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The Octadecyloxyethyl Ester of (*S*)-9-[3-Hydroxy-2-(Phosphonomethoxy) Propyl]Adenine Is a Potent and Selective Inhibitor of Hepatitis C Virus Replication in Genotype 1A, 1B, and 2A Replicons[▽]

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The octadecyloxyethyl (ODE) and hexadecyloxypropyl (HDP) esters of (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine (HPMPA) are potent inhibitors of orthopoxvirus, herpesvirus, human immunodeficiency virus type 1, and hepatitis B virus replication in vitro. HDP and ODE esters of (*S*)-HPMPA and (*R*)-HPMPA were evaluated for their activity in hepatitis C virus (HCV) replicon assays using luciferase (1B and 2A replicons) or RNA (1B) quantification. The ODE ester of (*S*)-HPMPA [ODE-(*S*)-HPMPA] was the most active compound, with 50% effective concentrations (EC_{50}s) in the 0.69 to 1.31 μM range. HDP and ODE esters of (*R*)-HPMPA were severalfold less active, while (*S*)-HPMPA and (*R*)-HPMPA were inactive. In genotype 1A and 1B replicons analyzed by HCV RNA analysis, ODE-(*S*)-HPMPA was the most active compound, with EC_{50}s of 1.8 and 2.1 μM, respectively.

It is estimated that 3 to 4 million persons in the United States are chronically infected with hepatitis C virus (HCV). Additionally, an increasing fraction of the HCV-infected population develops hepatic morbidity over time. Although combination therapy with peginterferon and ribavirin has improved the treatment options for this infection, highly effective and well-tolerated therapy has yet to be realized. The NS5B RNA polymerase of HCV is required for viral replication, and a number of nucleoside and nonnucleoside inhibitors of the enzyme have been described previously (7, 8, 14, 19, 23, 24). Antiviral nucleoside inhibitors that act at the polymerase active site are particularly attractive given that they are active across different HCV genotypes, present a high barrier to resistance (17), and have been successful in other chronic viral infections such as those with human immunodeficiency virus type 1 (HIV-1) and hepatitis B virus (HBV).

Acyclic nucleoside phosphonates, such as cidofovir, adefovir, and tenofovir, have been developed as effective therapy for a number of viral infections, including cytomegalovirus (CMV), HBV, and HIV-1 (6, 9). However, the negative charges associated with the phosphonate moiety limit their oral bioavailability and cellular penetration (5). This has required the development of the currently marketed prodrugs adefovir dipivoxil and tenofovir disoproxil fumarate. (*S*)-9-[3-Hydroxy-2-(phosphonomethoxy)-propyl]adenine [(*S*)-HPMPA] is active in vitro against herpesviruses (2) and orthopoxviruses (13). We have shown that the hexadecyloxypropyl (HDP) and octadecyloxyethyl (ODE) esters of HPMPA have multiple-log increases in antiviral activity in vitro compared with the unmodified compound against HIV-1, orthopoxviruses, and CMV (3, 10). Esterification with HDP or ODE greatly increases cell uptake and eventual conversion to the acyclic nucleotide diphosphate, the active metabolite of this class of agents (1, 16). This strategy also increases the oral bioavailability of these compounds, making them potentially attractive therapeutics (4, 20, 22).

We synthesized the HDP and ODE esters of (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine [HDP-(*S*)-HPMPA and ODE-(*S*)-HPMPA, respectively] as previously described (3). The HDP and ODE esters of (*R*)-9-[3-hydroxy-2-(phosphonomethoxy)-propyl]adenine [HDP-(*R*)-HPMPA and ODE-(*R*)-HPMPA, respectively] were synthesized as previously described (3) by substituting (*R*)-trityl glycidyl ether for (*S*)-trityl glycidyl ether. Structures of the alkoxyalkyl esters ODE-(*S*)-HPMPA and ODE-(*R*)-HPMPA are shown in Fig. 1. Compounds were assessed by high-performance liquid chromatography, thin-layer chromatography, proton nuclear magnetic resonance, and liquid chromatography-mass spectrometry and were judged to be >98% pure.

