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PSI-7851, a Pronucleotide of β-D-2′-Deoxy-2′-Fluoro-2′-*C*-Methyluridine Monophosphate, Is a Potent and Pan-Genotype Inhibitor of Hepatitis C Virus Replication▽

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The hepatitis C virus (HCV) NS5B RNA polymerase facilitates the RNA synthesis step during the HCV replication cycle. Nucleoside analogs targeting the NS5B provide an attractive approach to treating HCV infections because of their high barrier to resistance and pan-genotype activity. PSI-7851, a pronucleotide of β-D-2′-deoxy-2′-fluoro-2′-*C*-methyluridine-5′-monophosphate, is a highly active nucleotide analog inhibitor of HCV for which a phase 1b multiple ascending dose study of genotype 1-infected individuals was recently completed (M. Rodriguez-Torres, E. Lawitz, S. Flach, J. M. Denning, E. Albanis, W. T. Symonds, and M. M. Berry, Abstr. 60th Annu. Meet. Am. Assoc. Study Liver Dis., abstr. LB17, 2009). The studies described here characterize the *in vitro* antiviral activity and cytotoxicity profile of PSI-7851. The 50% effective concentration for PSI-7851 against the genotype 1b replicon was determined to be 0.075 ± 0.050 μM (mean ± standard deviation). PSI-7851 was similarly effective against replicons derived from genotypes 1a, 1b, and 2a and the genotype 1a and 2a infectious virus systems. The active triphosphate, PSI-7409, inhibited recombinant NS5B polymerases from genotypes 1 to 4 with comparable 50% inhibitory concentrations. PSI-7851 is a specific HCV inhibitor, as it lacks antiviral activity against other closely related and unrelated viruses. PSI-7409 also lacked any significant activity against cellular DNA and RNA polymerases. No cytotoxicity, mitochondrial toxicity, or bone marrow toxicity was associated with PSI-7851 at the highest concentration tested (100 μM). Cross-resistance studies using replicon mutants conferring resistance to modified nucleoside analogs showed that PSI-7851 was less active against the S282T replicon mutant, whereas cells expressing a replicon containing the S96T/N142T mutation remained fully susceptible to PSI-7851. Clearance studies using replicon cells demonstrated that PSI-7851 was able to clear cells of HCV replicon RNA and prevent viral rebound.

Hepatitis C virus (HCV) currently affects more than 170 million people worldwide. Approximately 70% of infected individuals develop chronic hepatitis, among whom about 20% will develop liver cirrhosis and fibrosis and up to 5% will progress to hepatocellular carcinoma (2). The current standard of care (SOC), which combines pegylated alpha interferon (PegIFN-α) and ribavirin (RBV), has limited efficacy in providing a sustained virological response (SVR), especially in individuals with HCV genotype 1 (~50%), the most prevalent genotype in Western countries (8, 11, 35). The impact of genetic diversity of HCV in patients receiving SOC therapy has been reviewed (26): SVR rates are higher in patients infected with genotype 2 or 3 (~80%), patients infected with genotype 4 appear to have a slightly better SVR rate (~60%) than patients infected with genotype 1, and patients infected with genotypes 5 and 6 may achieve an SVR at a level between those of genotypes 1 and 2/3. In addition to the variability in efficacy, the lengthy treatment (24 to 48 weeks) with SOC is frequently associated with undesirable side effects that may include anemia, fatigue, and depression (7). There is an urgent medical need to develop anti-HCV therapies that are safer and more effective. Direct-acting antivirals (DAAs) are compounds that target a specific viral protein. Currently, four major classes of DAAs are being investigated in phase II or III clinical trials: NS3 protease inhibitors, NS5A inhibitors, allosteric non-nucleoside NS5B polymerase inhibitors, and nucleoside/-tide NS5B polymerase inhibitors (21, 27, 46). Challenges for these DAAs include safety, pan-genotypic activity, and/or emergence of resistant viruses. An effective antiviral therapy against hepatitis C should encompass a broad spectrum of activity against all HCV genotypes, shorten treatment duration, have minimal side effects, and have a high barrier to resistance.

The HCV NS5B RNA-dependent RNA polymerase (Pol) is

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a critical component of the replicase complex and is responsible for initiating and catalyzing viral RNA synthesis (16, 32, 58). There is no human homolog of this protein, and it is absolutely required for viral infectivity (19). As a result, the HCV NS5B is an attractive target for the development of antiviral compounds. There are two major classes of NS5B inhibitors: nucleoside analogs, which are anabolized to their active triphosphates and act as alternative substrates for the polymerase, and nonnucleoside inhibitors (NNIs), which bind to allosteric regions on the protein. Two major drawbacks associated with NNIs are that the activity appears to vary significantly among different HCV genotypes and even subtypes (15, 33) and that there is a relatively low barrier for resistance as evidenced by the numerous naturally occurring resistant variants reported in the literature (18). In contrast, nucleoside analogs are similarly active across HCV genotypes (13, 15, 33) and have a higher barrier of resistance compared to the NNIs and NS3 protease inhibitors (36). To date only two amino acid changes within the NS5B polymerase that confer resistance to nucleoside inhibitors have been identified: S96T and S282T (1, 29). The S96T mutation confers resistance to 4′-azidocytidine (R1479), while the S282T mutation is resistant to a number of 2′-*C*-methyl-modified nucleoside inhibitors (1, 29, 38, 43).

