natural flavones and neuronal cell survival after oxidative stress: a structure–activity relationship study. J Agric Food Chem 58:2111–2115

Franklin JL, Johnson EM (1992) Suppression of programmed neuronal death by sustained elevation of cytoplasmic calcium. Trends Neurosci 15:501–508

García O, Massieu L (2001) Strategies for neuroprotection against L-trans-2,4-pyrrolidine dicarboxylate-induced neuronal damage during energy impairment in vitro. J Neurosci Res 64:418–428

Harborne JB (ed) (1994) The flavonoids, advances in research since 1986. Chapman and Hall, London

Heijnen CG, Haenen GR, Vekemans JA, Bast A (2001) Peroxynitrite scavenging of flavonoids: structure activity relationship. Environ Toxicol Pharmacol 10:199–206

Heim KE, Tagliaferro AR, Bobilya DJ (2002) Flavonoid antioxidants: chemistry, metabolism and structure–activity relationships. J Nutr Biochem 13:572–584

Juma BF, Yenese A, Midiwo JO, Waterman PG (2001) Flavones and phenylpropenoids in the surface exudate of *Psiadia punctulata*. Phytochemistry 57:571–574

Kruszewski M (2003) Labile iron pool: the main determinant of cellular response to oxidative stress. Mutat Res 531:81–92

Li S, Pu XP (2011) Neuroprotective effect of kaempferol against a 1-methyl-4-phenyl-1.2.3.6-tetrahydropyridine-induced mouse model of Parkinsońs disease. Biol Pharm Bull 34:1291–1296

Liu WB, Wang YP (2001) Magnesium lithospermate B inhibits hypoxia-induced calcium influx and nitric oxide release in endothelial cell. Acta Pharmacol Sin 22:1135–1142

Middleton E Jr, Kandaswami C, Theoharides TC (2000) The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev 52:673–751

Miller NJ, Rice-Evans C, Davies MJ, Gapinathan V, Milner A (1993) A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in prematures neonates. Clin Sci 84:407–412

Mira L, Fernández MT, Santos M, Rocha R, Florencio MH, Jennings KR (2002) Interactions of flavonoids with iron and copper ions: a mechanism for their antioxidant activity. Free Radic Res 36:1199–1208

Mueller H, Kassack MU, Wiese M (2004) Comparison of the usefulness of the MTT, ATP, and calcein assays to predict the potency of cytotoxic agents in various human cancer cell lines. J Biomol Screen 9:506–515

Nijveldt RJ, van Nood E, van Hoorn DE, Boelens PG, van Norren K, van Leeuwen PA (2001) Flavonoids: a review of probable mechanisms of action and potential applications. Am J Clin Nutr 74:418–425

Okimotoa Y, Watanabea A, Nikia E, Yamashitab T, Noguchia N (2000) A novel fluorescent probe diphenyl-1-pyrenylphosphine to follow lipid peroxidation in cell membranes. FEBS Lett 474:137–140

Ossola B, Kääriäinen TM, Männistö PT (2009) The multiple faces of quercetin in neuroprotection. Expert Opin Drug Saf 8:397–409

Peng W, Kuo SM (2003) Flavonoid structure affects the inhibition of lipid peroxidation in Caco-2 intestinal cells at physiological concentrations. J Nutr 133:2184–2187

Pietta PG (2000) Flavonoids as antioxidants. J Nat Prod 63:1035–1042

Rice-Evans C (2001) Flavonoid antioxidants. Curr Med Chem 8:797–807

Rice-Evans CA, Miller NJ, Paganga G (1996) Structure–antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med 20:933–956

Simonyi A, Wang Q, Miller RL, Yusof M, Shelat PB, Sun AY, Sun GY (2005) Polyphenols in cerebral ischemia: novel targets for neuroprotection. Mol Neurobiol 31:135–147

Spencer JP (2009) Flavonoids and brain health: multiple effects underpinned by common mechanisms. Genes Nutr 4:243–250

Walle T (2009) Methylation of dietary flavones increases their metabolic stability and chemopreventive effects. Int J Mol Sci 10:5002–5019

Wang H, Joseph JA (1999) Structure–activity relationships of quercetin in antagonizing hydrogen peroxide-induced calcium dysregulation in PC12 cells. Free Radic Biol Med 27:683–694

Williams RJ, Spencer JPE, Rice-Evans C (2004) Flavonoids: antioxidants or signalling molecules? Free Radic Biol Med 36:838–849

Yenesew A, Irungu B, Derese S, Midiwo JO, Heydenreich M, Peter MG (2003) Two prenylated flavonoids from the stem bark of *Erythrina burttii*. Phytochemistry 63:445–448

Neurochemistry International 89 (2015) 140–148

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

Quercetin in brain diseases: Potential and limits

Federico Dajas*, Juan Andrés Abin-Carriquiry, Florencia Arredondo, Fernanda Blasina, Carolina Echeverry, Marcela Martínez, Felicia Rivera, Lucía Vaamonde

Department of Neurochemistry, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay

ARTICLE INFO

Article history:
Received 31 January 2015
Received in revised form
7 May 2015
Accepted 1 July 2015
Available online 6 July 2015

Keywords:
Nutraceuticals
Flavonoids
Quercetin
Neuroprotection
Brain

ABSTRACT

Quercetin is a ubiquitous flavonoid present in beverages, food and plants that has been demonstrated to have a role in the prevention of neurodegenerative and cerebrovascular diseases.

In neuronal culture, quercetin increases survival against oxidative insults. Antioxidation appears to be a necessary but not sufficient condition for its neuroprotective action and modulation of intracellular signaling and transcription factors, increasing the expression of antioxidant and pro survival proteins and modulating inflammation, appears as important for neuronal protection. Quercetin also regulates the activity of kinases, changing the phosphorylation state of target molecules, resulting in modulation of cellular function and gene expression. Concentrations of quercetin higher than 100 μM consistently show cytotoxic and apoptotic effects by its autoxidation and generation of toxic quinones.

In vivo, results are controversial with some studies showing neuroprotection by quercetin and others not, requiring a drug delivery system or chronic treatments to show neuroprotective effects. The blood and brain bioavailability of free quercetin after ingestion is a complex and controversial process that produces final low concentrations, a fact that has led to suggestions that metabolites would be active by themselves and/or as pro-drugs that would release the active aglycone in the brain. Available studies show that in normal or low oxidative conditions, chronic treatments with quercetin contributes to re-establish the redox regulation of proteins, transcription factors and survival signaling cascades that promote survival. In the presence of highly oxidative conditions such as in an ischemic tissue, quercetin could become pro-oxidant and toxic. At present, evidence points to quercetin as a preventive molecule for neuropathology when administered in natural matrices such as vegetables and food. More research is needed to support its use as a lead compound in its free form in acute treatments, requiring new pharmaceutical formulations and/or structural changes to limit its pro-oxidant and toxic effects.

1. Introduction

The high and growing incidence of neurodegenerative and cerebrovascular diseases and the lack of effective therapies to treat them are a permanent challenge in the search of new therapeutic alternatives (De Lau and Breteler, 2006; Gustavsson et al., 2011; Reitz et al., 2011).

Although mechanisms resulting in neuronal death such as cellular energy failure, acidosis, glutamate release, intracellular calcium accumulation, lipid peroxidation, protein misfolding and neuro-inflammation have been known for some time (Bains and Shaw, 1997; Halliwell and Gutteridge, 1999; Kakizuka, 1998), this knowledge has not contributed to identify effective therapeutic targets. Despite intensive research aimed at developing neuroprotective treatments, no drugs have been proven successful for acute pharmacotherapy of stroke or treatment of Azheimer's Disease (Thompson and Ronaldson, 2014; Zemek et al., 2014).

In brain diseases, oxidative stress is involved in several of the identified pathological mechanisms ending in neuronal death. The excessive production of reactive oxygen and nitrogen species (ROS and NOS) has been recognized as critical in the beginning, maintenance and progression of neurodegenerative diseases (Gandhi and Abramov, 2012; Simonian and Coyle, 1996; Uttara et al., 2009). Nevertheless, trials with well-known antioxidant agents such as Vitamin E or C have shown negative and controversial results (Arts et al., 2004; Bjelakovic and Gluud, 2007; Devore et al., 2013; Emmert and Kirchner; Fata et al., 2014; Maxwell et al., 2005; Zhao et al., 2012).

* Corresponding author. Dept. of Neurochemistry, Instituto de Investigaciones Biológicas Clemente Estable, Avda Italia 3318, 11600, Montevideo, Uruguay.
E-mail address: fdajas@gmail.com (F. Dajas).

http://dx.doi.org/10.1016/j.neuint.2015.07.002
0197-0186/

In this disappointing scenario, numerous studies have consistently shown that the consumption of natural products in the form of beverages such as tea and wine as well as diets rich in fruits and vegetables, have a preventive role for cerebrovascular diseases and neurodegeneration. As an example, Devore and collaborators (Devore et al., 2013), have shown that an increase in long-term intake of blueberries and strawberries is associated with a slower rate of cognitive decline. Studies of this type are numerous, showing e.g. the protective actions of catechins of green tea (Mandel et al., 2005), the protective activity of flavonoids of red wine (Vauzour et al., 2008), the beneficial effects of flavonoids in flavonoid-rich diets or flavonoids in the Mediterranean diet (Miller and Ruiz-Larrea, 2002; Nijveldt et al., 2001). Fruit and vegetable juices may play also a role in delaying the onset of Alzheimer's disease (Dai et al., 2006; Nooyens et al., 2011). In a recent meta-analysis, 17 of 19 epidemiological studies reported significant benefits of fruit or vegetable consumption for cognitive performance (Lamport et al., 2014). Nevertheless, more research with carefully controlled trials is needed to answer since some inconsistencies have been reported (Halliwell, 2012; Mecocci et al., 2014). However, the available data suggest that chronic consumption of fruits and vegetables is beneficial for cognition in healthy older adults and could have a preventive role in brain pathology.

When researchers have tried to translate these protective and preventive actions into pharmacological interventions utilizing individual flavonoids contained in fruits and vegetables, controversial results have been observed in experimental neurodegenerative and cerebrovascular models (Arredondo et al., 2015; Ossola et al., 2008). The reasons for these inconsistencies between pharmacological effects of isolated flavonoids or chronic actions of the same molecules in complex mixtures are not simple and are not fully understood. Metabolism, bioavailability or final brain concentrations could be possible explanations (Dajas, 2012).

As a contribution to understand this matter we have chosen quercetin, a representative flavonoid, studying its neuroprotective effects in ischemic models, utilizing plant extracts with high concentrations of the flavonoid or assessing the protective capacity of the free aglycone *in vitro* and *in vivo*. We have studied biological markers of antioxidation, analyzing also actions on intracellular signaling, transcription factors and redox homeostasis. In this review we will analyze both beneficial and deleterious aspects of quercetin pharmacological profile, summarizing the results obtained, and comparing them with similar experimental approaches of different isolated flavonoids.

2. QUERCETIN

Quercetin (3, 3′, 4′, 5, 7-pentahydroxyflavone) is one of several naturally occurring dietary flavonol compounds belonging to a broad group of polyphenolic flavonoid substances (Fig. 1). Flavonoids are characterized by a phenyl benzo(c) pyrone-derived structure consisting of two benzene rings (A and B in Fig. 1) linked by a heterocyclic pyran or pyrone ring (C in Fig. 1) (Kühnau, 1976; Morand et al., 1998). In nature quercetin occurs primarily as quercetin glycoside which consists of the aglycone form conjugated to sugar moieties such as glucose or rutinose (Middleton, 1998). Major dietary sources include lettuce, chili pepper, cranberry, onion, tomato, broccoli and apple, which contribute to an estimated dietary intake of 16–25 mg/day in western countries (Hertog et al., 1993), and it is also present in a wide variety of plant-derived infusions (Farzaneh and Carvalho, 2015).

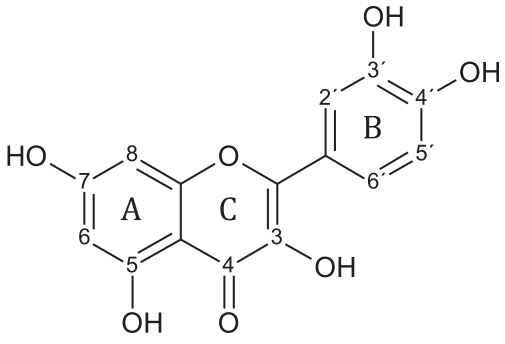


Fig. 1. Molecular structure of quercetin.

Quercetin exhibits numerous biological and pharmacological activities. Beyond neuropathology, a broad spectrum of beneficial properties have been described, including anti-cancer and modulation of cancer-related multi-drug resistance, anti-inflammatory effects, anti-atherosclerotic, anti-thrombotic, antihypertensive, as well as benefits for human endurance exercise capacity, among others (Bischoff, 2008; Chen et al., 2010; Formica and Regelson, 1995; Hertog et al., 1993; Kressler et al., 2011; Larson et al., 2010; Mendoza and Burd, 2011; Russo et al., 2012).

3. Mechanisms of quercetin neuroprotection in cell cultures: from intrinsic redox properties to cell signaling modulation

The beneficial effects of quercetin and related flavonoids have been attributed mainly to their antioxidant capacity, including their direct free radical scavenger and their metal chelating activity properties (Nijveldt et al., 2001; Rice-Evans, 2001). Additionally, quercetin presents indirect antioxidant effects through its capacity to interact and modulate antioxidant enzyme activities (Hanasaki et al., 1994).

Early work already demonstrated that the *in vitro* free radical scavenger activity of quercetin depends on the arrangement of functional groups on its core structure. Thus, the antioxidant potency has been linked to the ortho-dihydroxy substitution in the B-ring and the presence of 2,3-unsaturation and a 4-carbonyl in the C-ring (Fig. 1) (Bors et al., 1990; Heim et al., 2002; Rice-Evans, 2001). The presence of the ortho-dihydroxy structure on the B-ring confers a high degree of stability to the phenoxy radical, participating in electron delocalization. On the other hand, the activity as metal ion chelator appears to be defined by the presence of the ortho-dihydroxy substitution in the B-ring, the 4-carbonyl in the C-ring and a 5 or 3 hydroxy substitution (Mira et al., 2002). In this sense, quercetin fulfills all the structural requirements for effective radical scavenging activity, a fact that has been experimentally confirmed by us and other groups (Echeverry et al., 2015; Rice-Evans, 2001).

However, the structural requirements for more complex pharmacological functions such as the promotion of cell and neuronal survival have not been fully described yet. Thus, while the cytoprotective capacity of flavonoids has been mainly attributed to their antioxidant potency (Cainelli et al., 2008; Zhang et al., 2006) some studies (Dajas et al., 2003a,b; Takashima et al., 2014; Chen et al., 2015), assessing the cytoprotective potency of several structurally related flavonoids against oxidative stress, found that cytoprotection did not correlate with antioxidation. In particular, studying in cerebellar granule neurons the neuroprotective capacity of natural flavones, structurally related to quercetin, we found that the ortho-dihydroxy substitution in B-ring is not necessary to afford neuroprotection against an oxidative insult provoked by hydrogen peroxide (Echeverry et al., 2010). The hydroxyl substitutions in the position C3 (C-ring) and in C5 (A-ring) of the quercetin molecule would be also important for neuroprotection. Hence, our results showed that the structural features implicated in neuronal protection are different from those that provide the free radical

scavenging capacity (Echeverry et al., 2010).

A significant point for the global assessment of quercetin potential as a neuroprotective molecule is its pro-oxidant and cytotoxic properties observed at high concentrations in cell cultures. Although the concentration at which quercetin produces these negative effects depends on the cell type and exposure time, several studies have shown that concentrations higher than 100 μM can have pro-oxidant, apoptotic, antiproliferative, cytotoxic and genotoxic activities (Cotelle, 2001; Lambert et al., 2007). The pro-oxidant activity of quercetin is likely attributable to the presence of hydroxyls in the ortho-dihydroxy substitution in the B ring and its susceptibility to auto-oxidize or be converted to ortho-semiquinone and ortho-quinone/quinonemethide intermediates via enzymatic oxidation (Boots et al., 2003; Chang et al., 2008; Metodiewa et al., 1999; Walle, 2009). In this sense, we have shown that methylation in positions 3′ or 4′ of this catechol group (3′-O-methylquercetin (isorhamnetin) and 4′-O-methylquercetin (tamarixetin) produced compounds with less toxicity, keeping the neuroprotective capacity (Echeverry et al., 2010). These results would suggest that methylated metabolites of quercetin could be a lead structure for safer neuroprotective molecules with therapeutic potential.

Otherwise, at lower concentrations, quercetin pro-oxidant properties can alter the intracellular redox state through the generation of moderate amounts of ROS or indirectly through partial depletion of intracellular reduced glutathione (GSH). Both events may lead to a situation of mild oxidative stress, which in turn will influence antioxidant gene expression by modulation of redox sensitive transcription factors. This is the case of the transcription factor NF-E2-related factor-2 (Nrf2). Activation of Nrf2-dependent pathway results in the expression of genes with antioxidant responsive elements/electrophile responsive elements (ARE/EpRE) in their promoters, encoding for pro-survival, detoxifying and antioxidant proteins. Evidence suggests that flavonoids that retain a high intrinsic redox potential to generate ROS, like quercetin, would be potent inducers of ARE/EpRE-mediated Nrf2 activation (Lee-Hilz et al., 2006). Indeed, quercetin induces an Nrf2-dependent increase in gene expression of γ-glutamyl-cysteine-synthetase (GCS), the rate-limiting enzyme in the synthesis of GSH, one of the most important endogenous cellular antioxidants (Myhrstad et al., 2002; Lavoie et al., 2009; Arredondo et al., 2010). An increase in GSH levels through Nrf2 activation and neuroprotection by quercetin has been described in cerebellar granule neuronal cultures (Arredondo et al., 2010). Previous reports showed that primary cortical neurons also undergo ARE-mediated gene expression but appear to be constrained because of insufficient levels of Nrf2 protein (Shih et al., 2003). Alternatively these authors proposed that Nrf2-dependent enhancement of glial GSH release appears to be necessary and sufficient for cortical neuronal protection (Shih et al., 2003). Since it has been demonstrated that quercetin also induces Nrf2 activation in astrocytes, and microglia (Kang et al., 2013; Lavoie et al., 2009) it is likely that glial cells play a role in Nrf2-mediated neuroprotection by quercetin.

Furthermore, regarding the role of quercetin as a gene regulator, NF-κB is another key transcription factor regulated by quercetin and other flavonoids (Muraoka et al., 2002). This modulatory effect could improve neuro-inflammatory processes involved in diseases such as ischemic stroke and neurodegeneration, becoming a central mechanism of the beneficial effects of quercetin in these diseases (Chen et al., 2005). Additionally, AP-1 is also regulated by quercetin (Moon et al., 2003). This transcription factor regulates the expression of genes associated with cell growth and cellular stress. Other important cellular targets involved in cell repair and protective mechanisms are the sirtuins. In particular recent discoveries have shown that quercetin induces and activates SIRT1 regulating several stress related processes (Howitz et al., 2003, Vaziri et al., 2001). SIRT1 controls these processes by NAD+ dependent deacetylation of acetylated lysine groups of several transcription factors and other proteins such as histones, p53, FOXO transcription factors, among others (Vaziri et al., 2001).

In addition to its role as a potent antioxidant and transcription factor modulator, quercetin directly interacts with several cellular proteins, for example, by binding to ATP sites on enzymes and receptors. Furthermore quercetin has been considered a modulator of a broad number of protein kinases and lipid kinases signaling cascades such as phosphoinositide 3-kinase (PI3-kinase), Akt/PKB, tyrosine kinases, protein kinase C (PKC), and MAP kinases (Ishikawa and Kitamura, 2000; Kobuchi et al., 1999; Spencer, 2007; Williams et al., 2004). By such interaction with kinase-dependent signaling cascades, quercetin cellular effects may be further mediated through changes in the phosphorylation state of target molecules, resulting in modulation of cellular fate (Spencer, 2007; Williams et al., 2004). Indeed, The PI3K–Akt pathway is critical for trophic-factor-induced survival in neurons (Brunet et al., 2001). Some of the most well-characterized survival pathways include the MAP kinase-signaling pathway. Among these, extracellular signal-regulated protein kinase (ERK1/2) and c-Jun N-terminal kinase (JNK) are involved in apoptosis and various forms of cellular plasticity, and they are generally considered as exerting opposing actions. Interestingly, it has been shown that both pro-apoptotic and potentially anti-apoptotic pathways are activated in primary cortical neurons in response to quercetin stimulus or to a lesser extent, its O-methylated metabolites (Spencer et al., 2003). High concentrations of quercetin produced a sustained deactivation of Akt/PKB, leading to extensive caspase-3 activation and subsequent caspase-dependent cleavage of anti-apoptotic Akt/PKB, an event that effectively turns off the major survival signal and results in apoptotic neuronal death. Conversely, lower concentrations, caused a reversible inhibition of Akt phosphorylation and an attempted survival response was reflected in the increase in CREB phosphorylation (Spencer et al., 2003). Thus, in spite of diverse quercetin protective effects observed *in vitro*, caution should be taken in relation to its potential neurotoxicity, especially as *in vivo* concentrations still remain unclear.

Quercetin will have a beneficial or detrimental effect on cell survival depending on the cellular redox state, the flavonoid intracellular concentration and its subcellular localization. In this sense, cellular uptake studies in neurons, epithelial and lymphocyte cells have shown that quercetin is incorporated into cells immediately after its incubation, and that it accumulates in cellular structures like nucleus, nucleoli and mitochondria. Nevertheless, some discrepancies were found in relation of the rate of intracellular transformation of the flavonoid (Arredondo et al., 2010; Fiorani et al., 2010; Notas et al., 2012; Bandaruk et al., 2014). For example, while in neuronal cells quercetin completely disappeared after 24 h of incubation (most likely because of its susceptibility to auto-oxidation and/or its metabolization), in epithelial cells quercetin intracellular levels decreased more slowly and even persisted throughout 24 h (Arredondo et al., 2010; Notas et al., 2012; Bandaruk et al., 2014). Interestingly, these uptake studies strongly suggest that quercetin may exert a pleiotropic effect by modulating mechanisms related to gene transcription in discrete cell structures, i.e. nuclei and nucleoli, more than just only direct antioxidant effects. Furthermore, it was previously shown that quercetin accumulates in mitochondria (Fiorani et al., 2010), and protects against mitochondrial-linked pathologies (Carrasco-Pozo et al., 2012; Lagoa et al., 2011). Likewise, it modulates mitochondria redox state and either inhibits or enhances mitochondrial permeability transition pore (MPTP) permeability (De Marchi et al., 2009). Moreover, it was shown that quercetin enhances mitochondrial

biogenesis in muscle and brain (Davis et al., 2009). Mitochondrial dysfunction contributes to neuron degeneration by depletion of cellular ATP, through ROS generation and development of apoptosis by leading to the opening of the MPTP and the release of cytochrome c (Olanow and Tatton, 1999). Thus, mitochondrial targeted-effects appear to be a process by which quercetin could prevent neurodegeneration. Finally, endoplasmic reticulum (ER) may appear as another quercetin subcellular target. In this sense, disturbances in the normal functions of the ER, i.e. ER stress, leads to an evolutionarily conserved cell stress response, the unfolded protein response (UPR), which initially compensates for damage but can eventually trigger cell death if ER dysfunction is severe or prolonged. Important roles for ER stress-initiated cell death pathways have been recognized for several diseases, including hypoxia, ischemia/reperfusion injury, neurodegeneration, heart disease, and diabetes (Xu et al., 2005). Interestingly, it was shown that ER stress induces presenilin-1 expression through activating transcription factor 4 (ATF4), resulting in increased amyloid-β (Aβ) secretion by γ-secretase activity, which is in turn suppressed by quercetin by controlling ER stress (Ohta et al., 2011). Thus, ER stress preventing effects by quercetin could be a process by which this flavonoid could influence neuronal survival.

4. Quercetin bioavailability

The comprehension of how quercetin or flavonoids in general exert their beneficial effects at the systemic level and more specifically at the brain level requires deeper knowledge of their pharmacokinetics properties. Once ingested, flavonoids undergo several processes such as absorption, distribution, metabolism and excretion, which determine the fate of the aglycone and its metabolites.

The bioavailability of quercetin from different foods varies widely and several factors such as the food matrix, accompanying liquids and type of glycosylation (mainly the type of sugar and the position) can influence it (Arts et al., 2004; Hollman et al., 1997; Olthof et al., 2000).

Although there are several sources of the aglycone, dietary quercetin is mainly present in the glycosidic form, being the most frequent: 3-O-glucoside (isoquercetin), 3-O-rutinoside (rutin), 3-O-rhamnoside (quercitrin) and 3-O-galactoside (hyperoside), 4′-O-glucoside (spiraeoside) (Middleton, 1998).

It has been shown that glycosides are scarcely absorbed after their ingestion and they have to be hydrolyzed to their aglycone form as a first step to be absorbed. O-glucosides (quercetin-3-O-glucoside, quercetin-4′-O-glucoside) are cleaved efficiently in the small intestinal lumen by lactase phlorizin hydrolase or alternatively by the cytosolic β-glycosidase (Aziz et al., 1998; Day et al., 2003, 2000, 1998; Ioku et al., 1998; Németh et al., 2003; Pforte et al., 1999; Sesink et al., 2003; Shimoi et al., 1998; Walgren et al., 2000b, 2000a). On the other hand, quercetin rutinosides, rhamnosides and galactosides would not be efficiently hydrolyzed at the small intestinal level, and instead they are metabolized by resident enterobacteria of the large intestine. Moreover, only a small fraction of the released quercetin would be absorbed because most of it undergoes bacterial-mediated C-ring fission (Graefe et al., 2001; Jaganath et al., 2009, 2006).

Once the aglycone is free at the small intestine lumen, it is important to keep it soluble until it passes through enterocytes by passive transport. At this point the food matrix and accompanying liquids plays a crucial role. The aglycone is submitted to phase II reactions at the enterocytes such as methylation, glucuronidation and/or sulfation and then enters the portal circulation. A minor fraction remain back into the gastrointestinal tract (Day et al., 2000; Olthof et al., 2000).

As a summary of these processes it is clear that the dietary quercetin glucoside would be more efficiently absorbed than oral pharmaceutical preparations of the aglycone because of the very low solubility of the latter in small intestine lumen.

Once at the systemic circulation, quercetin and its metabolites undergo the first liver passage, being submitted to phase I reactions (mainly oxidation, reduction and/or hydrolysis) and making them available for additional phase II metabolization. Water-soluble metabolites could be excreted by the kidney or through the entero-hepatic circulation. Most of conjugated quercetin metabolites circulate in the bloodstream using serum albumin as a carrier protein (Murota et al., 2007) and normally, human total quercetin plasma concentrations are in the low nanomolar range, while quercetin aglycone and quercetin glycosides are scarcely present or undetectable in human and rat plasma (Day et al., 2001; De Boer et al., 2005). Upon quercetin supplementation, metabolites levels may increase to the high nanomolar or low micromolar range (Conquer et al., 1998; Hollman et al., 1996). It has been shown that the half-life of the quercetin metabolites are rather high, i.e. 11–28 h. This indicates that, upon repeated quercetin supplementation, they could attain a considerable plasma level (Hollman and Katan, 1997; Manach et al., 2005).

Finally, although there are numerous epidemiological and experimental evidences that show beneficial effects of dietary quercetin at the central nervous system (CNS) levels, it remains unclear if the aglycone or its metabolites are the responsible of these pharmacological effects (Bieger et al., 2008; De Boer et al., 2005; Ishisaka et al., 2011; Kawabata et al., 2010).

After orally quercetin treatment, the aglycone is scarcely present or absent at the CNS level while conjugated metabolites seems to be accumulated during the treatment (De Boer et al., 2005; Ishisaka et al., 2011) with a particular accumulation of quercetin-3-O-glucuronide in the epithelial cells of the choroid plexus (Ishisaka et al., 2014). Several experimental studies suggest that the metabolites exerts the beneficial effect by themselves (Ishisaka et al., 2011; Terao et al., 2011; Yoshino et al.). On the other hand, there are suggestions that they also may act as a source of aglycone, which could be released by the tissue β-glucuronidase during inflammatory processes (Ishisaka et al., 2014). In this sense, it was proposed that glucuronidated derivatives transport quercetin and its methylated forms, and deliver to the tissues the free aglycone, which would be the final effector (Perez-Vizcaino et al., 2012).

Thus, the complex metabolic processes described above may explain why results from *in vivo* studies do not necessarily reflect what happens *in vitro*. In this context, the utilization of quercetin as a putative therapeutic compound in neurodegenerative processes would mean to introduce pharmaceutical improvements affording brain availability.

5. Quercetin in experimental models of ischemia

The brain is highly susceptible to oxidative damage due to factors such as its high oxygen utilization rate, high iron content, presence of excess unsaturated fatty acids and decreased activities of detoxifying enzymes (Clarke and Sokoloff, 1999; Dringen, 2000; Gerlach et al., 1994; Halliwell, 2012). As mentioned above, a variety of cellular processes including excitotoxicity, metabolic unbalance, and inflammation, all involving the generation of ROS, would contribute to an increased neuronal death during the cerebral ischemic process (Liu et al., 1989; Kinouchi et al., 1991; Cao and Phillis, 1994). Therapeutic strategies to control the ischemic process have focused on one or another of these factors, and although experimental neuroprotective effects have been observed after, e.g. the utilization of NMDA receptor blockers or calcium channel blockers (Aoki et al., 2001; Williams et al., 2000), this therapeutic

approach of one target therapy at a time has largely failed.

On the other hand, as it was said previously, epidemiological evidence has provided support for the preventive use of natural beverages and polyphenol-rich foods in neurodegeneration and cerebrovascular diseases. Plant extracts have also shown to have CNS antioxidant and neuroprotective capacity and several plants extracts are rich in quercetin, mainly in its glycoside forms (Dajas, 2012). In order to compare the bioavailability and actions between flavonoids in complex mixtures and the same flavonoids given in their free form, we have studied *Achyrocline satureioides* (Lam.) D.C., Compositae, (*A. satureioides*), a plant that has a particular flavonoid profile. Widely distributed and utilized in Southern South America for its anti-inflammatory, antioxidant and immune–modulatory activities (Desmarchelier et al., 1998; Retta et al., 2012). *A. satureioides* has a rich flavonoid aglycone composition, including quercetin. After 21 days of oral pre-treatment with *A. satureioides*, rats subjected to permanent middle cerebral artery occlusion (pMCAo) improved their functional deficit, showing a significant decrease of the infarction volume (Rivera Megret et al., 2013). Concentrations of quercetin aglycone in the brain after oral administration of *A. satureioides* were consistently higher than quercetin given alone (unpublished data), showing that the components of the complex matrix of the extract may play a role in the bioavailability of the molecule, an effect previously observed for the food matrix (Bischoff, 2008). This effect could be important for the preventive effects of natural nutrients.

In models of neurotoxicity, chronic oral quercetin administration improved cognitive deficits and oxidative markers (Kumar et al., 2008; Lu et al., 2007). The need for repeated administration to obtain a neuroprotective effect would suggest a difficulty in the access of quercetin to the brain at effective concentrations. The results of chronic experiments would indicate the need for some accumulative concentrations (quercetin and/or metabolites) to obtain pharmacological efficiency (Ishisaka et al., 2011; Kumar et al., 2008; Lu et al., 2007). Time required for adaptive changes in gene expression (antioxidants, growth factors, mitochondrial biogenesis, etc.) to occur in brain may also account for the additional benefits of pre-dosing in models of ischemic brain damage.

Alternatively, a liposomal preparation with lecithin produced quantifiable cerebral amounts of quercetin that reduced significantly the cerebral damage provoked by pMCAo in rats when administered i/p 30 min after the artery occlusion (Rivera et al., 2004). When the protective effects of liposomal quercetin were investigated 1 and 4 h after arterial occlusion, no protective effects were observed. These results suggest that it would be necessary to utilize a systemic drug delivery system to reach measurable and effective brain quercetin concentrations for acute effects (Dajas et al., 2003a,b). Even in this case, quercetin protective effects would be observed only in early, likely not extended stages of brain damage.

On the other hand, quercetin has been studied in neurodegenerative and other CNS diseases (Table 1). As it can be observed in Table 1, regarding experimental Parkinson's Disease, while *in vitro* studies show regular protective effects, *in vivo* approaches are controversial. The same happens in models of Alzheimer's Disease.

Besides its activities at neurons and glia, quercetin plays an important role at the level of the vascular system in the brain, protecting vascular endothelial cells against oxidative and pro-inflammatory insults, potentiating the protective capacity. Evidences of these pharmacological actions are the protection by quercetin of endothelial cells against linoleic acid-mediated cell dysfunction (Reiterer et al., 2004), the down-regulation of cell adhesion molecule intercellular adhesion molecule-1 (ICAM-1) that plays a pivotal role in inflammatory responses (Kobuchi et al., 1999), the regulation of transcription factor hypoxia-inducible factor-1 (HIF-1), facilitating the transcription of numerous target genes involved in glycolysis and angiogenesis (Wilson and Poellinger, 2002). Furthermore, Aβ-peptides induced cytotoxicity in human brain microvascular endothelial cells can be relieved by quercetin treatment. At this level, quercetin strengthens the barrier integrity through the preservation of the trans-endothelial electrical resistance value (Li et al., 2015).

6. Nanosomal preparation of quercetin

As neurodegeneration and cerebrovascular diseases concentrate therapeutic research at final stages of life, perinatal asphyxia (PA) or lack of oxygen at birth is the research challenge at the beginning of life. The brain is the key system affected in PA since its neuropathological lesions can lead to sequelae.

Brain lesion after PA includes a long-lasting and complex process, where appropriate bioavailability of pharmacological agents in neuronal tissue is a key factor for the success of the pharmacological treatment. In this context, the quercetin multi-target action profile defined above would have potential for treatment of PA.

Provided the difficulties already mentioned for quercetin access to the brain, we developed a drug-delivery system consisting in a nanosomal preparation adequate for intravenous administration, capable of multiplying the concentration of quercetin in the brain. We provoked hypoxia in newborn piglets. After the severe hypoxic episode, nanosomal quercetin was applied and piglets were evaluated during three days for behavioral, brain activity and hemodynamic monitoring. Results have shown that the intravenous administration of nanosomes stabilized hemodynamic variables eliminating vasopressor requirements, significantly recovered brain activity and improved spontaneous breathing, motor and feeding behavior 72 hs after hypoxia (unpublished results). These results would show that nanosomes could be a good vehicle to obtain effective concentrations of quercetin in the brain.

