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Abstract

Hepatitis E Virus (HEV) infection is an emergent zoonotic disease, where chronic hepatitis E associated to solid organ transplant (SOT) recipients, related to genotype 3, is the clinical manifestation of major concern. In this setting, ribavirin (RBV) treatment is the only available therapy, though drug-resistant variants could emerge leading to a therapeutic failure. Crystallographic structures have not been reported for most of the HEV proteins, including the RNA-polymerase (RdRp). Therefore, the mechanism of action of RBV against HEV and the molecular interactions between this drug and RdRp are largely unknown.

In this work, we aimed to model *in silico* the 3D structure of a novel HEV3 RdRp (HEV_C1_Uy) from a chronically HEV infected-SOT recipient treated with RBV and to perform a molecular docking simulation between RBV triphosphate (RBVT), 7-methyl-guanosine-5'-triphosphate and the modelled protein.

The models were generated using I-TASSER server and validated with multiple bioinformatics tools. The docking analysis were carried out with AutoDock Vina and LeDock software.

We obtained a suitable model for HEV_C1_Uy (C-Score=-1.33, RMSD=10.4 ± 4.6 Å). RBVT displayed a binding affinity of -7.6 ± 0.2 Kcal/mol by molecular docking, mediated by 6 hydrogen-bonds (Q195-O14, S198-O11, E257-O13, S260-O2, O3, S311-O11) between the finger's-palm-domains and a free binding energy of 31.26 ± 16.81 kcal/mol by molecular dynamics simulations.

We identified the possible HEV RdRp interacting region for incoming nucleotides or analogs and provide novel insights that will contribute to better understand the molecular interactions of RBV and the enzyme and the mechanism of action of this antiviral drug.

1. Introduction

Hepatitis E virus (HEV) is a leading cause of acute hepatitis, a worldwide enterically-transmitted zoonotic disease [1, 2] that cause large waterborne epidemic outbreaks in developing countries [3–6]. Nonetheless, in industrialized countries, HEV has become an increasing public-health concern as autochthonous cases have raised drastically in the last few years [7]. HEV is a small, 32-34 nm, non or quasi-enveloped particle with a single-stranded positive-sense RNA genome of 7.2 Kb approximately [8]. The viral genome is capped at the 5'-end and polyadenylated at the 3'-end, containing three partially overlapping open reading frames (ORFs) [9]. ORF1 encodes a non-structural polyprotein of ~1693 amino acids (aa) with eight putative functional domains including methyltransferase, protease, RNA-helicase and RNA-dependent RNA polymerase (RdRp) [10]. ORF2 encodes for the capsid protein [11, 12] and ORF3 produces a multifunctional phosphoprotein essential for virion release [13, 14].

HEV is classified into the *Orthohepevirus A* genus of the *Hepeviridae* family and comprises eight distinct genotypes (HEV1 to HEV8) [15]. HEV3 genotype is globally distributed [16] and is more prevalent in high-income countries [17]. Outbreaks of this genotype are associated to the consumption of contaminated raw or undercooked meat and liver sausages derived from pigs, the main reservoir of the disease [18, 19]. HEV3 infection may evolve to a chronic hepatitis, particularly, in solid organ transplant (SOT) recipients and immunocompromised individuals with lymphoma, leukemia and HIV infection [20–23], which could result in liver fibrosis, cirrhosis or liver failure [24–27].

HEV belongs to the "alpha-like" supergroup III of positive-strand viruses, where HEV RdRp along with Rubella virus RdRp (RUBV) and Beet necrotic yellow vein virus RdRp (BNYVV) conform a distinct close cluster [10]. In this supergroup, eight conserved motifs (I-VIII) have been described [10]. It has been suggested that HEV RdRp can either initiate *de novo* synthesis from the RNA template or employ the template end to prime the synthesis from the 3´OH end [28]. Localization studies revealed that HEV RdRp is present in the endoplasmic reticulum (ER), suggesting the involvement of ER membrane in HEV replication [29].

Even though HEV RdRp is a crucial viral protein, information about its functional domains, template specificities, nucleotide selection, structure and functional role have not been described yet.

Ribavirin (RBV) (1-b-D-ribofuranosyl-1,2,4-triazole) is a guanosine/adenosine synthetic analog with broad-antiviral spectrum [30] and is phosphorylated intracellularly by a cellular adenosine kinase to monophosphate (RBVM), diphosphate (RBVD) and triphosphate (RBVT) forms [31]. RBVM is a competitive inhibitor of inosine monophosphate dehydrogenase that leads to a depletion in the GTP pools needed for RNA viral synthesis [31]. Moreover, RBVT can be misincorporated by the RdRp in the nascent viral RNA inducing base transitions,

producing an early chain termination and inhibition of replication by competitively inhibiting the binding of nucleotides [31]. Modeling studies revealed that the base moiety of ribavirin, 1,2,4-triazole-3-carboxamide, forms base pairs with both cytosine and uracil by a rotation of the carboxamide group [32]. Furthermore, it was observed that once RBV is in the RNA, it could be trapped in the "anti" conformation of the "pseudo-base" and is then able to bind base pairs with incoming pyrimidines [32]. For these reasons, RBV has been also considered a viral mutagen that could exceed the "error-catastrophe" threshold in RNA viruses, causing mainly A to G and U to A substitutions [33] that leads to lethal mutagenesis [31, 32, 34–37].