In order for nucleoside analogs to be active as alternative substrates, they must first be phosphorylated by cellular kinases to their corresponding 5′-triphosphates, which are active alternative substrate inhibitors for the NS5B polymerase. The efficiency of these metabolic steps, the stability of the triphosphates, and the affinity of the triphosphates for the NS5B polymerase are all important factors in determining the antiviral activities of nucleoside inhibitors. PSI-6130, 2′-F-2′-*C*-methylcytidine, was previously shown to be a specific inhibitor of HCV RNA replication in the replicon assay system (52). However, when the uridine analog, 2′-F-2′-*C*-methyluridine (referred to as PSI-6206), was tested in the replicon assay, it failed to inhibit HCV RNA synthesis due to the inability of cellular enzymes to metabolize PSI-6206 to its triphosphate, PSI-7409 (5, 34, 42). Biochemical studies with PSI-7409 showed that this compound was able to inhibit RNA synthesis mediated by the HCV replicase complex and by purified recombinant HCV NS5B polymerase (34, 42). Furthermore, *in vitro* stability studies using primary human hepatocytes demonstrated that PSI-7409 has a significantly longer half-life ($t_{1/2}$, 38 h) than PSI-6130-TP ($t_{1/2}$, 4.7 h), which could be a desirable pharmacologic benefit (34).

In order to bypass the initial nonproductive phosphorylation step of PSI-6206, the phosphoramidate prodrug methodology was explored as an approach to deliver 2′-F-2′-*C*-methyluridine monophosphate (47, 48). An extensive series of phosphoramidate prodrugs were synthesized, and PSI-7851 demonstrated the desired characteristics with regard to activity and *in vitro* toxicity. Herein we present the results of *in vitro* studies characterizing PSI-7851, a potent and specific anti-HCV compound with pan-genotype activity.

MATERIALS AND METHODS

Compounds. PSI-6130 (2′-deoxy-2′-fluoro-2′-*C*-methylcytidine), PSI-6206 (2′-deoxy-2′-fluoro-2′-*C*-methyluridine), PSI-7851 {CAS registry number 1064684-44-1; (*S*)-2-[(2*R*,3*R*,4*R*,5*R*)-5-(2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyl-tetrahydro-furan-2-ylmethoxy]-phenoxy-phosphorylamino propionic acid isopropyl ester}, D-ddFCTP (β-D-2′,3′-dideoxy-5′-F-cytidine-5′-triphosphate), R1479 (4′-azidocytidine), and the nonnucleoside NS5B inhibitor *N*-{3-[4-hydroxy-1-(3-methyl-butyl)-2-oxo-1,2-dihydro-pyrrolo[1,2-*b*]pyridazin-3-yl]-1,1-dioxo-1,4-dihydro-1λ^6-benzo(1,2,4)thiadiazin-7-yl}-*N*-methylmethanesulfonamide were synthesized at Pharmasset, Inc. PSI-6130-TP and PSI-7409 were synthesized by NuBlocks (Vista, CA). Gemcitabine and lamivudine were purchased from Hetero Drugs Ltd. (Hyderabad, India). Zalcitabine (2′,3′-dideoxycytidine [ddC]) was purchased from RI Chemicals (Orange, CA). Zidovudine (3′-azidothymidine [AZT]) was purchased from Samchully Pharm. Co., Ltd. (Seoul, South Korea). Infergen (a consensus interferon) was kindly provided by Larry Blatt (InterMune Inc., Brisbane, CA). Ribavirin was obtained from ICN Pharmaceuticals (Costa Mesa, CA). [α-^{32}P]dCTP, [α-^{32}P]UTP, and [α-^{32}P]GTP were purchased from Perkin-Elmer (Waltham, MA). 3′-dCTP was purchased from Trilink Biotechnology (San Diego, CA). Aphidicolin and α-amanitin were purchased from Sigma (St. Louis, MO).

Cells and viruses. Clone A HCV replicon cells (Apath LLC, Brooklyn, NY) and HepAD38 HBV cells (kindly provided by B. Korba, Georgetown University, Washington, DC) were maintained as described previously (52). ET-lunet cells, which stably express the ET subgenomic replicon that encodes the *firefly* luciferase gene (kindly provided by R. Bartenschlager, University of Heidelberg, Heidelberg, Germany), were maintained as described previously (31). Huh7 En5-3 cells containing the genotype 1a Htat, genotype 1b Btat, or Ntat and genotype 2a JFH-1 subgenomic replicon were cultured as described previously (59, 60). P4 cells (kindly provided by P. Charneau, Institut Pasteur, France), an HIV-1-infectible HeLa cell line expressing CD4/CXCR4 and a bacterial *LacZ* reporter gene under the control of the HIV-1 long terminal repeat promoter (4), were maintained in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 50 μg/ml penicillin, 2 mM L-glutamine, and 0.5 mg/ml G418. Huh7 and HepG2 cells (ATCC, Manassas, VA) were maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin. CEM and BxPC3 cells (ATCC) were maintained in RPMI 1640 (Invitrogen) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The New York strain of West Nile Virus (WNV) was kindly provided by N. Karabatsos and R. Lanciotti (Centers for Disease Control and Prevention [CDC], Atlanta, GA). The 17D strain of yellow fever virus (YFV) was obtained from ATCC. Influenza A/Brisbane/59/2007 (H1N1) and influenza A/Brisbane/10/2007 (H3N2) viruses were obtained from the CDC. Influenza A/Vietnam/1203/2004 (H5N1) hybrid virus [containing the nine core genes from influenza A/Ann Arbor/6/60 (H1N1)] was kindly provided by George Kemble (MedImmune, Mountain View, CA).

