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Flaviviral methyltransferase/RNA interaction: Structural basis for enzyme inhibition

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ABSTRACT

Flaviviruses are the causative agents of severe diseases such as Dengue or Yellow fever. The replicative machinery used by the virus is based on few enzymes including a methyltransferase, located in the N-terminal domain of the NS5 protein. Flaviviral methyltransferases are involved in the last two steps of the mRNA capping process, transferring a methyl group from S-adenosyl-L-methionine onto the N7 position of the cap guanine (guanine-N7 methyltransferase) and the ribose 2′O position of the first nucleotide following the cap guanine (nucleoside-2′O methyltransferase). The RNA capping process is crucial for mRNA stability, protein synthesis and virus replication. Such an essential function makes methyltransferases attractive targets for the design of antiviral drugs. In this context, starting from the crystal structure of Wesselsbron flavivirus methyltransferase, we elaborated a mechanistic model describing protein/RNA interaction during N7 methyl transfer. Next we used an in silico docking procedure to identify commercially available compounds that would display high affinity for the methyltransferase active site. The best candidates selected were tested in vitro to assay their effective inhibition on 2′O and N7 methyltransferase activities on Wesselsbron and Dengue virus (Dv) methyltransferases. The results of such combined computational and experimental screening approach led to the identification of a high-potency inhibitor.

1. Introduction

The genus *Flavivirus* comprises over 70 RNA viruses, many of which are important human pathogens. Dengue virus (Dv), as an example, is estimated to infect about 50 million people a year, causing 24,000 deaths (Guha-Sapir and Schimmer, 2005). Currently, no specific antiviral drugs are available against flaviviral infections, and their development has been an active field of research during recent years (Sampath and Padmanabhan, 2009).

The flaviviral genome consists of 11 kb; it is decorated with a 'cap-1' structure at the strictly conserved 5′ terminus $^{N7Me}GpppA_{2'OMe}$G-RNA. The genome encodes a 370-kDa polyprotein precursor, which is processed by viral and cellular proteases into three structural proteins and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) involved in virus replication (Fields et al., 2001). Among other proteins, the multifunctional protein NS5 is particularly important for viral replication. The C-terminal domain of NS5 is endowed with RNA-dependent RNA polymerase (RdRp) activity, while its N-terminal domain contains S-adenosyl-L-methionine (AdoMet)-dependent methyltransferase (MTase) activity. The MTase domain is involved in the last two steps of the capping process that starts with the conversion of the 5′-triphosphate end of the nascent RNA into a 5′-diphosphate (RNA triphosphatase activity), followed by the addition of a GMP unit *via* a 5′–5′ phosphodiester bond (guanylyltransferase activity). MTase, then, transfers a methyl group from AdoMet to the N7-atom of the cap guanine and, successively, to the ribose 2′O-atom of the first RNA nucleotide forming the 'cap 0' and 'cap 1' structures, respectively.

The crystal structures of the MTase domain from several viruses, in complex with AdoMet, S-adenosyl-L-homocysteine (the co-product of methyl transfer, AdoHcy), with GTP or with cap-analogues (Assenberg et al., 2007; Bollati et al., 2009; Egloff et al., 2002, 2007) are taken here as starting data for an analysis of the

Abbreviations: RdRp, RNA dependent RNA polymerase; N7 MTase, guanine N7 methyltransferase; 2′O MTase, nucleoside-2′O methyltransferase; AdoMet, S-adenosyl-L-methionine; AdoHcy, S-adenosyl-L-homocysteine; Wv, Wesselsbron virus; NS, non-structural protein; GMP, guanosine monophosphate; GTP, guanosine triphosphate; Dv, Dengue virus; WNv, West Nile virus; LBS, putative low affinity RNA binding site; HBS, high affinity RNA binding site; ATA, aurintricarboxylic acid; PPNDS, pyridoxal-5′-phosphate-6-(2′-naphthylazo-6′-nitro-4′,8′-disulfonate) tetrasodium salt; PMSF, phenylmethylsulphonyl fluoride; TLC, thin-layer chromatography.