Until several years ago, RBV was employed to treat Lassa fever virus infection [38–40] and in combination with interferon-α to treat hepatitis C virus (HCV) infection [41–43], vesicular stomatitis virus [44, 45], La Crosse encephalitis virus [46], reovirus [47], influenza [48, 49] and human immunodeficiency virus [50].

Currently, RBV is used as a monotherapy only to treat respiratory syncytial virus infection under certain clinical circumstances [51–53].

In the case of chronic HEV infection, in which viral RNA remains detectable for at least 3-6 months, there is no specific antiviral treatment for the patients. The first therapeutic line consists in decreasing the immunosuppression therapy, which exhibits good outcome in only 30% of the patients [25]. In a high proportion of cases where HEV clearance could not be achieved, RBV is administered for another 3 months [54], with good results [24, 55–58]. However, RBV treatment failure has been reported frequently due to HEV antiviral resistance likely associated to G1634R, Y1320H and K1383N substitutions in the RdRp [59]. The K1383N substitution is located in the F1-motif (177-180 aa in HEV RdRp) which is believed to bind the incoming nucleotide-triphosphate and select the correct one [60]. Hence, RBV has been proposed to be mutagenic to HEV during a prolonged treatment. Interestingly, the K1383N substitution could strongly decrease viral replication and increase RBV sensitivity *in vitro*, opposite to the observed clinical phenotype [59]. On the other hand, the Y1320H substitution increases HEV replication without altering RBV sensitivity, which may then be a compensatory change for the fitness loss resulting from K1383N. The G1634R substitution seemed to increase the replicative capacity of HEV and then reduce the efficiency of RBV [61].

In this work we pursued two goals. First, to obtain an HEV3 RdRp 3D *in silico* model of a strain coming from a chronic HEV-SOT patient treated with RBV [62], as there is no crystallographic structures available in databases, in order to thoroughly analyze HEV RdRp structural characteristics. Second, to perform a comprehensive molecular docking simulation between RBVT and the modelled HEV protein, aimed to provide novel insights on the enzyme-antiviral drug molecular interaction.

2.Materials and Methods

2.1. HEV strain and RNA extraction.

The HEV strain employed in this study came from a chronic hepatitis E case belonging to genotype 3 recently reported in Uruguay by our group (HEV_C1_Uy) [62]. The patient was a liver-transplanted (LT) 62-year-old man presenting an autochthonous chronic HEV3 infection with an altered liver enzymogram and histologic evidence of HEV infection (1 year and 6 months after LT), who was then treated with a 9-weeks course of RBV (1200 mg/day). After the RBV course he had a sustained virological response (SVR) in the 24 months follow-up.

Total RNA was extracted from a 10% fecal PBS suspension with *Quick*-RNA™ Miniprep Kit (Zymo Research Corp, USA) following manufacturer's instructions.

The consensus nucleotide sequence of the HEV RNA polymerase here described was submitted to GenBank under the accession number MT774175.

2.2. Next Generation Sequencing (NGS) and data analysis

Double stranded cDNA (dscDNA) was generated using Maxima H Minus Double-Stranded cDNA Synthesis Kit (ThermoFisher Scientific, USA) with random primers and 12 µL of extracted RNA. The dscDNA was amplified by Multiple Displacement Amplification (MDA) technology using REPLI-g Mini Kit (Qiagen, Germany) followed by purification and quantification using AMPure XP (Beckman Coulter, USA) and a Qubit fluorometer (Qubit™ DNA-HS Assay kit), respectively.

Nextera DNA Flex Library Preparation kit (Illumina, USA) with dual indexing was used from 50 ng of dscDNA. Control quality libraries were performed on a Fragment Analyzer 5200 system (Agilent Technologies, USA) using the Standard Sensitivity NGS Analysis Kit (Agilent Technologies, USA). Library was sequenced on an Illumina MiniSeq Genomic Platform at the Faculty of Sciences (UdelaR, Uruguay) using Mid Output Reagent Cartridge (300-cycles, 150 base-pair paired-end reads) by following standard Illumina protocols.

Sequencing raw reads were demultiplexed automatically on the MiniSeq platform with the default settings. Adapter/quality trimming and filtering were performed with BBDuk plugin and clean reads were mapped to a hepatitis E genome (FJ998008) using Geneious mapper (medium-low sensitivity) available in the Geneious Prime 2020.2.1 software (https://www.geneious.com).

Reference assembly and annotation was done with SeqMan NGen® Version 12.0 (DNASTAR. Madison, WI) using the reference genome retrieved from the GenBank database FJ998008.

2.3. RdRp sequence alignments

The RdRp domain was identified from the HEV_C1_Uy complete genome according to other HEV polymerase annotations in Uniprot database [63].

Multiple RdRp nucleotide and amino acids (aa) sequence alignments of complete RdRp from HEV strains and of the catalytic site from HEV and other viruses were carried out with ClustalW in MEGA v.7. [64]. HEV_C1_Uy complete RdRp and HEV3 selected subtype reference strains 1a-8a [65] were included for the alignment (Table S1).