The HCV genotype 1B replicon BM4-5 FEO has been previously described (26) and contains a firefly luciferase-neomycin phosphotransferase fusion protein. The SGR-JFH FEO replicon, based on the full-length JFH-1 genotype 2A HCV virus (25), was created by insertion of the PacI-KpnI fragment of the BM4-5 FEO plasmid into the PacI-KpnI-digested SGR-JFH1/Luc plasmid (Mighty Mix; Takara). The SGR-JFH1/Luc plasmid has been previously described (12) and is a bicistronic replicon with the HCV internal ribosome entry site directing translation of the firefly luciferase gene and an encephalomyocarditis virus internal ribosome entry site identical to the one found in the BM4-5 replicon. The sequence of the SGR-JFH FEO plasmid was verified by automated DNA sequencing.

The BM4-5 and SGR-JFH1 FEO plasmids were linearized with ScaI and XbaI, respectively; the SGR-JFH1 FEO plasmid was digested with mung bean nuclease to generate an authentic

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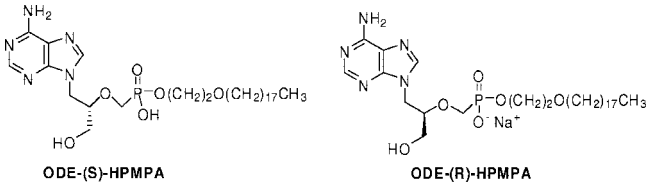

FIG. 1. Structures of ODE-(*S*)-HPMPA and ODE-(*R*)-HPMPA. The structure of the (*R*) and (*S*) isomers of ODE-HPMPA is shown. The structure of the (*R*) and (*S*) isomers of HDP-HPMPA (not shown) is identical to that of ODE-HPMPA except that the alkoxyalkyl moiety is $-(CH_2)_3O(CH_2)_{15}CH_3$.

3′ end (New England Biolabs). In vitro transcription and generation of cell lines stably expressing the BM4-5 FEO and SGR-JFH-1 FEO replicons were accomplished by electroporation of human hepatoma Huh-7.5.1 cells (a kind gift from Francis Chisari, Scripps Research Institute, La Jolla, CA) and selection with 500 μg/ml of G-418 as we have previously described (26).

Compound activity assays were carried out as previously described using 10,000 (BM4-5- or JFH-1-based) FEO replicon cells per well on 96-well plates (26). Cells and compounds were incubated for 48 to 72 h with all conditions run in triplicate. Additionally, three or four experimental replicates were completed per compound. Luciferase activity was determined using a microplate luminometer (Veritas microplate luminometer; Turner Biosystems) according to the manufacturer's instructions (Bright-Glo; Promega). Relative light units for each condition were used to generate a dose response for each compound by using Prism (version 4; GraphPad Software). The cytotoxicity of each compound was determined using a fluorescent cell viability and death assay (MultiTox-Fluor; Promega). All compounds were tested at concentrations of up to 100 μM (0.3% dimethyl sulfoxide final concentration). In other experiments, anti-HCV activity was determined in genotype 1A and 1B replicons by HCV RNA analysis as previously described (15, 18). Briefly, replicon cell lines were maintained as subconfluent cultures on 96-well plates. Compounds were added daily for 3 days in fresh medium. Twenty-four hours after the last dose of compound, antiviral activity was determined by blot hybridization analysis of intracellular HCV RNA, and cytotoxicity was assessed by neutral red dye uptake. Values for 50% effective concentration (EC_{50}) and for the minimal concentration required to induce 50% cleavage (CC_{50}) were calculated for each test compound by linear regression analysis, using data combined for all treated cultures. Antiviral and toxicity assays utilized triplicate cultures for each drug concentration; 12 untreated cultures were included in each assay. HCV and β-actin RNA quantitation standards were included on each individual hybridization blot (15, 18).