7. Concluding remarks

Evidence reviewed above shows quercetin as a potent antioxidant that increases survival in neurons in culture against a variety of oxidative insults. Interestingly, when compared with other flavonoids, antioxidation appears to be a necessary but not sufficient condition for neuroprotection and actions on intracellular signaling and transcription factors appear as crucial for the protection of neurons in culture. Activation of Nrf2, facilitating its translocation to the nucleus and increasing the expression of proteins of antioxidation and survival is particularly important. Increasing glutathione concentrations is one of the results of this activity and a key factor in the restoration of intracellular redox and survival. Activation of other transcription factors such as NF-κB or key molecules for survival such as sirtuins, as well as kinase inhibition and modulation of intracellular signaling are part of the complex pro-survival profile of quercetin. Accordingly, *in vitro* studies show a great potential of quercetin as a multi-target neuroprotective molecule.

Complex absorption and metabolism steps after oral intake difficult a direct translation of quercetin *in vitro* effects to *in vivo* neuroprotection and clinical applicability. Neuroprotection *in vivo* has been mainly observed after oral chronic treatments or by the acute utilization of drug delivery systems.

Besides, the narrow protective concentrations in neuronal culture or the short time therapeutic window in experimental focal ischemia would be a reflection of the “pro-oxidant” actions of quercetin when acutely positioned in presence of high oxidative stress in areas such as an ischemic tissue. The oxidation of quercetin generates quinones that could not be reduced back by the chain of

Table 1
Beneficial and derogative effects of quercetin in neurological and psychiatric diseases.

Experimental Parkinson's Disease	
No protection by quercetin in a 6-OHDA model of Parkinson's disease.	(Zbarsky et al., 2005)
Lack of robust protective effect of quercetin in two types of 6-hydroxydopamine-induced parkinsonian models in rats and dopaminergic cell cultures.	(Ossola et al., 2009)
Protective effects of quercetin against MPP+-induced oxidative stress in dopaminergic neurons.	(Bournival et al., 2009)
Cognitive-enhancing effect of quercetin in a rat model of Parkinson's disease	(Sriraksa et al., 2012)
Anti-parkinsonian properties of quercetin in MPTP model of Parkinson's Disease	(Lv et al., 2012)
Quercetin and desferrioxamine protect on 6-hydroxydopamine model in striatum of rats.	(Haleagrahara et al., 2013)
Quercetin up-regulates mitochondrial complex-I activity to protect against programmed cell death in rotenone model of Parkinson's disease in rats.	(Karuppagounder et al., 2013)
Protective effects of Quercetin glycoside (isoquercitrin), against 6-hydroxydopamine -induced toxicity in PC12 cells.	(Magalingam et al., 2014)
Fish Oil and Quercetin enhance neuroprotection in a rotenone rat model of Parkinson's Disease	(Denny Joseph and Muralidhara, 2015)
Quercetin glycosides induced neuroprotection by changes in the gene expression in a cellular model of Parkinson's disease.	(Magalingam et al., 2015)
Schizophrenia	
Quercetin reverses haloperidol-induced catalepsy	(Naidu and Kulkarni, 2004)
Inhibitory effects of quercetin on lipid peroxidation caused by antipsychotics	(Dietrich-Muszalska and Olas, 2010)
Alzheimer's disease	
Potent anti-amyloidogenic and fibril-destabilizing effects of quercetin *in vitro*.	(Ono et al., 2003)
Protective effect of quercetin in primary neurons against Aβ(1–42).	(Ansari et al., 2009)
Quercetin showed no effect on the expression of antioxidant and Alzheimer's disease relevant genes in mice.	(Huebbe et al., 2010)
Quercetin-3-O-glucuronide as an intervention for Alzheimer's disease.	(Ho et al., 2013)
Quercetin as a constituent of plants (*Acanthopanax henryi*) potential complementary source against Alzheimer's disease.	(Zhang et al., 2014)
Alzheimer's drug lead value of quercetin.	(Hassaan et al., 2014)
Quercetin ameliorates Alzheimer's disease pathology in aged triple transgenic Alzheimer's disease model mice.	(Sabogal-Guáqueta et al., 2015)
Experimental epilepsy	
Quercetin protects from oxidative stress and helps memory retrieval in kindled rats	(Nassiri-Asl et al., 2013)
Huntington's Disease	
Quercetin improves mitochondrial dysfunctions induced by 3-nitropropionic acid as a proposal therapy for Huntington's disease.	(Sandhir and Mehrotra, 2013)
Quercetin improves pathological markers in a 3-nitropropionic acid model of Huntington Disease.	(Chakraborty et al., 2014)

antioxidants such as tocopherol or ascorbate, becoming toxic to neurons. The activation of intracellular signaling such as e.g. modulation of kinases, could re-establish the cellular redox equilibrium, although these aspects have been much less explored *in vivo* and could have double-edge activities regarding neuronal survival in conditions of profound loss of redox equilibrium (quercetin mechanisms of action are summarized in Fig. 2).

These facts would limit an acute therapeutic utilization of quercetin in spite of its promising protective effects *in vitro* and *in vivo*. Modification of the molecule, replacing the toxic hydroxyl groups of the catechol moiety and thus reducing the formation of quinones could be a first important step to increase the weight of beneficial actions against the toxic ones.

The chronic administration of quercetin as part of complex

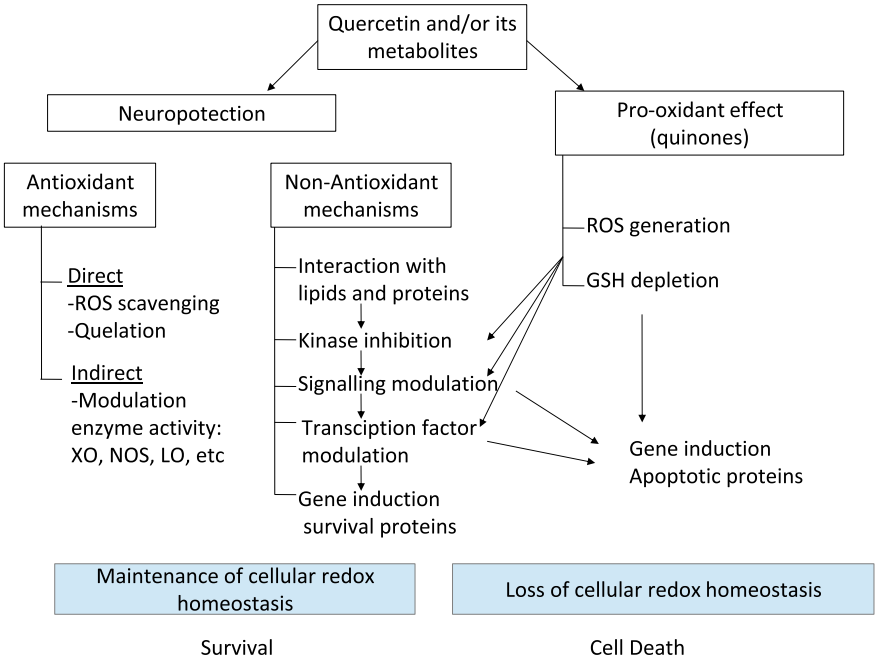


Fig. 2. Scheme summarizing mechanisms of action of quercetin and its metabolites.

natural mixtures would assure low amounts of the molecule in the brain where a low level of its pro-oxidant profile could potentiate antioxidant defenses. The potent and numerous direct and indirect antioxidant actions of quercetin would be effective in the initial redox unbalance stages of CNS diseases. As such, quercetin, as part of plant matrices would have an important role as a preventive nutraceutical agent. Much research is still needed to confirm its potential role as a therapeutic compound in acute conditions such as stroke or in advanced chronic neurodegenerative diseases.

References

Ansari, M.A., Abdul, H.M., Joshi, G., Opii, W.O., Butterfield, D.A., 2009. Protective effect of quercetin in primary neurons against Abeta(1-42): relevance to Alzheimer's disease. J. Nutr. Biochem. 20, 269–275.

Aoki, Y., Tamura, M., Itoh, Y., Ukai, Y., 2001. Cerebroprotective action of a Na+/Ca2+ channel blocker NS-7. I. Effect on the cerebral infarction and edema at the acute stage of permanent middle cerebral artery occlusion in rats. Brain Res. 890, 162–169.

Arredondo, F., Echeverry, C., Abin-Carriquiry, J.A., Blasina, F., Antúnez, K., Jones, D.P., Go, Y.-M., Liang, Y.-L., Dajas, F., 2010. After cellular internalization, quercetin causes Nrf2 nuclear translocation, increases glutathione levels, and prevents neuronal death against an oxidative insult. Free Radic. Biol. Med. 49, 738–747.

Arredondo, F., Echeverry, C., Blasina, F., Vaamonde, L., Díaz, M., Rivera, F., Martínez, M., Abin-Carriquiry, J.A., Dajas, F., 2015. Bioactive Nutraceuticals and Dietary Supplements in Neurological and Brain Disease. Elsevier.

Arts, I.C.W., Sesink, A.L.A., Faassen-Peters, M., Hollman, P.C.H., 2004. The type of sugar moiety is a major determinant of the small intestinal uptake and subsequent biliary excretion of dietary quercetin glycosides. Br. J. Nutr. 91, 841–847.

Aziz, A.A., Edwards, C.A., Lean, M.E., Crozier, A., 1998. Absorption and excretion of conjugated flavonols, including quercetin-4′-O-beta-glucoside and isorhamnetin-4′-O-beta-glucoside by human volunteers after the consumption of onions. Free Radic. Res. 29, 257–269.

Bains, J.S., Shaw, C.A., 1997. Neurodegenerative disorders in humans: the role of glutathione in oxidative stress-mediated neuronal death. Brain Res. Brain Res. Rev. 25, 335–358.

Bandaruk, Y., Mukai, R., Terao, J., 2014. Cellular uptake of quercetin and luteolin and their effects on monoamine oxidase-A in human neuroblastoma SH-SY5Y cells. Toxicol. Rep. 1, 639–649.

Bieger, J., Cermak, R., Blank, R., de Boer, V.C.J., Hollman, P.C.H., Kamphues, J., Wolffram, S., 2008. Tissue distribution of quercetin in pigs after long-term dietary supplementation. J. Nutr. 138, 1417–1420.

Bischoff, S.C., 2008. Quercetin: potentials in the prevention and therapy of disease. Curr. Opin. Clin. Nutr. Metab. Care 11, 733–740.

Bjelakovic, G., Gluud, C., 2007. Surviving antioxidant supplements. J. Natl. Cancer Inst. 99, 742–743.

Boots, A.W., Kubben, N., Haenen, G.R.M.M., Bast, A., 2003. Oxidized quercetin reacts with thiols rather than with ascorbate: implication for quercetin supplementation. Biochem. Biophys. Res. Commun. 308, 560–565.

Bors, W., Heller, W., Michel, C., Saran, M., 1990. Radical chemistry of flavonoid antioxidants. Adv. Exp. Med. Biol. 264, 165–170.

Bournival, J., Quessy, P., Martinoli, M.-G., 2009. Protective effects of resveratrol and quercetin against MPP+ -induced oxidative stress act by modulating markers of apoptotic death in dopaminergic neurons. Cell. Mol. Neurobiol. 29, 1169–1180.

Brunet, A., Datta, S.R., Greenberg, M.E., 2001. Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. Curr. Opin. Neurobiol. 11, 297–305.

Cainelli, G., Angeloni, C., Cervellati, R., Galletti, P., Giacomini, D., Hrelia, S., Sinisi, R., 2008. New polyphenolic beta-lactams with antioxidant activity. Chem. Biodivers. 5, 811–829.

Cao, X., Phillis, J.W., 1994. alpha-Phenyl-tert-butyl-nitrone reduces cortical infarct and edema in rats subjected to focal ischemia. Brain Res. 644, 267–272.

Carrasco-Pozo, C., Mizgier, M.L., Speisky, H., Gotteland, M., 2012. Differential protective effects of quercetin, resveratrol, rutin and epigallocatechin gallate against mitochondrial dysfunction induced by indomethacin in Caco-2 cells. Chem. Biol. Interact. 195, 199–205.

Chakraborty, J., Singh, R., Dutta, D., Naskar, A., Rajamma, U., Mohanakumar, K.P., 2014. Quercetin improves behavioral deficiencies, restores astrocytes and microglia, and reduces serotonin metabolism in 3-nitropropionic acid-induced rat model of Huntington's Disease. CNS Neurosci. Ther. 20, 10–19.

Chang, H., Mi, M., Ling, W., Zhu, J., Zhang, Q., Wei, N., Zhou, Y., Tang, Y., Yuan, J., 2008. Structurally related cytotoxic effects of flavonoids on human cancer cells in vitro. Arch. Pharm. Res. 31, 1137–1144.

Chen, C., Zhou, J., Ji, C., 2010. Quercetin: a potential drug to reverse multidrug resistance. Life Sci. 87, 333–338.

Chen, J.-C., Ho, F.-M., Pei-Dawn, Lee Chao, Chen, C.-P., Jeng, K.-C.G., Hsu, H.-B., Lee, S.-T., Wen, Tung Wu, Lin, W.-W., 2005. Inhibition of iNOS gene expression by quercetin is mediated by the inhibition of IkappaB kinase, nuclear factor-kappa B and STAT1, and depends on heme oxygenase-1 induction in mouse BV-2 microglia. Eur. J. Pharmacol. 521, 9–20.

Chen, P.-Y., Ho, Y.-R., Wu, M.-J., Huang, S.-P., Chen, P.-K., Tai, M.-H., Ho, C.-T., Yen, J.-H., 2015. Cytoprotective effects of fisetin against hypoxia-induced cell death in PC12 cells. Food Funct. 6, 286–295.

Clarke, D.D., Sokoloff, L., 1999. Circulation and Energy Metabolism of the Brain.

Conquer, J.A., Maiani, G., Azzini, E., Raguzzini, A., Holub, B.J., 1998. Supplementation with quercetin markedly increases plasma quercetin concentration without effect on selected risk factors for heart disease in healthy subjects. J. Nutr. 128, 593–597.

Cotelle, N., 2001. Role of flavonoids in oxidative stress. Curr. Top. Med. Chem. 1, 569–590.

Dai, Q., Borenstein, A.R., Wu, Y., Jackson, J.C., Larson, E.B., 2006. Fruit and vegetable juices and Alzheimer's disease: the Kame Project. Am. J. Med. 119, 751–759.

Dajas, F., 2012. Life or death: neuroprotective and anticancer effects of quercetin. J. Ethnopharmacol. 143, 383–396.

Dajas, F., Rivera, F., Blasina, F., Arredondo, F., Echeverry, C., Lafon, L., Morquio, A., Heizen, H., 2003a. Cell culture protection and in vivo neuroprotective capacity of flavonoids. Neurotox. Res. 5, 425–432.

Dajas, F., Rivera-Megret, F., Blasina, F., Arredondo, F., Abin-Carriquiry, J.A., Costa, G., Echeverry, C., Lafon, L., Heizen, H., Ferreira, M., Morquio, A., 2003b. Neuroprotection by flavonoids. Braz. Biol. Res. 36, 1613–1620.

Davis, J.M., Murphy, E.A., Carmichael, M.D., Davis, B., 2009. Quercetin increases brain and muscle mitochondrial biogenesis and exercise tolerance. Am. J. Physiol. Regul. Integr. Comp. Physiol. 296, R1071–R1077.

Day, A.J., Cañada, F.J., Díaz, J.C., Kroon, P.A., Mclauchlan, R., Faulds, C.B., Plumb, G.W., Morgan, M.R., Williamson, G., 2000. Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. FEBS Lett. 468, 166–170.

Day, A.J., DuPont, M.S., Ridley, S., Rhodes, M., Rhodes, M.J., Morgan, M.R., Williamson, G., 1998. Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver beta-glucosidase activity. FEBS Lett. 436, 71–75.

Day, A.J., Gee, J.M., DuPont, M.S., Johnson, I.T., Williamson, G., 2003. Absorption of quercetin-3-glucoside and quercetin-4′-glucoside in the rat small intestine: the role of lactase phlorizin hydrolase and the sodium-dependent glucose transporter. Biochem. Pharmacol. 65, 1199–1206.

Day, A.J., Mellon, F., Barron, D., Sarrazin, G., Morgan, M.R., Williamson, G., 2001. Human metabolism of dietary flavonoids: identification of plasma metabolites of quercetin. Free Radic. Res. 35, 941–952.

De Boer, V.C.J., Dihal, A.A., van der Woude, H., Arts, I.C.W., Wolffram, S., Alink, G.M., Rietjens, I.M.C.M., Keijer, J., Hollman, P.C.H., 2005. Tissue distribution of quercetin in rats and pigs. J. Nutr. 135, 1718–1725.

De Lau, L.M.L., Breteler, M.M.B., 2006. Epidemiology of Parkinson's disease. Lancet. Neurol. 5, 525–535.

De Marchi, U., Biasutto, L., Garbisa, S., Toninello, A., Zoratti, M., 2009. Quercetin can act either as an inhibitor or an inducer of the mitochondrial permeability transition pore: a demonstration of the ambivalent redox character of polyphenols. Biochim. Biophys. Acta 1787, 1425–1432.

Denny Joseph, K.M, Muralidhara, 2015. Combined oral supplementation of fish oil and quercetin enhances neuroprotection in a chronic rotenone rat model: relevance to Parkinson's disease. Neurochem. Res. 40 (5), 894–905.

Desmarchelier, C., Coussio, J., Ciccia, G., 1998. Antioxidant and free radical scavenging effects in extracts of the medicinal herb *Achyrocline satureioides* (Lam.) DC. ("marcela"). Braz. J. Med. Biol. Res. 31, 1163–1170.

Devore, E.E., Kang, J.H., Stampfer, M.J., Grodstein, F., 2013. The association of antioxidants and cognition in the Nurses' Health Study. Am. J. Epidemiol. 177, 33–41.

Dietrich-Muszalska, A., Olas, B., 2010. Inhibitory effects of polyphenol compounds on lipid peroxidation caused by antipsychotics (haloperidol and amisulpride) in human plasma in vitro. World J. Biol. Psychiatry 11, 276–281.

Dringen, R., 2000. Glutathione metabolism and oxidative stress in neurodegeneration. Eur. J. Biochem. 267, 4903.

Echeverry, C., Arredondo, F., Abin-Carriquiry, J.A., Midiwo, J.O., Ochieng, C., Kerubo, L., Dajas, F., 2010. Pretreatment with natural flavones and neuronal cell survival after oxidative stress: a structure-activity relationship study. J. Agric. Food Chem. 58, 2111–2115.

Echeverry, C., Arredondo, F., Martínez, M., Abin-Carriquiry, J.A., Midiwo, J., Dajas, F., 2015. Antioxidant activity, cellular bioavailability, and iron and calcium management of neuroprotective and nonneuroprotective flavones. Neurotox. Res. 27, 31–42.

Emmert, D.H., Kirchner, J.T. The role of vitamin E in the prevention of heart disease. Arch. Fam. Med. 8, 537–542.

Farzaneh, V., Carvalho, I.S., 2015. A review of the health benefit potentials of herbal plant infusions and their mechanism of actions. Ind. Crops Prod. 65, 247–258.

Fata, G. La, Weber, P., Mohajeri, M.H., 2014. Effects of vitamin E on cognitive performance during ageing and in Alzheimer's disease. Nutrients 6, 5453–5472.

Formica, J.V., Regelson, W., 1995. Review of the biology of quercetin and related bioflavonoids. Food Chem. Toxicol. Int. J. Publ. Br. Ind. Biol. Res. Assoc. 33, 1061–1080.

Fiorani, M., Guidarelli, A., Blasa, M., Azzolini, C., Candiracci, M., Piatti, E., Cantoni, O., 2010. Mitochondria accumulate large amounts of quercetin: prevention of mitochondrial damage and release upon oxidation of the extramitochondrial fraction of the flavonoid. J. Nutr. Biochem. 21, 397–404.

Gandhi, S., Abramov, A.Y., 2012. Mechanism of oxidative stress in neurodegeneration. Oxid. Med. Cell. Longev. 2012, 428010.

Gerlach, M., Ben-Shachar, D., Riederer, P., Youdim, M.B., 1994. Altered brain metabolism of iron as a cause of neurodegenerative diseases? J. Neurochem. 63,

793–807.
Graefe, E.U., Wittig, J., Mueller, S., Riethling, A.K., Uehleke, B., Drewelow, B., Pforte, H., Jacobasch, G., Derendorf, H., Veit, M., 2001. Pharmacokinetics and bioavailability of quercetin glycosides in humans. J. Clin. Pharmacol. 41, 492–499.
Gustavsson, A., Svensson, M., Jacobi, F., Allgulander, C., Alonso, J., Beghi, E., Dodel, R., Ekman, M., Faravelli, C., Fratiglioni, L., Gannon, B., Jones, D.H., Jennum, P., Jordanova, A., Jönsson, L., Karampampa, K., Knapp, M., Kobelt, G., Kurth, T., Lieb, R., Linde, M., Ljungcrantz, C., Maercker, A., Melin, B., Moscarelli, M., Musayev, A., Norwood, F., Preisig, M., Pugliatti, M., Rehm, J., Salvador-Carulla, L., Schlehofer, B., Simon, R., Steinhausen, H.-C., Stovner, L.J., Vallat, J.-M., Van den Bergh, P., van Os, J., Vos, P., Xu, W., Wittchen, H.-U., Jönsson, B., Olesen, J., 2011. Cost of disorders of the brain in Europe 2010. Eur. Neuropsychopharmacol. 21, 718–779. http://dx.doi.org/10.1016/j.euroneuro.2011.08.008.
Haleagrahara, N., Siew, C.J., Ponnusamy, K., 2013. Effect of quercetin and desferrioxamine on 6-hydroxydopamine (6-OHDA) induced neurotoxicity in striatum of rats. J. Toxicol. Sci. 38, 25–33.
Halliwell, B., 2012. Free radicals and antioxidants: updating a personal view. Nutr. Rev. 70, 257–265.
Halliwell, B., Gutteridge, J.M.C., 1999. Free Radicals in Biology and Medicine, Free Radical Biology and Medicine. Oxford University Press.
Hanasaki, Y., Ogawa, S., Fukui, S., 1994. The correlation between active oxygens scavenging and antioxidative effects of flavonoids. Free Radic. Biol. Med. 16, 845–850.
Hassaan, Y., Handoussa, H., El-Khatib, A.H., Linscheid, M.W., El Sayed, N., Ayoub, N., 2014. Evaluation of plant phenolic metabolites as a source of Alzheimer's drug leads. Biomed. Res. Int. 2014, 843263.
Heim, K.E., Tagliaferro, A.R., Bobilya, D.J., 2002. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. J. Nutr. Biochem. 13, 572–584.
Hertog, M.G., Hollman, P.C., Katan, M.B., Kromhout, D., 1993. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. Nutr. Cancer 20, 21–29.
Ho, L., Ferruzzi, M.G., Janle, E.M., Wang, J., Gong, B., Chen, T.-Y., Lobo, J., Cooper, B., Wu, Q.L., Talcott, S.T., Percival, S.S., Simon, J.E., Pasinetti, G.M., 2013. Identification of brain-targeted bioactive dietary quercetin-3-O-glucuronide as a novel intervention for Alzheimer's disease. FASEB J. 27, 769–781.
Hollman, P.C., Hertog, M.G., Katan, M.B., 1996. Role of dietary flavonoids in protection against cancer and coronary heart disease. Biochem. Soc. Trans. 24, 785–789.
Hollman, P.C., Katan, M.B., 1997. Absorption, metabolism and health effects of dietary flavonoids in man. Biomed. Pharmacother. 51, 305–310.
Hollman, P.C., Tijburg, L.B., Yang, C.S., 1997. Bioavailability of flavonoids from tea. Crit. Rev. Food Sci. Nutr. 37, 719–738.
Howitz, K.T., Bitterman, K.J., Cohen, H.Y., Lamming, D.W., Lavu, S., Wood, J.G., Zipkin, R.E., Chung, P., Kisielewski, A., Zhang, L.-L., Scherer, B., Sinclair, D.A., 2003. Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. Nature 425, 191–196.
Huebbe, P., Wagner, A.E., Boesch-Saadatmandi, C., Sellmer, F., Wolffram, S., Rimbach, G., 2010. Effect of dietary quercetin on brain quercetin levels and the expression of antioxidant and Alzheimer's disease relevant genes in mice. Pharmacol. Res. 61, 242–246.
Ioku, K., Pongpiriyadacha, Y., Konishi, Y., Takei, Y., Nakatani, N., Terao, J., 1998. beta-Glucosidase activity in the rat small intestine toward quercetin monoglucosides. Biosci. Biotechnol. Biochem. 62, 1428–1431.
Ishikawa, Y., Kitamura, M., 2000. Anti-apoptotic effect of quercetin: intervention in the JNK- and ERK-mediated apoptotic pathways. Kidney Int. 58, 1078–1087.
Ishisaka, A., Ichikawa, S., Sakakibara, H., Piskula, M.K., Nakamura, T., Kato, Y., Ito, M., Miyamoto, K., Tsuji, A., Kawai, Y., Terao, J., 2011. Accumulation of orally administered quercetin in brain tissue and its antioxidative effects in rats. Free Radic. Biol. Med. 51, 1329–1336.
Ishisaka, A., Mukai, R., Terao, J., Shibata, N., Kawai, Y., 2014. Specific localization of quercetin-3-O-glucuronide in human brain. Arch. Biochem. Biophys. 557, 11–17.
Jaganath, I.B., Mullen, W., Edwards, C.A., Crozier, A., 2006. The relative contribution of the small and large intestine to the absorption and metabolism of rutin in man. Free Radic. Res. 40, 1035–1046.
Jaganath, I.B., Mullen, W., Lean, M.E.J., Edwards, C.A., Crozier, A., 2009. In vitro catabolism of rutin by human fecal bacteria and the antioxidant capacity of its catabolites. Free Radic. Biol. Med. 47, 1180–1189.
Kakizuka, A., 1998. Protein precipitation: a common etiology in neurodegenerative disorders? Trends Genet. 14, 396–402.
Kang, C.-H., Choi, Y.H., Moon, S.-K., Kim, W.-J., Kim, G.-Y., 2013. Quercetin inhibits lipopolysaccharide-induced nitric oxide production in BV2 microglial cells by suppressing the NF-κB pathway and activating the Nrf2-dependent HO-1 pathway. Int. Immunopharmacol. 17, 808–813.
Karuppagounder, S.S., Madathil, S.K., Pandey, M., Haobam, R., Rajamma, U., Mohanakumar, K.P., 2013. Quercetin up-regulates mitochondrial complex-I activity to protect against programmed cell death in rotenone model of Parkinson's disease in rats. Neuroscience 236, 136–148.
Kawabata, K., Kawai, Y., Terao, J., 2010. Suppressive effect of quercetin on acute stress-induced hypothalamic-pituitary-adrenal axis response in Wistar rats. J. Nutr. Biochem. 21, 374–380.
Kinouchi, H., Epstein, C.J., Mizui, T., Carlson, E., Chen, S.F., Chan, P.H., 1991. Attenuation of focal cerebral ischemic injury in transgenic mice overexpressing CuZn superoxide dismutase. Proc. Natl. Acad. Sci. U. S. A. 88, 11158–11162.
Kobuchi, H., Roy, S., Sen, C.K., Nguyen, H.G., Packer, L., 1999. Quercetin inhibits inducible ICAM-1 expression in human endothelial cells through the JNK pathway. Am. J. Physiol. 277, C403–C411.
Kressler, J., Millard-Stafford, M., Warren, G.L., 2011. Quercetin and endurance exercise capacity: a systematic review and meta-analysis. Med. Sci. Sports Exerc. 43, 2396–2404.
Kühnau, J., 1976. The flavonoids. A class of semi-essential food components: their role in human nutrition. World Rev. Nutr. Diet. 24, 117–191.
Kumar, A., Sehgal, N., Kumar, P., Padi, S.S.V., Naidu, P.S., 2008. Protective effect of quercetin against lCV colchicine-induced cognitive dysfunctions and oxidative damage in rats. Phytother. Res. 22, 1563–1569.
Lagoa, R., Graziani, I., Lopez-Sanchez, C., Garcia-Martinez, V., Gutierrez-Merino, C., 2011. Complex I and cytochrome c are molecular targets of flavonoids that inhibit hydrogen peroxide production by mitochondria. Biochim. Biophys. Acta 1807, 1562–1572.
Lambert, J.D., Sang, S., Yang, C.S., 2007. Possible controversy over dietary polyphenols: benefits vs risks. Chem. Res. Toxicol. 20, 583–585.
Lamport, D.J., Saunders, C., Butler, L.T., Spencer, J.P., 2014. Fruits, vegetables, 100% juices, and cognitive function. Nutr. Rev. 72, 774–789.
Larson, A.J., Symons, J.D., Jalili, T., 2010. Quercetin: a treatment for hypertension?—a review of Efficacy and mechanisms. Pharmaceuticals 3, 237–250.
Lavoie, S., Chen, Y., Dalton, T.P., Gysin, R., Cuénod, M., Steullet, P., Do, K.Q., 2009. Curcumin, quercetin, and tBHQ modulate glutathione levels in astrocytes and neurons: importance of the glutamate cysteine ligase modifier subunit. J. Neurochem. 108, 1410–1422.
Lee-Hilz, Y.Y., Boerboom, A.-M.J.F., Westphal, A.H., Berkel, W.J.H., van Aarts, J.M.M.J.G., Rietjens, I.M.C.M., 2006. Pro-oxidant activity of flavonoids induces EpRE-mediated gene expression. Chem. Res. Toxicol 19, 1499–1505.
Li, Y., Zhou, S., Li, J., Sun, Y., Hasimu, H., Liu, R., Zhang, T., 2015. Quercetin protects human brain microvascular endothelial cells from fibrillar β-amyloid1–40-induced toxicity. Acta Pharm. Sin. B 5, 47–54.
Liu, J.J., Casley, D.J., Nayler, W.G., 1989. Ischaemia causes externalization of endothelin-1 binding sites in rat cardiac membranes. Biochem. Biophys. Res. Commun. 164, 1220–1225.
Lu, C., Lu, Y., Chen, J., Zhang, W., Wu, W., 2007. Synchronized and sustained release of multiple components in silymarin from erodible glyceryl monostearate matrix system. Eur. J. Pharm. Biopharm. 66, 210–219.
Lv, C., Hong, T., Yang, Z., Zhang, Y., Wang, L., Dong, M., Zhao, J., Mu, J., Meng, Y., 2012. Effect of quercetin in the 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine-induced mouse model of Parkinson's disease. Evid. Based. Complement. Altern. Med. 2012, 928643.
Magalingam, K.B., Radhakrishnan, A., Haleagrahara, N., 2014. Protective effects of flavonol isoquercitrin, against 6-hydroxy dopamine (6-OHDA)-induced toxicity in PC12 cells. BMC Res. Notes 7, 49.
Magalingam, K.B., Radhakrishnan, A., Ramdas, P., Haleagrahara, N., 2015. Quercetin glycosides induced neuroprotection by changes in the gene expression in a cellular model of Parkinson's disease. J. Mol. Neurosci. 55, 609–617.
Manach, C., Williamson, G., Morand, C., Scalbert, A., Rémésy, C., 2005. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. Am. J. Clin. Nutr. 81, 230S–242S.
Mandel, S.A., Avramovich-Tirosh, Y., Reznichenko, L., Zheng, H., Weinreb, O., Amit, T., Youdim, M.B.H., 2005. Multifunctional activities of green tea catechins in neuroprotection. Modulation of cell survival genes, iron-dependent oxidative stress and PKC signaling pathway. Neurosignals 14, 46–60.
Maxwell, C.J., Hicks, M.S., Hogan, D.B., Basran, J., Ebly, E.M., 2005. Supplemental use of antioxidant vitamins and subsequent risk of cognitive decline and dementia. Dement. Geriatr. Cogn. Disord. 20, 45–51.
Mecocci, P., Tinarelli, C., Schulz, R.J., Polidori, M.C., 2014. Nutraceuticals in cognitive impairment and Alzheimer's disease. Front. Pharmacol. 5, 147.
Mendoza, E.E., Burd, R., 2011. Quercetin as a systemic chemopreventative agent: structural and functional mechanisms. Mini Rev. Med. Chem. 11, 1216–1221.
Metodiewa, D., Jaiswal, A.K., Cenas, N., Dickancaité, E., Segura-Aguilar, J., 1999. Quercetin may act as a cytotoxic prooxidant after its metabolic activation to semiquinone and quinoidal product. Free Radic. Biol. Med. 26, 107–116.
Middleton, E.J., 1998. Effect of plant flavonoids on immune and inflammatory cell function. Adv. Exp. Med. Biol. 439, 175–182.
Miller, N.J., Ruiz-Larrea, M.B., 2002. Flavonoids and other plant phenols in the diet: their significance as antioxidants. J. Nutr. Environ. Med. 12, 39–51.
Mira, L., Fernandez, M.T., Santos, M., Rocha, R., Florêncio, M.H., Jennings, K.R., 2002. Interactions of flavonoids with iron and copper ions: a mechanism for their antioxidant activity. Free Radic. Res. 36, 1199–1208.
Moon, S.-K., Cho, G.-O., Jung, S.-Y., Gal, S.-W., Kwon, T.K., Lee, Y.-C., Madamanchi, N.R., Kim, C.-H., 2003. Quercetin exerts multiple inhibitory effects on vascular smooth muscle cells: role of ERK1/2, cell-cycle regulation, and matrix metalloproteinase-9. Biochem. Biophys. Res. Commun. 301, 1069–1078.
Morand, C., Crespy, V., Manach, C., Besson, C., Demigné, C., Rémésy, C., 1998. Plasma metabolites of quercetin and their antioxidant properties. Am. J. Physiol. 275, R212–R219.
Muraoka, K., Shimizu, K., Sun, X., Tani, T., Izumi, R., Miwa, K., Yamamoto, K., 2002. Flavonoids exert diverse inhibitory effects on the activation of NF-kappaB. Transpl. Proc. 34, 1335–1340.
Murota, K., Hotta, A., Ido, H., Kawai, Y., Moon, J.-H., Sekido, K., Hayashi, H., Inakuma, T., Terao, J., 2007. Antioxidant capacity of albumin-bound quercetin metabolites after onion consumption in humans. J. Med. Investig. 54, 370–374.
Myhrstad, M.C.W., Carlsen, H., Nordström, O., Blomhoff, R., Moskaug, J.Ø., 2002. Flavonoids increase the intracellular glutathione level by transactivation of the