An alignment including only the catalytic site of different viral RdRp was also carried out for HEV_C1_Uy and HEV reference strains (Table S1).

Additionally, diverse RdRp sequences were selected from several viruses for further analysis: Hepatitis C virus 1a-*Flaviviridae*, Hepatitis A virus IB- *Picornaviridae*, Coxsackievirus B1-*Picornaviridae*, Norwalk virus of the *Caliciviridae* family (former HEV classification), RUBV-*Matonaviridae* (reported to exhibit conserved motifs with HEV RdRp [10]) and BNYVV-*Benyviridae* a plant furovirus belonging to the "alpha-like" supergroup. Top first threading templates and structural analogs from I-TASSER modelling were also added to the alignment from RCSB:PDB database [66] (Table S1). Viral RdRp catalytic site annotated sequences were obtained from Uniprot database. P-distance pairwise matrices for RdRp catalytic site between HEV strains and the other viral sequences were performed in MEGA v. 7. A phylogenetic tree for the RdRp catalytic sites was constructed in MEGA v.7 by the Neighbor-Joining method with the Poisson model as the best substitution model. Bootstrap values were determined with 1000 replicates of the dataset.

2.4. HEV RdRp 3D modelling, structural analysis and validation

The amino acid sequences from the HEV_C1_Uy RdRp and a HEV3 RdRp from the swine Arkell strain (reference of subtype 3j) were employed to determine the 3D-structure. The secondary structure was predicted using PSIPRED 4.0 tool [67] (UCL-CS Bioinformatics). Annotation information was retrieved from ExPASy-PROSITE [68] and Pfam database [69]. Chemical and physical parameters for this protein were obtained from ExPASy-ProtParam tool [70].

Thorough sequence alignments analyses were performed in order to evaluate HEV RdRp homology modelling possibility, which was then discarded (data not shown). Therefore, the I-TASSER prediction server [71] was employed to obtain the 3D structure models and the best ranked structure in the hierarchical analysis in terms of the best C-Score and Root Mean Square Deviation (RMSD) were selected [71].

The models were assessed and validated using bioinformatics tools. ProSA-Web [72] was used to calculate the Z-score for the overall model quality which enables to establish whether the Z-score value of the model structure is located in the range of Z-scores exhibited by native proteins of similar size, with PDB as reference database. The Ramachandran plots (RAMPAGE) [73], were also constructed to establish amino acids in energetically favorable regions. Additionally, ERRAT [74], Verify 3D [75] and PROCHECK [76] software were employed. All these computational tools enable us to determine whether 3D models of HEV RdRp are reliable models to employ in molecular docking analysis.

The generated models were structurally aligned to the best ranked structures to establish the RMSD differences between the model and best template structures utilizing TM-Align based on TM-score [77]. The crystallized structures (6R1I, 1SH0, 3N6L, 3CDU) were downloaded from the PDB database.

Molecular graphics were performed with UCSF Chimera v. 1.8. [78].

2.5. Molecular docking

The 3D structure of RBV and RBVT were downloaded in PDB format from PubChem-NIH [79] (CID 37542 and 122108, respectively). 7-methyl-guanosine-5'-triphosphate (GTP) and Cinnamaldehyde (CIN) 3D structures were downloaded in PDB format from the Drugbank database [80] (Accession Numbers DB02716 and DB14184, respectively). The GTP physiological nucleotide was employed as an internal control for the molecular interaction analyses. The CIN organic compound, which is the main component of the cinnamon, was included as a negative control since no affinity towards RdRp has been reported for this compound [81].

Interaction analysis of HEV_C1_Uy RdRp and HEV Arkell RdRp with RBV, RBVT, GTP and CIN were carried out with AutoDock Vina v. 1.1.2 [82]. The grid box was defined with the online tool PeptiMap [83], which predicts the best ligand-binding site on the protein surface. The models were minimized adding charges with the default parameters in PMV v.1.5.6 (MGLTools-The Scripps Research Institute) [84] and saved in PDBQT file formats for the docking. Five runs of each docking assay were performed in a grid box with a spacing of 1 Å, presenting the dimensions x= 24, y= 28, z= 22 and its center located in x= 74.853, y= 63.244, z= 55.892 for HEV_C1_Uy RdRp. Concerning the HEV Arkell RdRp, conditions were the same as aforementioned but with dimensions x= 24, y= 24, z= 28 and the following coordinates x= 65.309, y= 56.220, z= 61.801. Additional docking assay was carried out involving the analog sites between HCV-ribonucleoside triphosphates (rNTPS) interaction and HEV_C1_Uy RdRp and HEV Arkell RdRp. The grid box for HEV_C1_Uy RdRp had the following characteristics, size: x= 20, y= 24, z= 28, center: x= 80.208 y= 63.928, z= 60.218, whereas for HEV

Arkell RdRp presented size: x= 30, y= 24, z= 20; center: x= 64.757, y= 52.581, z= 68.09. All the docking studies were also run five times in LeDock [85], employing the same grid box coordinates as in AutoDock Vina, with the ligand file format in SYBYL Mol2. In all cases, binding energies were reported as the mean Kcal/mol ± SD. The 2D protein-ligand interaction diagrams were built employing the software LigPlot+ v. 4.5.3 [86] and PoseView-Proteins*Plus* [87]. Analysis of the generated 3D docking interactions were performed in UCSF Chimera v. 1.8.

