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Flaviviridae viruses use a common molecular mechanism to escape nucleoside analogue inhibitors

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ABSTRACT

The RNA-dependent RNA polymerases of *Flaviviridae* viruses are crucial for replication. The *Flaviviridae* polymerase is organized into structural motifs (A-G), with motifs F, A, C and E containing interrogating, priming and catalytic substrate-interacting sites. Modified nucleoside analogues act as antiviral drugs by targeting *Flaviviridae* polymerases and integrating into the synthesized product causing premature termination. A threonine mutation of a conserved serine residue in motif B of *Flaviviridae* polymerases renders resistance to 2′-*C*-methylated nucleoside analogues. The mechanism how this single mutation causes *Flaviviridae* viruses to escape nucleoside analogues is not yet known. Given the pivotal position of the serine residue in motif B that supports motif F, we hypothesized the threonine mutation causes alterations in nucleoside exploration within the entry tunnel. Implementing a stochastic molecular software showed the all-atom 2′-*C*-methylated analogue reaction within the active sites of wild type and serine-threonine mutant polymerases from Hepacivirus and Flavivirus. Compared with the wild type, the serine-threonine mutant polymerases caused a significant decrease of analogue contacts with conserved interrogating residues in motif F and a displacement of metal ion cofactors. The simulations significantly showed that during the analogue exploration of the active site the hydrophobic methyl group in the serine-threonine mutant repels water-mediated hydrogen bonds with the 2′-*C*-methylated analogue, causing a concentration of water-mediated bonds at the substrate-interacting sites. Collectively, the data are an insight into a molecular escape mechanism by *Flaviviridae* viruses from 2′-*C*-methylated nucleoside analogue inhibitors.

1. Introduction

Every year millions of humans are infected by *Flaviviridae* viruses worldwide. The 2′-*C*-methylated nucleoside analogues are the most promising antiviral agents active against *Flaviviridae* viruses [1–6]. In particular, 2′-C-methyladenosine (CMA), and its derivatives, were found highly effective against *Flaviviridae* viruses [7], e.g., hepatitis C virus (HCV), of the genus Hepacivirus and members of the genus Flavivirus that include yellow fever virus, Kyasanur forest disease virus, Omsk hemorrhagic fevers virus [5] and tick-borne encephalitis virus (TBEV) [2]. *Flaviviridae* are Group IV, positive-sense ssRNA viruses that encode a RNA-dependent RNA polymerase (RdRp), an enzyme that catalyzes replication of viral RNA and is thus crucial for viral reproduction in a host cell. The overall tertiary structure of RdRps is a heart-shaped, right-hand conformation divided into the thumb, fingers and palm domain. These domains are sub-divided into structural motifs A-E [8]. The RdRp structural motifs G, F and the priming loop (PL) were identified later [9–11]. Therapeutic 2′-*C*-methylated nucleoside analogues target RdRps and are incorporated in the synthesized genetic product causing premature termination.

Flaviviridae family members have conserved RdRp residues responsible for substrate binding. Three nucleoside triphosphate (NTP) interacting sites have been identified in HCV as the catalytic,

Abbreviations: CMA, 2'-C-methyladenosine; CMA-TP, 2'-C-methyladenosine Triphosphate; CPUs, Computer-Processing Units; HCV, Hepatitis C Virus; JEV, Japanese Encephalitis Virus; NTP, Nucleoside Triphosphate; PELE, Protein Energy Landscape Exploration; PL, Priming Loop; PDB, Protein Databank; RdRp, RNA-dependent RNA polymerase; RMSD, Root Mean Square Deviation; TBEV, Tick-Borne Encephalitis Virus; VMD, Visual Molecular Dynamics; WT, Wild Type.

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priming and interrogating sites [12]. Structural conformational changes occur among the RdRp motifs depending on whether it is unbound (or apo-form), NTP-bound and/or primer-template-NTP-bound. The initial conformation during NTP-binding is weak and rapid, which has been attributed to substrate specificity, i.e., incorporating a correct NTP [13]. The geometry of a correct NTP is positioned to interact with electrostatic residues of motif F to collapse the RdRp structure. An incorrect NTP fails this positioning, resulting in an open motif F conformation and release of the incorrect NTP [13]. Motif F conformations are the most dynamic among the genus Flavivirus as described in several resolved RdRp structures. These include the Japanese encephalitis virus (JEV) [14,15], homologous to TBEV RdRp, Dengue virus [16] and West Nile virus [17].

Viruses display high mutation rates when introduced to selective pressures such as antiviral drugs. Migliaccio and colleagues treated HCV infected cells with CMA and selected a clone with a single Ser-Thr mutation that was resistant to the antiviral effects of CMA [7]. How this single mutation assists *Flaviviridae* viruses to escape nucleoside analogues is not yet understood. Static representations, such as crystal structures, are limited in providing insight into this escape mechanism. Additionally, crystallization of the complex is improbable since the Ser-Thr mutant RdRp is resistant to incorporate the nucleoside analogue [7]. The only means available to investigate this phenomenon is by molecular simulations. The current study uses a stochastic molecular software to investigate the all-atom initial reaction of the triphosphate form of CMA (CMA-TP) with *Flaviviridae* RdRps (wild type and Ser-Thr mutant) to identify the molecular escape mechanism from nucleoside analogue inhibitors.