gamma-glutamylcysteine synthetase catalytical subunit promoter. Free Radic. Biol. Med. 32, 386–393.
Naidu, P.S., Kulkarni, S.K., 2004. Quercetin, a bioflavonoid, reverses haloperidol-induced catalepsy. Methods Find. Exp. Clin. Pharmacol. 26, 323–326.
Nassiri-Asl, M., Moghbelinejad, S., Abbasi, E., Yonesi, F., Haghighi, M.-R., Lotfizadeh, M., Bazahang, P., 2013. Effects of quercetin on oxidative stress and memory retrieval in kindled rats. Epilepsy Behav. 28, 151–155.
Németh, K., Plumb, G.W., Berrin, J.-G., Juge, N., Jacob, R., Naim, H.Y., Williamson, G., Swallow, D.M., Kroon, P.A., 2003. Deglycosylation by small intestinal epithelial cell beta-glucosidases is a critical step in the absorption and metabolism of dietary flavonoid glycosides in humans. Eur. J. Nutr. 42, 29–42.
Nijveldt, R.J., van Nood, E., van Hoorn, D.E., Boelens, P.G., van Norren, K., van Leeuwen, P.A., 2001. Flavonoids: a review of probable mechanisms of action and potential applications. Am. J. Clin. Nutr. 74, 418–425.
Nooyens, A.C.J., Bueno-de-Mesquita, H.B., van Boxtel, M.P.J., van Gelder, B.M., Verhagen, H., Verschuren, W.M.M., 2011. Fruit and vegetable intake and cognitive decline in middle-aged men and women: the Doetinchem Cohort Study. Br. J. Nutr. 106, 752–761.
Notas, G., Nifli, A.-P., Kampa, M., Pelekanou, V., Alexaki, V.-I., Theodoropoulos, P., Vercauteren, J., Castanas, E., 2012. Quercetin accumulates in nuclear structures and triggers specific gene expression in epithelial cells. J. Nutr. Biochem. 23, 656–666.
Ohta, K., Mizuno, A., Li, S., Itoh, M., Ueda, M., Ohta, E., Hida, Y., Wang, M., Furoi, M., Tsuzuki, Y., Sobajima, M., Bohmoto, Y., Fukushima, T., Kobori, M., Inuzuka, T., Nakagawa, T., 2011. Endoplasmic reticulum stress enhances γ-secretase activity. Biochem. Biophys. Res. Commun. 416, 362–366.
Olanow, C.W., Tatton, W.G., 1999. Etiology and pathogenesis of Parkinson's disease. Annu. Rev. Neurosci. 22, 123–144.
Olthof, M.R., Hollman, P.C., Vree, T.B., Katan, M.B., 2000. Bioavailabilities of quercetin-3-glucoside and quercetin-4′-glucoside do not differ in humans. J. Nutr. 130, 1200–1203.
Ono, K., Yoshiike, Y., Takashima, A., Hasegawa, K., Naiki, H., Yamada, M., 2003. Potent anti-amyloidogenic and fibril-destabilizing effects of polyphenols in vitro: implications for the prevention and therapeutics of Alzheimer's disease. J. Neurochem. 87, 172–181.
Ossola, B., Kääriäinen, T.M., Raasmaja, A., Männistö, P.T., 2008. Time-dependent protective and harmful effects of quercetin on 6-OHDA-induced toxicity in neuronal SH-SY5Y cells. Toxicology 250, 1–8.
Ossola, B., Kääriäinen, T.M., Männistö, P.T., 2009. The multiple faces of quercetin in neuroprotection. Expert Opin. Drug Saf. 8, 397–409.
Perez-Vizcaino, F., Duarte, J., Santos-Buelga, C., 2012. The flavonoid paradox: conjugation and deconjugation as key steps for the biological activity of flavonoids. J. Sci. Food Agric. 92, 1822–1825.
Pforte, H., Hempel, J., Jacobasch, G., 1999. Distribution pattern of a flavonoid extract in the gastrointestinal lumen and wall of rats. Nahrung 43, 205–208.
Reiterer, G., Toborek, M., Hennig, B., 2004. Quercetin protects against linoleic acid-induced porcine endothelial cell dysfunction. J. Nutr. 134, 771–775.
Reitz, C., Brayne, C., Mayeux, R., 2011. Epidemiology of Alzheimer disease. Nat. Rev. Neurol. 7, 137–152.
Retta, D., Dellacassa, E., Villamil, J., Suárez, S.A., Bandoni, A.L., 2012. Marcela, a promising medicinal and aromatic plant from Latin America: a review. Ind. Crops Prod. 38, 27–38.
Rice-Evans, C., 2001. Flavonoid antioxidants. Curr. Med. Chem. 8, 797–807.
Rivera, F., Urbanavicius, J., Gervaz, E., Morquio, A., Dajas, F., 2004. Some aspects of the in vivo neuroprotective capacity of flavonoids: bioavailability and structure-activity relationship. Neurotox. Res. 6, 543–553.
Rivera Megret, F., Tejera Correa, D., Abin Carriquiri, JA., Prunell dos Santos, G., Martínez Busi, M., Dajas, F., 2013. *Achyrocline satureioides* (Lam.) DC. (marcela) reduces brain damage in permanent focal ischemia in rats. Rev. Cubana Plantas Med. 18 (3), 445–460.
Russo, M., Spagnuolo, C., Tedesco, I., Bilotto, S., Russo, G.L., 2012. The flavonoid quercetin in disease prevention and therapy: facts and fancies. Biochem. Pharmacol. 83, 6–15.
Sabogal-Guáqueta, A.M., Muñoz-Manco, J.I., Ramírez-Pineda, J.R., Lamprea-Rodriguez, M., Osorio, E., Cardona-Gómez, G.P., 2015. The flavonoid quercetin ameliorates Alzheimer's disease pathology and protects cognitive and emotional function in aged triple transgenic Alzheimer's disease model mice. Neuropharmacology 93C, 134–145.
Sandhir, R., Mehrotra, A., 2013. Quercetin supplementation is effective in improving mitochondrial dysfunctions induced by 3-nitropropionic acid: implications in Huntington's disease. Biochim. Biophys. Acta 1832, 421–430.
Sesink, A.L.A., Arts, I.C.W., Faassen-Peters, M., Hollman, P.C.H., 2003. Intestinal uptake of quercetin-3-glucoside in rats involves hydrolysis by lactase phlorizin hydrolase. J. Nutr. 133, 773–776.
Shih, A.Y., Johnson, D.A., Wong, G., Kraft, A.D., Jiang, L., Erb, H., Johnson, J.A., Murphy, T.H., 2003. Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress. J. Neurosci. 23, 3394–3406.
Shimoi, K., Okada, H., Furugori, M., Goda, T., Takase, S., Suzuki, M., Hara, Y., Yamamoto, H., Kinae, N., 1998. Intestinal absorption of luteolin and luteolin 7-O-beta-glucoside in rats and humans. FEBS Lett. 438, 220–224.
Simonian, N.A., Coyle, J.T., 1996. Oxidative stress in neurodegenerative diseases. Annu. Rev. Pharmacol. Toxicol. 36, 83–106.
Spencer, J.P.E., 2007. The interactions of flavonoids within neuronal signalling pathways. Genes. Nutr. 2, 257–273.
Spencer, J.P.E., Rice-Evans, C., Williams, R.J., 2003. Modulation of pro-survival Akt/protein kinase B and ERK1/2 signaling cascades by quercetin and its in vivo metabolites underlie their action on neuronal viability. J. Biol. Chem. 278, 34783–34793.
Sriraksa, N., Wattanathorn, J., Muchimapura, S., Tiamkao, S., Brown, K., Chaisiwamongkol, K., 2012. Cognitive-enhancing effect of quercetin in a rat model of Parkinson's disease induced by 6-hydroxydopamine. Evid. Based. Complement. Altern. Med. 2012, 823206.
Takashima, K., Matsushima, M., Hashimoto, K., Nose, H., Sato, M., Hashimoto, N., Hasegawa, Y., Kawabe, T., 2014. Protective effects of intratracheally administered quercetin on lipopolysaccharide-induced acute lung injury. Respir. Res. 15, 150.
Terao, J., Murota, K., Kawai, Y., 2011. Conjugated quercetin glucuronides as bioactive metabolites and precursors of aglycone in vivo. Food Funct. 2, 11–17.
Thompson, B.J., Ronaldson, P.T., 2014. Drug delivery to the ischemic brain. Adv. Pharmacol. 71, 165–202.
Uttara, B., Singh, A.V., Zamboni, P., Mahajan, R.T., 2009. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. Curr. Neuropharmacol. 7, 65–74.
Vauzour, D., Vafeiadou, K., Rodriguez-Mateos, A., Rendeiro, C., Spencer, J.P.E., 2008. The neuroprotective potential of flavonoids: a multiplicity of effects. Genes. Nutr. 3, 115–126.
Vaziri, H., Dessain, S.K., Ng Eaton, E., Imai, S.I., Frye, R.A., Pandita, T.K., Guarente, L., Weinberg, R.A., 2001. hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. Cell 107, 149–159.
Walgren, R.A., Karnaky, K.J., Lindenmayer, G.E., Walle, T., 2000a. Efflux of dietary flavonoid quercetin 4′-beta-glucoside across human intestinal Caco-2 cell monolayers by apical multidrug resistance-associated protein-2. J. Pharmacol. Exp. Ther. 294, 830–836.
Walgren, R.A., Lin, J.T., Kinne, R.K., Walle, T., 2000b. Cellular uptake of dietary flavonoid quercetin 4′-beta-glucoside by sodium-dependent glucose transporter SGLT1. J. Pharmacol. Exp. Ther. 294, 837–843.
Walle, T., 2009. Methylation of dietary flavones increases their metabolic stability and chemopreventive effects. Int. J. Mol. Sci. 10, 5002–5019.
Williams, J.M., Beckmann, A.M., Mason-Parker, S.E., Abraham, W.C., Wilce, P.A., Tate, W.P., 2000. Sequential increase in Egr-1 and AP-1 DNA binding activity in the dentate gyrus following the induction of long-term potentiation. Brain Res. Mol. Brain Res. 77, 258–266.
Williams, R.J., Spencer, J.P.E., Rice-Evans, C., 2004. Flavonoids: antioxidants or signalling molecules? Free Radic. Biol. Med. 36, 838–849.
Wilson, W.J., Poellinger, L., 2002. The dietary flavonoid quercetin modulates HIF-1 alpha activity in endothelial cells. Biochem. Biophys. Res. Commun. 293, 446–450.
Xu, C., Bailly-Maitre, B., Reed, J.C., 2005. Endoplasmic reticulum stress: cell life and death decisions. J. Clin. Investig. 115, 2656–2664.
Yoshino, S., Hara, A., Sakakibara, H., Kawabata, K., Tokumura, A., Ishisaka, A., Kawai, Y., Terao, J. Effect of quercetin and glucuronide metabolites on the monoamine oxidase-A reaction in mouse brain mitochondria. Nutrition 27, 847–852.
Zbarsky, V., Datla, K.P., Parkar, S., Rai, D.K., Aruoma, O.I., Dexter, D.T., 2005. Neuroprotective properties of the natural phenolic antioxidants curcumin and naringenin but not quercetin and fisetin in a 6-OHDA model of Parkinson's disease. Free Radic. Res. 39, 1119–1125.
Zemek, F., Drtinova, L., Nepovimova, E., Sepsova, V., Korabecny, J., Klimes, J., Kuca, K., 2014. Outcomes of Alzheimer's disease therapy with acetylcholinesterase inhibitors and memantine. Expert Opin. Drug Saf. 13, 759–774.
Zhang, J., Stanley, R.A., Adaim, A., Melton, L.D., Skinner, M.A., 2006. Free radical scavenging and cytoprotective activities of phenolic antioxidants. Mol. Nutr. Food Res. 50, 996–1005.
Zhang, X.D., Liu, X.Q., Kim, Y.H., Whang, W.K., 2014. Chemical constituents and their acetyl cholinesterase inhibitory and antioxidant activities from leaves of *Acanthopanax henryi*: potential complementary source against Alzheimer's disease. Arch. Pharm. Res. 37, 606–616.
Zhao, L., Wang, J.-L., Wang, Y.-R., Fa, X.-Z., 2012. Apigenin attenuates copper-mediated β-amyloid neurotoxicity through antioxidation, mitochondrion protection and MAPK signal inactivation in an AD cell model. Brain Res. 1492, 33–45.

Journal of Molecular Modeling

Quercetin Protein Targets Identification by Reverse Virtual Screening

--Manuscript Draft--

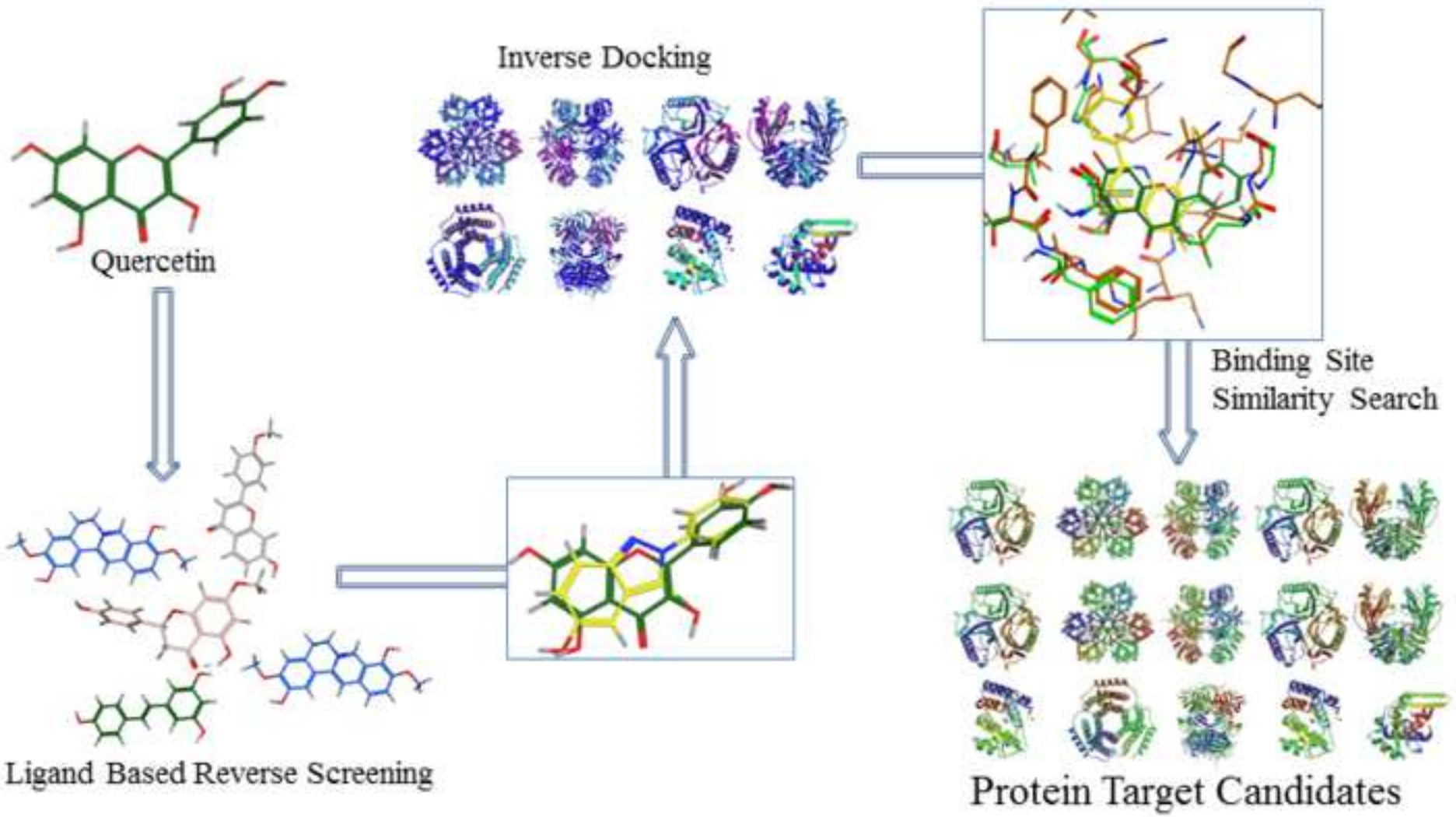
Manuscript Number:	
Full Title:	Quercetin Protein Targets Identification by Reverse Virtual Screening
Article Type:	Original paper
Keywords:	drug target prediction; molecular docking; flavonoids; quercetin.
Corresponding Author:	Margot Paulino, Professor Universidad de la República Faculty of Chemistry Montevideo, Montevideo URUGUAY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Universidad de la República Faculty of Chemistry
Corresponding Author's Secondary Institution:	
First Author:	Diego Carvalho, Lic.
First Author Secondary Information:	
Order of Authors:	Diego Carvalho, Lic.
	Margot Paulino, Professor
	Fabio Polticelli, Professor
	Florencia Arredondo, Lic
	Andrés Abin, Ph.D
Order of Authors Secondary Information:	
Funding Information:	
Abstract:	The ubiquitous flavonoid quercetin is broadly recognized for showing diverse biological and health-promoting effects, such as anticancer, anti-inflammatory and cytoprotective activities. The therapeutic potential of quercetin and similar compounds for preventing such diverse oxidative stress-related pathologies has been generally attributed to their direct antioxidant properties. Nevertheless, the accumulated evidence indicates that quercetin is able to interact with multiple cellular targets. In the present work we aimed to fully identify quercetin protein targets contained in the Protein Data Bank. For this purpose, we relied on a reverse screen strategy based on ligand similarity (SHAFTS) and target structure (idTarget, LIBRA). This work-flow allowed to identify a new set of candidate target proteins which show a wide structural and functional diversity including key proteins involved in neurodegeneration and cancer. This candidate set supports a multilevel and synergistic mechanism of action for quercetin protective effects.