2.6. Molecular dynamics simulation and binding free energy calculations.

mol2 files for Cinnamaldehyde (ZINC1532777), RBVT (ZINC12402860), and 7-methyl-GTP (ZINC15601432) were downloaded from ZINC Database [88]. These files were used for parametrization of the ligands with the help of SwissParam [89]. Using VMD, HEV_C1_Uy RdRp-ligand complexes were made, solvated, and, subsequently, the solvation box was ionized with 0.15 M of Na^+ and Cl^- [90]. Molecular Dynamics (MD) simulations followed; for this, we utilized NAMD 2.14 [91]. Minimization was carried out for 10,000 steps while a step itself was 2 fs long. Following minimization, the three systems were equilibrated for 2 ns, and, finally, longer MD simulations were carried out so that the total length of the simulations were at least 100 ns. CaFE [92] was used to perform molecular mechanics Poisson-Boltzmann surface area (MM/PBSA) calculations. CaFE utilized NAMD for the MM component, APBS for PB calculations [93], and VMD for SA calculations. Trajectories derived from the MD simulations were analyzed using CaFE. Default parameters of CaFE were used for the calculations.

3. Results

3.1. HEV_C1 _UY RdRp showed non-reported amino acidic substitutions

The HEV3 C1 Uy complete genome from the HEV-SOT chronic patient was successfully obtained by Illumina sequencing with a mean average coverage of sequencing depth of 71.7.

The RdRp domain was identified at the 1217-1703 position of the nonstructural protein. This protein contained 487 residues, a molecular weight of 53526.49 Da and an isoelectric point of 5.94.

Due to the high sequence divergence of the complete RdRp sequence among viral families, the alignments and identity matrices analyses were conducted separately for the catalytic site. Sequence analysis of the catalytic site revealed an identity for HEV_C1_Uy RdRp ranging from 17% for Coxsackievirus B1 and B3 to 29.8%-26.6% for BNYVV and RUBV, respectively. The percentage of identity of HEV_C1_Uy with HEV3 reference strains

ranges from 95.7% (3e) to 98.9% (3a, 3i and 3k). HEV_C1_Uy RdRp showed 86.89% of sequence identity with the closest related isolate (FJ705359).

HEV_C1_Uy presented several non-reported and infrequent substitutions, which were located outside the catalytic site. At the amino acidic level, a unique non-reported substitution V/I4A (V/I1220A referred to ORF1 position) was found in HEV_C1_Uy strain. The changes T/A392V (T/A1608V) and V443A (V1659A) in HEV_C1_Uy were exclusively found in HEV8a and 5a genotypes, respectively, which are very distantly related to HEV3, whereas the HEV_C1_Uy Q484H (Q1700H) change was previously reported in few strains from 2a, 5a and 6a genotypes (Fig. 1).

The eight reported conserved motifs (I-VIII) of the positive-strand viral RNA RdRp [10] were identified among the sequence alignments (Table 1). Motifs IV (D-x(4,5)-D) and VI (GDD) in the catalytic site were conserved among HEV strains and other viral families (Fig. 1).

HEV proteins have not clustered with any other RdRp viral sequence but were closely related and shared the highest homology with the RUBV and BNYVV proteins (Fig. 2). Analysis at the nucleotide level, exhibited that HEV_C1_Uy formed a separate cluster from other HEV3 subtypes strains (data not shown).

3.2. An acceptable HEV_C1_Uy RdRp 3D structure was obtained

HEV_C1_Uy RdRp and HEV Arkell RdRp models obtained had a C-Score of -1.33, RMSD score of 10.4 ± 4.6 Å and a C-Score of -1.10, respectively. C-score values determines the model quality and ranges from [-5, 2], where a higher value means a higher confidence. Additionally, a C-Score > -1.5 supports a model with correct global topology. The RMSD values determines the divergence in angstroms between the modelled protein and its template. Therefore, the obtained HEV RdRp models exhibited a suitable global topology.

The top first templates used by I-TASSER to model the proteins corresponded to the Porcine Aichi virus (Kobuvirus) from the *Picornaviridae* family (6R1I), Norwalk virus (1SH0) and Enterovirus A71 (EV71)-*Picornaviridae* (3N6L).The top first structural analog obtained with I-TASSER for HEV_C1_Uy RdRp corresponded to Coxsackievirus B3 (3CDU).

These models were further validated employing different bioinformatics tools. PROCHECK server, which verified the stereochemical quality of a protein structure, analyzing each residue geometry and the general geometry, showed the best results. Similarly, acceptable Ramachandran plots were observed between the models (86.60%-HEV_C1_Uy and 84.10%-Arkell corresponding to aa in favorable region for torsions and rotations).