2. Materials and methods

The respective apo-form and NTP-bound RdRp crystal structures for HCV (PDBs: 1NB4 and 1NB6) and JEV (PDBs: 4K6M and 4HDH) were downloaded from the Protein Databank (PDB) [18]. The apo-form TBEV RdRp was a homology-generated model used in previous reports [2,19] and the NTP-bound TBEV modelled structure was produced using the same method. The Ser-Thr mutant RdRps were generated using Schrödinger's Maestro software [20]. This study focuses only on RdRps, therefore the N-terminus methyltransferase is truncated. It should be noted that as a result the numeration is ~270–300 less than the full *Flaviviridae* RdRp sequences. For instance, Ser331 of TBEV in this study corresponds to Ser603 in the full RdRp sequence (GenBank accession #: U39292.1).

All structures were optimized using the Schrödinger's Maestro Protein Preparation Wizard [21]. Electrostatic potentials for the *Flaviviridae* RdRp structural motifs were calculated using the Poisson-Boltzmann equation implemented in the Schrödinger's Maestro software. The multiple sequence alignment was performed using the MAFFT server [22] set to default settings. Based on the multiple sequence alignment, the structural conformational change per residue between the apo-form and NTP-bound RdRps was performed by the MultiSeq plug-in that uses STAMP to align structures [23] - implemented in the Visual Molecular Dynamics (VMD) software [24]. The VMD software generated the structural representations.

The stochastic molecular simulations were performed using the Metropolis Monte Carlo-based Protein Energy Landscape Exploration server (PELE) [25]. The PELE server, accessed at https://pele.bsc.es/, provides ready-made scripts for unconstrained protein-ligand binding site search and induced fit. The PELE software and its many applications are thoroughly explained elsewhere [25,26]. The PELE methods using a few modified NTP analogues and several *Flaviviridae* RdRps have also been reported [19]. Water molecules and two metal ions were included within the binding cavity of the RdRps. The stochastic software implements a force field known as the optimized potentials for liquid simulations (OPLS-2005) [27]. The OPLS force field was previously shown suitable for metals [28], however, it treats metals as ions and the bonds as ionic, not as covalent bonds. For the simulations, the bonds between the metal ions (Mn^{2+}) and the ligating atoms from the RdRps were bridged and converted to zero-order bonds. Modifications to the unconstrained and induced fit ready-made PELE script are detailed in Supplemental Text S1.

An initial PELE run was performed to generate three random seeds for three independent unconstrained simulations for all RdRps. A total of 24 computer-processing units (CPUs) were used for each simulation. The frame that showed the closest migration towards the active site was chosen from each replicate and RdRp to initiate the induced fit simulations. Although this will be considered an additional three random replicates, both unconstrained and induced fit simulations were combined for each replicate. The combined unconstrained and induced fit simulations resulted in ~1500 frames per replication. For the bar charts and each replicate, a 2-sample test was made for equality of proportions with continuity correction [29] to test for differences between the wild type (WT) and Ser-Thr mutant RdRps during CMA-TP exploration.

3. Results and discussion

3.1. HCV, JEV and TBEV RdRp structural motifs and their electrostatic potential

The structural motifs are boxed and labeled in the alignment of Fig. 1A with the catalytic, priming and interrogating residues color-coded. The tertiary structural positions of the motifs are highlighted in Fig. 1B. The catalytic residues are conserved in JEV and TBEV, except for the HCV UTP-binding [12] substitution of the Leu (L) to an Ala (A) in motif F (indicated by the green arrow). The interrogating and priming sites also show substitutions. The interrogating substitutions for JEV and TBEV are the N-terminus Arg (R) to a Gly (G) and the motif F Lys (K) to a Phe (F). The substitution in the C-terminus priming site is the Thr (T) of HCV to a Glu (E) in JEV/TBEV. The full alignment in Supplemental Fig. S1 shows that the N- and C-terminus are less conserved than the sequences within the structural motifs. This causes a misalignment in the PL between HCV and JEV/TBEV and is not shown in Fig. 1A for clarity.

The single RdRp Ser-Thr mutation that causes HCV resistance to the nucleoside analogue CMA [7] is within motif B - the Ser is conserved in all three RdRps as indicated by the black arrow in Fig. 1A. The HCV RdRp, however, has large gaps flanking both ends of motif B compared with JEV/TBEV. The structurally conserved motifs are located within specific domains as shown by the tertiary representations in Fig. 1B. Motifs G and F are part of the fingers domain, A-E belong to the palm domain and the PL to the thumb domain. The NTP-tunnel sandwiched between motif F and the palm domain is clearly viewed 180° from the conventional right-hand orientation. Fig. 1C shows that the electrostatic potential within motif F and the palm domain differ between Flavivirus RdRps and HCV RdRp. The NTP-tunnel aperture of Flavivirus is also wider than HCV.

The α-carbon backbone root mean square deviation (RMSD) between the apo-forms of HCV and JEV/TBEV is 3.7 Å. The superposed apo-form structure in Fig. 2A shows the tertiary position of the catalytic, priming and interrogating residues (Fig. 1A) within motif F and the palm domain, specifically motifs A, C and E (i.e., the NTP-tunnel). The catalytic site within motifs A and C contains the conserved Asp residues that coordinate with the metal ion co-factors [12]. The residues of the priming site (motif E and the C-terminus) coordinate with the phosphate groups of NTPs and the

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