Manuscript

Click here to download Manuscript Reverse Screening7 JMolMod.doc

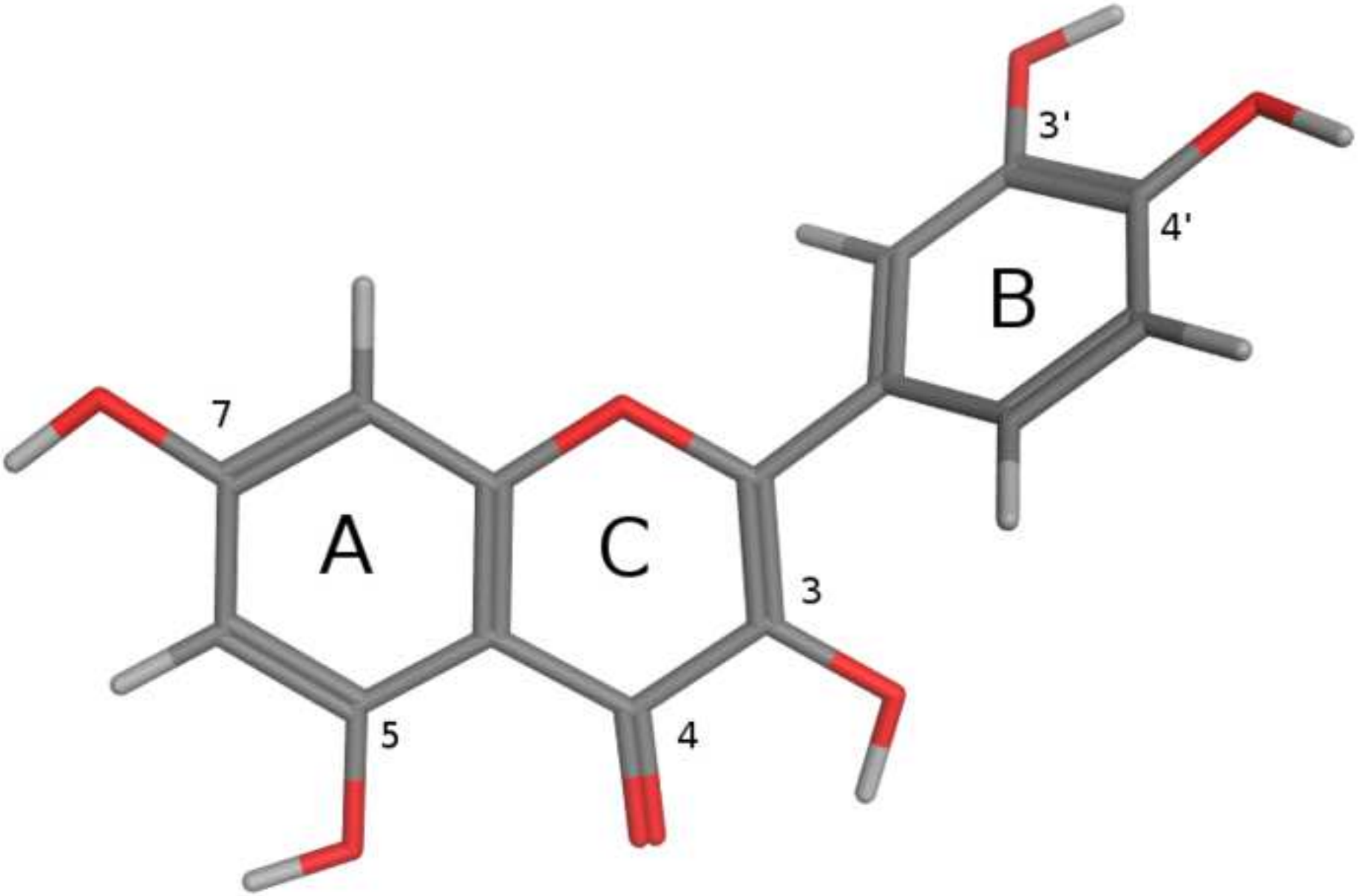
Click here to view linked References

Abstract graphic



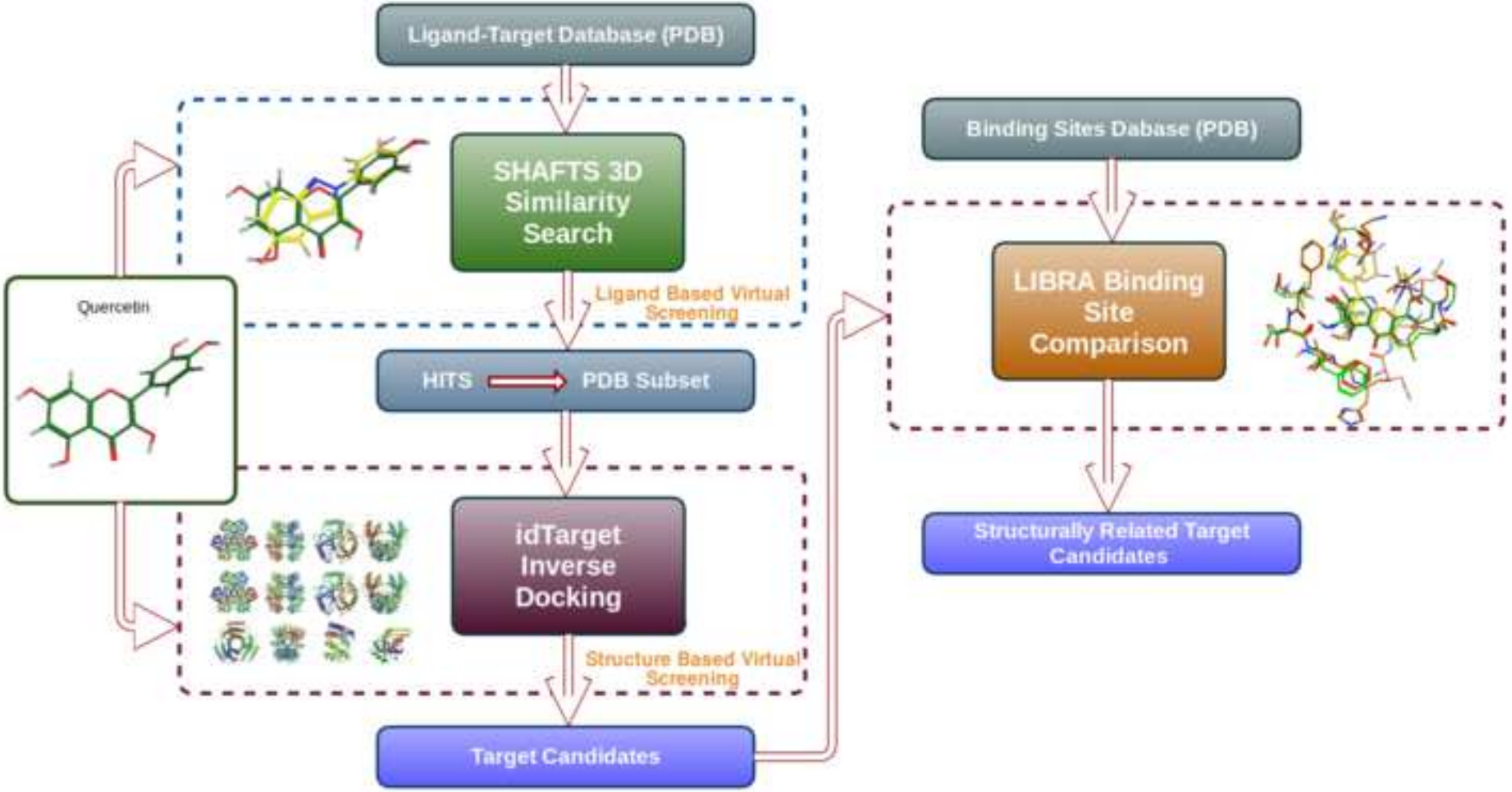
colour figure

Click here to download colour figure Figure1.tif



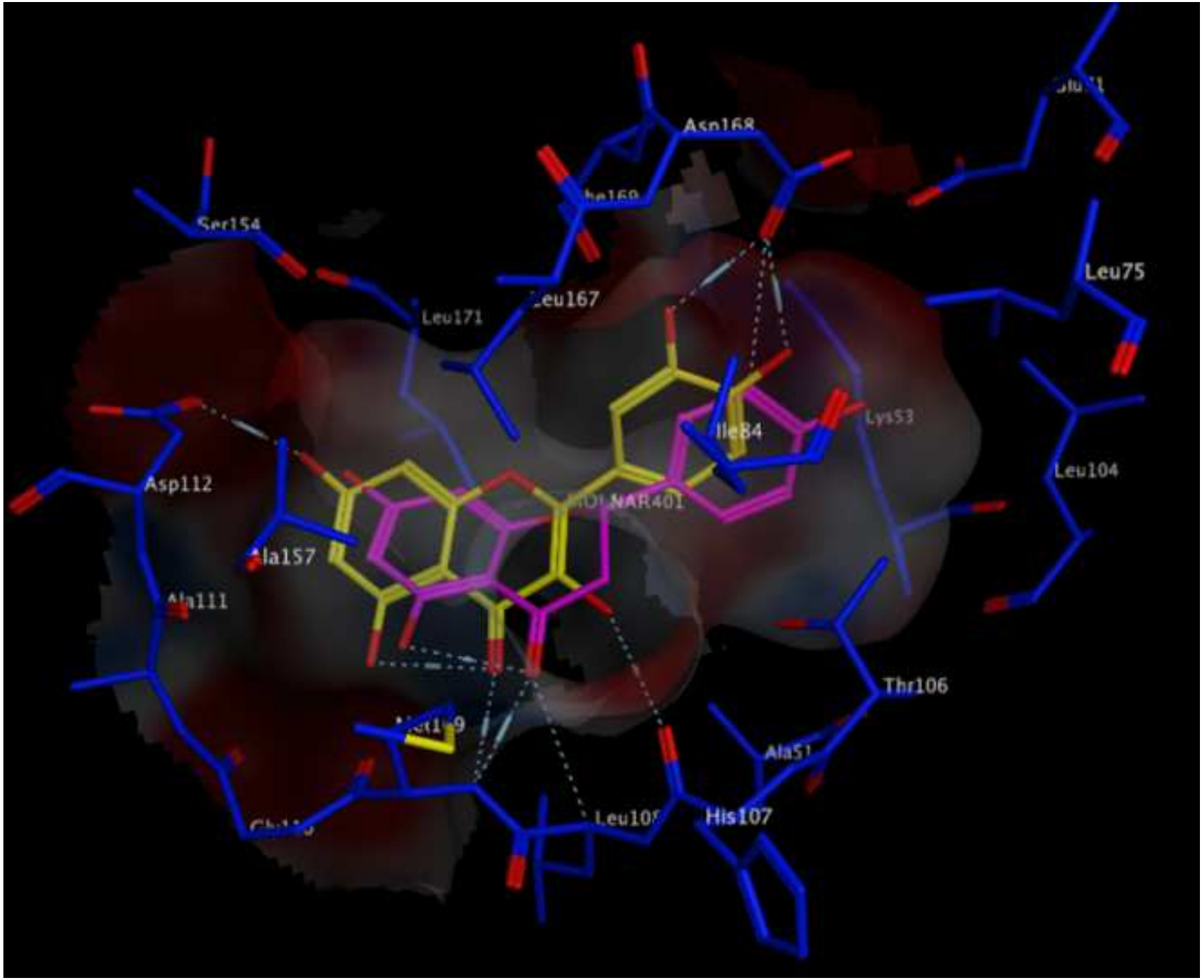
colour figure

Click here to download colour figure Figure2.tif



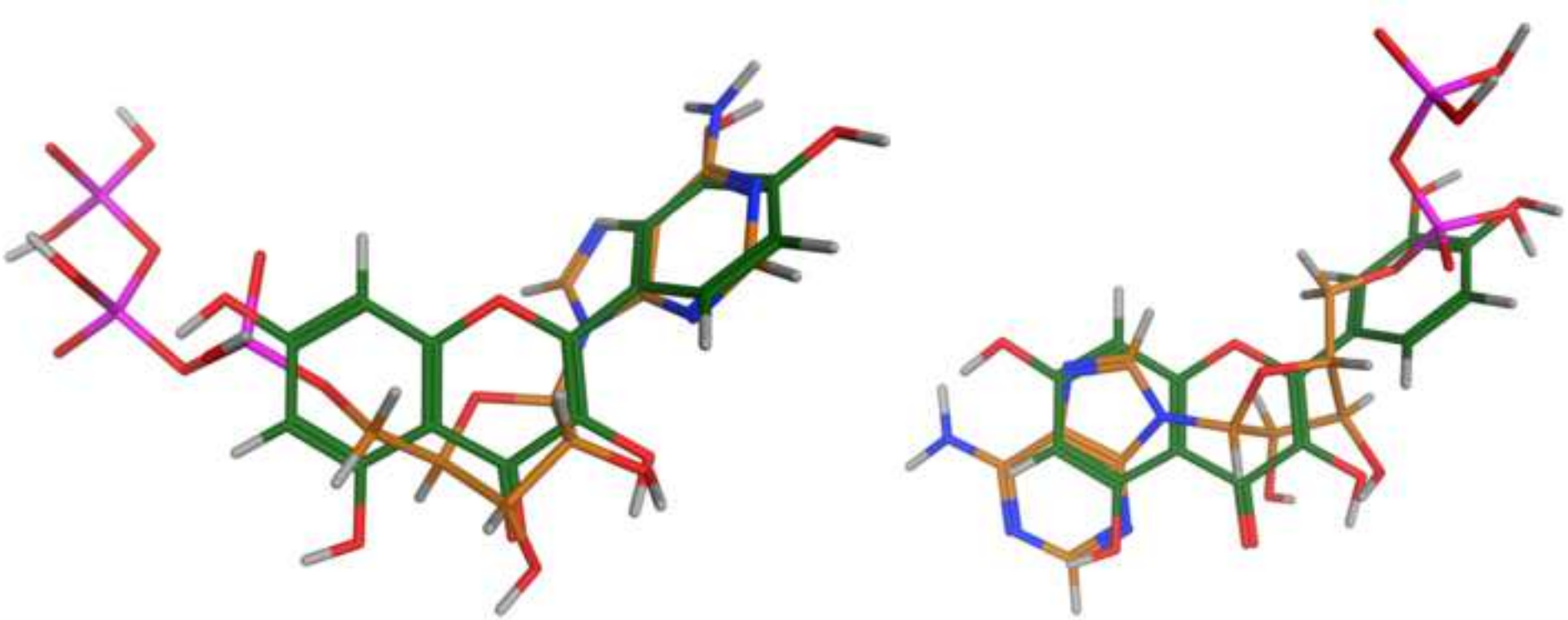
colour figure

Click here to download colour figure Figure3.tif



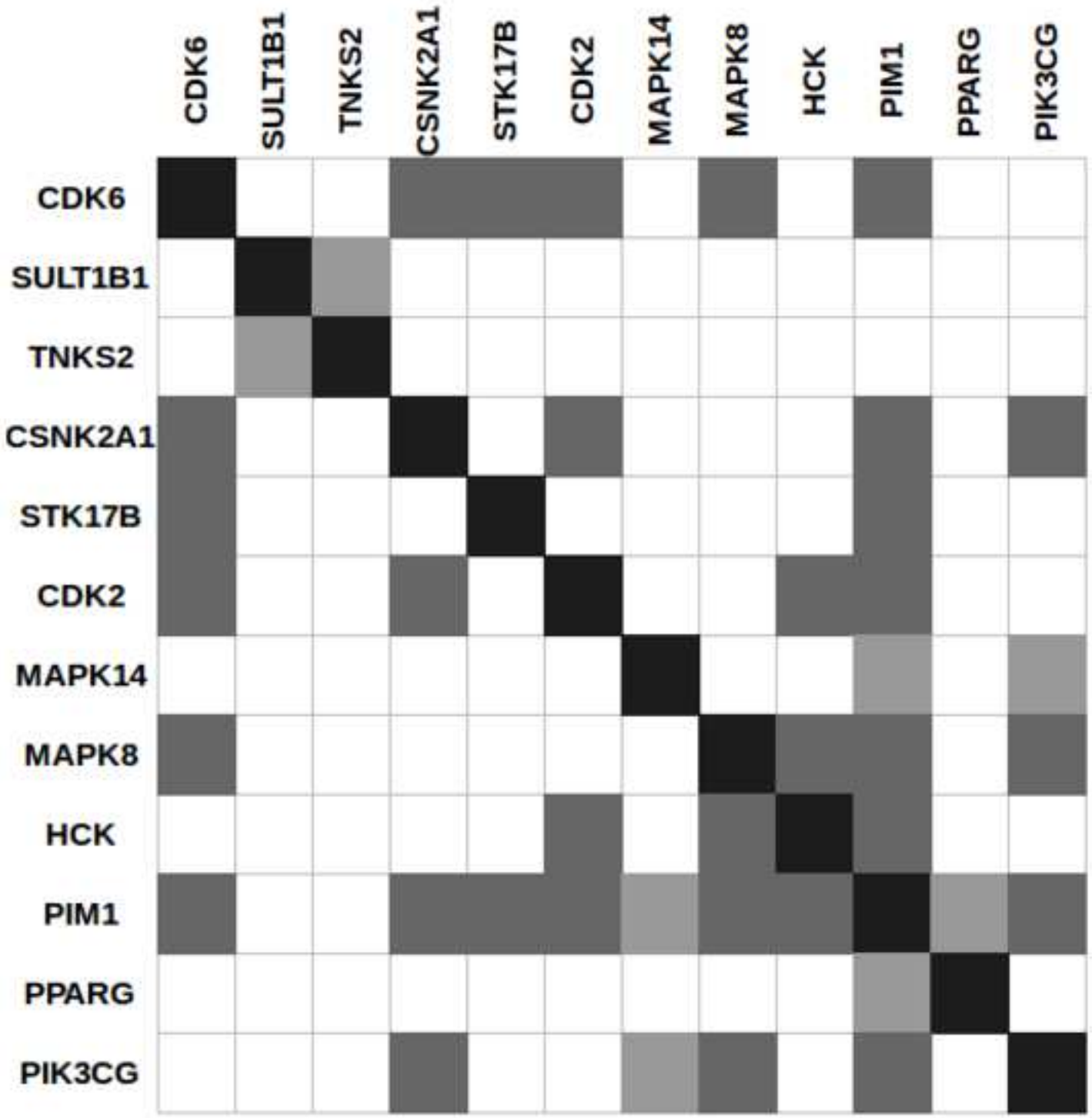
colour figure

Click here to download colour figure Figure4.tif



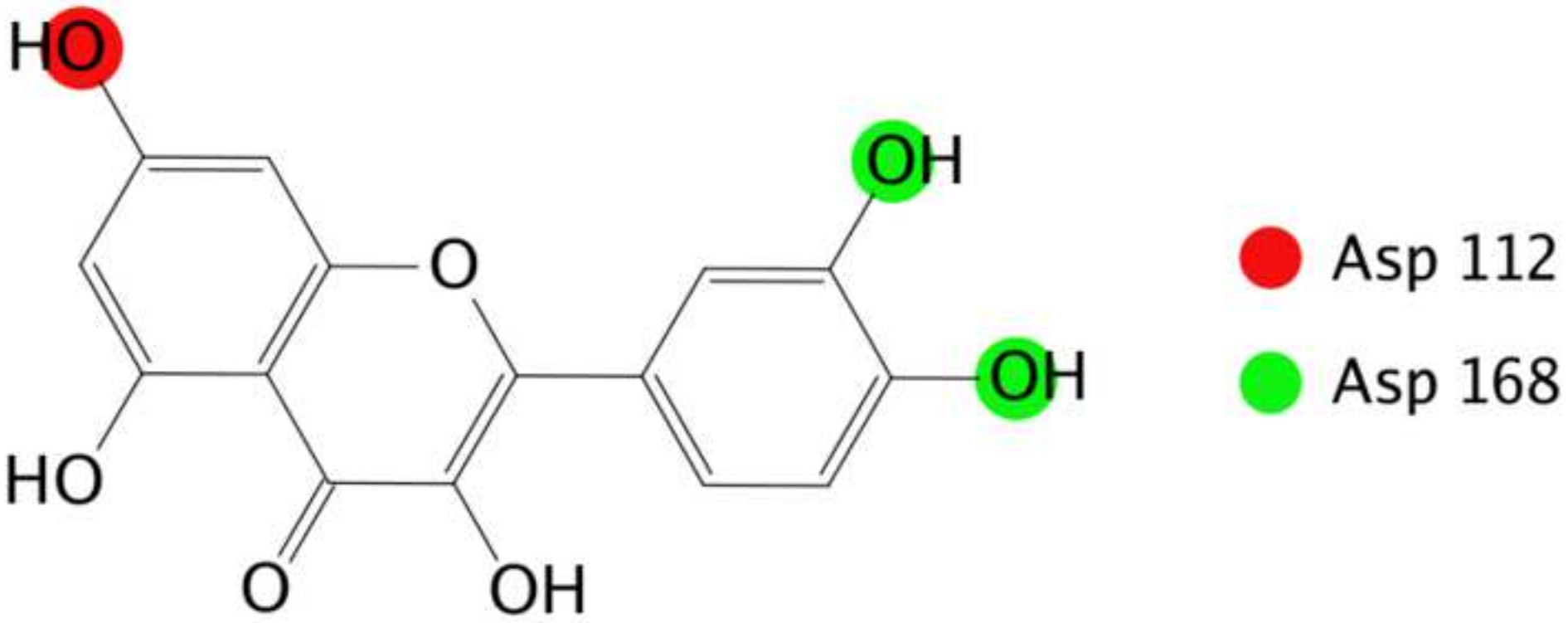
colour figure

Click here to download colour figure Figure5.tif



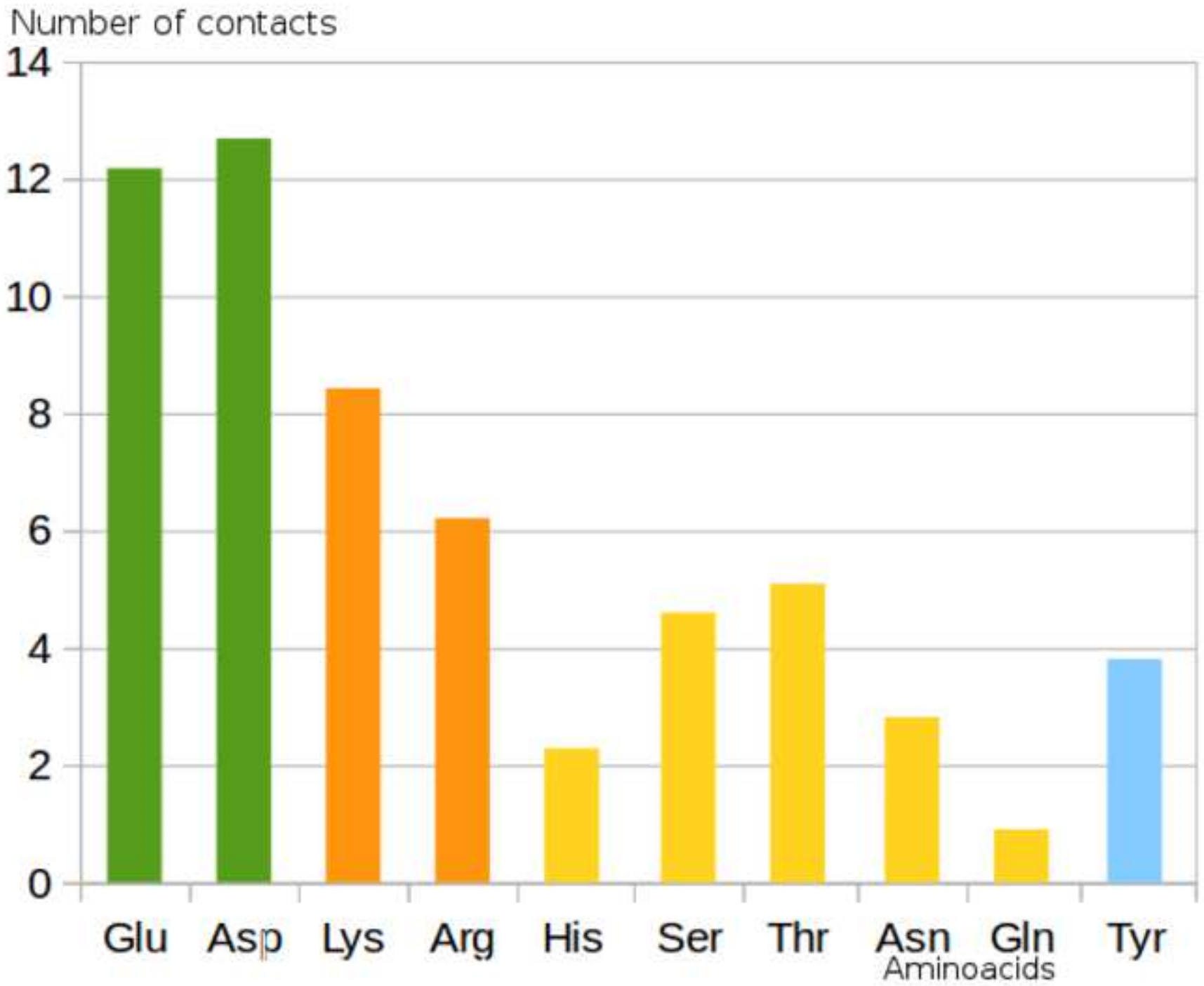
colour figure

Click here to download colour figure Figure6.tif



colour figure

Click here to download colour figure Figure7.tif



colour figure

Click here to download colour figure Figure8.tif

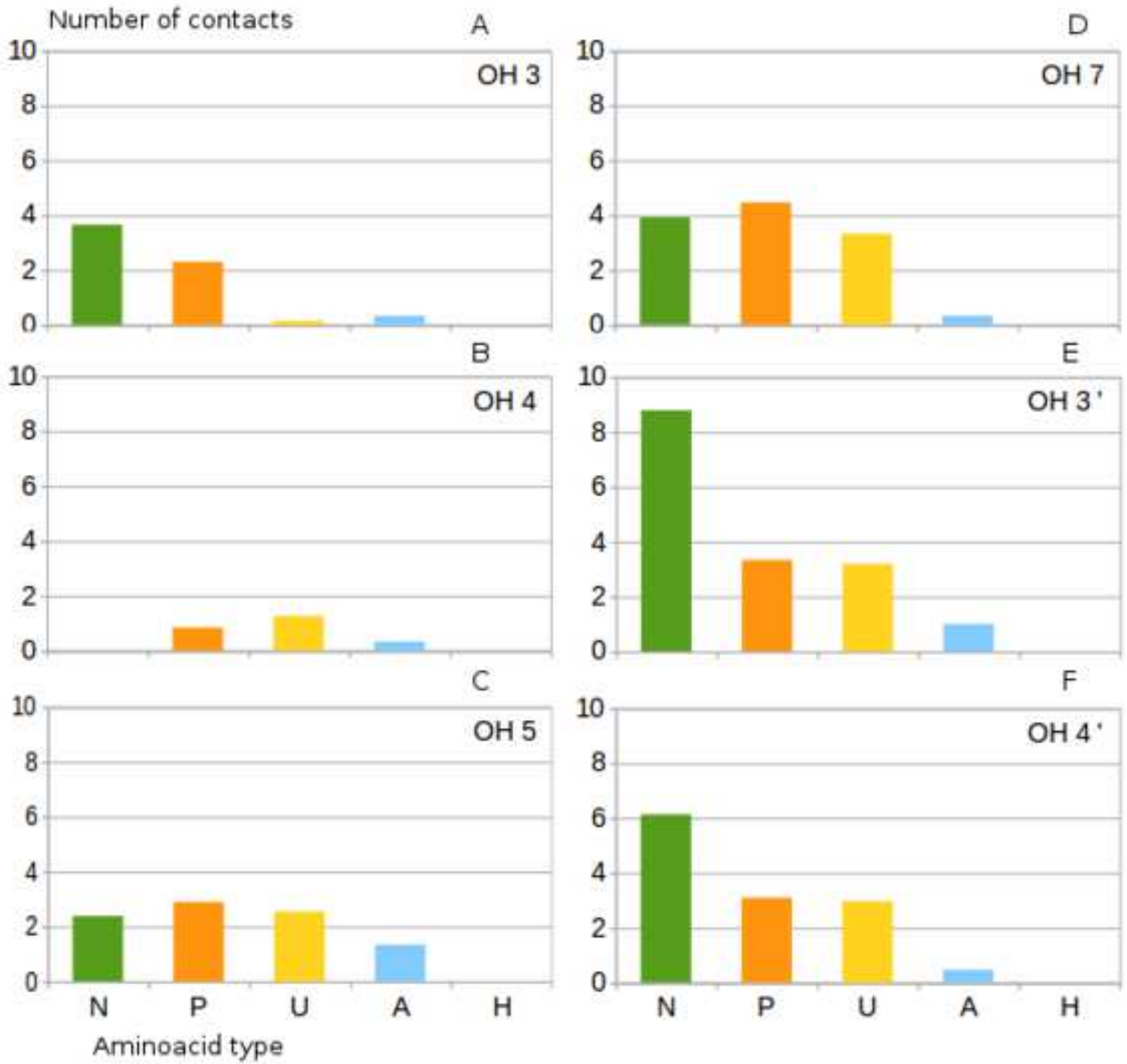


Table 1

PDB id	Ligand	Similarity	Name	Z-score	ΔG (kJ/mol)	Class
			Homo sapiens			
3bpt	QUE (Quercetin)	1,744	**3-Hydroxyisobutyryl-CoA hydrolase**	-1,03	-37,74	Hydrolase
1xo2	FSE (Fisetin)	1,832	Cyclin dependent kinase 6	-0,09	-44,18	Cell cycle/transferase
3ckl	STL (Resveratrol)	1,584	Sulfotransferase family cytosolic 1B member 1	0,08	-43,14	Transferase
4hl5	15W	1,542	Tankyrase-2	-0,10	-44,27	Transferase/transferase inhibitor
4hlf	15Z	1,569		0,00	-43,64	
4hlg	20B	1,524		1,99	-31,67	
4hmh	F94	1,512		1,78	-32,97	
4kzq	DFL	1,526		-0,17	-44,64	
4hkk	AGI (Apigenin)	1,727		0,11	-42,97	
4hkn	LU2 (Luteolin)	1,644		0,16	-42,68	
1sg0	STL (Resveratrol)	1,584	NRH dehydrogenase [quinone]	0,24	-42,22	Oxidoreductase
3ftu	RE2 (Dihydroresveratrol)	1,584	Leukotriene A-4 hydrolase	0,31	-41,80	Hydrolase
3ftx	RE2 (Dihydroresveratrol)	1,584		0,36	-41,46	
3fts	STL (Resveratrol)	1,584		0,13	-42,89	
3amy	AGI (Apigenin)	1,727	Casein kinase 2 subunit alpha	0,43	-41,05	Transferase
2ydx	STL (Resveratrol)	1,584	Methionine adenosyltransferase 2 subunit beta	0,45	-40,96	Oxidoreductase
3lm5	QUE (Quercetin)	1,744	**Serine/threonine-protein kinase 17B**	0,59	-40,08	Transferase
2duv	371	1,515	Cell division protein kinase 2	0,61	-39,96	Transferase
4eh3	NAR (Naringenin)	1,744	Mitogen-activated protein kinase 14	0,66	-39,71	Transferase/transferase inhibitor
3v3v	MYU (Quercetagetin)	1,660	Mitogen-activated protein kinase 8	0,69	-39,50	Transferase/transferase inhibitor
2hck	QUE (Quercetin)	1,744	**Hematopoetic cell kinase HCK**	0,71	-39,37	Transferase
2o65	MYF	1,646	**Serine/threonine-protein kinase PIM-1**	0,89	-38,33	Transferase
2o63	MYC (Myricetin)	1,744		0,93	-38,07	
2o64	MYU (Quercetagetin)	1,660		0,86	-38,45	
2o3p	QUE (Quercetin)	1,744		0,88	-38,37	
4lmu	QUE (Quercetin)	1,744		0,91	-38,16	
3l5r	47X	1,502	Macrophage migration inhibitory factor	1,00	-37,66	Isomerase

2l98	STL (Resveratrol)	1,584	Troponin C, slow skeletal and cardiac muscles	1,01	-37,57	Contractile protein
3sz1	LU2 (Luteolin)	1,644	Peroxisome proliferator-activated receptor gamma	1,16	-36,69	Transcription regulation
4jaz	STL (Resveratrol)	1,584		0,81	-38,79	
4gqr	MYC (Myricetin)	1,744	Pancreatic alpha-amylase	1,22	-36,32	Hydrolase/hydrolase inhibitor
4hd8	PIT (Piceatannol)	1,593	NAD-dependent protein deacetylase Sirtuin-3	1,60	-34,02	Hydrolase/hydrolase inhibitor
1thc	FL9	1,523	Transthyretin	1,40	-35,23	Transport protein
4des	57D (Chrysin)	1,617		1,31	-35,77	
4der	AGI (Apigenin)	1,727		1,83	-32,64	
4det	KMP (Kaempferol)	1,727		1,60	-34,06	
4dew	LU2 (Luteolin)	1,644		2,10	-31,05	
4deu	NAR (Naringenin)	1,744		1,44	-34,98	
1dvs	STL (Resveratrol)	1,584		1,96	-31,84	
			Oryctolagus cuniculus			
3ebo	57D (Chrysin)	1,617	Glycogen phosphorylase, muscle form	0,73	-39,29	Transferase/transferase inhibitor
			Sus scrofa			
1e90	MYC (Myricetin)	1,744	**Phosphatidylinositol 3-kinase catalytic subunit**	0,45	-40,92	Phosphoinositide 3-kinase gamma
1e8w	QUE (Quercetin)	1,744		0,45	-40,92	
			Bos taurus			
3nvy	QUE (Quercetin)	1,744	**Xanthine dehydrogenase/oxidase**	0,73	-39,25	Oxidoreductase
2jj2	QUE (Quercetin)	1,744	**ATP synthase**	1,44	-34,98	Hydrolase
2jj1	PIT (Piceatannol)	1,593		0,00	-37,49	
2jiz	STL (Resveratrol)	1,584		1,47	-34,81	

Table 2

Name	Associated disease	Biological process
Homo sapiens		
3-Hydroxyisobutyryl-CoA hydrolase		
Cell division protein kinase 6	Cancer	
Sulfotransferase family cytosolic 1b member 1		
Tankyrase-2		
Nrh dehydrogenase [quinone] 2	Cancer, Parkinson's disease	
Leukotriene a-4 hydrolase	Immune diseases	
Casein kinase II subunit alpha	Parkinson's disease	
Methionine adenosyltransferase 2 subunit beta		
Serine/threonine-protein kinase 17b	Cell death	
Cell division protein kinase 2	Cancer	
Mitogen-activated protein kinase 14	Parkinson's disease, Amyotrophic Lateral Sclerosis	Cell death, neuron related
Mitogen-activated protein kinase 8	Cancer, Parkinson's disease	Cell death, neuron related
Hematopoetic cell kinase HCK	Immune diseases, Parkinson's disease	Cell death
Serine/threonine-protein kinase PIM-1		Cell death
Macrophage migration inhibitory factor	Immune diseaases, Alzheimer's disease	Cell death
Troponin C, slow skeletal and cardiac muscles		Cell death
Peroxisome proliferator-activated receptor gamma	Diabetes, Cancer, Alzheimer's disease, Huntington's disease	Cell death
Pancreatic alpha-amylase	Alzheimer's disease, Parkinson's disease	
Nad-dependent protein deacetylase sirtuin-3, mitochondrial	Aging	
Transthyretin	Amyloidosis	
Oryctolagus cuniculus		
Glycogen phosphorylase, muscle form		
Sus scrofa		
Phosphatidylinositol 3-kinase catalytic subunit	Aging, Cancer	Cell death, neuron related
Bos taurus		
Xanthine dehydrogenase/oxidase		Cell death
ATP synthase	Alzheimer's disease, Parkinson's disease, Huntington's disease	

Table 3

Uniprot id	Name	Associated disease	Biological process
	Homo sapiens		
O14965	Aurora kinase A	Cancer	Cell death/neuron related
Q96RR4	Calcium/calmodulin-dependent protein kinase kinase 2	Anxiety disorder	
Q13554	Calcium/calmodulin-dependent protein kinase type 2 beta	Cancer	Cell death/neuron related
O00311	Cell division cycle 7-related protein kinase		Proliferation
P50750	Cell division protein kinase 9		Proliferation
O43293	Death-associated protein kinase 3	Cancer	Cell death/neuron related
P49759	Dual specificity protein kinase CLK1		Proliferation
Q13627	Dual specificity tyrosine-phosphorylation-regulated kinase 1A		
P49841	Glycogen synthase kinase-3 beta	Alzheimer's disease, cancer	Cell death/neuron related
P49137	MAP kinase-activated protein kinase 2		Neuron related
P53779	Mitogen-activated protein kinase 10	Epileptic encephalopathy	Neuron related
P45984	Mitogen-activated protein kinase 9	Cancer	Cell death/neuron related
Q99683	Mitogen-activated protein kinase kinase kinase 5	Amyotrophic Lateral Sclerosis	Cell death/neuron related
O14936	Peripheral plasma membrane protein CASK	Microcephaly	Proliferation
O15530	Phosphoinositide-dependent kinase-1	Cancer	Cell death/neuron related
Q13464	Rho-associated protein kinase 1	Cancer	Cell death/neuron related
Q9Y6E0	Serine/threonine-protein kinase 24		Cell death/neuron related
P15056	Serine/threonine-protein kinase B-raf	Cancer	Cell death/proliferation/neuron related
O14757	Serine/threonine-protein kinase Chk1	Cancer	Proliferation
P49888	Estrogen sulfotransferase	Cancer	
P09874	Poly [ADP-ribose] polymerase 1	Alzheimer's disease	
Q9UGN5	Poly [ADP-ribose] polymerase 2		Cell death
Q9Y6F1	Poly [ADP-ribose] polymerase 3	Cancer	
Q53GL7	Poly [ADP-ribose] polymerase 10		Proliferation
Q9H0J9	Poly [ADP-ribose] polymerase 12		
Q460N5	Poly [ADP-ribose] polymerase 14		
Q460N3	Poly [ADP-ribose] polymerase 15		
Q8N5Y8	Poly [ADP-ribose] polymerase 16		Cell death
P50225	Sulfotransferase 1A1	Cancer	
P0DMM9	Sulfotransferase 1A3	Cancer	
O00338	Sulfotransferase 1C2	Cancer	
Q6IMI6	Sulfotransferase 1C3		
O75897	Sulfotransferase 1C4		
O95271	Tankyrase-1		

P51813	Cytoplasmic tyrosine-protein kinase BMX		Cell death/proliferation
P29320	Ephrin type-A receptor 3		Neuron related
P11362	Fibroblast growth factor receptor 1	Cancer	Cell death/proliferation/neuron related
P21802	Fibroblast growth factor receptor 2	Cancer	Cell death/proliferation/neuron related
P12931	Proto-oncogene tyrosine-protein kinase Src	Cancer	Cell death/proliferation/neuron related
P00519	Tyrosine-protein kinase ABL1	Cancer	Cell death/proliferation/neuron related
Q08881	Tyrosine-protein kinase ITK/TSK	Immune diseases	Proliferation
P23458	Tyrosine-protein kinase JAK1	Cancer	Proliferation
O60674	Tyrosine-protein kinase JAK2	Cancer	Cell death/proliferation/neuron related
P52333	Tyrosine-protein kinase JAK3		Cell death/proliferation
P06239	Tyrosine-protein kinase LCK	Diabetes	Cell death/proliferation/neuron related
P35968	Vascular endothelial growth factor receptor 2	Cancer	Cell death/proliferation
Mus musculus			
P54763 (P29323)	Ephrin type-B receptor 2	Cancer	
Rattus norvegicus			
P63086 (P28482)	Mitogen-activated protein kinase 1	Alzheimer's disease, cancer	Cell death/proliferation/neuron related
Bos taurus			
Q28021 (O75116)	Rho-associated protein kinase 2	Cancer	Cell death/neuron related

Table 4

Uniprot id	Name
Homo sapiens	
P11309	**Serine/threonine-protein kinase pim-1**
O60669	Monocarboxylate transporter 2
O94768	**Serine/threonine-protein kinase 17B**
O94956	Solute carrier organic anion transporter family member 2B1
P08183	Multidrug resistance protein 1
P08631	**Hematopoietic cell kinase HCK**
P10632	Cytochrome P450 2C8
P25705	**ATP synthase**
P48736	**Phosphatidylinositol 3-kinase catalytic subunit**
P53985	Monocarboxylate transporter 1
Q6NUS8	UDP-glucuronosyltransferase 3A1
Q6NVY1	**3-Hydroxyisobutyryl-CoA hydrolase**
Q92887	Canalicular multispecific organic anion transporter 1
Q9UNQ0	ATP-binding cassette sub-family G member 2
Q16678	Cytochrome P450 1B1
P04798	Cytochrome P450 1A1
P68400	**Casein kinase 2 alpha**
P11511	Cytochrome P450 19A1
P15121	Aldehyde reductase
P12931	**Tyrosine-protein kinase SRC**
P35968	**Vascular endothelial growth factor receptor 2**
P08069	Insulin-like growth factor 1 receptor
Rattus norvegicus	
P21588 (P21589)	5'-nucleotidase
Bos taurus	
P80457 (P47989)	**Xanthine dehydrogenase**
P21398 (P21397)	Monoamine Oxidase A

Fig. 1S STITCH interaction network among protein candidates and protein related candidates found in the Protein Data Bank. Nodes are either colored (if they are directly linked to the input) or white (nodes of a higher iteration/depth). Edges, i.e. predicted functional links, consists of up to eight lines: one color for each type of evidence. For further information please visit: http://stitch.embl.de/

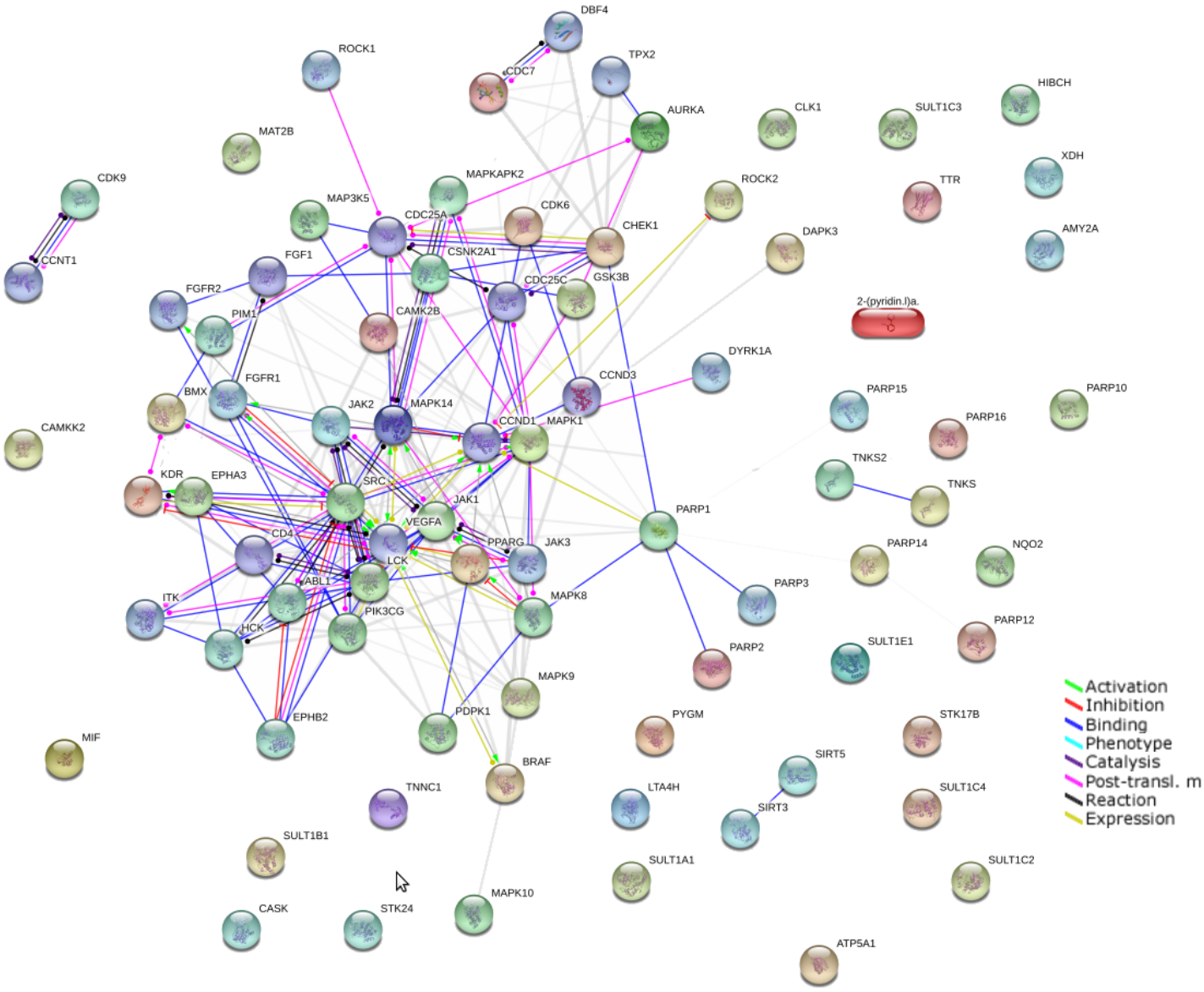
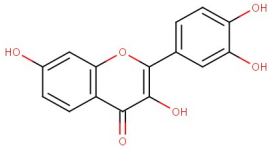
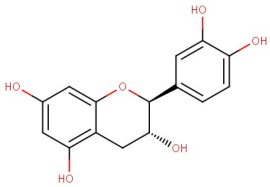
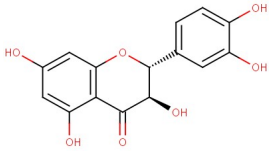
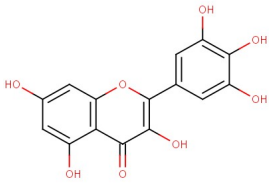
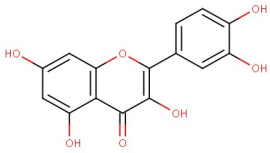
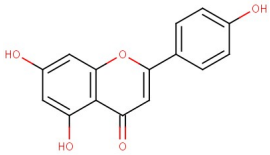
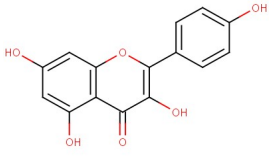
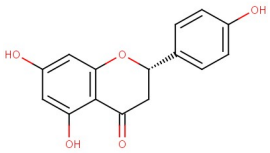
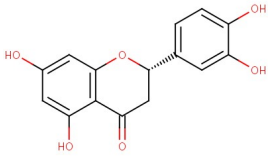
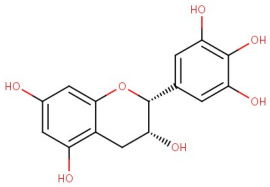
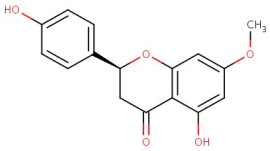
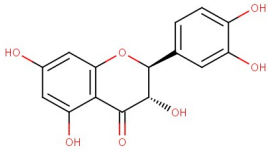
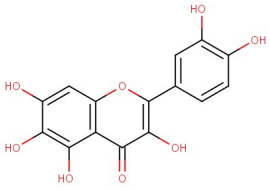
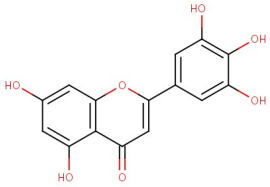
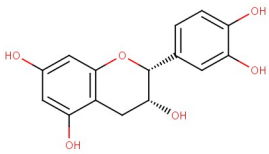
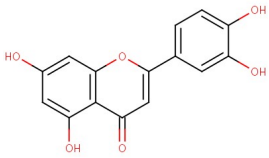
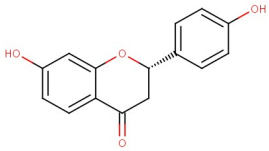
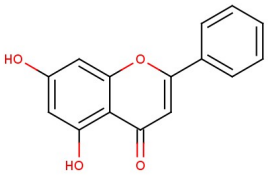
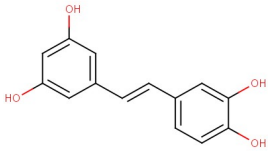
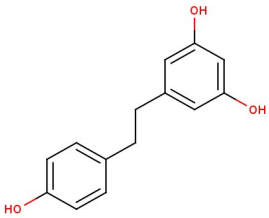
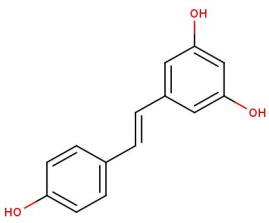
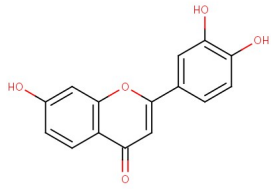
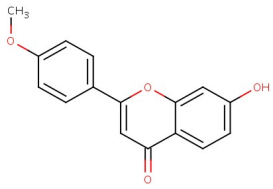
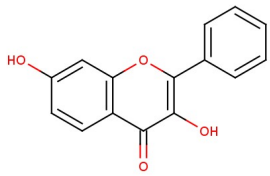
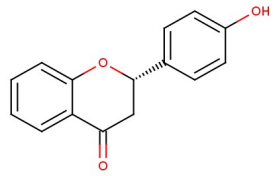
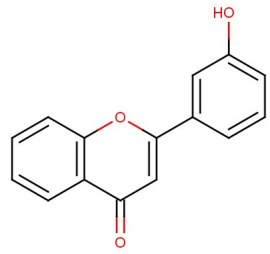
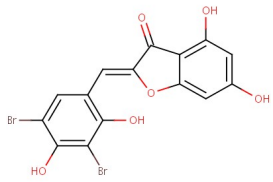
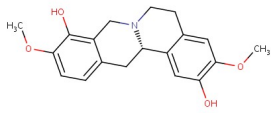


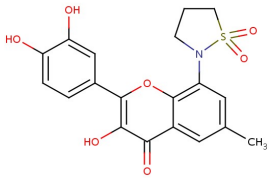
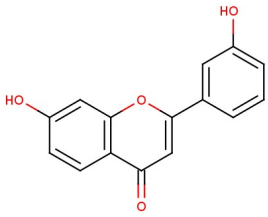
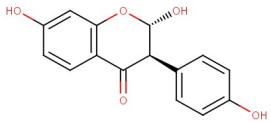
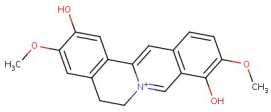
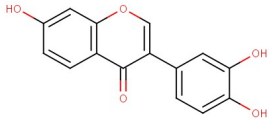
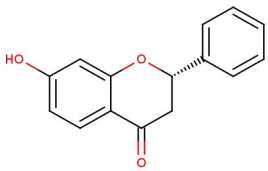
Table 1S Library of quercetin-similar ligands identified by SHAFTS in the first stage of the reverse screening work-flow. Ligands are depicted according to their Protein Data Bank id, common name, SHAFTS similarity score, whether it is co-crystallized with a mammal protein or not and structure

PDB id	Name	Similarity score	Mammal protein	2D Representation
FSE	Fisetin	1,832	Yes	
KXN	-	1,785	-	
DQH	-	1,767	-	
MYC	Myricetin	1,744	Yes	
QUE	Quercetin	1,744	Yes	
AGI	Apigenin	1,727	Yes	
KMP	Kaempferol	1,727	Yes	

NAR	Naringenin	1,712	Yes	
ERD	-	1,699	-	
EGT	Epigallocatechin	1,695	-	
SAK	-	1,671	-	
DH2	-	1,669	-	
MYU	Quercetagetin	1,66	Yes	
MYF	-	1,646	Yes	

28E	Epicatechin	1,645	-	
LU2	Luteolin	1,644	Yes	
DFV	-	1,643	-	
57D	Chrysin	1,617	Yes	
PIT	Piceatannol	1,593	Yes	
RE2	Dihydroresveratrol	1,584	Yes	
STL	Resveratrol	1,584	Yes	

15Z	-	1,569	Yes	
15W	-	1,542	Yes	
HHF	-	1,537	-	
DFL	-	1,526	Yes	
20B	-	1,524	Yes	
FL9	-	1,523	Yes	
SLX	(S)-scoulerine	1,518	-	

371	-	1,515	Yes	
F94	-	1,512	Yes	
2HI	-	1,509	-	
DEH	-	1,503	-	
47X	-	1,502	Yes	
DDC	-	1,501	-	

Quercetin Protein Targets Identification by Reverse Virtual Screening

Diego Carvalho[a,b], Margot Paulino[a*], Fabio Polticelli[c,d], Florencia Arredondo[b], Juan A. Abin-Carriquiry[b*]

[a]*Center of Bioinformatics, Facultad de Química - UdelaR, 11800 Montevideo, Uruguay*

[b]*Department of Neurochemistry, Instituto de Investigaciones Biológicas Clemente Estable, 11600 Montevideo, Uruguay*

[c]*Department of Sciences, Roma Tre University, 00146 Rome, Italy*

[d]*National Institute of Nuclear Physics, Roma Tre Section, 00146 Rome, Italy*

** Corresponding authors. E-mail address: margot@fq.edu.uy, Phone: +598 29291558, Fax: +598 29241960; E-mail address: jaabin@iibce.edu.uy, Phone: +598 099597502, Fax: +598 24872603*

ABSTRACT

The ubiquitous flavonoid quercetin is broadly recognized for showing diverse biological and health-promoting effects, such as anticancer, anti-inflammatory and cytoprotective activities. The therapeutic potential of quercetin and similar compounds for preventing such diverse oxidative stress-related pathologies has been generally attributed to their direct antioxidant properties. Nevertheless, the accumulated evidence indicates that quercetin is able to interact with multiple cellular targets. In the present work we aimed to fully identify quercetin protein targets contained in the Protein Data Bank. For this purpose, we relied on a reverse screen strategy based on ligand similarity (SHAFTS) and target structure (idTarget, LIBRA). This work-flow allowed to identify a new set of candidate target proteins which show a wide structural and functional diversity including key proteins involved in neurodegeneration and cancer. This candidate set supports a multilevel and synergistic mechanism of action for quercetin protective effects.

