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Selective inhibition of the West Nile virus methyltransferase by nucleoside analogs

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ABSTRACT

The flavivirus methyltransferase (MTase) sequentially methylates the N-7 and 2′-O positions of the viral RNA cap (GpppA-RNA→m^7GpppA-RNA→m^7GpppAm-RNA), using S-adenosyl-L-methionine (SAM) as a methyl donor. We report here the synthesis and biological evaluation of a series of novel nucleoside analogs. Two of these compounds can effectively and competitively inhibit the WNV MTase with IC_{50} values in micromolar range and, more importantly, do not inhibit human MTase. The compounds can also suppress the WNV replication in cell culture.

1. Introduction

Most flaviviruses, such as dengue viruses (DENVs), West Nile virus (WNV), yellow fever virus (YFV), Japanese encephalitis virus (JEV), and tick-borne encephalitis virus (TBEV), cause significant human disease. Approximately 2.5 billion people are at risk of DENV infection, with an estimated 500,000 cases of life-threatening disease per year. In addition, WNV is now the leading cause of arboviral encephalitis in the US (USGS, 2010), resulting in more than a thousand human deaths (CDC, 2010). However, vaccines for humans currently are available only for YFV, JEV, and TBEV (Burke and Monath, 2001); and more importantly no clinically approved antiviral therapy is available for treatment of flavivirus infection. Therefore, it is a public health priority to develop antiviral agents for post-infection treatment (Kramer et al., 2007; Sampath and Padmanabhan, 2009).

Most eukaryotic and viral mRNAs possess a 5′-cap that is important for mRNA stability and efficient translation (Furuichi and Shatkin, 2000). We and others have shown that recombinant NS5 proteins from various flaviviruses possess both N-7 and 2′-O MTase activities (Dong et al., 2007; Egloff et al., 2002; Kroschewski et al., 2008; Ray et al., 2006; Zhou et al., 2007). Flavivirus MTase has been shown to be essential for WNV (Dong et al., 2007; Zhou et al., 2007), KUNV (Khromykh et al., 1998), YFV (Bhattacharya et al., 2008), and DENV replication (Kroschewski et al., 2008). Several MTase inhibitors, some of which showed antiviral efficacy in cell cultures, have been reported (Benarroch et al., 2004; Dong et al., 2008b; Lim et al., 2008, 2011; Luzhkov et al., 2007; Milani et al., 2009; Podvinec et al., 2010; Selisko et al., 2010). Some of these potential inhibitors targeted the co-factor SAM-binding site. However, it was known that SAM is also a methyl donor for host RNA and protein methylations. Inhibitors targeting the SAM pocket may nonspecifically suppress host MTases, resulting in toxicity. We have identified a flavivirus-specific pocket near the SAM-binding site of flavivirus MTase (Dong et al., 2010). Recently, a highly selective inhibitor of the flavivirus MTase but not against human MTases was reported to target this pocket, although the antiviral efficacy of the compound has not been characterized (Lim et al., 2011).

The aim of the present study was to investigate whether selective inhibitors can be identified. We chose five nucleoside analogs and found that these nucleoside analogs can inhibit the methyltransferase activities of the WNV MTase to various degrees. Two compounds were found to have higher potency than others and they did not inhibit the activity of human RNA MTase.

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2. Materials and methods

2.1. Compounds

The nucleoside analogs were synthesized as previously described (Ghosh and Kass, 2010). Sinefungin (SIN) was purchased from Sigma–Aldrich. S-adenosyl-methionine (SAM) was purchased from New England Biolabs. [α-^{32}P]GTP was purchased from MP Biomedicals.

2.2. In vitro MTase inhibition assay

The 5′-end-labeled substrates G*pppA-RNA and m^{7}G*pppA-RNA, representing the first 90 nucleotides of the WNV genome (the asterisk indicates that the following phosphate is ^{32}P labeled), were prepared as described previously (Dong et al., 2008b; Zhou et al., 2007). The N-7 and 2′-O methylation inhibition assays were performed as described previously (Dong et al., 2008b; Ray et al., 2006). The N-7 methylation was measured by conversion of G*pppA-RNA→m^{7}G*pppA-RNA. The 2′-O methylation was monitored by conversion of m^{7}G*pppA-RNA→m^{7}G*pppAm-RNA. Both methylation assays were performed with 1.5 μM WNV MTase, 80 μM SAM, 0.36 μM G*pppA-RNA or m^{7}G*pppA-RNA substrate, and various concentrations of each compound. The methylation reactions were digested with nuclease P1 to release cap moieties (m^{7}G*pppAm, m^{7}G*pppA, and G*pppA). The cap molecules were separated on a thin-layer chromatograph (TLC), and quantified by a PhosphorImager (Dong et al., 2008b; Ray et al., 2006). The percentage of activity was determined after quantification of m^{7}G*pppA, m^{7}G*pppAm, and G*pppA. The IC_{50} value, unless specified, was determined by fitting of the dose–response curve using the ORIGIN software package. K_i was calculated according to the Cheng–Prusoff equation (Cheng and Prusoff, 1973) ($K_i = IC_{50}/(1 + [S]/K_m)$, where K_i is the inhibition constant of the inhibitor, [S] is substrate concentration and K_m is the concentration of substrate at which enzyme activity is at half maximal (Chung et al., 2010)).