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main structural and mechanistic features related to MTase activity. Since flaviviral MTase displays both N7 and 2′O activities (Ray et al., 2006; Zhou et al., 2007), in order for capped RNA to be methylated at the two different sites the nucleic acid substrate must adopt two distinct binding modes relative to the enzyme active site. In particular, many structural results (Assenberg et al., 2007; Bollati et al., 2009; Egloff et al., 2002, 2007) show that small capped RNA analogues bind to a MTase high affinity binding site (HBS), where they are supposed to be held during 2′O methylation. On the contrary, structural details on a distinct binding site occupied by capped RNA during N7 methylation are missing. Structural considerations, however, suggest the existence of a secondary, putative low affinity binding site (LBS) (Dong et al., 2008a; Mastrangelo et al., 2007) located in a positively charged region close to the AdoMet binding site. Here, we first present a model of a short capped RNA (GpppAGUp) bound to the LBS of Wesselsbron virus MTase (^{Wv}MTase), that is used to explore the details of protein/RNA interaction during N7 methyl transfer. Based on this analysis, we then discussed an in silico search that allowed to select three synthetic compounds, predicted to display low free energy of binding to ^{Wv}MTase. The compounds selected were tested in vitro on ^{Wv}MTase and ^{Dv}MTase in 2′O and N7 methyltransferase activity assays. The results of the combined computational and experimental screening allowed us to identify aurintricarboxylic acid (ATA) as the most potent flaviviral MTase inhibitor known to date. The inhibitory effect of ATA is discussed here on the basis of existing models of the flaviviral MTase mechanism for N7 and 2′O cap methylation. Since flavivirus MTase has been shown by mutagenesis to be crucial for viral replication (Zhou et al., 2007), shedding light on MTase inhibition may prove a productive path towards the development of therapeutics against life threatening flaviviral diseases (Dong et al., 2008a,b).

2. Materials and methods

2.1. Chemical database for virtual screening and reagents

The virtual Library of Pharmacologically Active Compounds (LOPAC) used for the docking analysis was provided by Sigma–Aldrich, and included 1280 commercially available compounds (www.sigmaaldrich.com). AdoMet was purchased from New England BioLabs. The compounds tested in vitro, ATA, nilutamide and pyridoxal-5′-phosphate-6-(2′-naphthylazo-6′-nitro-4′,8′-disulfonate) tetrasodium salt (PPNDS), were from Sigma–Aldrich. Compounds were dissolved at 20 mM in H_2O, or at 8 mM in 0.1 mM NaOH (ATA), and stored at −20 °C.