Keywords: drug target prediction; molecular docking; flavonoids; quercetin.

1. Introduction

Phytochemical rich diets are correlated with increased longevity and wide range of health benefits including a decreased incidence of cardiovascular diseases and a slowed progression of cerebrovascular diseases [1–5]. Flavonoids constitute one of the largest families of phytochemicals found in plants. Alongside their role in the flavor and the color of fruits and vegetables, flavonoids are widely recognized for their direct antioxidant properties [6–11]. In particular, many flavonoids display anti-inflamatory, anti-carcinogenic, and neuroprotective effects properties among other bioactivities [12–25].

This ubiquitous polyphenolic group comprises several subclasses such as flavones (including flavonols), flavanones, flavans (including flavanols), isoflavones, and anthocyanidins. Flavonols are by far the most abundant and widely distributed in nature, being quercetin (*2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one;* Figure 1) the prototypical flavonol. In relation to the previously cited flavonoids neuroprotective or anticancer effects, several studies suggest that they may be mediated by their interaction with specific proteins involved in intracellular signaling cascades, rather than direct antioxidant mechanisms [26–34]. A complete explanation of quercetin broad range of biological effects requires the description of its whole target space.

While wet-lab studies in this field such as affinity chromatography, expression cloning and protein microarrays are work- and time-intensive, *in silico* target prediction is a valuable inexpensive and complementary technique [35]. ReverseScreen3D, INVDOCK, Tarfisdock, PharmMapper and idTarget are some of the *in silico* target fishing tools available [36–40]. By employing these platforms many favorable experiences have led to the discovery of protein targets of novel compounds like saffron bioactive agents and approved drugs like mebendazol [41, 42]. For a comprehensive review on the topic please refer to Valls et. al. [43].

In the present study, we proposed a hierarchical screening of quercetin protein targets based on the sequential arrangement of ligand similarity search and inverse docking. Hierarchical screening takes

advantages of the fastest method (ligand similarity) in the first stage of the screening process, reducing the search space for the most expensive approach (docking) and the number of false positives obtained [44]. For each step in the screening we chose two of the most recent publicly available software for *in silico* target fishing (Figure 2). Additionally we further amplified the search by including proteins with similar binding site recognized by our in-house-developed software LIBRA (Ligand Binding Site Recognition Application) [45].

2. Methods

The current *in silico* strategies for target identification include ligand-based, structure-based, machine learning-based and biochemical network-based approaches. When searching for potential target proteins, the query ligand is compared against a library of known interacting compounds [46]. Inverse docking is a docking scheme that screens and ranks a protein library instead of a ligand library.

2.1 The ligand-target database

A database of suitable ligands contained in the Protein Data Bank was developed for ligand based screening [47]. Firstly, all the ligands contained in the PDB were downloaded from the 2014 version of *LigandExpo database*, a repository for co-crystalized structures and hetero-atoms in general indexed with the corresponding protein entry [48]. The molecular structures were downloaded in SMILES-Stereo format as a whole database and rebuilt in MOE software [49]. Starting from 17969 PDB ligand entries, a series of hierarchical filters has been applied with MOE to construct the database as follows: 1) Hydrogenation was corrected. 2) Undesirable small molecular weight ligands (salts and water molecules) and repeated entries were removed. 3) Molecules below the molecular weight of the flavonoid frame were deleted, and Oprea rules were applied to restrict the database to flavonoid-like molecules and drug-like molecules respectively. Oprea rules are a refined version of the classical Lipinski's rules of five to determine drug likeliness [50]. 4) Molecules containing Se or As were also deleted. The current molecular database was

energy minimized in MOE with a Merck molecular force field (MMFF94x) until 0.01 kcal/mol RMS gradient and saved in mol2 format. The final database contained 9924 different molecules.

2.2 Ligand based screening

Taking quercetin as a probe, we searched our in-house ligand-target database for similar co-crystallized ligands in the PDB. Quercetin molecular structure was built in MOE, energy minimized (MMFF94x, 0.01 kcal/mol RMS gradient) and saved in mol2 format for screening processes. SHAFTS (SHApe-Feature Similarity) algorithm implemented in its stand-alone version was used to screen quercetin against the conformers obtained from the ligand-target database. SHAFTS is an algorithm for tridimensional structural similarity calculation and ligand-based virtual screening [51]. SHAFTS has demonstrated satisfactory active compounds enrichment and scaffold hopping capability against several representative kinases in retrospective virtual screening studies [52–54]. It adopts a similarity metrics considering both molecular shape and pharmacophoric characteristics (hydrophobic center, positive or negative charge center, hydrogen bond acceptor and donor, and aromatic rings). A feature triplet hashing method is used for the fast rigid alignment of molecular structure. It finally returns a sorted list of molecule identifiers associated with structural similarity score against the query and the corresponding structural alignment.

Because SHAFTS uses a semi-rigid strategy for structural alignment, the conformational analysis program Cindy was used to generate a maximum of 100 lowest-energy conformers for each molecule in the ligand-target database [55]. SHAFTS was then implemented with its default configuration. Protein complex identifiers were retrieved for ligands with a similarity score against quercetin above 1.5 (maximum 2.0) and subjected to the next stage of the screening.

2.3 Structure based screening

The optimized structure of quercetin was uploaded onto the online inverse dock server idTarget for screening against the selected list of protein candidates obtained in the previous stage.

idTarget performs inverse molecular docking for a query molecule against the whole PDB or a

custom list of protein structures [40]. It applies a divide and conquer algorithm to search the protein surface for suitable docking sites and a state-of-the-art scoring function optimized for binding energy prediction. A protein target *i* for a ligand *j* is selected considering the affinity profile for the ligand. A Z-score is calculated according to:

where E_{ij} is the dock score of ligand *j* to the protein pocket *i*, E_i and sd_i are the center and width of the affinity profile of protein *i*.

We selected the server scanning mode as it is the most exhaustive, where usual molecular docking procedures are carried out for each protein structure. Proteins were considered to be possible ligand targets if the idTarget binding energy was lower than -37.6 kJ/mol (250 nM) with a Z-score of 1.0 or lower (adapted from Nikolić et. al. [56]).

2.4 Binding site comparison

2.4.1 Binding site similarity searching among target candidates

The protein candidates selected by idTarget were subjected to binding site comparison all-against-all by our in-house-developed software LIBRA (LIgand Binding site Recognition Application) [45]. LIBRA is a tool for searching local structural similarities between a protein structure and a collection of functional sites and their environment. LIBRA employs a graph-based approach in conjunction with a database of more than 170 thousand ligand binding sites generated by extracting residues surrounding the ligand from approximately 75 thousand structures of protein-ligand complexes deposited in PDB. To compare the candidates set we generated a separated database of their corresponding binding sites, every site was then screened one by one against the whole set. Similar sites were defined according to these requirements: 70 % of similar residues given by blosum62 scoring, minimum motif size of 5 residues, and maximum structural alignment RMSD of 1 Å (Ångström). Sites with the same characteristics but a RMSD between 1 and 1.5 Å (Ångström) were defined as less similar.

2.4.2 Target candidates amplification based on binding site similarity

Delineating binding site similarities for proteins is another possible route for finding new targets for existing ligands. The protein candidates selected by idTarget were subjected to binding site comparison against a representative set of proteins contained in the PDB using LIBRA. Similar sites were defined according to these requirements: 100 % of similar residues (blosum62 scoring), minimum motive size of 5 residues, maximum structural alignment RMSD of 1 Å (Ångström) and no steric clashes between the ligand from the query protein and the known protein.

2.5 Interaction pattern assessment

The interaction pattern between idTarget docking poses for quercetin and the protein candidate group was assessed by Protein Ligand Interaction Fingerprint (PLIF) module of MOE. PLIF is a simple method to represent and analyze the three-dimensional protein-ligand interaction. The method converts the interaction pattern into a one dimensional binary string and can be utilized as postdocking molecular description and filter tool to reorganize docking poses. Every protein was considered as its biological unit downloaded from PDB. The structure was optimized before the analysis, hydrogen atoms were adjusted and charges assigned with Amber99 force field. For the fingerprint calculation every docking pose given by idTarget was used and the total contacts were normalized by the number of poses. No surrounding water molecules were considered.

2.6 Functional and structural characterization

The final set of protein candidates was annotated according to protein structure and family, activity and involvement in disease. The corresponding human homologue was taken for proteins belonging to other mammals. The information was obtained from publicly available databases and tools like PDB, Uniprot [57], DAVID (gene ontology terms and literature information)[58], KEGG (biological pathways) [59], GO TermMapper [60, 61] (gene ontology terms) and FlyMine (gene ontology terms and literature information) [62]. Different sources were considered as they bring complementary data. KEGG pathways are defined as molecular interaction/reaction network

diagram including genes, proteins, RNAs, chemical compounds, glycans, chemical reactions, disease genes and drug targets. On the other hand, GO biological processes are strictly linked to genes, and represent a recognized series of events or molecular functions with a defined beginning and end, whose alteration can lead to a variation of the phenotype.

Already described quercetin targets were collected from different platforms like DrugBank, ChEMBL, BindingDB and SuperTarget [63–66]. Once quercetin targets are identified by literature and *in silico* predictive approaches, they can be mapped onto specific disease-associated networks or pathways and target databases of known bioactive compounds to construct drug associated networks. The search tool for interactions of chemicals (STITCH) database integrates information about interactions of chemicals and proteins from different types of databases and was used for this purpose [67].

3. Results and discussion

3.1 Ligand based screening

We first screened the PDB to construct a database of pharmacologically relevant ligands associated with protein complexes (see Methods section for details). Using quercetin as input molecule SHAFTS was then applied to select quercetin-like compounds, based on a structural similarity score. The resulting ligand set comprised 34 molecules, mainly flavonoids with the exception of the molecules identified by PDB as FL9, PIT, RE2, SLX, STL and DEH (see Supplementary material). The co-crystallized protein set comprised 25 mammalian proteins. Proteins from plants and other organisms were not considered for the subsequent analysis.

3.2 Structure based screening

The set of 25 proteins (Table 1) was then subjected to structure-based screening against quercetin using idTarget. This procedure allowed us to obtain protein target candidates associated with docking poses for quercetin and ranked according to their predicted affinity. This new protein set included 24 of the 25 different mammalian proteins previously selected. Their predicted affinity

ranges from 15.1 nM to 494.1 nM, and has minor variations depending on the specific crystal structure for a given protein. IdTarget server only rejected 1 mammalian protein, sirtuin 5, which confirmed the reliability of the molecular similarity comparison. It is also remarkable that all known complexes for quercetin available in the PDB at the time of the assay were retrieved.

According to the docking poses obtained by idTarget, quercetin reaches the known binding site of every protein which is generally a catalytic or an allosteric site (Figure 3). While idTarget accurately predicts the protein binding sites, docking poses do not resemble in general the co-crystallized ligand. Frequently quercetin appears in the same plane but with opposite orientation. This divergence can be explained by the quercetin inner symmetry. Additionally, some docking simulations show peculiarities: a few sirtuin 3 docking poses overlap with the protein substrate, glycogen phosphorylase docking poses are located in a known allosteric site normally occupied by the endogenous ligand AMP, sulfotransferase 1b (Sult1b) and ATPase docking poses bind in the ATP site in contrast to the co-crystallized ligands, which bind in an adjacent allosteric site. Both the latter proteins have been co-crystallized with ATP or a related molecule. Except for Sult1b all these proteins have unfavorable Z-scores and predicted affinities. Proteins in this latter group have wide and exposed binding sites in contrast with the best scoring candidates. According to idTarget score and taking into account the qualitative considerations expressed above, the following proteins should not be prioritized as target candidates: macrophage migration inhibitory factor, troponin C, peroxisome proliferator-activated receptor gamma, pancreatic alpha amylase, sirtuin 3, transthyretin, glycogen phosphorylase and ATP synthase.

The new candidate target set comprises proteins with a broad range of macromolecular folds and biological activities including enzymes, transcription factors and transport proteins, and can be divided into proteins with direct metabolic action and proteins involved in intracellular signaling pathways. The first group includes 3-hydroxyisobutyryl-CoA hydrolase, sulfotransferase 1b, pancreatic alpha amylase, transthyretin, glycogen phosphorylase and xanthine oxidase. In the

second group, it is worthwhile to mention protein kinases, highlighted in Table 1 in bold, which have been previously involved in cell death/survival activities, and have been proposed as putative targets in cancer and neurodegeneration [68–71]. In particular p38 MAPK is a key protein kinase involved in neuronal apoptosis and inflammation, whose inhibition has been proposed as a treatment against ischemic damage and neurodegeneration [69]. Another interesting group is that of Tankyrases, which are a particular group of poly(ADP-ribosyl)transferases that differ by their overall domain structure and functions. It has been suggested that tankyrase inhibitors could improve anti-cancer effects if combined with other kinase inhibitors such as MEK, epidermal growth factor receptor or phospho-inositide 3-kinase inhibitors in cancer treatment [72].

The structural and functional information obtained (Table 1) can be related to the available information on the involvement of the target candidates in biological processes and disease (Table 2). The spectrum of biological activities of the target candidates is in agreement with the multi-target mechanism of action proposed for quercetin. Thus quercetin may represent a multi-target drug.

3.3 Structural comparison

An interesting finding of the present study is that most of the endogenous ligands, substrates or cofactors related to the resulting protein set were nucleotide derivatives like ATP/ADP, FAD, NAD, pyridoxal phosphate, PAPS, PAP, HIBYL-CoA and xanthine. Structural similarity between quercetin and ATP/ADP was thus analyzed. The result of this analysis (Figure 4) indicated that quercetin may work as an ATP mimetic. In this regard, previous studies suggested a correspondence between the benzopyranone moiety of the flavonoid and the adenine part of the ATP as well as the ortho-dihydroxyphenyl and the phosphates of ATP [73].

The protein target candidates greatly vary in sequence length, fold and quaternary structure. This fact suggests that the structural similarity found within the group is local rather than global. For this reason, a binding site comparison with LIBRA was conducted whose results are presented in

Figure 5.

As expected, the algorithm was able to find strong similarities within the kinases group. Most of these proteins share the same fold according to PFAM (protein kinase domain, PF00069). Other domains present are tyrosine kinase domain (PF07714) and PI3 kinase domain (PF00613). During the last years kinases have been recognized as promising drug targets for tumor therapy including MAP kinases, receptor tyrosine kinases, and kinases related to the PI3-kinase/Akt/mTOR signaling pathway [74–77]. On this regard, previous studies have shown that quercetin treatment leads to pro-apoptotic effects in cancer models through modulation of these kinase pathways [78]. Thus, our results are in agreement with such proposed quercetin effect.

It is also remarkable the minor similarity found among PPAR gamma and PIM 1 kinase. Currently it is unclear whether quercetin and related compounds are direct or indirect activators of PPAR gamma pathway. *In vitro* evidence favors a mechanism involving quercetin inhibition of cyclin dependent kinase 5 (CDK5) [79]. Lastly, it is worthwhile to mention that the similarity found among sult 1b and tankyrase 2 corresponds to a different site than the flavonoid binding site.

3.4 Amplification: binding site similarity search

Based on LIBRA analysis on local similarity at the binding site of the protein candidates found in the PDB we proposed an additional set of proteins (Table 3). This new set of candidates participates also in cellular metabolism and signaling cascades that can be related to previously described flavonoid biological effects. Interestingly, most of the proteins identified bind ligands similar to quercetin and may have been found on the first stage of the screening, but their similarity score ranged from 1.0 to 1.3. This result suggests that additional reliable candidates could have been initially found with a less stringent cut-off ligand based screening.

3.5 Quercetin interaction pattern

To assess the existence of any general trend in the interaction of quercetin with the protein candidates, their interaction pattern with quercetin docking poses was analyzed (ex. Figure 6). The

pattern showed a preference for polar and charged amino acids, especially glutamate and aspartate, which interact with quercetin hydroxyl groups (Figure 7). Hydroxyl groups in positions 3' and 7 are the most frequently involved in hydrogen bonds with protein residues (Figure 8). This general pattern appears stronger in the kinase group of protein candidates (not shown).

Interestingly related compounds like fisetin, myricetin and resveratrol which have the required hydroxyl groups for this pattern also show remarkable similarities in their biological effects [11]. The case of flavonoids like rutin remains elusive as the disaccharide moiety in position 3 could cause steric clashes in the binding. This would imply that either the bioactive form loses this substituent or another binding orientation than that suggested by docking is possible.

3.6 Functional characterization

Lastly, we analyzed the functional distribution of the final set of candidate targets (Table 2, Table 3). The significantly enriched gene ontology (GO) functional annotations of these targets include regulation of cell death and proliferation. KEGG pathways highlight their involvement in cancer and neurodegeneration as well. It is also remarkable the role of some target proteins in neuronal physiology, given their involvement in neurotrophin signaling, neuronal differentiation and synapse function. These are already reported effects of flavonoids [80–82]. Nevertheless, such enriched pathways and GO functional annotations provide important clues for understanding the molecular mechanisms underlying flavonoids effects.

STITCH protein-protein interaction network analysis also showed that among the target candidates, the kinase group, poly ADP-ribose polymerase group and PPAR-gamma receptor constitute a separate interacting network (see Supplementary material) supporting their participation in related biological processes. In particular PARP-1 facilitates diverse inflammatory responses by promoting inflammation-relevant gene expression through NFkappB and promotes mitochondria-associated cell death in injured tissues [83]. Also, it is widely accepted that PARP-1 signals the MAPK pathway by modulating the phosphorylation of ERK1/2, p38, and c-Jun NH2-terminal kinase. Thus

far, the studies on the relationship between PARP-1 and MAPK suggest that they might stimulate each other in a positive feedback cycle to propagate the responses to the long-lasting stress signals [83]. Such crosstalk between protein targets reinforces the concept of multilevel and synergistic mechanisms of action underlying flavonoid biological effects. Another relevant point is the pleiotropism of many of the protein targets in both cancer and neuronal protection. Glycogen synthase kinase 3b is a perfect example of a promising target candidate which is involved in cancer survival and also promotes nuclear export and degradation of Nrf2 (Nuclear Factor-erythroid 2 (NF-E2) p45-related Factor-2), a transcription factor that triggers antioxidant and cytoprotective responses [78, 84, 85].

Finally we made a list of proteins previously reported by pre-clinical studies as putative quercetin targets, and we compared it with our resulting *in silico* target candidates (Table 4). Many of the enlisted proteins do not have crystallographic structures deposited in the PDB, so they may constitute false negatives by our virtual screening. Nevertheless, there are several enlisted proteins with structural data that were found as target candidates by our virtual screening. In this group we found: serine/threonine protein kinase PIM 1, serine/threonine protein kinase 17B, hematopoietic cell kinase HCK, ATP synthase, phosphatidylinositol 3 kinase, 3-hydroxyisobutiryl CoA hydrolase, casein kinase II, xanthine dehydrogenase, tyrosine protein kinase SRC and vascular endothelial growth factor receptor 2. It is noteworthy that our virtual screening showed a group of target candidates that were not previously reported as putative quercetin targets, and thus the results of the present study could be of interest for drug discovery and future target validation.

4. Conclusions

In conclusion, in this study, a hierarchical inverse screening approach using SHAFTS and idTarget was employed to identify the potential protein targets of quercetin. The screening successfully retrieved every quercetin target contained in the PDB and expanded the list to new putative quercetin targets. Most of the predicted panel of targets fall under conventional clinical targets with

anti-tumour and neuro-protective effects or target enzymes of drug design, and some of them have been well established in experimental settings. In addition, some of these protein targets are involved at different stages in the same signaling pathways or in interconnected signaling pathways, supporting a pleiotropic, multilevel and synergistic mechanism of action of quercetin.

Conversely, some issues still need to be addressed. Apart from docking scoring limitations in protein-target ranking, the under representation of rare proteins in the PDB or the absence of proteins for which three-dimensional structures are not available can cause false negatives [86, 87]. In addition, to further refine these results other interaction repositories like DrugBank could be incorporated. Moreover, the interactions in a ligand-protein complex could also be analyzed by means of molecular dynamics simulations to ensure that flexibility of proteins is considered.

Overall, we believe the present results may broaden the understanding of quercetin mechanism of action and generate new hypothesis about its therapeutic potential. In addition, our integrated approach is immediately valuable for the drug discovery and development process providing valuable information for future *in vitro* and *in vivo* studies.

Acknowledgments

This work was supported by the National Agency of Research and Innovation and the Programme for the Development of Basic Science (PEDECIBA)-Master of Bioinformatics.

Table Captions

Table 1 Protein target candidates found in the Protein Data Bank (PDB) by reverse virtual screening based on ligand similarity with SHAFTS (SHApe-Feature Similarity) algorithm and inverse docking with idTarget web server (*http://idtarget.rcas.sinica.edu.tw/*) [40, 51]. Candidates are segregated by source organism and depicted according to the corresponding PDB entries, co-crystalized ligand and its structural similarity score with quercetin, as well as protein name, idTarget scoring (Z-score and predicted affinity) and protein class given by the PDB [47]. Proteins co-crystallized with quercetin are depicted in bold

Table 2 Disease association and relevant biological process for the target candidates found in the Protein Data Bank (PDB). The information was gathered from publicly available databases like PDB, Uniprot and gene ontology repositories [47, 57, 61] . Depicte source organism. Proteins co-crystallized with quercetin are depicted in bold

Table 3 Structurally related proteins to the candidates found in the Protein Data Bank (PDB) defined by LIBRA (Ligand Binding site Recognition Application) binding site comparison. Proteins are depicted according to the corresponding Uniprot entry (human homologue in parenthesis), name, related disease and biological process [45]. The information was gathered from publicly available databases like PDB, Uniprot and gene ontology repositories [47, 57, 61]

Table 4. Top ranking interactions reported in different repositories of in-vitro protein-ligand interaction assay data like DrugBank, ChEMBLE, BindingDB and Supertarget [64–66, 88]. Proteins are segregated by source organism and depicted according to the corresponding Uniprot entry, and name [57]. Proteins highlighted in bold were previously identified in this work by in-silico reverse virtual screening

Figure Captions

Fig. 1 Quercetin structure optimized by MMFF94x until 0.01 kcal/mol RMS gradient with MOE (Molecular Operating Environment) software [49]. Flavonoid ABC ring notation, and substitution numeration are indicated. Oxygen atoms are depicted in red

Fig. 2 Reverse screening work-flow. The strategy involved a similarity search with SHAFTS (SHApe-Feature Similarity) algorithm of co-crystalized ligands from the Protein Data Bank (PDB) followed by reverse docking with idTarget web server (*http://idtarget.rcas.sinica.edu.tw/*) [40, 51]. An amplification stage is added to identify related proteins by LIBRA (Ligand Binding site Recognition Application) binding site comparison [45]

Fig. 3. Result of quercetin (yellow sticks) molecular docking with mitogen-activated protein kinase 14 (p38 MAP kinase) co-crystallized with naringenin (violet sticks) (Protein Data Bank accession id 4eh3) according to IdTarget web server (*http://idtarget.rcas.sinica.edu.tw/*) [40]. A dotted electrostatic surface was drawn around quercetin at a Van der Waals distance, areas depicted in red color correspond to a negative charged surface and white surface to a neutral charge. A good overlapping between naringenin a quercetin docking pose is observed confirming the reliability of the docking method

Fig. 4 Structural alignment between ATP/ADP and quercetin given by SHAFTS (SHApe-Feature Similarity) algorithm [52]. The alignment shows two different quercetin orientations (ATP alignment similarity score: 0.8693, ADP alignment similarity score: 0.9790) that can overlap functional groups of these endogenous ligands and suggests a quercetin ATP/ADP mimetic action

Fig. 5 Binding site similarities among the protein target candidates found in the Protein Data Bank (PDB) as defined by LIBRA (Ligand Binding site Recognition Application) parameters (see Methods section) [45]. Equal sites are indicated by black squares, similar sites by grey squares, less similar sites as pale grey squares (1<RMSD<1.5). Proteins are denoted by their gene name. The strongest similarity is found among the ATP binding site of protein kinases

Fig. 6 Quercetin molecular contacts with mitogen-activated protein kinase 14 (p38 MAP kinase) assessed by Protein Ligand Interaction Fingerprint (PLIF) with MOE (Molecular Operating Environment) software (Protein Data Bank accession id 4eh3) [49]

Fig. 7 Quercetin interaction pattern among protein candidates found in the Protein Data Bank (PDB). Quercetin molecular contacts with the protein candidates were assessed by Protein Ligand Interaction Fingerprint (PLIF) with MOE (Molecular Operating Environment) software (Protein Data Bank accession id 4eh3) [49]. Number of contacts was then calculated as the accumulated contacts per amino acid residue from all protein candidates weighted by docking conformations

Fig. 8 Quercetin interaction pattern among protein candidates found in the Protein Data Bank (PDB). Quercetin molecular contacts with the protein candidates were assessed by Protein Ligand Interaction Fingerprint (PLIF) with MOE (Molecular Operating Environment) software (Protein Data Bank accession id 4eh3) [49]. Number of contacts was then calculated as the accumulated contacts per amino acid residue from all protein candidates weighted by docking conformations. Number of contacts were segregated by the corresponding quercetin hydroxyl and oxygen substitutions (squares A to B) and groped by amino acid type (N: negative charged, P: positive charged, U: polar uncharged, A: aromatic, H: hydrophobic)

References

1. Burr ML (1995) Explaining the French paradox. J R Soc Health 115:217–9.

2. McCullough ML, Peterson JJ, Patel R, Jacques PF, Shah R, Dwyer JT (2012) Flavonoid intake and cardiovascular disease mortality in a prospective cohort of US adults. Am J Clin Nutr 95:454–64. doi: 10.3945/ajcn.111.016634

3. Hollman PCH, Geelen A, Kromhout D (2010) Dietary Flavonol Intake May Lower Stroke Risk in Men and Women. J Nutr 140:600–604. doi: 10.3945/jn.109.116632

4. Kuriyama S, Shimazu T, Ohmori K, Kikuchi N, Nakaya N, Nishino Y, Tsubono Y, Tsuji I (2006) Green tea consumption and mortality due to cardiovascular disease, cancer, and all causes in Japan: the Ohsaki study. JAMA J Am Med Assoc 296:1255–1265. doi: 10.1001/jama.296.10.1255

5. Commenges D, Scotet V, Renaud S, Jacqmin-Gadda H, Barberger-Gateau P, Dartigues JF (2000) Intake of flavonoids and risk of dementia. Eur J Epidemiol 16:357–63.

6. Cao G, Sofic E, Prior RL (1997) Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. Free Radic Biol Med 22:749–760.

7. Lotito SB, Frei B (2006) Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: cause, consequence, or epiphenomenon? Free Radic Biol Med 41:1727–1746. doi: 10.1016/j.freeradbiomed.2006.04.033

8. Fuhrman B, Aviram M (2001) Flavonoids protect LDL from oxidation and attenuate atherosclerosis. Curr Opin Lipidol 12:41–8.

9. Benavente-García O, Castillo J (2008) Update on uses and properties of citrus flavonoids: new findings in anticancer, cardiovascular, and anti-inflammatory activity. J Agric Food Chem 56:6185–205. doi: 10.1021/jf8006568

10. Kale A, Gawande S, Kotwal S (2008) Cancer phytotherapeutics: role for flavonoids at the cellular level. Phytother Res 22:567–77. doi: 10.1002/ptr.2283

11. Bisht K, Wagner K-H, Bulmer AC (2010) Curcumin, resveratrol and flavonoids as anti-inflammatory, cyto- and DNA-protective dietary compounds. Toxicology 278:88–100. doi: 10.1016/j.tox.2009.11.008

12. Cazarolli LH, Zanatta L, Alberton EH, Figueiredo MS, Folador P, Damazio RG, Pizzolatti MG, Silva FR (2008) Flavonoids: cellular and molecular mechanism of action in glucose homeostasis. Mini Rev Med Chem 8:1032–8.

13. Spencer JPE, Vauzour D, Rendeiro C (2009) Flavonoids and cognition: the molecular mechanisms underlying their behavioural effects. Arch Biochem Biophys 492:1–9. doi: 10.1016/j.abb.2009.10.003

14. Si H, Fu Z, Babu PV, Zhen W, Leroith T, Meaney MP, Voelker KA, Jia Z, Grange RW, Liu D (2011) Dietary epicatechin promotes survival of obese diabetic mice and Drosophila melanogaster. J Nutr 141:1095–100. doi: 10.3945/jn.110.134270

15. Maher P, Dargusch R, Ehren JL, Okada S, Sharma K, Schubert D (2011) Fisetin lowers methylglyoxal dependent protein glycation and limits the complications of diabetes. PLoS One 6:e21226. doi: 10.1371/journal.pone.0021226

16. Reed J (2002) Cranberry flavonoids, atherosclerosis and cardiovascular health. Crit Rev Food Sci Nutr 42:301–16. doi: 10.1080/10408390209351919

17. Arab L, Liebeskind DS (2010) Tea, flavonoids and stroke in man and mouse. Arch Biochem Biophys 501:31–6. doi: 10.1016/j.abb.2010.03.015

18. Vidya Priyadarsini R, Senthil Murugan R, Maitreyi S, Ramalingam K, Karunagaran D, Nagini S (2010) The flavonoid quercetin induces cell cycle arrest and mitochondria-mediated apoptosis in human cervical cancer (HeLa) cells through p53 induction and NF-κB inhibition. Eur J Pharmacol 649:84–91. doi: 10.1016/j.ejphar.2010.09.020

19. Cherry JJ, Rietz A, Malinkevich A, Liu Y, Xie M, Bartolowits M, Davisson VJ, Baleja JD, Androphy EJ (2013) Structure based identification and characterization of flavonoids that disrupt human papillomavirus-16 E6 function. PLoS One 8:e84506. doi: 10.1371/journal.pone.0084506

20. García-Saura MF, Galisteo M, Villar IC, Bermejo A, Zarzuelo A, Vargas F, Duarte J (2005) Effects of chronic quercetin treatment in experimental renovascular hypertension. Mol Cell Biochem 270:147–55.

21. Calderone V, Chericoni S, Martinelli C, Testai L, Nardi A, Morelli I, Breschi MC, Martinotti E (2004) Vasorelaxing effects of flavonoids: investigation on the possible involvement of potassium channels. Naunyn Schmiedebergs Arch Pharmacol 370:290–8. doi: 10.1007/s00210-004-0964-z

22. Hämäläinen M, Nieminen R, Vuorela P, Heinonen M, Moilanen E (2007) Anti-inflammatory effects of flavonoids: genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-kappaB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-kappaB activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages. Mediators Inflamm 2007:45673. doi: 10.1155/2007/45673

23. Davis JM, Murphy EA, McClellan JL, Carmichael MD, Gangemi JD (2008) Quercetin reduces susceptibility to influenza infection following stressful exercise. AJP Regul Integr Comp Physiol 295:R505–R509. doi: 10.1152/ajpregu.90319.2008

24. Arredondo F, Echeverry C, Abin-Carriquiry JA, Blasina F, Antúnez K, Jones DP, Go YM, Liang YL, Dajas F (2010) After cellular internalization, quercetin causes Nrf2 nuclear translocation, increases glutathione levels, and prevents neuronal death against an oxidative insult. Free Radic Biol Med 49:738–47. doi: 10.1016/j.freeradbiomed.2010.05.020

25. Jung SH, Kang KD, Ji D, Fawcett RJ, Safa R, Kamalden TA, Osborne NN (2008) The flavonoid baicalin counteracts ischemic and oxidative insults to retinal cells and lipid peroxidation to brain membranes. Neurochem Int 53:325–37. doi: 10.1016/j.neuint.2008.09.004

26. Echeverry C, Arredondo F, Abin-Carriquiry JA, Midiwo JO, Ochieng C, Kerubo L, Dajas F (2010) Pretreatment with natural flavones and neuronal cell survival after oxidative stress: a structure-activity relationship study. J Agric Food Chem 58:2111–5. doi: 10.1021/jf902951v

27. Dajas F (2012) Life or death: Neuroprotective and anticancer effects of quercetin. J Ethnopharmacol 143:383–96. doi: 10.1016/j.jep.2012.07.005

28. Williams RJ, Spencer JPE, Rice-Evans C (2004) Flavonoids: antioxidants or signalling molecules? Free Radic Biol Med 36:838–49. doi: 10.1016/j.freeradbiomed.2004.01.001

29. Pandey AK, Hazari PP, Patnaik R, Mishra AK (2011) The role of ASIC1a in neuroprotection elicited by quercetin in focal cerebral ischemia. Brain Res 1383:289–99. doi: 10.1016/j.brainres.2011.01.085

30. Lee KW, Kang NJ, Heo YS, Rogozin EA, Pugliese A, Hwang MK, Bowden GT, Bode AM, Lee HJ, Dong Z (2008) Raf and MEK protein kinases are direct molecular targets for the chemopreventive effect of quercetin, a major flavonol in red wine. Cancer Res 68:946–55. doi: 10.1158/0008-5472.CAN-07-3140

31. Mandel S, Weinreb O, Amit T, Youdim MBH (2004) Cell signaling pathways in the neuroprotective actions of the green tea polyphenol (-)-epigallocatechin-3-gallate: implications for neurodegenerative diseases. J Neurochem 88:1555–1569.