2.3. Inhibition of human RNA MTase (hRNMTase)

The human guanine N-7 RNA MTase was overexpressed as a GST-fusion protein in *Escherichia coli*. BL21 (DE3) cells as previously described (Pillutla et al., 1998). Recombinant hRNMTase was purified through a glutathione Sepharose 4B affinity column (GE Healthcare), followed by gel filtration chromatography using a Superdex S-200 column (GE Healthcare) to ensure that protein purity was >99%. The protein was concentrated using an Amicon stirred cell (Millipore), aliquoted and stored at −80 °C.

The inhibition assay of hRNMTase was performed with the same condition used for the WNV N-7 MTase inhibition assay, except that the WNV MTase was replaced by hRNMTase. The hRNMTase reaction contained 0.3 μM hRNMTase, 80 μM SAM, 0.36 μM 5′-end-labeled 90nt WNV substrates G*pppA-RNA, and compounds at various concentrations.

2.4. SAM binding assay

A G25 filtration assay was used to evaluate compound competitive inhibition of SAM binding to the WNV MTase. A 50-μl reaction mixture contains 0.6 μM WNV MTase (or 0.12 μM hRNMTase), 1 μl of 10-fold diluted [methyl-^{3}H]-SAM (78 Ci/mmol, Perkin Elmer) (about 14.1 nM final concentration), and each compound at a concentration series of 3-fold dilution of 60 μM (or 300 μM for hRNMTase). The reaction mixtures were incubated at room temperature for 2 h, loaded to the Biomax Spin-25 Mini-column, and centrifuged at 5000 rpm for three minutes. Eluted samples were quantified by Geiger scintillation counter (Beckman LS6500). All data points were done in duplicate.

2.5. Ligand docking

The initial 3D coordinates for compound 2 were generated using the Frog2.1 server (http://bioserv.rpbs.jussieu.fr/cgi-bin/Frog2) (Miteva et al., 2010) from the SMILES description for compound 2, with no additional optimization. The parameters used were: no disambiguation, no stage 2 Monte-Carlo, no minimization, a single conformation requested, Ewindow 50.0, Emax 500.0, and RMSD 0.8. CHARMM General Force Field (CGENFF) topology and parameters (Vanommeslaeghe et al., 2010) for the compound were then generated using the ParamChem server (https://www.paramchem.org/) (Malde et al., 2011), with the parameters for the silicon atom obtained by transfer of parameters from an alkyl tetrahedral carbon. All further optimization and docking of the compound were performed using the CHARMM program, version 35b3 (Brooks et al., 2009). The initial compound conformation was optimized using 10,000 steps of steepest descent (SD) and adopted basis Newton Raphson (ABNR) minimizations with a harmonic constraint of 0.5 kcal/mol on all non-hydrogen atoms and a convergence tolerance of 0.0001 kcal/mol between successive steps. The compound was then oriented in the binding pocket of the WNV MTase using corresponding non-hydrogen atoms of the furanose sugar entity in the inhibitor sinefungin (PDB ID: 3LKZ, chain A). The sugar atoms used in the orientation were then fixed and the rest of the compound was optimized in isolation using optimization protocol 1, which consisted of (a) 10,000 SD and ABNR steps with a tolerance of 0.01 kcal/mol, (b) 2000 steps of Langevin dynamics at 1000 K with a friction coefficient of 1.0/ps, (c) 10,000 SD and ABNR steps with a tolerance of 0.01 kcal/mol. The compound was then optimized further in the presence of the protein by simulated annealing, first with its sugar non-hydrogen atoms and all protein atoms held fixed, then with the same sugar atoms and all non-hydrogen atoms of the protein held by weak harmonic constraints of 1 kcal/mol. The simulated annealing consisted of successive application of protocol 1 with a temperature bath for the Langevin dynamics set to 100, 200, 300, 200, and 100 K. It was observed that the sidechain of His110 reoriented significantly from the original crystal structure due to steric clashes with the 2′ sugar substituent in compound 2. The interaction of this sidechain was further optimized in isolation using protocol 1 and additional miscellaneous mean field potential (MMFP) distance restraints between atoms ND1 and NE2 of His110 and the nearest oxygen atom of compound 2. The vacuum interaction energy between compound 2 and the WNV MTase in this final docked conformation was −97.8 kcal/mol, which decomposed into a van der Waals component of −58.6 kcal/mol and an electrostatic component of −39.2 kcal/mol.

2.6. Cytotoxicity assay

Cytotoxicity was measured by a MTT cell proliferation assay using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide method(ATCC). Approximately 2×10^4 human A549 cells in 100 μl of media were seeded into 60 wells of a 96 well plate, the remaining wells held media. Plates were held at RT for 1 h and then incubated for 20–24 h. The media was removed and 100 μl of media containing decreasing concentrations of antiviral compound in 1% DMSO were added to the wells. All determinations were performed in triplicate. After 42 h incubation at 37 °C, 10 μl of MTT was added to the wells and incubated another 3 h. Detergent (100 μl) was placed in the wells and the plate was incubated for 3 h at room temperature in the dark. A microtiter plate reader (Ely808, BioTek Instruments, Inc.) with a 570 nm filter was used