279 Moreover, the crystallographic structures used as templates were also validated for comparison. Overall, the two
280 models exhibited acceptable validation values (Table 2).
281 Additionally, structural alignments between the two models, and between the two models and the templates were
282 carried out. TM-Score for HEV_C1_Uy and HEV Arkell was 0.86, similar values were observed for the PDB
283 templates. The highest TM-Scores registered were 0.88 for HEV_C1_Uy and the Enterovirus A71 and of 0.91 for
284 HEV Arkell and Porcine Aichi virus. TM-Score are in the range of (0, 1], where 1 corresponds to a perfect match
285 between two structures and values greater than 0.5 indicates the same fold. Therefore, all the TM-Scores obtained
286 suggested that the structure of the models and the templates had the same fold. These results reinforce the
287 confidence in the obtained structural models.
288 The secondary structure prediction chart for the HEV_C1_Uy model exhibited the distribution of the helix, strand
289 and coil throughout the protein sequence and the confidence of prediction for each section (Fig. 3).
290 The finger´s, thumb and palm domains were identified in the 3D models (Fig. 4). Also, the 3D structural alignment
291 revealed that HEV_C1_Uy and HEV Arkell, as well as HEV_C1_Uy and EV71 had similar structural folding as
292 observed by the TM-align Scores (Fig. 4).
293
294 **3.3. HEV_C1_Uy exhibited very favorable binding affinity with RBVT.**
295 Molecular docking studies between RBVT and the modelled RdRp were executed in order to evaluate their
296 interaction interface. HEV_C1_Uy RdRp and the control protein HEV Arkell showed very favorable binding
297 energies with RBVT in AutoDock Vina (-7.6 ± 0.2 Kcal/mol and -8.0 ± 0.1 Kcal/mol, respectively) and LeDock
298 (-8.01 ± 0.18 Kcal/mol and -8.16 ± 0.14 Kcal/mol, respectively) (Table 3). In fact, these values were under the -
299 7.0 Kcal/mol threshold [94], and are indicative of a strongly binding ligand; this threshold was defined for a set
300 of diverse antivirals ligands in Auto Dock against Human Immunodeficiency Virus (HIV). The interactions were
301 mediated by 6 hydrogen bonds (H-bond) in the case of HEV_C1_Uy involving residues Q195-O14, S198-O11,
302 E257-O13, S260-O2, O3, S311-O11 and 9 H-bonds for HEV Arkell. The closest distance between atoms of RBVT
303 and HEV_C1_Uy was of 2.88 Å and the more distant one was of 3.07 Å (Fig. 5).
304 The GTP interaction used as control showed the best binding affinity for HEV_C1_Uy. On the other hand, in
305 HEV Arkell the most favorable interaction for AutoDock Vina corresponded to RBVT. However, according to
306 LeDock the best score obtained was for GTP. Furthermore, the negative control (CIN) exhibited a weak binding
307 energy (-5.0 ± 0 Kcal/mol and -2.99 ± 0.03 Kcal/mol with AutoDock Vina and LeDock, respectively), indicating
308 a non-specific interaction. Unphosphorylated RBV was also evaluated by molecular docking as control, (data not

shown), though further 3D interaction analyses were carried out with RBVT, since it is the active form for RdRp interaction.

Additionally, since the residues for HCV and rNTP H-bond interaction have been previously identified [95], through sequence alignment we extrapolated them to HEV_C1_Uy RdRp to carry out RBVT docking studies in those sites for comparison purposes. The analog sites HEV-HCV were: S367-Q262, R386-R291, R394-G305 and T390-E301. Highly similar favorable binding affinity was observed (-7.7 ± 0.2 Kcal/mol for RBVT with AutoDock Vina) compared to the previously mentioned molecular docking with the Peptimap prediction sites (-7.6 ± 0.2 Kcal/mol). The interaction of RBVT-HEV_C1_Uy in this case was mediated by 7 H-bonds, I197-N4, S198-O14, N4, S260-O4, K309-O12, O13, S311-O13, with 2.82 Å and 3.08 Å as the closest and furthest distance between atoms, respectively. Several interacting amino acids are the same that those observed with Peptimap sites.

3.4. Molecular dynamics simulation analysis.

To a large degree, MM/PBSA results are in agreement with the docking results. For CIN, the estimated free binding energy to HEV_C1_Uy RdRp is in the expected range (-7.65 ± 4.16 kcal/mol) when standard deviation (SD) values are taken into account. For GTP, when taken together with a considerable SD value, the free binding energy estimate confirms expectations (5.38 ± 12.69 kcal/mol). As for RBVT, the MM/PBSA results predict a binding affinity of 31.26 ± 16.81 kcal/mol to HEV_C1_Uy RdRp.

All simulations reached equilibrium states (Fig. 6 A-C). Little fluctuation was seen in the residues, except for the range 50-200 (which contains the residues that form the binding pocket and some flexible loops) and, as would be expected, the termini (Fig. 6 D-F).

4. Discussion

Chronic hepatitis E in SOT recipients and immunocompromised individuals, frequently associated to severe extrahepatic manifestations, is a disease of major concern in high-income and non-endemic countries where HEV3 is prevalent. In the last few years, product of a sharp increment of HEV cases and an improvement of the diagnosis, many aspects of chronic HEV infection have been uncovered and much more information is now available [96]. In this clinical setting, antiviral treatment with RBV, the only approved drug to treat HEV infection, is the main option since the risk of acute rejection prevents the use of pegylated interferon alpha as a therapeutic option [55,

57, 58]. In fact, RBV administered for at least 3 months has shown favorable outcomes with confirmed efficacy in acute and chronic HEV cases [24, 55–58].