32. Huang WW, Chiu YJ, Fan MJ, Lu HF, Yeh HF, Li KH, Chen PY, Chung JG, Yang JS (2010) Kaempferol induced apoptosis via endoplasmic reticulum stress and mitochondria-dependent pathway in human osteosarcoma U-2 OS cells. Mol Nutr Food Res 54:1585–1595. doi: 10.1002/mnfr.201000005

33. Lee YK, Hwang JT, Kwon DY, Surh YJ, Park OJ (2010) Induction of apoptosis by quercetin is mediated through AMPKalpha1/ASK1/p38 pathway. Cancer Lett 292:228–36. doi: 10.1016/j.canlet.2009.12.005

34. Lee KW, Bode AM, Dong Z (2011) Molecular targets of phytochemicals for cancer prevention. Nat Rev Cancer 11:211–8. doi: 10.1038/nrc3017

35. Hart CP (2005) Finding the target after screening the phenotype. Drug Discov Today 10:513–9. doi: 10.1016/S1359-6446(05)03415-X

36. Kinnings SL, Jackson RM (2011) ReverseScreen3D: a structure-based ligand matching method to identify protein targets. J Chem Inf Model 51:624–34. doi: 10.1021/ci1003174

37. Chen YZ, Zhi DG (2001) Ligand-protein inverse docking and its potential use in the computer search of protein targets of a small molecule. Proteins 43:217–26.

38. Li H, Gao Z, Kang L, Zhang H, Yang K, Yu K, Luo X, Zhu W, Chen K, Shen J, Wang X, Jiang H (2006) TarFisDock: a web server for identifying drug targets with docking approach. Nucleic Acids Res 34:W219–24. doi: 10.1093/nar/gkl114

39. Liu X, Ouyang S, Yu B, Liu Y, Huang K, Gong J, Zheng S, Li Z, Li H, Jiang H (2010) PharmMapper server: a web server for potential drug target identification using pharmacophore mapping approach. Nucleic Acids Res 38:W609–14. doi: 10.1093/nar/gkq300

40. Wang J, Chu P, Chen C-M, Lin J (2012) idTarget: a web server for identifying protein targets of small chemical molecules with robust scoring functions and a divide-and-conquer docking approach. Nucleic Acids Res 40:W393–9. doi: 10.1093/nar/gks496

41. Bhattacharjee B, Vijayasarathy S, Karunakar P, Chatterjee J (2012) Comparative reverse screening approach to identify potential anti-neoplastic targets of saffron functional components and binding mode. Asian Pac J Cancer Prev 13:5605–11.

42. Dakshanamurthy S, Issa NT, Assefnia S, Seshasayee A, Peters OJ, Madhavan S, Uren A, Brown ML, Byers SW (2012) Predicting new indications for approved drugs using a proteochemometric method. J Med Chem 55:6832–48. doi: 10.1021/jm300576q

43. Cereto-Massagué A, Ojeda MJ, Valls C, Mulero M, Pujadas G, Garcia-Vallve S (2015) Tools for in silico target fishing. Methods 71:98-103. doi: 10.1016/j.ymeth.2014.09.006

44. Kumar A, Zhang KY (2015) Hierarchical virtual screening approaches in small molecule drug discovery. Methods 71:26-37. doi: 10.1016/j.ymeth.2014.07.007

45. Viet Hung L, Caprari S, Bizai M, Toti D, Polticelli F (2015) LIBRA: LIgand Binding site Recognition Application. Bioinformatics. doi: 10.1093/bioinformatics/btv489

46. Klabunde T (2007) Chemogenomic approaches to drug discovery: similar receptors bind similar ligands. Br J Pharmacol 152:5–7. doi: 10.1038/sj.bjp.0707308

47. Berman HM (2000) The Protein Data Bank. Nucleic Acids Res 28:235–242. doi: 10.1093/nar/28.1.235

48. Feng Z, Chen L, Maddula H, Akcan O, Oughtred R, Berman HM, Westbrook J (2004) Ligand Depot: a data warehouse for ligands bound to macromolecules. Bioinformatics 20:2153–5. doi: 10.1093/bioinformatics/bth214

49. Molecular Operating Environment (MOE 2011.10) Chemical Computing Group Inc, Montreal Quebec Canada http://www.chemcomp.com. 2011

50. Oprea TI, Tropsha A, Faulon JL, Rintoul MD (2007) Systems chemical biology. Nat Chem Biol 3:447-50

51. Liu X, Jiang H, Li H (2011) SHAFTS: a hybrid approach for 3D molecular similarity calculation. 1. Method and assessment of virtual screening. J Chem Inf Model 51:2372–85. doi: 10.1021/ci200060s

52. Lu W, Liu X, Cao X, Xue M, Liu K, Zhao Z, Shen X, Jiang H, Xu Y, Huang J, Li H (2011) SHAFTS: a hybrid approach for 3D molecular similarity calculation. 2. Prospective case study in the discovery of diverse p90 ribosomal S6 protein kinase 2 inhibitors to suppress cell migration. J Med Chem 54:3564–74. doi: 10.1021/jm200139j

53. Bai F, Liu H, Tong L, Zhou W, Liu L, Zhao Z, Liu X, Jiang H, Wang X, Xie H, Li H (2012) Discovery of novel selective inhibitors for EGFR-T790M/L858R. Bioorg Med Chem Lett 22:1365–70. doi: 10.1016/j.bmcl.2011.12.067

54. Kong X, Qin J, Li Z, Vultur A, Tong L, Feng E, Rajan G, Liu S, Lu J, Liang Z, Zheng M, Zhu W, Jiang H, Herlyn M, Liu H, Marmorstein R, Luo C (2012) Development of a novel class of B-Raf(V600E)-selective inhibitors through virtual screening and hierarchical hit optimization. Org Biomol Chem 10:7402–17. doi: 10.1039/c2ob26081f

55. Liu X, Bai F, Ouyang S, Wang X, Li H, Jiang H (2009) Cyndi: a multi-objective evolution algorithm based method for bioactive molecular conformational generation. BMC Bioinformatics 10:101. doi: 10.1186/1471-2105-10-101

56. Nikolić AR, Petri ET, Klisurić OR, Ćelić AS, Jakimov DS, Djurendić EA, Penov Gaši KM, Sakač MN (2015) Synthesis and anticancer cell potential of steroidal 16,17-seco-16,17a-dinitriles: identification of a selective inhibitor of hormone-independent breast cancer cells. Bioorg Med Chem 23:703–11. doi: 10.1016/j.bmc.2014.12.069

57. Consortium TU (2014) UniProt: a hub for protein information. Nucleic Acids Res 43:D204–D212. doi: 10.1093/nar/gku989

58. Dennis G, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA (2003) DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol 4:P3.

59. Kanehisa M, Goto S (2000) KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res 28:27–30.

60. Boyle EI, Weng S, Gollub J, Jin H, Botstein D, Cherry JM, Sherlock G (2004) GO::TermFinder - Open source software for accessing Gene Ontology information and finding significantly enriched Gene Ontology terms associated with a list of genes. Bioinformatics 20:3710–3715. doi: 10.1093/bioinformatics/bth456

61. Harris MA, Clark J, Ireland A, Lomax J, Ashburner M, Foulger R, Eilbeck K, Lewis S, Marshall B, Mungall C, Richter J, Rubin GM, Blake JA, Bult C, Dolan M, Drabkin H, Eppig JT, Hill DP, Ni L, Ringwald M, Balakrishnan R, Cherry JM, Christie KR, Costanzo MC, Dwight SS, Engel S, Fisk DG, Hirschman JE, Hong EL, Nash RS, Sethuraman A, Theesfeld CL, Botstein D, Dolinski K, Feierbach B, Berardini T, Mundodi S, Rhee SY, Apweiler R, Barrell D, Camon E, Dimmer E, Lee V, Chisholm R, Gaudet P, Kibbe W, Kishore R, Schwarz EM, Sternberg P, Gwinn M, Hannick L, Wortman J, Berriman M, Wood V, de la Cruz N, Tonellato P, Jaiswal P, Seigfried T, White R; Gene Ontology Consortium (2004) The Gene Ontology (GO) database and informatics resource. Nucleic Acids Res 32:D258–D261. doi: 10.1093/nar/gkh036

62. Lyne R, Smith R, Rutherford K, Wakeling M, Varley A, Guillier F, Janssens H, Ji W, Mclaren P, North P, Rana D, Riley T, Sullivan J, Watkins X, Woodbridge M, Lilley K, Russell S, Ashburner M, Mizuguchi K, Micklem G (2007) FlyMine: an integrated database for Drosophila and Anopheles genomics. Genome Biol 8:R129. doi: 10.1186/gb-2007-8-7-r129

63. Wishart DS, Knox C, Guo AC, Shrivastava S, Hassanali M, Stothard P, Chang Z, Woolsey J (2006) DrugBank: a comprehensive resource for in silico drug discovery and exploration. Nucleic Acids Res 34:D668–72. doi: 10.1093/nar/gkj067

64. Davies M, Nowotka M, Papadatos G, Dedman N, Gaulton A, Atkinson F, Bellis L, Overington JP (2015) ChEMBL web services: streamlining access to drug discovery data and utilities. Nucleic Acids Res. doi: 10.1093/nar/gkv352

65. Liu T, Lin Y, Wen X, Jorissen RN, Gilson MK (2007) BindingDB: a web-accessible database of experimentally determined protein-ligand binding affinities. Nucleic Acids Res 35:D198–201. doi: 10.1093/nar/gkl999

66. Günther S, Kuhn M, Dunkel M, Campillos M, Senger C, Petsalaki E, Ahmed J, Urdiales EG, Gewiess A, Jensen LJ, Schneider R, Skoblo R, Russell RB, Bourne PE, Bork P, Preissner R (2008) SuperTarget and Matador: resources for exploring drug-target relationships. Nucleic Acids Res 36:D919–22. doi: 10.1093/nar/gkm862

67. Kuhn M, Szklarczyk D, Franceschini A, von Mering C, Jensen LJ, Bork P (2012) STITCH 3: zooming in on protein-chemical interactions. Nucleic Acids Res 40:D876–80. doi: 10.1093/nar/gkr1011

68. Schroeter H, Boyd C, Spencer JP, Williams RJ, Cadenas E, Rice-Evans C (2002) MAPK signaling in neurodegeneration: influences of flavonoids and of nitric oxide. Neurobiol Aging 23:861–80.

69. Zhang XM, Zhang L, Wang G, Niu W, He Z, Ding L, Jia J (2015) Suppression of mitochondrial fission in experimental cerebral ischemia: The potential neuroprotective target of p38 MAPK inhibition. Neurochem Int 1–8. doi: 10.1016/j.neuint.2015.06.010

70. Han D, Scott EL, Dong Y, Raz L, Wang R, Zhang Q (2015) Attenuation of mitochondrial and nuclear p38α signaling: a novel mechanism of estrogen neuroprotection in cerebral ischemia. Mol Cell Endocrinol 400:21–31. doi: 10.1016/j.mce.2014.11.010

71. Zhao YF, Zhang Q, Xi JY, Li YH, Ma CG, Xiao BG (2015) Multitarget intervention of Fasudil in the neuroprotection of dopaminergic neurons in MPTP-mouse model of Parkinson’s disease. J Neurol Sci 353:28–37. doi: 10.1016/j.jns.2015.03.022

72. Lehtiö L, Chi NW, Krauss S (2013) Tankyrases as drug targets. FEBS J 280:3576–3593. doi: 10.1111/febs.12320

73. Teillet F, Boumendjel A, Boutonnat J, Ronot X (2008) Flavonoids as RTK inhibitors and potential anticancer agents. Med Res Rev 28:715–745

74. Sebolt-Leopold JS, Herrera R (2004) Targeting the mitogen-activated protein kinase cascade to treat cancer. Nat Rev Cancer 4:937–47. doi: 10.1038/nrc1503

75. Hynes NE, Lane HA (2005) ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer 5:341–54. doi: 10.1038/nrc1609

76. Zhou H-Y, Huang S-L (2012) Current development of the second generation of mTOR inhibitors as anticancer agents. Chin J Cancer 31:8–18. doi: 10.5732/cjc.011.10281

77. Granville CA, Memmott RM, Gills JJ, Dennis PA (2006) Handicapping the race to develop inhibitors of the phosphoinositide 3-kinase/Akt/mammalian target of rapamycin pathway. Clin Cancer Res 12:679–89. doi: 10.1158/1078-0432.CCR-05-1654

78. Russo GL, Russo M, Spagnuolo C, Tedesco I, Bilotto S, Iannitti R, Palumbo R (2014) Quercetin: A Pleiotropic Kinase Inhibitor Against Cancer. Cancer Treat Res 159:185-205. doi: 10.1007/978-3-642-38007-5_11

79. Beekmann K, Rubió L, de Haan LH, Actis-Goretta L, van der Burg B, van Bladeren PJ, Rietjens IM (2015) The effect of quercetin and kaempferol aglycones and glucuronides on peroxisome proliferator-activated receptor-gamma (PPAR-γ). Food Funct 6:1098-107. doi: 10.1039/c5fo00076a

80. Blasina MF, Vaamonde L, Morquio A, Echeverry C, Arredondo F, Dajas F (2009) Differentiation induced by Achyrocline satureioides (Lam) infusion in PC12 cells. Phytother Res 23:1263–9. doi: 10.1002/ptr.2607

81. Chen PY, Wu MJ, Chang HY, Tai MH, Ho CT, Yen JH (2015) Up-Regulation of miR-34a Expression in Response to the Luteolin-Induced Neurite Outgrowth of PC12 Cells. J Agric Food Chem 63:4148–59. doi: 10.1021/acs.jafc.5b01005

82. Spencer JPE (2009) Flavonoids and brain health: multiple effects underpinned by common mechanisms. Genes Nutr 4:243–50. doi: 10.1007/s12263-009-0136-3

83. Ba X, Garg NJ (2011) Signaling mechanism of poly(ADP-ribose) polymerase-1 (PARP-1) in inflammatory diseases. Am J Pathol 178:946–955. doi: 10.1016/j.ajpath.2010.12.004

84. Yoshino Y, Ishioka C (2015) Inhibition of glycogen synthase kinase-3 beta induces apoptosis and mitotic catastrophe by disrupting centrosome regulation in cancer cells. Sci Rep 5:13249. doi: 10.1038/srep13249

85. Jain AK, Jaiswal AK (2007) GSK-3beta acts upstream of Fyn kinase in regulation of nuclear export and degradation of NF-E2 related factor 2. J Biol Chem 282:16502–16510.

86. Warren GL, Andrews CW, Capelli AM, Clarke B, LaLonde J, Lambert MH, Lindvall M, Nevins N, Semus SF, Senger S, Tedesco G, Wall ID, Woolven JM, Peishoff CE, Head MS

(2006) A critical assessment of docking programs and scoring functions. J Med Chem 49:5912–31. doi: 10.1021/jm050362n

87. Xie L, Bourne PE (2005) Functional coverage of the human genome by existing structures, structural genomics targets, and homology models. PLoS Comput Biol 1:e31. doi: 10.1371/journal.pcbi.0010031

88. Law V, Knox C, Djoumbou Y, Jewison T, Guo AC, Liu Y, Maciejewski A, Arndt D, Wilson M, Neveu V, Tang A, Gabriel G, Ly C, Adamjee S, Dame ZT, Han B, Zhou Y, Wishart DS (2014) DrugBank 4.0: shedding new light on drug metabolism. Nucleic Acids Res 42:D1091–7. doi: 10.1093/nar/gkt1068

Send Orders of Reprints at reprints@benthamscience.net

30 *Central Nervous System Agents in Medicinal Chemistry*, 2013, *13*, 30-35

Neuroprotective Actions of Flavones and Flavonols: Mechanisms and Relationship to Flavonoid Structural Features

Federico Dajas*, Abin-Carriquiry Juan Andrés, Arredondo Florencia, Echeverry Carolina and Rivera-Megret Felicia

Department of Neurochemistry, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay

Abstract: Epidemiological studies have shown positive preventive action of flavonoids on cardiovascular and neurodegenerative events. Among the six groups in which flavonoids are classified, the flavones and flavonols, based on the backbone of 2-phenylchromen-4-one (2-phenyl-1-benzopyran-4-one) are the most commonly encountered within the families and genera of the higher plants. Numerous studies support a neuroprotective activity of flavones such as luteolin and flavonols such as kaempherol and quercetin in experimental focal ischemia and models of neurodegeneration. Antioxidation, modulation of signaling cascades and gene expression as well as anti-inflammation appear as the main protective mechanisms and mitochondria are a likely main target mediating the preventive actions against oxidative stress. Flavones and flavonols re-establish the redox regulation of proteins, transcription factors and signaling cascades that are otherwise inhibited by elevated oxidative stress. The final survival or death of the neuron depends on flavone and flavonol concentrations, time of exposure and, mainly, metabolic and oxidative neuronal circumstances. Neuroprotection appears to be linked to specific structural motifs, beyond those involved in antioxidation. By themselves or as templates for synthetic compounds, flavone and flavonol molecules show potential as multi-targeted therapeutic tools for protecting the brain. Nonetheless, more research needs to be done on the correlation of potential beneficial effects of flavones and flavonols and their mechanisms of action.

Keywords: Flavones, flavonols, neuroprotection, oxidative stress, quercetin, signalling.

1. INTRODUCTION

1.1. Brain Pathology, Redox Homeostasis and Oxidative Stress

It is known that brain pathology in the form of cerebrovascular and neurodegenerative disease is a leading cause of death all over the world, with an incidence of about 2/1000 and an 8% total death rate [1, 2]. Moreover, stroke and dementia are a source of high individual and family suffering mainly because of the lack of efficient therapeutic alternatives. The latter motivates research efforts to identify the mechanisms of neuronal death and to discover new compounds to prevent them. Neuronal death in these neuropathologies is a complex phenomenon involving failure of metabolic processes, protein impaired mitochondrial function, increased oxidative damage, defects in the proteasome system, protein aggregation, changes in iron metabolism, and events of excitotoxicity and inflammation [3]. The interaction between all these cellular processes would not be necessarily a cascade but a cycle of events, of which oxidative stress is a major component [4].

Under physiologic conditions, the balance between the generation and the elimination of reactive oxygen and nitrogen species (ROS, NOS) maintains a redox homeostasis to ensure the correct function of redox-sensitive signaling proteins. However, when homeostasis is disturbed, oxidative stress may take place leading to damage of lipids, proteins and nucleic acids and disruption of redox signaling [5, 6]. Aberrant redox cell signaling may in turn cause cell death, contributing to disease onset [7-10].

With only 2% of the body weight, the brain represents almost 20% of the O_2 consumption of the organism [11]. Indeed, because of its high metabolic rate and relatively reduced capacity for cellular defense and regeneration compared with other organs, the brain is believed to be particularly susceptible to oxidative stress events. In this sense, the maintenance of cellular redox homeostasis appears as a cue to the control and the prevention of oxidative stress-related brain diseases [12, 13].

The above-described situation has led to the search of a variety of antioxidant approaches to attenuate acute ischemic and chronic neurodegenerative disease injuries [14, 15]. Nevertheless, promising results showing strong neuroprotective effects in different preclinical models, failed systematically in clinical trials [16]. In this context, it is likely that molecules acting on multiple targets and involved in all the events of the neurodegenerative processes would be required to be therapeutically effective [3].

1.2. Flavonoids: Flavones and Flavonols

Flavonoids represent the most common group of polyphenolic compounds in the human diet and are widely

*Address correspondence to this author at the Department of Neurochemistry, Instituto de Investigaciones Biológicas Clemente Estable, Avda Italia 3318, 11600, Montevideo, Uruguay; Tel:/Fax: 5982 4872603; E-mail: fdajas@gmail.com

1875-6166/13 $58.00+.00

found in plants. They consist of two aromatic carbon rings benzopyran (A and C-rings) and a benzene (B-ring), and can be divided into different groups depending on the degree of oxidation of the C-ring, the hydroxylation pattern, and the substitution of the C3-position. The main sub-classes of flavonoids are: flavonols, flavones, flavanones, flavan-3-ols, anthocyanidins, and isoflavones. Recently, there has been great interest in the potential of flavonoids to prevent neurodegeneration [17-19]. Although there are diverse reports on neuroprotective effects by different classes of flavonoids on cellular cultures and animal models [20, 21], in this review we will focus on the neuroprotection by flavones (*e.g.*: luteolin, apigenin, jaceosidin and eupatilin) and flavonols (*e.g.*: myricetin, kaempferol and quercetin) in degenerative pathologies and particularly in cerebral ischemia. The flavones and flavonols (Fig. **1**) are the most commonly encountered flavonoids, being ubiquitous within the families and genera of the higher plants [22]. In a previous study [23] we found that some flavones and flavonols such as quercetin have specific structural features that assure their protective effects. We will focus on evidence of neuroprotection by these flavonoid groups in particular, and we will further review evidence about mechanisms of action and structural features underlying their neuroprotective effects. A better understanding of the action of these two groups of flavonoids in neuroprotection could open new strategies for synthetizing leading molecules for the development of beneficial therapies in acute and chronic neurodegenerative diseases.

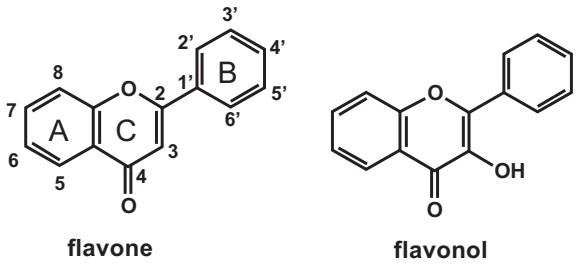


Fig. (1). Flavone and Flavonol structure.

2. EVIDENCES OF NEUROPROTECTION BY FLAVONES AND FLAVONOLS

Numerous studies *in vivo* describe the beneficial effects of the flavonoids in diseases such as cerebral ischemia, Parkinson´s and Alzheimer´s Disease [24, 25]. Thus, luteolin could prevent the behavioral and histological damage induced by ischemia/reperfusion in rats after a 13 days post-ischemic treatment [26]. A recent study confirmed these beneficial effects of luteolin in chronic experimental ischemia [27]. In another study, rats were sensitized for acute and chronic experimental allergic encephalomyelitis, an experimental model of Multiple Sclerosis (MS). In MS disease, migration of monocytes across the blood-brain barrier is a crucial step in the formation of new lesions in the brain. Luteolin substantially suppressed clinical symptoms and prevented recurrence when administered either before or after disease onset resulting in reduced inflammation and axonal damage in the CNS by preventing monocyte migration across the brain endothelium [28].

Besides luteolin some flavones such as jaceosidin ameliorated neuroinflammation in a mouse model of experimental allergic encephalomyelitis [29] and eupatilin was neuroprotective against transient global cerebral ischemia in mice by increased Akt (also known as Protein Kinase B) phosphorylation [30].

When reviewing the neuroprotective activities of flavonols, kaempferol is one of the most studied molecules. Numerous preclinical studies have shown that kaempferol and some of its glycosides have a wide range of pharmacological activities, including antioxidant, anti-inflammatory, anticancer, neuroprotective, anxiolytic, and analgesic actions [31]. An intravenous injection of kaempferol, decreased the brain damage in the neocortex and striatum in transient focal cerebral ischemia model (60 min) and after reperfusion [32]. In a mouse model of Parkinson's disease, Li and Pu showed that kaempferol treatment could prevent the loss of TH-positive neurons induced by 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), improving motor coordination, raising striatal dopamine and its metabolite levels [33]. In a model of Alzheimer´s Disease, kaempferol significantly reversed the effects of beta amyloid peptide (Aβ) administered by intra-cerebro-ventricular injection to induce memory impairment [34].

Quercetin has been undoubtedly the most extensively studied flavonol in their neuroprotective potential *in vivo*. In this respect our group demonstrated that a quercetin/lecithin preparation administered 30 minutes prior to permanent middle cerebral artery occlusion (pMCAo), reduced significantly the motor impairments and the cerebral infarct volume caused by arterial occlusion during 24 hours with respect to not treated ischemic rat [35, 36]. Other recent experimental evidence confirms the neuroprotective effects of quercetin against cerebral ischemia [37].

During cerebral ischemia there is an activation of matrix metalloproteinases (MMPs), an event that results in the breakdown of the Blood Brain Barrier (BBB). Therefore, inhibition of MMPs may be a potential protective therapy against secondary ischemic damage [38]. When the role of quercetin was studied in a photo-thrombosis focal ischemia model in rats, it was found that treatment decreased the over-expression of MMP-9 at 24 and 48 hours after ischemic injury. Also the increased permeability of the BBB and brain oedema was significantly reduced in animals treated with quercetin [38]. The protective role of quercetin has been also demonstrated in the spinal cord damage induced by trauma [39].

3. WORKING MECHANISMS UNDERLYING NEURONAL PROTECTION BY FLAVONES AND FLAVONOLS

3.1. Antioxidative Properties

Traditionally, the beneficial effects of these compounds have been attributed mainly to their antioxidant capacity, including their direct free radical scavenger and their metal chelating activity properties [26, 28, 40]. Additionally, they show broader antioxidant effects through their capacity to interact and modulate enzyme activities such as xanthine oxidase and nitric oxide synthase [22, 40]. More recently, evidence from cell studies suggests that flavones and flavonols

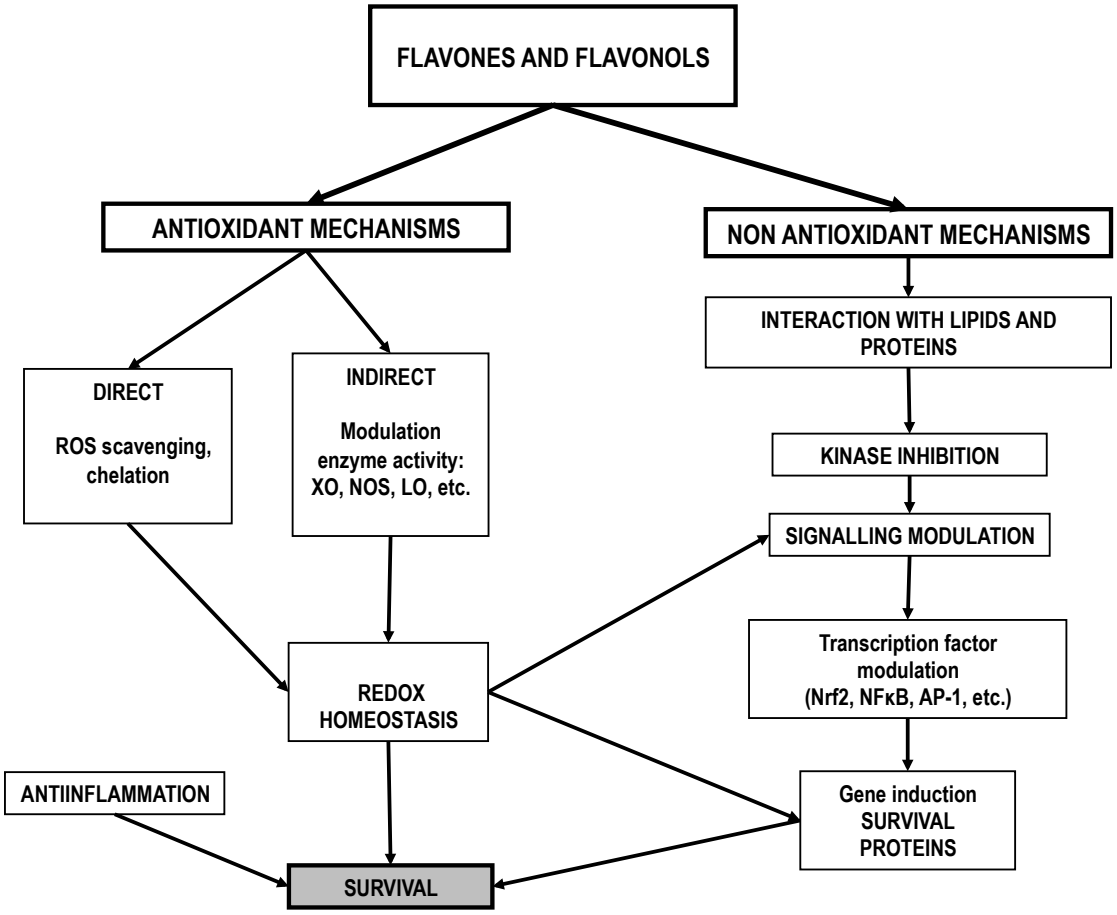


Fig. (2). Schematic synthesis of neuroprotective flavones and flavonols neuroprotective mechanisms.

can influence antioxidant gene expression by modulation of redox sensitive transcription factors such as Nuclear factor κB (NF κB) and Nf E2 related factor-2 (Nrf2). These mechanisms result in the induction of genes encoding for pro-survival, detoxifying and antioxidant proteins such as SODs, glutathione peroxidases and NADPH:quinone oxidoreductases [41-43]. As an important result of antioxidant activities, flavones and flavonols would re-establish the redox regulation of proteins, transcription factors and survival signaling cascades otherwise altered by elevated ROS.

3.2. Modulation of Intracellular Signalling Pathways

Besides their pharmacological role as potent antioxidants, flavones and quercetin show an important additional pharmacological activity represented by their capacity to modulate a number of protein kinases and lipid kinases signalling cascades, such as the phosphatidylinositol 3-kinase (PI3K)/Akt, tyrosine kinase, protein kinase C (PKC) and mitogen-activated protein kinase (MAP kinase) signalling pathways. In this sense, attention turned towards the modulation of these intracellular cascades as a therapeutic approach against neurodegeneration, since these signalling pathways have been involved in neuronal survival, neurogenesis, and cognitive performance through changes in synaptic plasticity. By their interaction with these intracellular signaling cascades, the beneficial cellular effects of flavones and flavonols may be mediated through changes in the phosphorylation state of target molecules, resulting in modulation of cellular function and gene expression, and leading to cell survival [44-50]. Thus, one of the most well characterized MAPK pathways is governed by the mitogenic extracellular signal-regulated protein kinase (ERK). ERK1/2 and c-JunN-terminal kinase (JNK) are involved in apoptosis and various forms of cellular plasticity, and they are generally considered as exerting opposing actions. ERK1/2 is usually associated with pro-survival signaling through mechanisms that may involve the activation of the cyclic AMP regulatory binding protein (CREB) and the up regulation of antiapoptotic proteins. Conversely, JNK has been strongly linked to transcription-dependent apoptotic signaling possibly through the activation of c-Jun [48].

3.3. Role in Mitochondria Dysfunction

Numerous studies have identified a mitochondrial dysfunction that could contribute to neuron degeneration by depletion of cellular ATP, through ROS generation and development of apoptosis by leading to the opening of the mitochondrial permeability transition pore (MPTP) and the release of cytochrome C [51]. It is known that quercetin accumulates in the mitochondria [52] and could protect against mitochondrial-linked pathologies [53]. In Caco-2 cells quercetin was beneficial against indomethacin-induced

mitochondrial dysfunction, precisely by its ability to enter cells and accumulate in mitochondria. Further experimental work by Lagoa and collaborators [54] revealed that mitochondria are a plausible main target of flavones (e.g.: quercetin, kaempferol, apigenin) mediating, at least in part, their preventive actions against oxidative stress and mitochondrial dysfunction-associated pathologies. Closure or opening of MPTP is critical for cell survival or death [55]. In a series of experiments, De Marchi and collaborators [56] have shown that quercetin was able to modulate the redox state of mitochondria and to either inhibit or enhance MPTP permeability. This protective activity point to a potential benefit of flavones and flavonols treatment for conditions involving mitochondrial dysfunction associated with increased oxidative stress.

4. STRUCTURAL FEATURES UNDERLYING NEUROPROTECTIVE EFFECTS OF FLAVONES AND FLAVONOLS

The structural diversity of flavones and flavonols offers the possibility to identify the molecular substitutions required for different biological actions. The *in vitro* free radicals scavenger activity depends on the arrangement of functional groups on its core structure. The configuration and total number of hydroxyl groups is important for this activity. Thus, their antioxidant potency has been linked to the *ortho*-dihydroxy substitution in the B-ring and the presence of 2, 3-unsaturation and a 4-carbonyl in the C-ring [57, 58]. Their activity as metal ion chelators appears to be defined by the presence of the *ortho*-dihydroxy substitution in the B-ring, the 4-carbonyl in the C-ring and a 5 or 3 hydroxy substitutions [59]. According to these studies the most effective radical scavengers are the flavonols with catechol groups (quercetin), followed by flavone groups, a fact which has been experimentally confirmed by our and other groups [24, 58].

Although there is an agreement in the literature on some of the molecular features that determine particular activities of flavonoids such as a scavenger activity, the critical structural requirements for more complex activities as cytoprotection are controversial. In general, the cytoprotective capacity of flavonoids against different insults has been mainly attributed to their antioxidant potency [60, 61]. Nonetheless, other studies [35], assessing the cytoprotective potency of several structurally related flavonoids against oxidative stress, found that cytoprotection did not correlate with the antioxidation potency. Recently, Echeverry and co-workers (2010) performed a structure-activity relationship study focusing on the protective effects of flavones against oxidative stress in neuronal cultures [23]. They showed that only 4 of 13 flavones studied exhibited neuronal protective activity, strongly indicating that specific structural features are related to this action. Indeed, the hydroxy substitutions in the A-ring (C5 and C7) and in position C3 (C-ring) of the flavones would be necessary to afford neuroprotection, indicating that the structural requirements for neuronal protection are different from those that afford antioxidant capacity [23]. The work by Echeverry *et al.* demonstrated for the first time the grouped structural features important for neuroprotection. Separately, the hydroxy substitution in position C3 (C-ring) had been previously shown as important in scavenger activity and anti-inflammatory effects [62, 63]. It had been also shown that OH substitutions on the C5 or/and C7 significantly affect the anti-apoptotic properties of flavonoids [64] and the inhibition of xanthine oxidase [65, 66]. The number of hydroxyl groups on the B ring and the degree of saturation of the C ring are important determinants in the inhibition of signal transduction enzymes such as PKC. Ferriola *et al.* also found that the minimal essential features required for PKC inhibition is a coplanar flavone structure with free hydroxyl substituents at 3′, 4'and 7 positions [45]. In relation to the coplanarity, Echeverry *et al.* showed that it would not be a determinant factor for neuroprotection, since some of the coplanar flavones studied were not protective. Additionally, other studies showed that flavonoids, lacking coplanarity (*e.g.* epigalocathechin) were neuroprotective [20, 23, 67]. On the other hand, Hodnick *et al.* found that the number and hydroxyl configurations of flavonoid structures, especially in the B-ring, drastically alter its effects on the mitochondrial respiratory chain [68]. Many authors, who studied flavonoids, including flavones with different hydroxyl group substituents, found that all the flavonoids tested were more potent inhibitors of the NADH-oxidase system than succinoxidase, indicating that the primary site of inhibition is in complex I (NADH coenzyme Q reductase) [68]. These authors suggested that the planarity of the chromone (pyrone) structure enhances inhibitory activity [69].