ODE-(*S*)-HPMPA was the most active compound in the luciferase assays with EC_{50}s of 1.31 and 0.69 μM in 1B and 2A replicons, respectively, while the HDP esters were generally less active (Table 1). In general, the EC_{50} of the alkoxyalkyl compounds is about fourfold higher in the genotype 2 replicon system (compared to those in genotype 1B) with the exception of ODE-(*S*)-HPMPA, which shows similar activity levels in both 1B and 2A replicons. (*S*)-HPMPA and (*R*)-HPMPA were inactive in both genotype 1 and 2 luciferase replicon systems, consistent with the poor penetration of cells as reported previously for (*S*)-HPMPA (16). ODE-(*R*)-HPMPA and HDP-(*R*)-HPMPA were also active in vitro but were significantly less active than the corresponding (*S*)-HPMPA enantiomers. However, the (*R*) enantiomers were less cytotoxic, with CC_{50} values of >100 μM versus 35.6 and 85.3 μM for ODE-(*S*)-HPMPA and HDP-(*S*)-HPMPA, respectively. The selectivity index for ODE-(*S*)-HPMPA was 27 to 52 (Table 1). The selectivity indices for the current compounds are moderate, in the range of 20 to 40, but encouraging for lead compounds. It should be kept in mind that the cytotoxicity testing was only performed in Huh-7.5.1 cells, a liver cancer cell line, which may be more prone to the antiproliferative effects of nucleosides than are primary hepatocytes or non-tumor-based cell lines (11). In HCV RNA assay results of genotype 1A and 1B replicons, ODE-(*S*)-HPMPA was active with EC_{50} values of 1.8 ± 0.3 and 2.1 ± 0.2 μM, similar to those observed in genotypes 1B and 2A with the luciferase assay (Table 1). This finding rules out the possibility that the antiviral activity noted in luciferase assays is artifactual due to effects of the compounds on luciferase activity or ATP pools.

There are currently few anti-HCV nucleosides in clinical studies; the development of NM283 (2′-*C*-methylcytidine prodrug) and R1626 (4′-azidocytidine prodrug) was recently discontinued. Additionally, most compounds currently or recently under clinical investigation select for one of two polymerase resistance mutations—the S282T mutation for 2′-*C*-methyl compounds (19) or the S96T mutation for 4′-azidocytidine (17). Thus, new anti-HCV nucleoside candidates are needed, particularly if they possess unique resistance profiles.

Taken together the alkoxyalkyl derivatives of HPMPA offer low micromolar potency with reasonable toxicity profiles and are therefore potential lead compounds which can be modified in attempts to enhance potency and/or decrease toxicity. HDP-(*S*)-HPMPA and ODE-(*S*)-HPMPA are orally bioavailable and are active in lethal animal models of orthopoxvirus disease (21) and murine and human cytomegalovirus (22). HDP-(*S*)-HPMPA provides excellent drug exposure in the liver when

TABLE 1. Effect of HDP-(*S*)-HPMPA and ODE-(*S*)-HPMPA on HCV replication in vitro

Compound[a]	EC_{50} (μM)	CC_{50} (μM)	Selectivity (CC_{50}/EC_{50})
BM4-5 FEO, genotype 1B			
(*S*)-HPMPA	>100	>100	
(*R*)-HPMPA	>100	>100	
ODE-(*S*)-HPMPA (4)	1.31 ± 0.66	35.6 ± 6.8	27
ODE-(*R*)-HPMPA (4)	7.0 ± 8.21	>100	>14
HDP-(*S*)-HPMPA (4)	2.02 ± 0.95	85.3 ± 6.8	42
HDP-(*R*)-HPMPA (4)	6.71 ± 2.95	>100	>15
SGR-JFH-1 FEO, genotype 2A			
(*S*)-HPMPA	>100	>100	
(*R*)-HPMPA	>100	>100	
ODE-(*S*)-HPMPA (4)	0.69 ± 0.71	35.6 ± 6.8	52
ODE-(*R*)-HPMPA (4)	26.7 ± 14.3	>100	>4
HDP-(*S*)-HPMPA (4)	6.5 ± 12.5	85.3 ± 20.8	13
HDP-(*R*)-HPMPA (4)	41.0 ± 3.61	>100	>2

[a] Data are the means ± standard deviations. The number in parentheses is the number of replicate determinations.

given orally and is orally active in transgenic HBV mice (J. D. Morrey, B. E. Korba, J. R. Beadle, D. L. Wyles, and K. Y. Hostetler, submitted for publication). Studies are currently under way to isolate and characterize drug-resistant mutants and to evaluate whether replicon cells can be cured by longer drug exposures. We are also evaluating the activity of acyclic nucleoside phosphonates containing other nucleobases and other lipid ester side chains to better define the spectrum of antiviral activity, oral pharmacokinetics, and mechanism of action and to determine resistance profiles.

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