Unfortunately, though significant advances have been achieved in terms of cell-culture isolation of HEV [97, 98] the lack of an efficient and standardized model has hampered the study of HEV viral cycle and therefore prevented the comprehension of the antiviral mechanisms of action, of RBV. Viral RdRp is believed to interact with RBV, though the molecular details of HEV-RBV interaction have not been identified yet. Remarkably, no HEV RdRp enzyme have been crystallized and the likely ligand-binding pocket for nucleotides or any other molecule is not known. This contrast with other viral RdRp which have been co-crystallized with nucleoside triphosphates or oligonucleotides to map the substrate-binding sites, as reported for HCV-rNTPs complex [95]. Hence, *in silico* computational approaches, which are widely employed to predict and evaluate molecule-target interactions for drug discovery [99, 100], might be an useful tool for afford this knowledge gap. Structural studies on replicative complexes of RdRp and NTPs or analogs are currently needed to better understand the enzymes low copying fidelity and the mutagenic activity of the analogs on the viral replication [101].

In this work, we pursued two goals. First, to model *in silico* the HEV3 RdRp protein of a HEV chronic strain and second, to perform a detailed molecular docking study and molecular dynamics simulations, with the aim to identify the interacting domain of the viral enzyme and its binding affinity with the drug RBV.

Herein, we report a complete HEV RdRp sequence (HEV_C1_Uy), corresponding to a LT patient chronically infected with HEV3. The patient had been successfully treated with RBV in a 9-weeks course, and had an SVR during the 24 months follow-up. Sequence analysis identified the HEV_C1_Uy catalytic site corresponding to the region spanning residues 248 to 359. This catalytic site showed elevated sequence divergence among virus families, being the highest aa percentage identity observed with BNYVV and RUBV (29.8%-26.6%, respectively). These results support the notion that these viruses constitute a distinct monophyletic group in the "alpha-like" supergroup of positive-strand RNA viruses [10]. However, HEV3 strains formed a separate independent cluster from the RUBV and BNYVV group in the phylogenetic group reconstruction.

RNA and DNA polymerases share a basic structure, where RdRp are more similar to each other than to other different polymerases. There is almost no detectable sequence similarity between viral RdRps with the exception of some conserved motifs [102, 103]. Indeed, this was the case with HEV and other virus, since sequence alignments needed to be performed including only the catalytic site due to the extreme divergence observed. Interestingly, all these diverse viruses have two conserved motifs, D-x(4,5)-D and GDD. It has been shown, that *in vitro* substitutions in the GDD motif abolished the RdRp activity of HEV [104], HCV [105], RUBV [106],

calicivirus [107] and poliovirus [108]. Thus, the GDD motif plays a crucial role in the catalytic activity and metal ion coordination [109, 110], which could therefore explain its conservation among a wide range of RdRps.

On the other hand, there is enough sequence conservation in order to perform alignments and identify motifs within some viral families [102]. Certainly, this was observed for HEV and RUBV alignment, where eight conserved motifs (I-VIII) associated to positive-strand viral RNA RdRps were successfully identified. Furthermore, sequence comparison between HEV_C1_Uy with HEV genotypes and HEV3 subtypes revealed several differences, where few unique (V/I1220A) and infrequent substitutions (T/A1608V, V1659A and Q1700H) were observed. Single nucleotide variants at protein level have been reported to be less abundant at ligand binding sites and less rare variants were found to be located apart from enzyme active sites, resulting in moderate changes of the physic-chemical properties of the aa [111]. Moreover, it has been reported that mutations in the functional sites would alter the enzyme's catalytic activity, even mutations with no significant effects can modify the affinity of protein-drug interactions [112]. Current *in vitro* research is being conducted to address if these HEV_C1_Uy substitutions, which were distantly located from the catalytic site, are involved in RBV sensitivity or in the enzymatic activity. Debing et al. [59] reported three substitutions likely associated to RBV resistance (Y1320H, K1383N and G1634R) located outside the catalytic site. Additionally, other substitutions have been reported in HEV infected patients (D1384G, K1398R, V1479I, Y1587F), which were suggested to be replication competent and to possibly affect the HEV replication by modulating the RdRp activity [61, 113–115]. Recently, substitutions mutants C1483W and N1530T isolated from HEV acute liver failure patients have been strongly associated to high viral load and mortality [116].

Notably, a RdRp substitution was reported (F1439Y) to be significantly associated to HEV fulminant liver failure patients [117]. Among the reported substitutions, HEV_C1_Uy RdRp presented F1439Y (F233Y in HEV_C1_Uy RdRp) and V1479I (V273I). The V1479I substitution has been previously reported in chronic HEV-SOT patients exhibiting RBV resistance, suggesting that this substitution could modulate the RdRp activity [115].

Interestingly, the HEV3 Arkell swine strain selected for modelling and docking comparison, presented the G1634R and the F1439Y substitutions.