In summary, although reviewed evidence shows that some flavones and flavonols exhibit potential as therapeutic modalities in neuropathology, the data highlight the need to elucidate issues such as bioavailability. Since many polyphenols have very limited bioavailability and are extensively metabolised, the mechanisms globally supporting the beneficial effects of these flavonoids in the brain *in vivo* remain to be elucidated. It is uncertain that flavone and flavonol concentrations in the brain- that in the case of quercetin are in the sub nano molar concentration range after chronic administration [70] - would have the same effects detected in *in vitro* experiments.

This low bioavailability might be related to the human studies that have shown, *e.g.*, that quercetin has small effects on plasma antioxidant biomarkers and no effects on antioxidant indices, such as antioxidant status, oxidized LDL, inflammation or metabolism [71-73] Nonetheless, provided that neuronal death prevention and anti-inflammatory activities have been shown *in vivo* experimentally, it is likely that the concentrations reached by flavones and flavonols *in vivo* would influence survival cascades and transcription factors by modulating redox potential of neurons and glia. It is also likely that chronic flavone and flavonol intake - in the diet or after use of plants with ethnopharmacological profile - could reach pharmacological levels in the brain by an accumulative effect.

Nonetheless, reviewed studies emphasise the need for further research on several and different aspects of flavones and flavonol activities, such as the extent and conditions for antioxidation in neurons, its relationship with nuclear effects on gene expression, the exploration of the active molecules and the improvement of their bioavailability through the use of transporters such as liposomes. In any case, by themselves,

or, more likely, as a template for synthetic compounds, the flavone/flavonol molecule shows potential as a multi-targeted therapeutic tool for neuropathologies involving oxidative damage.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

Declared none.

PATIENT CONSENT

Declared none.

REFERENCES

[1] Kolominsky-Rabas, P.L.; Sarti, C.; Heuschmann, P.U.; Graf, C.; Siemonsen, S.; Neundoerfer, B.; Katalinic, A.; Lang, E.; Gassmann, K.G.; von Stockert, T.R. A prospective community-based study of stroke in Germany. The Erlangen Stroke Project (ESPro): incidence and case fatality at 1, 3, and 12 months. *Stroke*, **1998**, *29*(12), 2501-06.

[2] Samsa, G.; Bian, J.; Lipscomb, J.; Matchar, D. Epidemiology of recurrent cerebral infarction: a medicare claims-based comparison, of first and recurrent strokes on 2-year survival and cost. *Stroke*, **1999**, *30*, 338-49.

[3] Dajas, F.; Arredondo, F.; Echeverry, C.; Ferreira, M.; Morquio, A. Flavonoids and the brain: Evidences and putative mechanisms for a protective capacity. *Curr. Neuropharmacol.*, **2005**, *3*(3), 193-205.

[4] Andersen, J.K. Oxidative stress in neurodegeneration: cause or consequence? *Naure Re.v Neurosci.,* **2004**, *5*, S18-S25.

[5] Sies, H.; Jones, D. P. Oxidative Stress. In: Fink G. ed. Encyclopedia of Stress, 2nd edition. San Diego: Academic Press; pp. 45-48, **2007**.

[6] Jones, D. P. Redefining oxidative stress. *Antioxid. Redox Signaling.* **2006**, *8*(9-10), 1865-79.

[7] Finkel, T. Oxygen radicals and signaling. *Curr. Opin. Cell Biol.,* **1998**, *10*, 248-53.

[8] Halliwell, B.; Gutteridge, J. M. C. eds. Free Radicals in Biology and Medicine. Oxford, Oxford University Press; **2007**.

[9] Suzuki, Y. J.; Forman, H. J.; Sevanian, A. Oxidants as stimulators of signal transduction. *Free Radic. Biol. Med.,* **1997**, *22*(1-2), 269-85.

[10] Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M. T.; Mazur, M.; Telser, J. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.,* **2007**, *39*(1), 44-84.

[11] Halliwell, B.; Gutteridge, J.M.C. Free radicals in Biology and Medicine. Third ed. Oxford: Oxford University Press; **1998**.

[12] Bains, J. S.; Shaw, C. A. Neurodegenerative disorders in humans: the role of glutathione in oxidative stress-mediated neuronal death. *Brain Res. Brain Res. Rev*., **1997**, *25*(3), 335-58.

[13] Ballatori, N.; Krance, S. M.; Notenboom, S.; Shi, S.; Tieu, K.; Hammond, C. L.Glutathione dysregulation and the etiology and progression of human diseases. *Biol. Chem.,* **2009**, *390*, 191-214.

[14] Clark, W.M.; Rinker, L.G.; Lessov, N.S.; Lowery, S.L.; Cipolla, M.J. Efficacy of antioxidant therapies in transient focal ischemia in mice. *Stroke*, **2001**, *32*, 1000-4.

[15] Clemens, J.A.; Panetta, J.A. Neuroprotection by antioxidants in models of global and focal ischemia. *Ann. N Y Acad. Sci*., **1994**, *738,* (Nov 17), 250-56.

[16] Lakhan, S.E.; Kirchgessner, A.; Hofer, M. Inflammatory mechanism in ischemic stroke: therapeutic approaches. *J. Transl. Med.,* **2009**, *7*(Nov 17), 97.

[17] Spencer, J. P. The impact of flavonoids on memory: physiological and molecular considerations. *Chem. Soc. Rev.,* **2009**, *38*, 1152-61.

[18] Zhao, B. Natural antioxidants protect neurons in Alzheimer's disease and Parkinson's disease. *Neurochem. Res.,* **2009**, *34*(4), 630-38.

[19] Spencer, J. P. Flavonoids and brain health: multiple effects underpinned by common mechanisms. *Genes Nutr.,* **2009**, *4*(4), 243-50.

[20] Levites, Y.; Weinreb, O.; Maor, G.; Youdin, M.B. ; Mandel, S. Green teapolyphennol (-)-epigallocatechin-3-gallate prevents N-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine-induced dopaminergic neurodegeneration. *J. Neurochem.,* **2001**, *78*(5), 1073-82.

[21] Pietta, P.G. Flavonoids as antioxidants. *J. Nat. Prod.,* **2000**, *63*(7), 1035-42.

[22] Middleton, E. Jr.; Kandaswami, C.; Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.,* **2000**, *52*(4), 673-751.

[23] Echeverry, C.; Arredondo, F.; Abin-Carriquiry, J.A.; Midiwo, J.O.; Ochieng, C.; Kerubo, L.; Dajas, F. Pretreatment with natural flavones and neuronal cell survival after oxidative stress: A structure-activity relationship study. *J. Agric. Food Chem.,* **2010**, *58*, 2111-15.

[24] Dajas, F.; Rivera-Megret, F.; Blasina, F.; Abin-Carriquiry, A.; Cosata, G.; Echeverry, C.; Lafon, L.; Heizen, H.; Ferreira, M.; Morquio, A. Neuroprotection by flavonoids. *Braz. J. Med. Biol. Res*., **2003a**, *36*(12), 1613-20.

[25] Simonyi, A.; Wang, Q.; Miller, R.L.; Yusof, M.; Shelat, P.B.; Sun, A.Y.; Sun, G.Y. Polyphenols in cerebral ischemia_ novel targets for neuroprotection. *Mol. Neurobiol.,* **2005**, *31*, 135-47.

[26] Zhao, G.; Zang, S.Y.; Jiang, Z.H.; Chen, Y.Y.; Ji, X.H.; Lu, B.F.; Wu, J.H.; Qin, G.W.; Guo, L.H. Postischemic administration of liposome-encapsulated luteolin prevents against ischemia-reperfusion injury in a rat middle cerebral artery occlusion model. *J. Nutr. Biochem.,* **2011**, *22*(10), 929-36.

[27] Qiao, H.; Dong, L.; Zhang, X.; Zhu, C.; Zhang, X.; Wang, L.; Liu, Z.; Chen, L.; Xing, Y.; Wang, C.; Li, Y. Protective effect of Luteolin in experimental ischemic stroke_ Upregulated SOD1, CAT, Bcl-2 and Claudin-5, down-regulated MDA and Bax expression. *Neurochem. Res.*, **2012**, DOI: 10.1007/s11064-012-0822-1.

[28] Hendriks, J.J.; Alblas, J.; Van der Pol, S.M.; van Tol, E.A.; Dijkstra, C.D.; de Vries, H.E. Flavonoids influence monocytic GTPase activity and are protective in experimental allergic encephalitis. *J. Exp. Med.,* **2004**, *200*(12), 1667-72.

[29] Nam, Y.; Choi, M.; Hwang, H.; Lee, M.G.; Kwon, B.M.; Lee, M.G.; Kwon, B.M.; Lee, W.H.; Suk, K. Natural Flavone Jaceosidin is a Neuroinflammation Inhibitor. *Phytother. Res.,* **2012** (May 23)10-1002/ptr.4737.

[30] Cai, M.; Phan, P.T.; Hong, J.G.; Kim, D.H.; Kim, J.M.; Park, S.J.; Liu, X.; Han, J.E.; Park, H.; Choi, J.W.; Ryu, J.H. The neuroprotective effect of eupatilin against ischemia/reperfusion-induced delayed neuronal damage in mice. *Eur. J. Pharmacol.,* **2012**, *689*(1-3), 104-10.

[31] Calderón-Montaño, J.M.; Burgos-Morón, E.; Pérez-Guerrerp. C.; López-Làzaro, M. A. Review on the dietary flavonoid kaempferol. *Mini Rev. Med. Chem*., **2011**, *11*(4), 298- 344.

[32] López-Sánchez, C.; Martín-Romero, F.J.; Sun, F.; Luis, L.; Samhan-Arias, A.K.; García-Martínez, V.; Gutiérrez-Merino, C. Blood micromolar concentrations of kaempferol afford protection against ischemia/reperfusion-induced damage in rat brain. *Brain Res.,* **2007**, *1182*, 123-37.

[33] Li, S.; and Pu, X.P. Neuroprotective effect of kaempferol against a 1-methyl-4-phenyl-1.2.3.6-tetrahydropyridine-induced mouse model of Parkinson´s disease. *Biol Pharm Bull.,* **2011**, *34*(8), 1291-96.

[34] Kim, J.; Choi, S.; Cho, H.Y.; Hwang, H.J.; Kim, Y.; Lim, S.T.; Kim, C. J.; Kim, H. K.; Peterson, S.; Shin D. H. Protective effects of kaempferol against Amyloid Beta (Aβ)-induced Neurotoxicity in ICR mice. *Biosci. Biotechnol. Biochim.,* **2010**, *74*(2), 397-401.

[35] Dajas, F.; Rivera, F.; Blasina, F.; Arredondo, F.; Echeverry, C.; Lafon, L.; Morquio, A.; Heizen, H. Cell culture protection and *in vivo* neuroprotective capacity of flavonoids. *Neurotox. Res.,* **2003b**, *5*(6), 425-32.

[36] Rivera, F.; Urbanavicius, J.; Gervaz, E.; Morquio, A.; Dajas, A. Some Aspects of the *in vivo* Neuroprotective Capacity of flavonoids: Bioavailability and Structure-Activity Relationship. *Neurotox Res.*, **2004**, *6*(7-8), 543-53.

[37] Ahmad, A.; Khan, M.; Hoda, M.N.; Raza, S.S.; Khan, M.B.; Javed, H.; Ishrat, T.; Ashafaq, M.; Ahmad, M.E.; Safhi, M.M.; Islam, F. Quercetin protects against oxidative stress associated damages in a

rat model of transient focal cerebral ischemia and reperfusion. *Neurochem. Res.,* **2011**, *36*, 1360-71.

[38] Lee, J.K.; Kwak, H.J.; Piao, M.S.; Jang, J.W.; Kim, S.H.; Kim, H.S. Quercetin reduces the elevated matrix metalloproteinases-9 level and improves functional outcome after cerebral focal ischemia in rats. *Acta Neurochir. (Wien.),* **2011**, *153*(69), 1321-29.

[39] Schültke, E.; Kamencic, H.; Skihar, V.M.; Griebel, R.; Juurlink, B. Quercetin in an animal model of spinal cord compression injury: correlation of treatment duration with recovery of motor function. Spinal Cord., **2010**, *48*(2), 112-17.

[40] Nijveldt, R.J.; van Nood, E.; van Hoorn, D.E.C.; Boelens, P.G.; van Norren, K.; van Leeuwen, P.A. Flavonoids: a review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.,* **2001**, *74*(4), 418-25.

[41] Granado-Serrano, A. B., Martín, M. A., Bravo, L., Goya, L., & Ramos, S. Quercetin induces apoptosis via caspase activation, regulation of Bcl-2, and inhibition of PI-3-kinase/Akt and ERK pathways in a human hepatoma cell line (HepG2). *J. Nutr.*, **2006**, *136*(11), 2715-21.

[42] Seelinger, G., Merfort, I., Schempp, C.M.. Anti-oxidant, anti-inflammatory and anti-allergic activities of luteolin. *Planta Med.,* **2008**, *74*(14), 1667-77.

[43] Arredondo, F.; Echeverry, C.; Abin-Carriquiry, J.A.; Blasina, F.; Antúnez, K.; Jones, D.P.; Go, Y.M.; Liang, Y.L.; Dajas, F. After cellular internalization, quercetin causes Nrf2 nuclear translocation, increases glutathione levels, and prevents neuronal death against an oxidative insult. *Free Radic Biol Med.,* **2010**, *49*(5), 738-47.

[44] Spencer, JP. Flavonoids: modulators of brain function? *Br. J. Nutr.,* **2008**, *99,* E Suppl 1:ES60-77.

[45] Ferriola, P.C.; Cody, V.; Middleton, E. Jr. Protein kinase C inhibition by plant flavonoids. Kinetic mechanisms and structure-activity relationships. *Biochem. Pharmacol.,* **1989**, *15*(38), 1617-24.

[46] Agullo, G.; Gamet-Payrastre, L.; Manenti, S.; Viala, C.; Rémésy, C.; Chap, H.; Payrastre, B. Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition. *Biochem. Pharmacol.*, **1997**, *53*(11), 1649-57.

[47] Brunet, A.; Datta, S.R.; Greenberg, M.E. Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. *Curr. Opin. Neurobiol.*, **2011**, *1*(3), 297-305.

[48] Williams, R. J; Spencer, J. P. E.; Rice-Evans, C. Flavonoids: antioxidants or signalling molecules? *Free radical Biol. Med.*, **2004**, *36*(7), 838-49.

[49] Vauzour, D.; Vafeiadou, K.; Rodriguez-Mateos, A.; Rendeiro, C.; Spencer, J.P. The neuroprotective potential of flavonoids: a multiplicity of effects. *Genes Nutr.*, **2008**, *3*(3-4), 115-26.

[50] Lee, J.; Li, Y.; Chen, H.; Lin, R. Protective effects of luteolin against lipopolysaccharide-induced acute lung injury involve inhibition of MEK/ERK and PI3K/Akt pathways in neutrophils. *Acta Pharmacol. Sinica*, **2010**, *31*, 831-38.

[51] Olanov, C.W.; Tatton W., G. Etiology and pathogenesis of Parkinson´s Disease. *Annual Rev. Neurosci.,* **1999**, *22*, 123-44.

[52] Fiorania, M.; Guidarellib, A.; Blasaa, M.; Azzolinia, C.; Candiraccia, M.; Piattia, E.; Canton, H. Mitochondria accumulate large amounts of quercetin: prevention of mitochondrial damage and release upon oxidation of the extramitochondrial fraction of the flavonoid. *J. Nutr. Biochem.,* **2010**, *21*(5), 397-404.

[53] Carrasco-Pozo, C.; Mizgier, M.L.; Speisky, H.; Gotteland, M. Differential protective effects of quercetin, resveratrol, rutin and epigallocatechin gallate against mitochondrial dysfunction induced by indomethacin in Caco-2 cells. *Chem. Biol. Interact.,* **2012**, *195*, (3), 199-205.

[54] Lagoa, R.; Graziani, I.; Lopez-Sanchez, C.; Garcia-Martinez, V.; Gutierrez-Merino, C. Complex I and cytochrome c are molecular targets of flavonoids that inhibit hydrogen peroxide production by mitochondria. *Biochim. Biophys. Acta*, **2011**, *1807*(12), 1562-72.

[55] Baines, C.P.; Kaiser, R.A.; Purcell, N.H.; Blair, N.S.; Osinska, H.; Hambleton, M.A.; Brunskill, E.W.; Sayen, M.R.; Gottlieb, R.A.; Dorn, G.W.; Robbins, J.; Molkentin, J.D. Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature,* **2005**, *434*(7033), 658-62.

[56] De Marchi, U.; Biasutto, L.; Garbisa, S.; Toninello, A.; Zoratti, M. Quercetin can act either as an inhibitor or an inducer of the mitochondrial permeability transition pore: A demonstration of the ambivalent redox character of polyphenols. *Biochim. Biophys Acta*, **2009**, *1787*(12), 1425-32.

[57] Bors, W.; Heller, W.; Michel, C.; Saran, M. Radical chemistry of flavonoid antioxidants. *Adv. Exp. Med. Biol.,* **1990**, *264*, 165-70.

[58] Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic. Biol. Med.,* **1996**, *20(7)*, 933-56.

[59] Mira, L.; Fernández, M. T.; Santos, M.; Rocha, R.; Florencio, M. H.; Jennings, K. R. Interactions of flavonoids with iron and copper ions: a mechanism for their antioxidant activity. *Free Radic. Res.,* **2002**, *36*(11), 1199-208.

[60] Cainelli, G.; Angeloni, C.; Cervellati, R.; Galletti, P.; Giacomini, D.; Hrelia, S.; Sinisi, R. New polyphenolic beta-lactams with antioxidant activity. *Chem. Biodivers.,* **2008**, *5*(5), 811-29.

[61] Zhang, J.; Stanley, R.A.; Adaim, A.; Melton, L.D.; Skinner, M.A. Free radical scavenging and cytoprotective activities of phenolic antioxidants. *Mol. Nutr. Food Res*., **2006**, *50*(11), 996-1005.

[62] Arora, A.; Nair, M.G.; Strasburg, G.M. Structure-activity relationships for antioxidant activities of a series of flavonoids in a liposomal system. *Free Radic. Biol. Med*., **1998**, *24*(9), 1355-63.

[63] Theoharides, T.C.; Alexandrakis, M.; Kempuraj, D.; Lytinas, M. Anti-inflammatory actions of flavonoids and structural requirements for new design. *Int. J. Immunopathol. Pharmacol*., **2001**, *14*(3), 119-27.

[64] Lee, E.R.; Kang, Y.J.; Kim, J.H.; Lee, H.T.; Cho, S. G. Modulation of apoptosis in HaCaT keratinocytes via differential regulation of ERK signaling pathway by flavonoids. *J. Biol. Chem.,* **2005**, *280*(36), 31498-507.

[65] Van Hoorn, D.; Nijveldt, R. J.; Van Leeuwen, P. A. M.; Hofman, Z.; Rabet, L. M.; De Bont, D. B. A.; Van Norren, K. Acurate prediction of xanthine oxidase ihibition based on the structure of flavonoids. *Eur. J. Pharmacol.,* **2002**, *451*(2), 111-18.

[66] Varga, Z.; Seres, I.; Nagy, E.; Ujhelyi, L.; Balla, G.; Balla, J.; Antus, S. Structure prerequisite for antioxidant activity of silybin in different biochemical systems *in vitro*. *Phytomedicine,* **2006**, *13*, (1-2), 85-93.

[67] Ogborne, R.M.; Rushworth, S.A.; O'Connell, M.A. Epigallocatechin activates haem oxygenase-1 expression via protein kinase Cδ and Nrf2. *Biochem. Biophys. Res. Commun*., **2008**, *373*(4), 584-88.

[68] Hodnick, W.F.; Duval, D.L.; Pardini, R.S. Inhibition of mitochondrial respiration and cyanide-stimulated generation of reactive oxygen species by selected flavonoids. *Biochem. Pharmacol.,* **1994**, *47*(3), 573-80.

[69] Hodnick, W.F.; Milosavljevic, E.B.; Nelson, J.H.; Pardini, R.S. Relationships between redox potentials, inhibition of mitochondrial respiration, and production of oxygen radicals by flavonoids, *Biochem. Pharmacol*., **1987**, *37*(13), 2607-11.

[70] Schaffer, S.; Halliwell, B. Do polyphenols enter the brain and does it matter? Some theoretical and practical considerations. *Genes Nutr.,* **2012**, *7*(2), 99-109.

[71] Williamson, G.; Manach, C. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention Studies. *Am. J. Clin. Nutr*. **2005**, *81*, 2435S-2455S.

[72] Egert, S.; Wolffram, S.; Bosy-Westphal, A.; Boesch-Saadatmandi, C.; Wagner, E.A.; Frank, J.; Rimbach, G.; Mueller, M. Daily quercetin supplementation dose-dependently increases plasma quercetin concentrations in healthy humans. *J. Nutr.* **2008**, *138*, 1615-21.

[73] Kelly, G.S. Quercetin. *Altern. Med. Rev.,* **2011**, *16*, 172-93.

Received: July 27, 2012 **Revised: September 17, 2012** **Accepted: October 09, 2012**

19 The Neuroprotective Capacity of *Achyrocline Satureioides* (Lam) D.C. and its Flavonoids
Mechanisms of Action

Federico Dajas, Juan A. Abin-Carriquiry, Florencia Arredondo, Fernanda Blasina, Marcela Diaz, Carolina Echeverry, Felicia Rivera-Megret, and Lucia Vaamonde

CONTENTS

19.1 INTRODUCTION

Humans relied for countless generations on plant extracts to recover the biological equilibrium lost from infections, wounds, tumors, and all type of ailments. Even today, a high percent of the world's population still rely on plant preparations as the only therapeutic choice. The last century brought scientific knowledge to the popularly accepted beneficial effects of plant extracts. Diverse biological actions such as anticancer, anti-inflammation, anti-atherosclerotic, or neuroprotective effects were

described for plants as well as for their main components (Geleijnse et al., 1999; Middleton et al., 2000; Aruoma et al., 2003).

Mortality and morbidity by cerebrovascular diseases are prominent among human pathologies. These diseases are a major cause of long-term disability which is associated with prolonged and severe clinical and socio-economical repercussions. Indeed, the American Heart Association estimates that there are more than 795.000 strokes per year in USA and that 5% of them result in death (Pardridge, 2001; Lloyd-Jones et al., 2010). Besides, the number of people with dementia increases every year because of the sustained growth of the older population (Alzheimer Association, 2009). These facts have prompted an intensive research work on the neuronal lesion mechanisms occurring after stroke, ischemia, or neurodegenerative pathologies such as Alzheimer's or Parkinson's diseases, and the therapeutic strategies to attenuate or stop them. The processes leading to neuronal death are extremely complex and activate several cascades of events including oxidative stress, inflammation, apoptosis, and excitotoxicity (Yu et al., 1998; Dajas et al., 2003a,b; Rivera et al., 2004). With the extended human longevity and the consequent increase in brain diseases, the search for molecules that could interfere with these pathological processes and promote neuronal survival, is a great challenge.

19.2 BRAIN DEGENERATIVE PROCESSES

With few exceptions, the causes of neurodegeneration are essentially unknown, and even when they have been identified, the mechanisms by which they initiate the process remain speculative. Nevertheless, there are three main mechanisms of neuronal cell death acting both separately or cooperatively which may underlie neurodegeneration. This lethal triplet is metabolic compromise, excitotoxicity, and oxidative stress, which converges in neuronal cell death (Alexi et al., 2000). Strategies to rescue or protect injured neurons usually involve promotion of neuronal growth and function or prevention of neurotoxic processes. Besides, tissue inflammation can be an additional process in this triplet of cooperative pathways explaining the wide range of lesions observed in the CNS pathologies (Howes et al., 2003; Dajas et al., 2005, Jyoti and Sharma, 2006).

Under physiological conditions, the balance between generation and elimination of reactive oxygen and nitrogen species (ROS/RNS) maintains a redox homeostasis that ensures the correct function of redox-sensitive signaling proteins, critical for normal cell life. Redox status is tightly controlled by the cell through antioxidant defenses including enzymes like catalase, peroxidases, and superoxide dismutase (SOD) or molecules such as ascorbic acid (Halliwell and Gutteridge, 1986; Schafer and Buettner, 2001). A regular provision of exogenous antioxidants could contribute to retain a physiological redox environment. With only 2% of the body weight, the brain represents almost 20% of the O_2 consumption of the organism (Halliwell and Gutteridge, 1986). This high metabolic rate is the basis for the high brain generation of ROS/RNS and the concomitant need for important brain antioxidant defenses. In this context, it is understandable that numerous research papers point to oxidative

stress as having a critical role in the pathology of neurodegenerative and vascular diseases of the brain (Schmidley, 1990; Siesjo et al., 1995; Gorelick, 2002).

Besides, as mentioned above, other relevant cellular processes occur during neurodegeneration such as metabolic failure, and release of neurotransmitters, particularly glutamate with NMDA receptor activation, and massive entry of calcium into the cells (Nicotera and Lipton, 1999; Alexi et al., 2000; Pardridge, 2001), leading to a general activation of intracellular enzymes, further ROS generation, lipid-peroxidation, DNA damage and cell membrane disruption, resulting in neuronal death (Halliwell and Gutteridge, 1986). Therapeutic strategies to treat neuropathologies have focused on one or more of these events, and neuroprotective effects at an experimental level have been observed after, for example, the use of NMDA receptor antagonists (Williams et al., 2000) or calcium channel blockers (O'Neill et al., 2001), and even anti-inflammatory compounds (Welton et al., 1986).

In particular, the widespread generation of ROS/RNS has also prompted the development and application of a variety of antioxidant approaches to attenuate neuronal injury (Clemens and Panetta, 1994; Clark et al., 2001). Nevertheless, promising results showing strong neuroprotective activity after the use of single target compounds in different experimental models, failed in clinical trials (Lakhan et al., 2009). In this context, it is likely that molecules acting on multiple targets of the complex pathological process would be required to be therapeutically effective (Dajas et al., 2005). Plant extracts with complex mixture of compounds or synergistic molecules could be an answer to these multitarget mechanisms of action.

19.3 MEDICINAL PLANTS AND NEUROPROTECTION

Early evidences pointed out to plants, fruits, beverages like wine and tea, and their main compounds such as flavonoids, as meaningful candidates in the search for protective principles due to their high antioxidant capacities (Nijveldt et al., 2001; Kim et al., 2002, El-Sherbiny et al., 2003; da Luz and Coimbra, 2004). In this sense, plant extracts with a high antioxidant capacity have shown improvements in cognitive function (Youdim and Joseph, 2001) or appear to inhibit neuro-inflammatory processes in the brain (Chen et al., 2005).

Plant extracts have been utilized for thousands of years to control the consequences of CNS disorders in countries like China and India, where, for example, Ayurvedic medicine has utilized *Bacopa monniera* for almost 3000 years to improve memory and intellect (Jyoti and Sharma, 2006). Many more examples could be mentioned such as *Centella asiatica*, utilized for memory and cognition improvement (Husain et al., 2007) in a context of a growing interest for the use of medicinal plants in the western world. Among the numerous plant families, those classified in the genre *Asteracea* are widely utilized for medicinal purposes related to the brain. As an example, Wang et al. (2009) have demonstrated that Hand-Mazz, a Chinese herbal medicine, significantly reduced the infarct volume and ameliorated the neurological deficit (Wang et al., 2009). In America, although native people utilized plants with strong CNS effects such as *Erythroxylon coca* (coca) (Biscoping and Bachmann-Mennenga, 2000), curare (Reynolds, 2005), or nicotine

Q1

(Lu et al., 2009) for hundreds of years, the extended use of local plant extracts for brain disorders is less prominent than in Asian populations. Nevertheless, plants for symptoms of dementia have been widely utilized by indigenous people (Chapter 26 this book) from diverse families, mainly *Apocinaceae*, *Leguminosae*, and *Bignoniaceae*.

19.4 *ACHYROCLINE SATUREIOIDES* AND ITS FLAVONOIDS

Achyrocline satureioides (Lam) D.C. (*Asteraceae*) is a native medicinal herb known by the popular name of "marcela" that grows in extensive regions of Uruguay, Paraguay, Brazil, and Argentina. Its infusion is widely utilized for the treatment of gastrointestinal disorders, as a menstrual regulator, as a sedative and as an antispasmodic (Da Silva and Langeloh, 1994). *A. satureioides* has been shown to present a potent free radical scavenging ability (Desmarchelier et al., 1998), and a recent paper by Cosentino et al. (2008) has shown that *A. satureioides* has a modulatory role in the production of inflammatory signals in human polynuclear blood cells (Cosentino et al., 2008). Up to now, reported studies have focused on hepatoprotection, antitumor, antiviral, cytotoxic, and immunomodulatory properties of *A. satureioides* extracts (Puhlmann et al., 1992; Zanon et al., 1999; Kadarian et al., 2002), and its infusions are also utilized as anti-atherosclerotic and for some nervous system disorders (Ruffa et al., 2002; Taylor, 2002).

In particular, a study by Arredondo et al. (2004) was the first to show the cytoprotective capacity of *A. satureioides* against an oxidative insult in cell culture models. In this study, treatments with other plant extracts such as *Ginkgo biloba* or *Epilobium parviflorum* infusions showed no cytoprotective activity. An important contribution of this work was to show that in spite of similar scavenging activities against the ABTS radical, *A. satureioides* and *Ginkgo biloba* infusions had different protective properties. Furthermore, *Epilobium parviflorum* infusion, which showed the highest scavenger potency, did not protect cells from the oxidative insult (Arredondo et al., 2004). Accordingly, in this work the scavenger activity of infusions did not correlate with cytoprotective activity, suggesting that other mechanisms could be involved in the *A. satureioides* infusion protective effect (Arredondo et al., 2004). The first clue for the understanding of the different cytoprotective actions of *A. satureioides*, *Ginkgo biloba*, and *Epilobium parviflorum* infusions was given by the analysis of the flavonoid content which revealed a completely different profile. The total content of flavonoids in *A. satureioides* infusion was more than 10-fold higher than the flavonoid content of the other two infusions, which could be a clue for the differential cytoprotective activity found.

Dietary flavonoids have received much attention over the past decade, and increasing work is highlighting their potential health beneficial properties, including: anti-inflammatory, antimutagenic, antiangiogenic, cardioprotective, and neuroprotective effects (Benavente-Garcia and Castillo, 2008; Singh et al., 2008). They are the most abundant of the polyphenolic compounds found throughout the *Plantae* kingdom (Harborne, 1993; Andersen and Markham, 2006). Their chemical structure comprises two aromatic rings connected by a three carbon bridge, resulting in a C6–C3–C6 skeleton (Williams, 1995; Figure 19.1). The main sub-classes of flavonoids

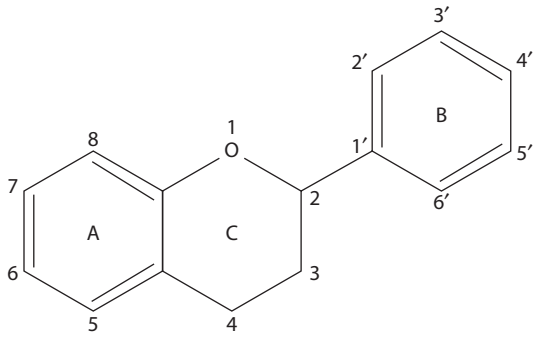


FIGURE 19.1 Flavonoids basic structure.

are: flavonols, flavones, flavanones, flavan-3-ols, anthocyanidins, and isoflavones. They can be found as free aglycones, although they mostly exist conjugated to glycosides. Due to the extensive substitutions of their basic structure (sugars, hydroxyl and alkyl groups, etc.), a diverse number of flavonoids can be found in nature (Rice-Evans, 2001).

Focusing on flavonoid content of the three infusions in the study of Arredondo et al. (2004), study, chromatographic analysis showed that while *A. satureioides* flavonoids were similarly distributed in free and glycosylated forms, those included in *Ginkgo biloba* and *Epilobium parviflorum* infusions were mainly glycosides. Interestingly, further experiments with isolated flavonoids showed that rutin (a quercetin glycoside) did not protect cells from the oxidative insult. Additionally, two of the aglycones present in *A. satureioides* (quercetin and luteolin) showed cytoprotective activity (Arredondo et al., 2004). These results supported the hypothesis that aglycones could be the principal components responsible for the *A. satureioides* cytoprotective activity, a hypothesis that could be extended to the *Asteraceae* family which, in contrast with other plant families, contains mainly aglycone flavonoids (Dajas et al., 2005). Furthermore, chronic pre-treatment during 21 days with an *A. satureioides* decoction had neuroprotective effects in a permanent middle cerebral artery occlusion (pMCAo) model in rats, reducing the cerebral infarct size. The pre-treatment with the decoction during 7, 14, or 21 days also improved significantly the behavioral deficits caused by pMCAo in all experimental groups (F. Rivera, personal communication). In the same model, results obtained by Rivera et al. (2004) gave support to the hypothesis that *A. satureioides* flavonoids could be the active compounds involved in the neuroprotective potency of this extract, by demonstrating that the administration of liposomal quercetin to rats submitted to the focal ischemia by pMCAo, significantly decreased the lesion volume and resulted in a clear-cut improvement of the behavioral indexes (Rivera et al., 2004).

Globally, these studies suggest that, similarl to *A. satureioides*, extracts of medicinal plants with high concentrations of flavonoids, particularly quercetin or quercetin-related flavones could afford brain protection (Dajas et al., 2003a,b; Rivera et al., 2004).

19.5 PLANT FLAVONOIDS AS PROMISING THERAPEUTIC MOLECULES AGAINST NEURODEGENERATION

Traditionally, the beneficial effects of flavonoids have been attributed mainly to their direct antioxidant free radical scavenger properties (Castelluccio et al., 1995; Rice-Evans, 2001; Schroeter et al., 2002), although this concept seems to be an oversimplification. Indeed, there is now an accumulating evidence that flavonoids can act as indirect antioxidants through the modulation of cell signaling pathways, and even they can regulate cellular functions by mechanisms beyond antioxidant activity (Nijveldt et al., 2001; Rice-Evans, 2001; Mandel et al., 2008; Spencer, 2009).