HCV inhibition assay. Clone A (1,500 cells/well) or ET-lunet (3,000 cells/well) cells were incubated for 4 days with serially diluted test compounds prepared as described previously (52). Inhibition of HCV RNA replication was determined by measuring the levels of luminescence expressed via the luciferase reporter gene encoded within the ET replicon using the Bright-Glo reagent (Promega, Madison, WI) and/or by real-time PCR (RT-PCR) (53). The 50% and 90% effective concentration (EC_{50} and EC_{90}) values, the concentrations at which 50% and 90% inhibition were achieved, were determined using the GraphPad Prism software (San Diego, CA). Anti-HCV assays against the Btat, Ntat, Htat, and JFH-1 subgenomic replicons and the H77sV2 and JFH-1 infectious viruses were performed as previously described (3, 59–61).

WNV, YFV, IVA, HBV, and HIV inhibition assays. Antiviral assays against WNV, YFV, and influenza A virus (IVA) were performed using the neutral red dye uptake assay described by McManus et al. (37). Infergen, a consensus interferon, was included as a positive control for assays against WNV and YFV. Ribavirin was the positive control for IVA studies. Assays against HBV were performed using HepAD38 cells, which replicate HBV under conditions that can be regulated with tetracycline (22), as previously described (12, 51). Assays against HIV were performed using P4 cells seeded at 10,000 cells/well in 96-well plates and incubated overnight at 37°C in a humidified 5% CO_2 atmosphere prior to the addition of PSI-7851 or AZT (positive control). Cells were incubated with compounds for 4 h, followed by HIV infection using "P24-equivalent" of the HIV virus strain NL4-3 (4 ng/well) in the presence of DEAE-dextran (20 μg/ml). After HIV infection, P4 cell assay plates were incubated for an additional 40 to 42 h. Beta-galactosidase activity was then assayed using the Galacto-Star kit (Applied BioSystems, Foster City, CA). Luminescence was measured using a Victor3 plate reader (Perkin-Elmer, Boston, MA). Percent inhibition of HIV replication was determined by comparing the change in luminescence of the drug-treated wells versus the no-drug controls.

HCV NS5B polymerase assay. Purification of recombinant genotype 1b_Con1 NS5BΔ21 polymerase has been described previously (53). The genotype

2a_JFH1 NS5BΔ21 expression plasmid was constructed from pYSGR-JFH (obtained from Brett Lindenbach, Yale University, CT), using the following primers: 5′-CTA ACT AGT ATG TCA TAC TCC TGGTACC GG-3′ and 5′-CCG CTC GAG GCG GGG TCG GGC GCG CGA CA-3′ (SpeI and XhoI sites underlined). The digested PCR product was ligated into the pET23a vector (EMD Chemicals Inc., Darmstadt, Germany) to generate a C-terminal His-tagged expression construct. 2a_JFH1 NS5BΔ21 was expressed in Rosetta (DE3) *Escherichia coli* and purified as described previously (13). Genotype 3a and 4a NS5B polymerases were constructed from human serum samples containing genotype 3a or 4a HCV (SeraCare Life Sciences, Milford, MA), from which viral RNA was isolated using the QIAamp MinElute virus spin kit (Qiagen, Valencia, CA). Isolated viral RNA was reverse transcribed using random hexamers and the Thermoscript RT-PCR system (Invitrogen). Full-length genotype 4a NS5B was amplified with a mixture of two forward primers (5′-GGG ATC CTC GAT GTC ATA CTC GTG GAC TGG and 5′-GGG ATC CTC AAT GTC ATA TTC ATG GAC TGG GGC) containing BamHI restriction sites (underlined) and a single reverse primer (5′-ATT TGC GGC CGC CCG AGC AGG CAG CAG GAA GAT G) containing an EagI site (underlined). Full-length genotype 3a NS5B was amplified by a nested PCR method as described previously (15). Briefly, four forward primers and two reverse primers were used for the primary PCR step (3aFor1, 5′-CAG TCC AGC ACT RCT TCC AAG G; 3aFor2, 5′-GAG TCC GAC TCA GAG TCA TGC TC; 3a For3, 5′-CCG GAC TTG AGT TGC GAC; 3a For4, 5′-CCG GAC TTG AGT TGC GAC TC; 3aRev1, 5′-CAA AAA AGR AAT GGA GTG TTA TC; and 3aRev2, 5′-TGG AGT GTT ATC YTA CCA GC). The primary PCR product was further amplified using primers 3aFor4 and 3aRev1 and TA cloned into the pCR8/GW/TOPO vector by using the pCR8/GW/TOPO TA cloning kit (Invitrogen). Sequences were confirmed, and the NS5B regions were amplified to create genotypes 3a and 4a NS5BΔ21 in the pET28a(+) vector with a C-terminal His tag (EMD Chemicals Inc.) and expressed in Rosetta (DE3) *E. coli* cells. Genotype 3a and 4a NS5BΔ21 polymerases were each purified using a His-trap HP column (GE Healthcare, Piscataway, NJ). Polymerase reactions using the purified NS5BΔ21 polymerases were performed in a 20-μl mixture containing various concentrations of the test compound, a 1 μM concentration of all four natural ribonucleotides, an appropriate amount of [α-^{32}P]UTP, 20 ng/μl of genotype 1b(-) internal ribosome entry site (IRES) RNA template, 1 unit/μl of SUPERase-In (Ambion, Austin, TX), 40 ng/μl of NS5B, 1 mM $MgCl_2$, 0.75 mM $MnCl_2$, and 2 mM dithithreitol (DTT) in 50 mM HEPES buffer (pH 7.5). After incubating at 27°C (30 min with genotype 1b and 2a NS5BΔ21 and 3 h with genotype 3a and 4a NS5BΔ21), the reaction was quenched by adding 80 μl of stop solution (12.5 mM EDTA, 2.25 M NaCl, and 225 mM sodium citrate). The radioactive RNA products were applied to a Hybond N$^+$ membrane (GE Healthcare) and quantified using a phosphorimager (Perkin-Elmer) as described previously (41). All reactions were performed in duplicate, and the 50% inhibitory concentrations (IC_{50}s) were calculated using the GraphFit program (Erithacus Software, Horley, Surrey, United Kingdom).