Moreover, RBV has been suggested to act as a mutagen in patients chronically infected with HCV [118]. Several studies showed that *in vitro* growing of poliovirus (*Picornaviridae*) in the presence of RBVT promotes the selection of the mutant G64S, that showed a lower affinity to RBVT, thus increasing template copying fidelity [119–122]. Furthermore, it has been demonstrated that the G64R, G64T and S264L substitutions confer RBV resistance in EV71 by increasing the RdRp replication fidelity [123]. Therefore, RBV may indeed exert its

antiviral activity through a mutagenic effect also for HEV [124–126], as it has been reported that RBV increases HEV quasispecies heterogeneity [59, 61, 127, 128].

Validated HEV3 C1 Uy RdRp and HEV Arkell models were obtained through bioinformatics *de novo* strategies. These models exhibited a correct global topology and share the same folding with a high structural alignment score (0.91).

Previous reports have demonstrated that it is possible to acquire a reliable 3D RdRp model *in silico*, even when there is low sequence identity with modelling templates [129]. The widely employed I-TASSER server is a powerful platform based on sequence-to-structure-to-function prediction paradigm, where the software first generates three-dimensional atomic models from multiple threading alignments and iterative structural assembly simulation. The threading methodology is used for identifying template proteins from solved structure databases that have a similar structure or similar structural motifs [130].

RdRps share a similar overall structure with the finger's, thumb and palm domain arranged in a cupped right-hand configuration, with an N-terminal domain bridging the finger's and thumb region [131], which were successfully identified in the HEV models.

Molecular docking analysis for RBVT with HEV_C1_Uy and HEV Arkell revealed a favorable binding affinity under the established threshold (-7.0 Kcal/mol) [94], with similar good values observed for the GTP control. The RBVT interactions were mediated by 6 H-bond for HEV_C1_Uy (Q195-O14, S198-O11, E257-O13, S260-O2, O3, S311-O11) and 9 H-bonds for HEV Arkell (H6-O7, M67-O12, Q69-O12, E70-O12, K177-O3, Q195-O3, G196-O10, S198-O7, K309-O9). These interaction sites were very similar between both models since similar regions were identified as best candidates for docking by Peptimap and were found to be buried between the finger's-palm domains of the HEV RdRp. Notably, the GTP binding region was located within the same interacting site for RBVT in the HEV models, suggesting that this is the region that could directly interact with incoming nucleotides or analogs.

The RdRp structure of poliovirus (*Picornaviridae*) has a N-terminal glycine residue buried in a pocket at the base of the finger's domain, forming 4 H-bonds that reposition the catalytic residue Asp238 into the active site (palm domain). The Asp238 residue was then able to establish a long H-bond interaction (2.8 Å) with the 2′OH of the incoming rNTP, as part of a flexible interdomain linker, a common molecular mechanism to most picornaviruses [132, 133]. A similar atom distance was obtained for RBVT-HEV_C1_Uy (2.82-3.07 Å). Indeed, HEV_C1_Uy and EV71 (*Picornaviridae*) exhibited the best structural alignment score, as well as HEV Arkell and Porcine Aichi virus, another *Picornaviridae* family member.

Substitutions V/I1220A, T/A1608V, V1659A and Q1700H identified in HEV_C1_Uy, occur in the finger's, thumb, thumb and in the protruding coil-thumb domain, respectively, while the substitutions F233Y and V273I were located in the bridge thumb-palm domain and in the finger's domain, respectively. These positions and particularly the domains where they are located were described in several viral families. To gain insight into the mutational effect of RBV, the X-ray structure of the foot-and-mouth disease virus (FMVD) (*Picornaviridae*) with natural substrates (ATP, UTP) and RBV had been obtained and reported [101]. It was shown that the loop β9-α11 of the finger's domain, can be flexible and necessary to adapt its conformation and interactions to the size and shape of the incoming nucleotides and, additionally, it contains the M296I substitution found in RBV resistant FMDV strains [101]. Conversely, the HCV 3D RdRp modelling revealed that the F415Y RBV resistant variant is located at the P helix region of the thumb domain, which is suggested to interact with the minor groove of the template-primer duplex in the putative-RNA binding site [33]. Furthermore, HCV-rNTPs reported binding sites [95] were close to the RBV interaction region in our HEV models, with favorable docking simulation scores.

To explore into the stability of the HEV_C1_Uy-ligand complexes, further analysis was performed by a 100 ns MD simulation. The binding free energy calculations by MM/PBSA confirmed the previous docking results obtained for GTP and CIN taking the SD values into consideration. However, the MM/PBSA results for RBVT did not correspond to the favorable binding affinity observed by molecular docking. This observation is very interesting and raises additional questions concerning the role of selected mutations in the RBV antiviral activity. One explanation of this result might be that the HEV_C1_Uy strain presents several unique and infrequent substitutions in the RdRp protein (V/I1220A, T/A1608V, V1659A and Q1700H), as well as one associated with fulminant liver failure (F1439Y) and one identified in RBV resistant patients (V1479I), suggested to be involved in RdRp activity modulation. Therefore, this rare combination of substitutions might affect the binding stability of RBVT-HEV_C1_Uy complex. Particularly, the presence of the substitution V/I1220A, not previously reported in an HEV-RdRp, was located within the RBVT binding-pocket analyzed, which could then alter this complex binding affinity. Further reverse genetics-based *in vitro* research will be needed to shed light on this issue.