2.2. Locating a short capped RNA in the LBS

In order to produce an MTase model suitable for the analysis of the N7 methylation process, the crystal structure of ^{Wv}MTase in its complex with AdoMet and with an HBS-bound small capped RNA analog, ^{N7Me}GpppG, solved at 1.9 Å resolution (pdb 3ELW) (Bollati et al., 2009) was used. Hydrogen atoms and computed Gasteiger charges (Gasteiger and Marsili, 1978) were added after removing the HBS-bound RNA analog, maintaining the AdoMet cofactor bound to the protein. A squared grid (30 Å side) centered roughly in the LBS region between Ser56 and Arg84 (Autogrid4; step size 0.375 Å, 80 × 80 × 80 = 512,000 points) (Goodford, 1985) was subsequently built and used as the volume explored in the capped RNA docking searches. The short capped RNA GpppAGUp molecule was then built using the program Ghemical (http://www.bioinformatics.org/ghemical). Seventy genetic algorithm searches were performed using Autodock4, moving GpppAGUp within the described grid and using 32 active torsions in the ligand molecule (with 150 individuals in population and 27,000 generations; Morris et al., 1998). The search produced a list of 70 capped RNA positions within the explored LBS volume, ranked by means of the Estimated Free Energy of Binding (ΔG) that varied between −3.32 and −12.43 kcal/mol. Among the four best ΔG values (in the −11.74, −12.43 kcal/mol range) the model with the cap Guanine N7 atom closer to the AdoMet methyl group was chosen (−11.86 kcal/mol; AdoMet-CH3–N7-RNA distance 7.3 Å). Such model (^{Wv}MTase/GpppAGUp) was then used as starting structure for molecular dynamics (MD) simulations, performed using the program GROMACS (van der Spoel et al., 2005). Briefly, the protein with AdoMet and the docked capped RNA (GpppAGUp) were enclosed in a box of 61 Å × 68 Å × 91 Å filled with 11,688 water molecules and 6 Cl^- ions for charge equilibration. Using the GROMACS force field (GROMOS-87 with corrections as in Mark et al., 1994), the energy was minimized with a steepest descent algorithm, and the system was then equilibrated at 10 K for 1 ps and at 100 K for 5 ps. The MD simulation was carried out with time step of 1 fs using a leap-frog algorithm and periodic boundary conditions; electrostatic interactions were treated with Fast Particle-Mesh Ewald algorithm, while van der Waals interactions were cut-off at 12 Å; the simulation was performed at fixed T (300 K) and P (1 atm) using Berendsen coupling (Berendsen et al., 1984). The structure produced after 9 ns was used as final model for the analysis of protein/RNA interaction.

2.3. In silico search for MTase inhibitors

The AutoDock4 software package (Morris et al., 1998) was used for a docking search using compounds from the LOPAC library, and Python Molecule Viewer 1.4.5 (MGL-tools package http://www.mgltools.scripps.edu/) to analyze the data. As docking model the atomic coordinates of ^{Wv}MTase in complex with AdoMet and ^{N7Me}GpppG were chosen (pdb 3ELW) (Bollati et al., 2009), keeping both the protein and the AdoMet molecule and removing water and the cap-analogue. Hydrogen atoms and computed Gasteiger charges were added using the program Autodock4 (Gasteiger and Marsili, 1978). The protein model was then used to build a discrete grid within a box with dimensions 23 Å × 15 Å × 26 Å (Autogrid4; step size 0.375 Å, 60 × 40 × 70 = 168,000 points) (Goodford, 1985) for the compound docking search. The search (i.e. the grid center position) was centered in a wide crevice located between the AdoMet binding site and the RNA HBS, more precisely between residues Lys182 and Arg213. In this way the protein active site, where the methyl transfer from AdoMet occurs, is completely included in the search grid chosen. Twenty genetic algorithm searches were run using Autodock4 for each compound in the LOPAC library (with 150 individuals in the population and 27,000 generations) (Morris et al., 1998).

2.4. In vitro synthesis of capped RNA

The ^{7Me}GpppAC$_5$ RNA substrate, used for the 2′O MTase assay, was synthesized by incubating the DNA oligonucleotide CCCCGGGTCT$_{25}$ with the bacteriophage T7 DNA primase in the presence of ^{7Me}GpppA and CTP as described (Peyrane et al., 2007). The reaction products obtained after a 48 h incubation period were purified by reverse phase chromatography in HPLC. We collected the peak corresponding to ^{7Me}GpppAC$_5$ as described (Peyrane et al., 2007).

The RNA substrate used for N7 MTase assay corresponding to the authentic 5′-terminal 351 nucleotides of the Dv genome (Dv$_{1-351}$ RNA) was obtained as follows. The 5′ UTR of Dv serotype 2, New Guinea C strain was amplified by PCR using the primers BamH1-Φ2.5Dv-5′(s) (CGGGATCCCAGTAATACGACTCACTATTAGTTGTTAG-TCTACGTGGACC) and EcoR1-Dv-351(as) (GGAATTCGGTGGTGCA-GATGAACTTCAG), and cloned in the pUC18 (Fermentas) plasmid