In the following paragraphs, we will present an overview of the potential working mechanisms of flavonoids in the cell, which may underlie their beneficial effects in the prevention and/or treatment of brain pathology. Besides, we will enumerate the structural features that clearly identified a group of flavonoids that selectively protect neurons. Regarding the myriad of additional functions of flavonoids in the organism, we refer the reader to the many comprehensive reviews available (Middleton et al., 2000; Nijveldt et al., 2001; Spencer et al., 2004).

19.5.1 Working Mechanisms of Flavonoids

Focusing on *antioxidant properties* of flavonoids, the presence of phenolic groups gives their direct antioxidant free radical scavenging capacity (Rice-Evans, 2001). Besides, some flavonoids can chelate metals, preventing metal-catalyzed free radical formation (Mandel et al., 2006). Additionally they can confer indirect antioxidant effects through their capacity to interact and modulate enzyme activities such as xanthine oxidase (XO), nitric oxide synthase (NOS), and so on (Middleton et al., 2000). Interestingly, many of these mechanisms result in an anti-inflammatory effect. Recently, evidence from cell studies suggests that flavonoids can influence gene expression by modulation of redox-sensitive transcription factors such as Nuclear factor κB (NFκB), NF-E2-related factor-2 (Nrf2), and so on. These mechanisms result in part, in the induction of genes encoding for pro-survival, detoxifying, and antioxidant proteins such as SODs, glutathione peroxidases (GPx's), glutathione-S-transferases (GST), NADPH : quinone oxidoreductases (NQOs), heme oxygenases (HOs), and γ-glutamylcysteine synthetase (γ-GCS) (Seelinger et al., 2008; Granado-Serrano et al., 2010).

These direct and indirect antioxidant properties may, at least in part, contribute to the disease-preventing effects of flavonoids as a result of the neutralization of the cascade of oxidative events and inflammatory processes occurring in neurodegenerative and neurovascular diseases (Muir et al., 2007).

On the other hand, accumulating evidence suggests that the beneficial cellular effects of flavonoids may be mediated by their *nonantioxidant properties*, for example, their interactions with specific proteins crucial for intracellular signaling cascades likely to affect cellular function and/or regulate gene expression (Williams et al., 2004). Indeed, flavonoids are believed to act by modulating a number of protein kinase and lipid kinase signaling cascades, such as PI3 Kinase (PI3K/Akt), tyrosine kinase, protein kinase C (PKC), and MAPK signaling pathways (Middleton et al., 2000; Williams et al., 2004). Modulation of these pathways is likely to affect

cellular function, leading to changes in caspase activity and gene expression. As an example, it has been observed that, some flavonoids inhibit oxidant-induced neuronal apoptosis via a mechanism involving the activation or phosphorylation of signaling proteins important in the pro-survival pathways (Bourival et al., 2009). Furthermore, flavonoids have the potential to bind to the ATP-binding sites of a large number of proteins including mitochondrial ATPase, calcium plasma membrane ATPase, protein kinase A and C, and topoisomerases (Spencer, 2007). In addition, interaction with binding sites of several receptors has been reported, including nuclear estrogenic receptors (Middleton et al., 2000).

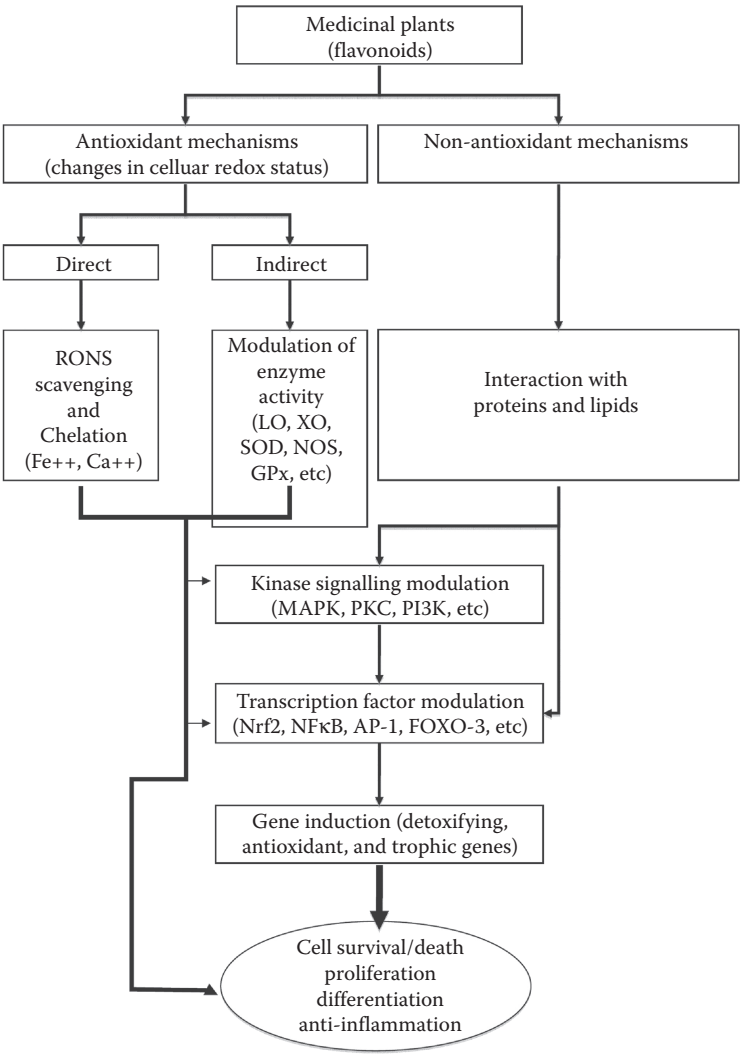


FIGURE 19.2 Scheme presenting antioxidant and nonantioxidant working mechanisms of plant flavonoids and their cellular effects.

The mechanisms enlisted above are proposed to be responsible of the numerous effects of flavonoids within the brain including: pro and anti apoptotic effects, neurotrophism, modulation of memory and learning, and cognitive function (Spencer, 2009) (see Figure 19.2 for a schematic view of flavonoid mechanisms of action).

19.5.2 Structural Characteristics Underlying Flavonoids Effects

The structural diversity of these natural compounds offers the possibility to identify the molecular substitutions required for different biological actions. For example, some studies have demonstrated that the protective properties of flavonoids against oxidative stress are structure-dependent. Indeed, their antioxidant potency has been linked to the *ortho*-dihydroxy substitution in the B-ring and the presence of 2,3-unsaturation and a 4-carbonyl in the C-ring (see Figure 19.1) (Bors et al., 1990, Rice-Evans et al., 1996). Their activity as metal ion chelators appears to be defined by the presence of the *ortho*-dihydroxy substitution in the B-ring, the 4-carbonyl in the C-ring and a 5-hydroxy substitution in the A-ring (Mira et al., 2002), while their pro-oxidant properties would be associated with the number of hydroxyl groups present in the molecule (Heim et al., 2002). In particular, the cytoprotective capacity of flavonoids against different insults has been mainly attributed to their antioxidant potency (Zhang et al. 2006; Cainelli et al., 2008). However, another work (Dajas et al., 2003a,b), assessing the cytoprotective potency of several structurally related flavonoids against oxidative stress, found that cytoprotection did not correlate with the antioxidation potency. Recently, Echeverry et al. (2010) performed a structure–activity relationship (SAR) study focusing on the protective effects of flavones against oxidative stress in neuronal cultures. These results showed that only four of 13 flavones studied exhibited neuronal protective activity, strongly indicating that specific structural features are related to this action. Indeed, the hydroxy substitutions in the A-ring (C5 and C7) and in position C3 (C-ring) of the flavones would be necessary to afford neuroprotection, indicating that the structural requirements for neuronal cell protection are different from those that afford antioxidant capacity (Echeverry et al., 2010). The hydroxy substitution in position C3 (C-ring) has been previously shown important in a wide range of biological activities such as scavenger activity and anti-inflammatory effects (Arora et al., 1998; Theoharides et al., 2001). An important and novel contribution of this work was that the *ortho*-dihydroxy substitution in the B-ring was not needed for protection (Echeverry et al., 2010).

While these results can be taken as strong evidence for a neuroprotective role of flavonoids and for their structural characteristics underlying these effects, they also open the question on how significant these experiments could be for a putative protection *in vivo*. It has to be taken into account that flavonoids undergo several and important metabolic changes before and after they reach the blood.

19.6 BIOAVAILABILITY OF FLAVONOIDS IN THE BRAIN *IN VIVO*

Following the oral ingestion of flavonoids (aglycones or glycoside conjugates), only a small percentage reaches the systemic circulation and tissues, and very little of the absorbed compounds retains its original structure (Clifford, 2004; Figure 19.3).

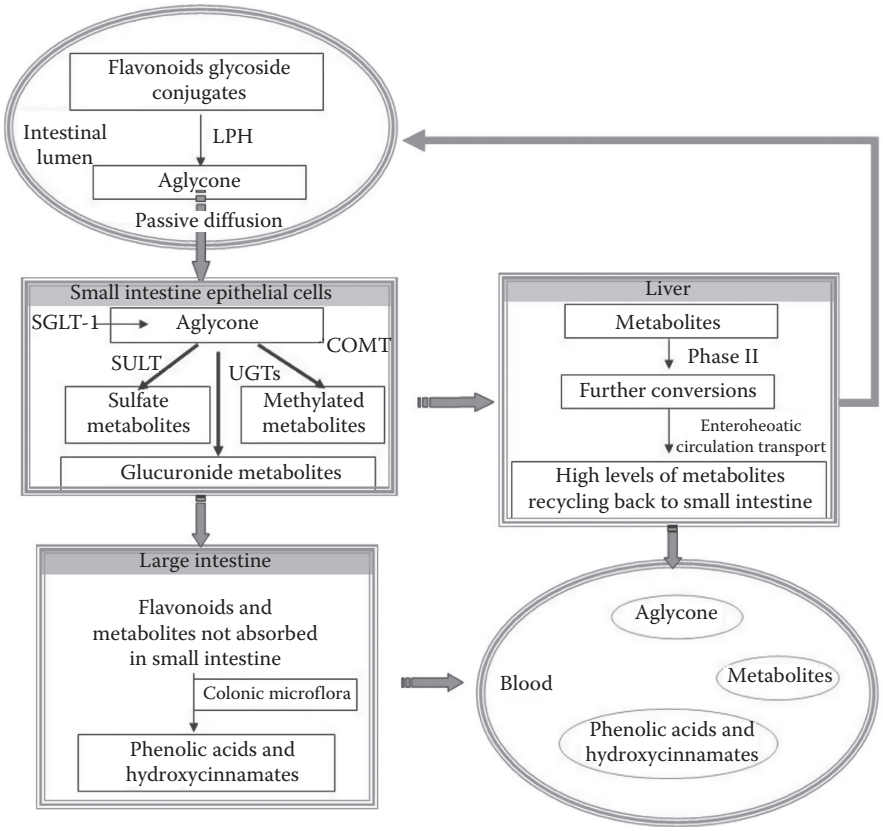


FIGURE 19.3 Plant flavonoids metabolization after oral intake. Low bioavailability of dietary flavonoids is mainly due to highly efficient metabolization briefly described as follows. Glycoside conjugates hydrolysis by lactase phloridizin hydrolase (LPH) in the brush-border of the small intestine epithelidal cells releases aglycones. LPH exhibits broad substrate specificity for flavonoid-glucosides, and the released aglycone may then enter the epithelial cells by passive diffusion as a result of its increased lipophylicity. An alternative hydrolysis could take place by the cytosolic β glucosidase (CBG) following the active sodium-dependent glucose transporter SGLT1 within the epithelial cells. Prior to passage into the blood stream the aglycones undergo metabolism, forming sulfate, glucuronide, and/or methylated metabolites through the respective action of sulfotransferases (SULT), uridine-5′-diphosphate glucuronosyltransferases (UGTs), and catechol-O-methyltransferases (COMT). There is also efflux of at least some of the metabolites back into the lumen of the small intestine and this is thought to involve the multidrug resistance protein (MRP) and P-glycoprotein (P-gp). Once in the bloodstream, metabolites can be subjected to phase II metabolism with further conversions occurring in the liver, with high enterohepatic circulation transport, which results in high levels of metabolites recycling back to the small intestine. Flavonoids and their metabolites which are not absorbed in the small gut can be then absorbed in the large intestine but will be subjected to the action of the colonic microflora.

The percentage of intake that is absorbed varies with the structure of the aglycone and its glycosides substitutions, and the food matrix. Each single flavonoid generates several metabolites, although a few are majors. The exact yields and proportions between them will vary with the individual's genetic profile and its intestinal microflora, and even more, Kawai et al. (2009) have recently demonstrated that the profiles for quercetin metabolites could differ between two oral administration procedures. Moreover, in regular dietary circumstances its half-life is shorter than the gap between frequent consumption (Kawai et al., 2009).

When considering brain bioavailability, compounds have to pass through the digestive system, to cross through the circulation, and to pass through the blood brain barrier (BBB) to be taken by the CNS. This long process could explain some controversial results. For example, it was demonstrated that after 8 weeks of supplementation with blueberries, anthocyanins were present in the cortex and hippocampus in rats (Shukitt-Hale et al., 2008). However, another similar work was not able to detect the same compounds in rat brain after berry consumption (Borges et al., 2007). Besides, few studies have shown the presence of free flavonoids or their metabolites in the brain in normal or pathological conditions (Abd El Mohsen et al., 2002; Youdim et al., 2004). In the case of ischemic processes, neuroprotection could be explained by the facilitated entry of the molecules into the brain once the selective permeability of the BBB is lost. On the other hand, some of the papers showing neuroprotection in acute conditions utilize a systemic route (Ossola et al., 2009). In these conditions, high concentrations of aglycones in blood for a short period of time would facilitate the crossing of a continuous or damaged BBB. The saturation of the conjugation systems is a possibility that has already been raised by some authors (Manach et al., 2004). When these experimental results are considered together with the bioavailability studies analyzed above, one of the first questions raised refers to the nature of the active molecules in the brain: aglycones or metabolites? Different works show the predominant presence of metabolites in the brain in normal and pathological conditions (Abd El Mohsen et al., 2002; Youdim et al., 2004) suggesting that they could be the bioactive molecules. On the other hand, numerous *in vitro* or a few *in vivo* studies (Abd El Mohsen et al., 2002; Youdim et al., 2004) support the beneficial effects of nonmetabolized flavonoids. This point could be better explored in case of studying the effect on the brain of a preparation with free flavonoids in a drug carrier, such as liposomes, which would delay flavonoid metabolization, facilitating BBB crossing. Rivera et al. (2004) demonstrated that while aqueous preparations of quercetin, fisetin, and catechin were undetected in the brain of rats up to 4 hours after an intraperitoneal single dose, quercetin and fisetin were detected in the brain after 30 minutes if a liposomal preparation was administered by the same way (Rivera et al., 2004). Furthermore, quercetin and fisetin liposomal preparations demonstrated to decrease the volume of the ischemic lesion and the recovery of the cyto-architecture in ischemic areas of striatum and cortex in rats after pMCAo. However, catechin failed to protect brain tissue in this model. These results indicate again the relevance of structural features of flavonoids for neuroprotective effects.

19.7 CONCLUDING REMARKS

To summarize, data reviewed in this work, either *in vitro* or *in vivo*, could be considered a strongly suggestive evidence for a protective role of *Asteraceae* plants, particularly *A. satureioides*, and its flavonoids on neurons in culture and on the brain *in vivo*. The conditions for this protection are far from being homogeneous and very important issues, such as the identification of the protective molecules and mechanisms of action remain opened. Though much research still needs to be done in several domains from toxicity to bioavailability, from the fundamental to the clinic, medicinal plants could generate entirely new preparations exhibiting a potent family of neuroprotective molecules in the brain.

ACKNOWLEDGMENTS

Some experimental concepts described in this paper are based on the work conducted in the laboratories of the authors. These experimental studies were supported by grants from OPCW (Organization for the Prohibition of the Chemical Weapons), Gautier Laboratories (Uruguay) and PEDECIBA (Program for the Development of Basic Sciences, Uruguay). The authors thank past and present members of the Department of Neurochemistry, Instituto de Investigaciones Biológicas "Clemente Estable," Montevideo, who contributed with data and discussions to the ideas presented here.

REFERENCES

Abd El Mohsen, M. M., Kuhnle, G., Rechner, A. R. et al. 2002. Uptake and metabolism of epicatechin and its access to the brain after oral ingestion. *Free Radical Biology & Medicine* 33, 1693–1702.

Alexi, T., Borlongan, C. V., Faull, R. L. et al. 2000. Neuroprotective strategies for basal ganglia degeneration: Parkinson's and Huntington's diseases. *Progress in Neurobiology* 60, 409–470.

Alzheimer Association. 2009. Alzheimer's disease facts and figures. *Alzheimers Dementia* 5, 234–270.

Andersen, M. and Markham, K. R. 2006. *Flavonoids: Chemistry, Biochemistry, and Applications.* Boca Raton: Taylor & Francis.

Arora, A., Nair, M. G., and Strasburg, G. M. 1998. Structure–activity relationships for antioxidant activities of a series of flavonoids in a liposomal system. *Free Radical Biology & Medicine* 24, 1355–1363.

Arredondo, M. F., Blasina, F., Echeverry, C. et al. 2004. Cytoprotection by Achyrocline satureioides (Lam) D.C. and some of its main flavonoids against oxidative stress. *Journal of Ethnopharmacology* 91, 13–20.

Aruoma, O. I., Bahorun, T., and Jen, L. S. 2003. Neuroprotection by bioactive components in medicinal and food plant extracts. *Mutation Research* 544, 203–215.

Benavente-Garcia, O. and Castillo, J. 2008. Update on uses and properties of citrus flavonoids: New findings in anticancer, cardiovascular, and anti-inflammatory activity. *Journal of Agricultural and Food Chemistry* 56, 6185–6205.

Biscoping, J. and Bachmann-Mennenga, M. B. 2000. [Local anesthetics from ester to isomer] *Anasthesiol Intensivmed Notfallmed Schmerzther* 35, 285–292.

Borges, G., Roowi, S., Rouanet, J. M., Duthie, G. G., Lean, M. E., and Crozier, A. 2007. The bioavailability of raspberry anthocyanins and ellagitannins in rats. *Molecular Nutrition and Food Research* 51, 714–725.

Bors, W., Heller, W., Michel, C., and Saran, M. 1990. Radical chemistry of flavonoid antioxidants. *Advances in Experimental and Medicinal Biology* 264, 165–170.

Bourival, J., Quessy, P., and Martinoli, M. G. 2009. Protective effects of resveratrol and quercetin against MPP+ -induced oxidative stress act by modulating markers of apoptotic death in dopaminergic neurons. *Cell Molecular Neurobiology* 29, 1169–1180.

Cainelli, G., Angeloni, C., Cervellati, R. et al. 2008. New polyphenolic beta-lactams with antioxidant activity. *Chemistry and Biodiversity* 5, 811–829.

Castelluccio, C., Paganga, G., Melikian, N. et al. 1995. Antioxidant potential of intermediates in phenylpropanoid metabolism in higher plants. *FEBS Letters* 368, 188–192.

Chen, J. C., Ho, F. M., Pei-Dawn Lee, C. et al. 2005. Inhibition of iNOS gene expression by quercetin is mediated by the inhibition of IkappaB kinase, nuclear factor-kappa B and STAT1, and depends on heme oxygenase-1 induction in mouse BV-2 microglia. *European Journal of Pharmacology* 521, 9–20.

Clark, W. M., Rinker, L. G., Lessov, N. S., Lowery, S. L., and Cipolla, M. J. 2001. Efficacy of antioxidant therapies in transient focal ischemia in mice. *Stroke* 32, 1000–1004.

Clemens, J. A. and Panetta, J. A. 1994. Neuroprotection by antioxidants in models of global and focal ischemia. *Annals of the New York Academy of Sciences* 738, 250–256.

Clifford, M. N. 2004. Diet-derived phenols in plasma and tissues and their implications for health. *Planta Medica* 70, 1103–1014.

Cosentino, M., Bombelli, R., Carcano, E. et al. 2008. Immunomodulatory properties of Achyrocline satureioides (Lam.) D.C. infusion: A study on human leukocytes. *Journal of Ethnopharmacology* 116, 501–507.

da Luz, P. L. and Coimbra, S. R. 2004. Wine, alcohol and atherosclerosis: Clinical evidences and mechanisms. *Brazilian Journal of Medicine and Biological Research* 37, 1275–1295.

Da Silva, F. and Langeloh, M. 1994. Comparative activity of hydroalcoholic 80% (v/v) of *Achyrocline satureioides* (Lam.) DC (Asteraceae) with papaverine and atropine on rat isolated jejunum. *Acta Farmaceutica Bonaerense* 13, 35–40.

Dajas, F., Arredondo, F., Echeverry, C., Ferreira, M., Morquio, A., and Rivera, F. 2005. Flavonoids and the brain: Evidences and putative mechanisms for a protective capacity. *Current Neuropharmacology* 3, 95–114.

Dajas, F., Rivera, F. Blasina, F. et al. 2003a. Cell culture protection and in vivo neuroprotective capacity of flavonoids. *Neurotoxicology Research* 5, 425–432.

Dajas, F., Rivera-Megret, F., Blasina, F. et al. 2003b. Neuroprotection by flavonoids. *Brazilian Journal of Medicine and Biological Research* 36, 1613–1620.

Desmarchelier, C., Coussio, J., and Ciccia, G. 1998. Antioxidant and free radical scavenging effects in extracts of the medicinal herb Achyrocline satureioides (Lam.) DC. ("marcela"). *Brazilian Journal of Medicine and Biological Research* 31, 1163–1170.

Echeverry, C., Arredondo, F., Abin-Carriquiry, J. A. et al. 2010. Pretreatment with natural flavones and neuronal cell survival after oxidative stress: A structure–activity relationship study. *Journal of Agriculture and Food Chemistry* 58, 2111–2115.

El-Sherbiny, D. A., Khalifa, A. E., Attia, A. S., and Eldenshary Eel, D. 2003. Hypericum perforatum extract demonstrates antioxidant properties against elevated rat brain oxidative status induced by amnestic dose of scopolamine. *Pharmacology, Biochemistry and Behavior* 76, 525–533.

Geleijnse, J. M., Launer, L. J., Hofman, A., Pols, H. A., and Witteman, J. C. 1999. Tea flavonoids may protect against atherosclerosis: The Rotterdam Study. *Archives of Internal Medicine* 159, 2170–2174.

Gorelick, P. B. 2002. Stroke prevention therapy beyond antithrombotics: Unifying mechanisms in ischemic stroke pathogenesis and implications for therapy: An invited review. *Stroke* 33, 862–875.

Granado-Serrano, A. B., Martin, M. A., Haegeman, G., Goya, L., Bravo, L., and Ramos, S. 2010. Epicatechin induces NF-kappaB, activator protein-1 (AP-1) and nuclear transcription factor erythroid 2p45-related factor-2 (Nrf2) via phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) and extracellular regulated kinase (ERK) signalling in HepG2 cells. *British Journal of Nutrition* 103, 168–179.

Halliwell, B. and Gutteridge, J. M. 1986. Oxygen free radicals and iron in relation to biology and medicine: Some problems and concepts. *Archives of Biochemistry and Biophysics* 246, 501–514.

Harborne, J. B. 1993. *The Flavonoids; Advances in Research Since l986. Phytochemical Methods.* Chapman and Hall: London.

Heim, K. E., Tagliaferro, A. R., and Bobilya, D. J. 2002. Flavonoid antioxidants: Chemistry, metabolism and structure–activity relationships. *Journal of Nutrition and Biochemistry* 13, 572–584.

Howes, M. J., Perry, N. S., and Houghton, P. J. 2003. Plants with traditional uses and activities, relevant to the management of Alzheimer's disease and other cognitive disorders. *Phytotherapy Research* 17, 1–18.

Husain, G., Deepa, M., Singh, P. N., Rao, C., and Kumar, V. 2007. Ethnopharmacological review of native traditional medicinal plants for brain disorders. *Pharmacognosy Review* 1, 19–28.

Jyoti, A. and Sharma, D. 2006. Neuroprotective role of Bacopa monniera extract against aluminium-induced oxidative stress in the hippocampus of rat brain. *Neurotoxicology* 27, 451–457.

Kadarian, C., Broussalis, A. M., Mino, J., Lopez, P., Gorzalczany, S., Ferraro, G., and Acevedo, C. 2002. Hepatoprotective activity of Achyrocline satureioides(Lam) D. C. *Pharmacology Research* 45, 57–61.

Kawai, Y., Saito, S., Nishikawa, T., Ishisaka, A., Murota, K., and Terao, J. 2009. Different profiles of quercetin metabolites in rat plasma: Comparison of two administration methods. *Bioscience, Biotechnology and Biochemistry* 73, 517–523.

Kim, S. R., Park, M. J., Lee, M. K. et al. 2002. Flavonoids of Inula britannica protect cultured cortical cells from necrotic cell death induced by glutamate. *Free Radical Biology and Medicine* 32, 596–604.

Lakhan, S. E., Kirchgessner, A., and Hofer, M. 2009. Inflammatory mechanisms in ischemic stroke: Therapeutic approaches. *Journal of Translation Medicine* 7, 97.

Lloyd-Jones, D., Adams, R. J., Brown, T. M. et al. 2010. Heart disease and stroke statistics–2010 update: A report from the american heart association. *Circulation* 121: e46–e215.

Lu, L., Liu, Y., Zhu, W., Shi, J., Ling, W., and Kosten, T. R. 2009. Traditional medicine in the treatment of drug addiction. *American Journal of Drug and Alcohol Abuse* 35, 1–11.

Manach, C., Scalbert, A., Morand, C., Remesy, C., and Jimenez, L. 2004. Polyphenols: Food sources and bioavailability. *American Journal of Clinical Nutrition* 79, 727–747.

Mandel, S., Weinreb, O., Reznichenko, L., Kalfon, L., and Amit, T. 2006. Green tea catechins as brain-permeable, non toxic iron chelators to "iron out iron" from the brain. *Journal of Neural Transmission* (*Suppl.*) 249–257. **Q3**

Mandel, S. A., Amit, T., Weinreb, O., Reznichenko, L., and Youdim, M. B. 2008. Simultaneous manipulation of multiple brain targets by green tea catechins: A potential neuroprotective strategy for Alzheimer and Parkinson diseases. *CNS Neuroscience and Therapeutics* 14, 352–365.

Middleton, E., Jr., Kandaswami, C., and Theoharides, T. C. 2000. The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. *Pharmacological Reviews* 52, 673–751.

Mira, L., Fernandez, M. T., Santos, M., Rocha, R., Florencio, M. H., and Jennings, K. R. 2002. Interactions of flavonoids with iron and copper ions: A mechanism for their antioxidant activity. *Free Radical Research* 36, 1199–1208.

Muir, K. W., Tyrrell, P., Sattar, N., and Warburton, E. 2007. Inflammation and ischaemic stroke. *Current Opinion in Neurology* 20, 334–342.

Nicotera, P. and Lipton, S. A. 1999. Excitotoxins in neuronal apoptosis and necrosis. *Journal of Cerebral Blood Flow and Metabolism* 19, 583–591.

Nijveldt, R. J., van Nood, E., van Hoorn, D. E., Boelens, P. G., van Norren, K., and van Leeuwen, P. A. 2001. Flavonoids: A review of probable mechanisms of action and potential applications. *American Journal of Clinical Nutrition* 74, 418–425.

O'Neill, M. J., Hicks, C. A., Ward, M. A. et al. 2001. LY393615, a novel neuronal Ca(2+) and Na(+) channel blocker with neuroprotective effects in models of *in vitro* and *in vivo* cerebral ischemia. *Brain Research* 888, 138–149.

Ossola, B., Kääriäinen, T. M., and Männistö, P. T. 2009. The multiple faces of quercetin in neuroprotection. *Expert Opinion on Drug Safety* 4, 397–409.

Pardridge, W. M. 2001. Neuroprotection in stroke: Is it time to consider large-molecule drugs? *Drug Discovery Today* 6, 751–753.

Puhlmann, J., Knaus, U., Tubaro, L., Schaefer, W., and Wagner, H. 1992. Immunologically active metallic ion-containing polysaccharides of Achyrocline satureioides. *Phytochemistry* 31, 2617–2621.

Reynolds, T. 2005. Hemlock alkaloids from Socrates to poison aloes. *Phytochemistry* 66, 1399–1406.

Rice-Evans, C. 2001. Flavonoid antioxidants. *Current Medicinal Chemistry* 8, 797–807.

Rice-Evans, C. A., Miller, N. J., and Paganga, G. 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine* 20, 933–956.

Rivera, F., Gervaz, E., Sere, C., and Dajas, F. 2004. Toxicological studies of the aqueous extract from Achyrocline satureioides (Lam.) DC (Marcela). *Journal of Ethnopharmacology* 95, 359–362.

Rivera, F., Urbanavicius, J., Gervaz, E., Morquio, A., and Dajas, F. 2004. Some aspects of the in vivo neuroprotective capacity of flavonoids: Bioavailability and structure–activity relationship. *Neurotoxicology Research* 6, 543–553.

Ruffa, M. J., Ferraro, G., Wagner, M. L., Calcagno, M. L., Campos, R. H., and Cavallaro, L. 2002. Cytotoxic effect of Argentine medicinal plant extracts on human hepatocellular carcinoma cell line. *Journal of Ethnopharmacology* 79, 335–339.

Schafer, F. Q. and Buettner, G. R. 2001. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radical Biology and Medicine* 30, 1191–1212.

Schmidley, J. W. 1990. Free radicals in central nervous system ischemia. *Stroke* 21, 1086–1090.

Schroeter, H., Boyd, C., Spencer, J. P., Williams, R. J., Cadenas, E., and Rice-Evans, C. 2002. MAPK signaling in neurodegeneration: Influences of flavonoids and of nitric oxide. *Neurobiology and Aging* 23, 861–880.

Seelinger, G., Merfort, I., and Schempp, C. M. 2008. Anti-oxidant, anti-inflammatory and anti-allergic activities of luteolin. *Planta Medica* 74, 1667–1677.

Shukitt-Hale, B., Lau, F. C., and Joseph, J. A. 2008. Berry fruit supplementation and the aging brain. *Journal of Agriculture and Food Chemistry* 56, 636–641.

Siesjo, B. K., Zhao, Q., Pahlmark, K., Siesjo, P., Katsura, K., and Folbergrova, J. 1995. Glutamate, calcium, and free radicals as mediators of ischemic brain damage. *Annals of Thoracic Surgery* 59, 1316–1320.

Singh, M., Arseneault, M., Sanderson, T., Murthy, V., and Ramassamy, C. 2008. Challenges for research on polyphenols from foods in Alzheimer's disease: Bioavailability, metabolism, and cellular and molecular mechanisms. *Journal of Agriculture and Food Chemistry* 56, 4855–4873.

Spencer, J. P. 2007. The interactions of flavonoids within neuronal signalling pathways. *Genes Nutrition* 2, 257–273.

Spencer, J. P. 2009. Flavonoids and brain health: Multiple effects underpinned by common mechanisms. *Genes Nutrition* 4, 243–250.

Spencer, J. P. 2009. The impact of flavonoids on memory: Physiological and molecular considerations. *Chemical Society Reviews* 38: 1152–1161.

Spencer, J. P., Abd-el-Mohsen, M. M., and Rice-Evans, C. 2004. Cellular uptake and metabolism of flavonoids and their metabolites: Implications for their bioactivity. *Archives in Biochemistry and Biophysics* 423, 148–161.

Taylor, E. M. 2002. The impact of efflux transporters in the brain on the development of drugs for CNS disorders. *Clinical Pharmacokinetics* 41, 81–92.

Theoharides, T. C., Alexandrakis, M., Kempuraj, D., and Lytinas, M. 2001. Anti-inflammatory actions of flavonoids and structural requirements for new design. *International Journal of Immunopathology and Pharmacology* 14, 119–127.

Wang, Y. L., Li, R. P., Guo, M. L., Zhang, G., Zhang, N., and Ma, Y. L. 2009. Bakkenolides from Petasites tricholobus and their neuroprotective effects related to antioxidant activities. *Planta Medica* 75, 230–235.

Welton, A. F., Tobias, L. D., Fiedler-Nagy, C. et al. 1986. Effect of flavonoids on arachidonic acid metabolism. *Progress in Clinical Biology and Research* 213, 231–242.

Williams, A. J., Dave, J. R., Phillips, J. B., Lin, Y., McCabe, R. T., and Tortella, F. C. 2000. Neuroprotective efficacy and therapeutic window of the high-affinity N-methyl-D-aspartate antagonist conantokin-G: In vitro (primary cerebellar neurons) and in vivo (rat model of transient focal brain ischemia) studies. *Journal of Pharmacology and Experimental Therapeutics* 294, 378–386.

Williams, C. L. 1995. Healthy eating: Clarifying advice on fruit and vegetable consumption. *British Medical Journal* 310, 1453–1455.

Williams, R. J., Spencer, J. P., and Rice-Evans, C. 2004. Flavonoids: Antioxidants or signalling molecules? *Free Radical Biology and Medicine* 36, 838–849.

Youdim, K. A. and Joseph, J. A. 2001. A possible emerging role of phytochemicals in improving age-related neurological dysfunctions: A multiplicity of effects. *Free Radical Biology and Medicine* 30, 583–594.

Youdim, K. A., Shukitt-Hale, B., and Joseph, J. A. 2004. Flavonoids and the brain: Interactions at the blood-brain barrier and their physiological effects on the central nervous system. *Free Radical Biology and Medicine* 37, 1683–1693.

Yu, Z. F., Bruce-Keller, A. J., Goodman, Y., and Mattson, M. P. 1998. Uric acid protects neurons against excitotoxic and metabolic insults in cell culture, and against focal ischemic brain injury *in vivo. Journal of Neuroscience Research* 53, 613–625.

Zanon, S. M., Ceriatti, F. S., Rovera, M., Sabini, L. J., and Ramos, B. A. 1999. Search for antiviral activity of certain medicinal plants from Cordoba, Argentina. *Rev Latinoam Microbiol*. 41, 59–62.

Zhang, J., Stanley, R. A., Adaim, A., Melton, L. D., and Skinner, M. A. 2006. Free radical scavenging and cytoprotective activities of phenolic antioxidants. *Molecular Nutrition and Food Research* 50, 996–1005.

Cat #: K11713 **Chapter: 19**

TO: CORRESPONDING AUTHOR
AUTHOR QUERIES – TO BE ANSWERED BY THE AUTHOR

The following queries have arisen during the typesetting of your manuscript. Please answer these queries by marking the required corrections at the appropriate point in the text.

Query No.	Query	Response
Q1	Please check the edit made to the sentence, "Plant extracts have been utilized …"	
Q2	Please check the sense of the sentence.	
Q3	Please provide volume number for Mandel et al. (2006).	