Cellular, mitochrondrial, and human bone marrow toxicity assays. Huh7, HepG2, BxPC3, and CEM cells were treated with PSI-7851 (serially diluted from 100 μM in medium containing dimethyl sulfoxide [DMSO]), Gemcitabine (1 μM), or DMSO control for 8 days as described previously (52). A cell viability assay was performed using the CellTiter 96 Aqueous One Solution cell proliferation assay kit as recommended by the manufacturer (Promega). The absorbance at 490 nm was read with a Victor3 plate reader (Perkin Elmer) using the medium-only control wells as blanks. The IC_{50} was determined by comparing the absorbance in wells containing cells and test compound to the absorbance in the DMSO control wells. Mitochrondrial toxicity was examined by incubating HepG2 and CEM cells for 14 days with PSI-7851 or ddC (up to 100 μM). On days 7 and 11 medium was removed and replenished with fresh medium containing the appropriate concentration of compound. The levels of mitochondrial cytochrome *c* oxidase subunit II (COXII) gene and ribosomal DNA (rDNA) were determined using a multiplex quantitative RT-PCR protocol as described previously (51). The proliferation of human erythroid and myeloid hematopoietic progenitor cells derived from normal bone marrow (Lonza, Walkersville, MD) was assessed in a semisolid methylcellulose-based medium (R&D Systems, Minneapolis, MN) containing recombinant human (rh) stem cell factor (50 ng/ml), rh-interleukin-3 (10 ng/ml), rh-granulocyte-monocyte colony-stimulating factor (10 ng/ml), and rh-erythropoietin (3 U/ml). Increasing concentrations of PSI-7851 (up to 50 μM) diluted in DMSO were added to progenitor cells seeded at 2×10^4 cells per culture in the methylcellulose-based medium. PSI-7851 or DMSO (0.1%) control was incubated with the progenitor cells for 14 to 16 days in culture, after which the proliferated colonies were assessed and scored based on size and morphology to determine the 50% inhibition concentration as described previously (50).

Inhibition of human DNA and RNA polymerases. Human DNA polymerase α, β, or γ (CHIMRx, Milwaukee, WI) was assayed in a 10-μl reaction mixture containing 50 mM Tris (pH 7.5), 50 mM NaCl, 3 mU/μl activated calf thymus DNA (GE Healthcare), a 20 μM concentration of all four natural deoxynucleoside triphosphates, 4 μCi [α-^{32}P]dCTP, 5 mM $MgCl_2$, and increasing concentrations of PSI-7409 (up to 1 mM), D-ddFCTP, or aphidicolin. DNA polymerase α, β, or γ was added to the reaction mixture to give final concentrations of 20, 18, and, 50 μg/ml, respectively. All reactions were run at 37°C and quenched at 30 min by mixing with 1 μl of 0.5 M EDTA. The radiolabeled products were quantified using the Whatman DE81 paper binding assay as described previously (40). A nonlinear fit was performed to determine the IC_{50} using GraphFit. The activity of RNA polymerase II was determined in a 25-μl *in vitro* transcription reaction mixture containing 100 ng of cytomegalovirus (CMV) immediate-early promoter DNA, 400 μM ATP, CTP, and UTP, 16 μM GTP, 10 μCi [α-^{32}P]GTP, 3 mM $MgCl_2$, and various concentrations of PSI-7409 (up to 1 mM), 3′-dCTP, or α-amanitin in transcription buffer (20 mM HEPES [pH 7.9], 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, and 20% glycerol). The reactions were initiated by adding 8 units of HeLaScribe nuclear extract (Promega). All reactions were run at 30°C and quenched at 60 min by mixing with 125 μl of stop solution (0.3 M Tris-HCl [pH 7.4], 0.3 M sodium acetate, 0.5% SDS, 2 mM EDTA, and 3 μg/ml tRNA). The RNA product was purified using an RNeasy kit (Qiagen) according to the manufacturer's instructions. The resulting samples contained 12 μl and the same volume of gel loading dye (98% formamide, 10 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue) was added. The samples were heated at 90°C for 5 min and loaded onto a 6% polyacrylamide sequencing gel. After running, the gel was exposed to a phosphorscreen, and the product was visualized and quantified by using a phosphorimager.