On the other hand, the MD force field may not represent the highly polar phosphate groups well, as for instance, it takes no account of the possibility of different ionization states or dynamic polarization effects induced by the interacting protein groups. MD simulations are also heavily dependent on the initial ligand conformation, so even a slight difference in ligand conformation would affect the binding affinity values [134, 135].

In summary, by using a bioinformatics approach, we obtained the first acceptable models of HEV RdRp belonging to a viral strain isolated from a chronically infected patient and the reference HEV3 Arkell swine strain, in order to perform molecular docking studies and MD simulations with RBVT.

Results described here showed that RBVT could bind to the HEV3 RdRp finger's-palm domains, and the possible interaction site and H-bonds involved are described in detail. We also showed that RBVT and GTP might share the same binding site in the RdRp, suggesting that this could be the interacting region for incoming nucleotides or analogs. However, MM/PBSA results differed from the binding affinities obtained by molecular docking for the reasons previously mentioned.

Even tough additional research efforts should be performed *in vitro* aimed to corroborate all these data, our findings will contribute to better understand the mechanism of action of RBV in HEV RdRp, and therefore, this validated model could be an useful tool for the development of new potential HEV antiviral drugs on a rational basis by inferring the possible ligand-target interaction.

Author Contributions

Conceptualization, C.Q-G and F.C.; methodology, F.C., S.R-M., Y.P., R.P., D.R.H; software, F.C., S.R-M. D.R.H., G.G.; validation, F.C. and S.R-M.; formal analysis, F.C., S.R-M., C.Q-G. D.R.H., G.G and S.M.,; investigation, F.C.; S.M.; resources, S.M..; data curation, Y.P., R.P..; writing—original draft preparation, F.C.; writing—review and editing, C.Q-G., S.R-M., Y.P., R.P., D.R.H., G.G. and S.M.; visualization, F.C., S.M..; supervision, C.Q-G. and S.M.; project administration, S.M, J.A.; funding acquisition, S.M. and J.A. All authors have read and agreed to the published version of the manuscript.

Disclosure statement

The authors declare that there are no conflicts of interest.

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Ethical approval

The study was approved by the Ethics Committee from the Hospital Central de las Fuerzas Armadas.

Ethical approval number 07/CE/19.

Informed consent

The patient gave written informed consent

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Figure Captions

Fig. 1. Multiple sequence alignment of viral RNA-dependent RNA polymerase (RdRp). A. Complete RdRp sequence alignment for Hepatitis E virus (HEV) reference genotypes (HEV1-HEV8), subtypes (HEV 3a-3m) and HEV_C1_Uy. The black dot indicates the non-reported substitution V/I4A and the infrequent ones T/A392V, V443A and Q484H. B. Sequence alignment for the RdRp catalytic site for Porcine Aichi virus (Kobuvirus), Enterovirus A71, Coxsackie B3, B1, Rubella virus, Norwalk virus, Hepatitis A, Beet necrotic yellow vein virus (BNYVV) and HEV_C1_Uy. The conserved motifs (D-x(4,5)-D) and GDD are highlighted in yellow and green, respectively.

Fig. 2. Phylogenetic tree based on the RdRp catalytic site constructed by the Neighbour-Joning method with Poisson model as the substitution model. HEV strain coming from the HEV-SOT recipient (♦) was compared to other viral RdRp. Only bootstrap values >60% are shown.

Fig. 3. Secondary structure prediction for HEV_C1_Uy RdRp model employing PSIPRED. The Strand, Helix, Coil distribution and its confidence of prediction is shown for each residue throughout the sequence.

Fig. 4. 3D Structural models of HEV RdRp obtained by I-TASSER. A. HEV_C1_Uy RdRp model. B. HEV Arkell RdRp model. C. Structural alignment between HEV_C1_Uy (green) and HEV Arkell (red). D. Structural alignment between HEV_C1_Uy (green) and the top threading template Porcine Aichi virus (Kobivirus) (blue) (PDB: 6R1I). The finger's, thumb and palm domains are indicated in all the models. Graphs were obtained with UCSF Chimera v. 1.8.

Fig. 5. Molecular docking analysis between HEV_C1_Uy RdRp and RBVT. A. 3D interaction of the HEV RdRp with ligand RBVT in the defined finger's-palm domains binding pocket. Hydrogen-bonds interacting residues are indicated in purple with single-letter amino acid code. Graphs were obtained with UCSF Chimera v. 1.8. B. 2D diagram of the HEV RdRp-RBVT showing the residues forming hydrogen-bonds and the distance between atoms, employing LigPlot+ v. 4.5.3.

Fig. 6. The RMSD and RMSF analysis of the MD trajectories. A – C. RMSD values expressed in Angstroms for the whole duration of the MD simulation for the complexes containing Cinnamaldehyde, GTP, and RBVT respectively. D – F. RMSF values expressed in Angstroms for the whole duration of the MD simulation and all the protein residues of complexes containing Cinnamaldehyde, GTP, and RBVT respectively. RMSD values were calculated for the protein backbone while RMSF values were calculated for Cα atoms of each residue.