Cross-resistance studies. HCV clone A cells containing the NS5B S282T replicon mutant were established previously by selecting the cells with 2′-*C*-methyladenosine (52). To generate the NS5B S96T/N142T mutant replicon, the S96T and N142T mutations were introduced into the ET replicon by using the QuikChange II XL site-directed mutagenesis kit (Stratagene/Agilent Technologies, Cedar Creek, TX), and mutations were confirmed by sequencing (Cogenics/Beckman Coulter Genomics, Danvers, MA). The primers used to generate the mutations were as follows: S96T (+), 5′-GTA AGC TGA CGC CCC CAC ATA CGG CCA GAT CTA AAT TTG GCT ATG G; S96T (-), 5′-CCA TAG CCA AAT TTA GAT CTG GCC GTA TGT GGG GGC GTC AGC TTA C; N142T (+), 5′-CAC CAT CAT GGC AAA AAC TGA GGT TTT CTG CGT CCA ACC AGA GAA GG; N142T (-), 5′-CCT TCT CTG GTT GGA CGC AGA AAA CCT CAG TTT TTG CCA TGA TGG TG (Integrated DNA Technologies, Coralville, IA). The ET-S96T/N142T replicon plasmid DNA was linearized with ScaI, and the replicon RNA was generated by *in vitro* transcription using the Ribomax large-scale RNA production system (Promega) and electroporated into Huh-lunet cells by using a BTX ECM 830 square wave electroporator (Genetronics, San Diego, CA) as described previously (24). A stable cell line was selected by culturing in the presence of 0.2 mg/ml G418. Anti-HCV activity in clone A wild-type or S282T-resistant replicon cells was evaluated using RT-PCR. Anti-HCV activity in ET-lunet wild-type or S96T/N142T-resistant replicon cell lines was evaluated using a luciferase-based replicon assay.

HCV replicon clearance and rebound studies. ET-lunet replicon cells were seeded at 1×10^5 cells per well in a six-well plate in culture medium without G418. DMSO, PSI-7851, or a benzothiadiazine NS5B nonnucleoside inhibitor was added to cells at 0.1, 1, and 2 μM so that the final DMSO concentration was 0.5% for each compound. Cells were passaged at ratios of 1:3 or 1:4 every 3 to 4 days for 3 weeks and replenished with fresh medium containing the appropriate amount of compounds. At each passage an aliquot of cells was harvested for RNA analysis. After 3 weeks, cells were passaged into culture medium containing 0.25 mg/ml G418 without inhibitor and cultured for an additional 3 weeks. Cells were split when they reached about 90% confluence, and an aliquot was harvested for RNA analysis. At the end of the experiment, total RNA was extracted from all cell samples and levels of HCV RNA and rRNA were determined using RT-PCR. To determine the relative log HCV RNA reduction, HCV RNA was normalized to rRNA by calculating the difference in threshold cycles (ΔC_t; HCV C_t − rRNA C_t). $\Delta\Delta C_t$ was calculated by subtracting the average DMSO controls from each ΔC_t value. The level of HCV RNA was calculated as the $\log(2^{-\Delta\Delta Ct})$. Results were expressed as the log HCV RNA change in the compound-treated cells compared to the HCV RNA level in DMSO-treated control cells. The effect of compound treatment was also examined by colony formation. Cells were washed with phosphate-buffered saline, fixed using 7% formaldehyde, and stained with 1.25% crystal violet at the end of the 6-week experiment.

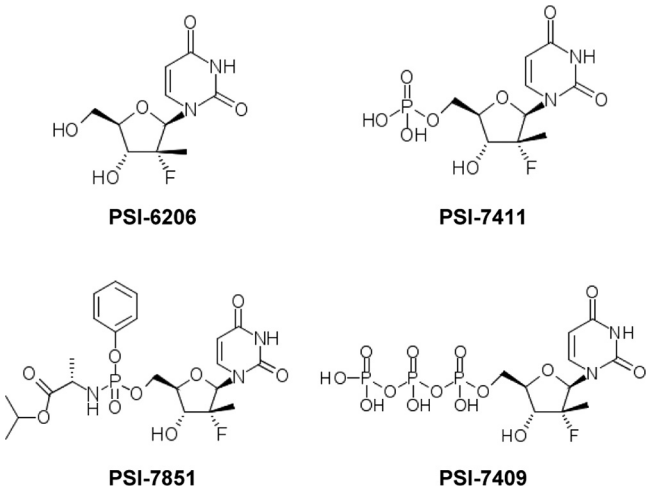


FIG. 1. Chemical structures of PSI-6206, PSI-7411, PSI-7851, and PSI-7409. PSI-6206 is the 2′-F-2′-*C*-methyluridine nucleoside analog. PSI-7411 is the monophosphate of PSI-6206. PSI-7851 is the pronucleotide of PSI-7411. PSI-7409 is the active 5′-triphosphate.

RESULTS

Antiviral activity of PSI-7851. PSI-6206, β-D-2′-deoxy-2′-fluoro-2′-*C*-methyluridine, failed to inhibit HCV replicon replication (5) because it could not be converted to its monophosphate form (PSI-7411) (42). However, biochemical studies showed that PSI-7411 could be phosphorylated to the corresponding di- and triphosphate forms (42). PSI-7409, the active 5′-triphosphate form, has been shown previously to inhibit both the NS5B polymerase and the replicase complex (34, 42). Therefore, PSI-7851, a pronucleotide of PSI-7411, was synthesized in order to bypass the initial nonproductive phosphorylation step. The structures of PSI-6206, PSI-7851, PSI-7411, and PSI-7409 are shown in Fig. 1.

The anti-HCV activity of PSI-7851 was evaluated using both clone A and ET-lunet cells, both of which contained the Con1-derived genotype 1b subgenomic replicon. Results in the ET-lunet cells showed that PSI-7851 inhibited HCV replication with an EC_{50} of 0.075 ± 0.050 μM, and an EC_{90} of 0.52 ± 0.25 μM, and in clone A replicon cells the EC_{90} was 0.45 ± 0.19 μM (Table 1). PSI-7851 was more active than PSI-6130 when tested in parallel in both ET-lunet and clone A cells. As shown in Table 1, the EC_{50} for PSI-7851 suggested that the compound was up to 18-fold more active than PSI-6130.

To evaluate the spectrum of anti-HCV activity, PSI-7851 was tested against a panel of genotype 1a, 1b, and 2a replicons and in the genotype 1a and 2a infectious virus systems. The genotype 1a replicon, Htat, was derived from the H77 strain (60), the Btat and Ntat cells stably express a replicon derived from genotype 1b Con1 and N strains, respectively (59), and the genotype 2a replicon was derived from the JFH-1 strain (17). The two infectious virus systems were derived from genotypes 1a (H77sV2) and 2a (JFH-1) HCV (56, 61). The corresponding EC_{50} and EC_{90} values are summarized in Table 2. The activities of PSI-7851 against the genotype 1b Btat and Ntat replicon were similar, with EC_{50}s of 0.090 ± 0.048 and 0.093 ± 0.083 μM and EC_{90} values of 0.65 ± 0.068 μM and 0.75 ± 0.12 μM, respectively. In the genotype 1a Htat replicon assay, the EC_{50} and EC_{90} values for PSI-7851 were 0.43 ± 0.026 μM and 1.22 ± 0.86 μM, respectively. In the genotype 2a JFH-1 replicon assay, the EC_{50} and EC_{90} values for PSI-7851 were 0.28 ± 0.073 μM and 1.36 ± 0.12 μM, respectively. Cytotoxicity was evaluated by quantifying the level of cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Table 2). The results indicated that PSI-7851 was not cytotoxic in any of the four cell lines at the highest concentration tested, with CC_{50} values of >50 μM. PSI-7851 effectively inhibited HCV replication in the infectious virus assays. The EC_{50} and EC_{90} values for PSI-7851 were 0.19 ± 0.018 μM and 0.34 ± 0.16 μM against the H77sV2 infectious virus and 0.18 ± 0.041 μM and 0.52 ± 0.20 μM against the JFH-1 infectious virus.

To assess the specificity of PSI-7851, the compound was tested against a number of closely related and unrelated DNA and RNA viruses. The closely related viruses included WNV and YFV, which belong to the same *Flaviviridae* family as HCV. The unrelated viruses included the H1N1, H5N1, and H3N2 strains of IVA, HBV, and HIV. Infected cells were treated with either PSI-7851 or the appropriate positive control: ribavirin for IVA, Infergen for WNV and YFV, lamivudine for HBV, and AZT for HIV. As summarized in Table 2, PSI-7851 was inactive against these viruses, with EC_{50}s of >100 μM.

Activity of PSI-7409 against the NS5B polymerase from HCV genotypes 1 to 4. In order to further examine the genotype dependency of PSI-7851, its active 5′-triphosphate metabolite, PSI-7409 (Fig. 1), was tested for its ability to inhibit HCV NS5B polymerases isolated from genotypes (GT) 1 to 4. The 21-amino-acid C-terminal truncated recombinant NS5B polymerases were cloned, expressed, and purified from cDNA or patient serum samples containing HCV from GT 1b (Con1), 2a (JFH-1), 3a, or 4a. We observed that the NS5BΔ21 polymerases from GT 3a and 4a were much less catalytically efficient than the polymerases from GT 1b_Con1 and GT 2a_JFH1. As a result, the reactions for GT 3a and GT 4a NS5BΔ21 polymerases were allowed to proceed for 3 h, while the reaction mixtures with for GT 1b_Con1 and GT 2a_JFH1 NS5BΔ21 polymerases were incubated for 30 min. As shown in Fig. 2, PSI-7409 inhibited the enzymatic activities of these NS5BΔ21 polymerases in a dose-dependent manner. The IC_{50}s for PSI-7409 against GT 1b, 2a, 3a, and 4a NS5B polymerases were determined to be 1.6 ± 0.2 μM, 2.8 ± 0.5 μM, 0.7 ± 0.3 μM, and 2.6 ± 1.2 μM, respectively.

***In vitro* safety profile of PSI-7851.** Three different cell-based assays were performed in order to assess the effects of PSI-7851 on cell viability. PSI-7851 was tested in an 8-day cytotox-

TABLE 1. Activities of PSI-7851 and PSI-6130 in the subgenomic 1b replicon within ET-lunet and clone A cells[a]

Compound	ET-lunet cells		Clone A cells EC_{90} (μM)
	EC_{50} (μM)	EC_{90} (μM)	
PSI-7851	0.075 ± 0.050	0.52 ± 0.25	0.45 ± 0.19
PSI-6130	1.34 ± 0.54	3.81 ± 2.02	4.32 ± 2.17

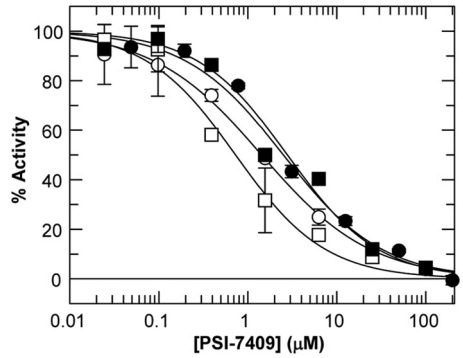
[a] Data are averages from at least four independent experiments ± standard deviations. Cells were incubated with PSI-7851 or PSI-6130 for 4 days before determination of HCV inhibition. Quantitative real-time PCR and/or luciferase-based reporter assays were performed in ET-lunet cells. Quantitative real-time PCR was used in clone A cells.

TABLE 2. Activities of PSI-7851 against genotype 1a, 1b, and 2a HCV replicon cells, genotype 1a and 2a infectious virus systems, and other closely related and unrelated DNA and RNA viruses

In vitro HCV system	PSI-7851			Positive controls[d]	
	EC_{50} (μM)	EC_{90} (μM)	CC_{50} (μM)	EC_{50}	CC_{50}
Replicons[a]					
GT1b_Con1	0.090 ± 0.048	0.65 ± 0.068	>50		
GT1b_N	0.093 ± 0.083	0.75 ± 0.12	>50		
GT1a_H77	0.43 ± 0.026	1.22 ± 0.86	>50		
GT2a_JFH-1	0.28 ± 0.073	1.36 ± 0.19	>50		
Infectious viruses[b]					
GT1a_H77	0.19 ± 0.018	0.34 ± 0.16			
GT2a_JFH-1	0.18 ± 0.041	0.52 ± 0.20			
Other DNA and RNA viruses[c]					
WNV	>100		>100	1.3 ± 1.0 ng/ml	>10 ng/ml
YFV	>100		>100	0.015 ± 0.005 ng/ml	>10 ng/ml
IVA (H1N1)	>100		>100	38 ± 18 μM	>1,000 μM
IVA (H5N1)	>100		>100	32 ± 7.8 μM	>1,000 μM
IVA (H3N2)	>100		>100	18 ± 13 μM	>1,000 μM
HBV	>100		>100	0.052 ± 0.004 μM	>100 μM
HIV	>100		>100	0.11 ± 0.09 μM	>100 μM

[a] Cells were treated with PSI-7851 for 3 days prior to determining HCV inhibition. Quantitative real-time PCR was used to quantify levels of HCV RNA and GAPDH RNA, and percent inhibition was compared to no-drug control cells.
[b] Infectious foci were determined using a primary HCV core mouse monoclonal antibody with a fluorescein isothiocyanate-labeled secondary antibody.
[c] Activities of antiviral compounds against WNV, YFV, and IVA were determined using the neutral red dye uptake assay. Activities of antiviral compounds against HBV were determined by quantitative real-time PCR. Activities of antiviral compounds against HIV were determined by performing a beta-galactosidase-based reporter assay.
[d] Positive controls: Infergen for WNV and YFV, ribavirin for IVA, lamividine for HBV, and AZT for HIV. All values are reported as averages of triplicates ± standard deviations.

icity assay using four different cell lines derived from various organs: Huh7 (human hepatoma), HepG2 (human hepatoma), BxPC3 (human pancreatic cancer), and CEM (human T lymphoblast) cells. Gemcitabine was included as a positive control for all four cell lines. Results showed PSI-7851 was not cytotoxic to Huh7, HepG2, BxPC-3, or CEM cells at 100 μM, the highest concentration tested (CC_{50}, >100 μM). Cytotoxicity was observed with gemcitabine at all concentrations (CC_{50}, <1 μM).



NS5BΔ21 polymerase	IC_{50} (μM)
GT 1b_Con1	1.6 ± 0.2
GT 2a_JFH-1	2.8 ± 0.5
GT 3a	0.7 ± 0.3
GT 4a	2.6 ± 1.2

FIG. 2. Effect of PSI-7409 on the activity of recombinant NS5B polymerases from GT 1b (○), 2a (●), 3a (□), and 4a (■). Increasing concentrations of PSI-7409 were added to NS5BΔ21 polymerases isolated from GT 1b_Con1, GT 2a_JFH-1, GT 3a, and GT 4a. Polymerase activity was determined by measuring the incorporation of [α-^{32}P]UTP into an HCV IRES template by using phosphorimaging. IC_{50}s were calculated from the dose-response curves, and error bars represent standard deviations from at least two independent experiments performed in duplicate.

Mitochondrial toxicity has been described as an adverse effect associated with the long-term use of certain nucleoside/-tide analogs (6, 30, 39, 54). A 14-day assay was performed using HepG2 and CEM cells in order to evaluate whether PSI-7851 was toxic to mitochondria. To measure mitochondrial toxicity the level of the mitochrondrial cytochrome *c* oxidase subunit II gene (mtDNA) was determined using a real-time PCR assay. In addition, cell toxicity was examined in this assay by measuring the levels of the ribosome RNA gene. ddC was used as a positive control for mitochondrial toxicity. Results showed PSI-7851 was neither cytotoxic nor mitochondrially toxic at concentrations up to 100 μM, the highest concentration tested. However, ddC inhibited mitochondrial DNA synthesis in both cell lines, with CC_{90} values of <3 μM for both HepG2 and CEM cells. ddC also demonstrated cytotoxicity for HepG2 and CEM cells, with CC_{90} values of 12.77 and 28.50 μM, respectively.

Bone marrow toxicity has been associated with a number of nucleoside antiviral analogs (49, 50). The effects of PSI-7851 on the proliferation of human erythroid and myeloid progenitor cells were evaluated. Colony formation assays were used to measure the differentiation of the hematopoietic progenitors into granulocyte-myeloid or erythroid cell lineages over 14 to 16 days. Results showed that the CC_{50} for PSI-7851 was >50 μM for both erythroid and myeloid progenitor cells.

Effects of PSI-7409 on human DNA and RNA polymerases. To further characterize the toxicity profile of PSI-7851, its

TABLE 3. Activities of PSI-7409, D-ddFCTP, and aphidicolin against human DNA Pol α, β, and γ[a]

Compound	IC_{50} (μM)		
	Pol α	Pol β	Pol γ
PSI-7409	550 ± 120	>1,000	>1,000
D-ddFCTP	>500	<10	85 ± 15
Aphidicolin	16 ± 2	>100	>100

[a] Human DNA polymerases α, β, and γ were incubated with compounds for 30 min at 37°C prior to quantifying the synthesized radiolabeled products using a filter binding assay. Values represent averages of triplicates ± standard deviations.

active metabolite, PSI-7409, was tested for the ability to inhibit the activity of human DNA Pol α, β, and γ and RNA Pol II. As shown in Table 3, PSI-7409 was a weak inhibitor of DNA Pol α (IC_{50}, 550 μM). DNA Pol β and γ were not inhibited by 1 mM PSI-7409, the highest concentration tested. D-ddFCTP, a known inhibitor of DNA Pol β and Pol γ (20), inhibited DNA Pol β and Pol γ with IC_{50}s of <10 μM and 85 ± 15 μM, respectively. Pol α was not inhibited by 500 μM D-ddFCTP. Aphidicolin, a specific inhibitor for DNA Pol α (9, 57), inhibited DNA Pol α with an IC_{50} of 16 ± 2 μM, but had no effect on Pol β and γ.

An *in vitro* transcription reaction using HeLa nuclear extract was employed to examine the effect of PSI-7409 on human RNA Pol II activity. Both 3′-dCTP and α-amanitin were included as controls. As shown in Fig. 3, analysis of the product formed by gel electrophoresis showed that the reaction mixture containing the HeLa nuclear extract and no compound gave a single major RNA product band. A significant amount of RNA product was made in the presence of 500 μM PSI-7409 or 3′-dCTP, about 85% and 67%, respectively. Therefore, the IC_{50} for both PSI-7409 and 3′-dCTP in this assay was greater than 500 μM. In contrast, the known Pol II inhibitor α-amanitin completely inhibited the reaction at 1 μM.

Cross-resistance studies. Two replicon mutant cell lines that contained either the NS5B S282T or S96T/N142T mutations were tested in order to determine the resistance profile of PSI-7851. The S96T mutation confers resistance to 4′-azidocytidine (R1479), while the S282T mutation confers resistance to various 2′-*C*-methyl-modified nucleoside analogs (1, 29, 38). Cells containing the S282T mutant were generated previously by passaging clone A cells in the presence of increasing concentrations of 2′-*C*-methyladenosine. The S96T/N142T replicon mutant cells were established by electroporating the highly permissive Lunet cells with ET replicon containing the S96T/N142T mutations. It was previously reported that the N142T mutation was selected together with S96T and that the N142T mutation could slightly improve the replication efficiency of a replicon containing the S96T mutation (29). In addition to PSI-7851, the 4′-azidocytidine compound was included as a positive control for the S96T/N142T mutated replicon.

As expected, the S96T/N142T replicon cells were less susceptible to 4′-azidocytidine (EC_{90}, 80.1 μM) than the wild-type cells (EC_{90}, 17.0 μM). PSI-7851 was similarly active against cells containing the ET-lunet S96T/N142T mutant or the wild-type replicon, with EC_{90} values of 0.39 μM and 0.52 μM, respectively. Clone A cells containing the S282T replicon did confer resistance to PSI-7851 (EC_{90}, 7.4 μM) but remained fully susceptible to 4′-azidocytidine. As summarized in Table 4, PSI-7851 was about 16-fold less active against replicons containing the NS5B S282T mutation, while R1479 was about 5-fold less active against the S96T/N142T replicon-containing cells. Results also indicate that no cross-resistance exists between PSI-7851 and R1479.

PSI-7851 clears HCV replicon RNA and prevents viral rebound. A successful anti-HCV therapy should efficiently clear HCV and prevent rebound of the virus even after drug removal. Using the replicon system, PSI-7851 was evaluated for its ability to clear the HCV replicon from ET-lunet cells and prevent viral rebound. An NS5B NNI, *N*-{3-[4-hydroxy-1-(3-methyl-butyl)-2-oxo-1,2-dihydro-pyrrolo[1,2-b]pyridazin-3-yl]-1,1-dioxo-1,4-dihydro-1λ^6-benzo[1,2,4]thiadiazin-7-yl}-*N*-methyl-methanesulfonamide, a benzothiadiazine compound that binds to the palm region of the NS5B polymerase, was included for comparison (55). The EC_{50} for the benzothiadiazine compound was determined to be 0.11 ± 0.038 μM in the ET-lunet replicon assay. Replicon cells were treated with PSI-7851 or the NS5B benzothiadiazine NNI at approximately 1, 10, and 20 times their EC_{50}s (0.1, 1, and 2 μM) for 3 weeks in the absence of G418, followed by withdrawal of compounds and G418 treatment for an additional 3 weeks. In the absence of inhibitor, cells maintained a stable level of HCV RNA throughout the course of the experiment. By the end of the first 3 weeks, cells treated with 0.1 μM (1× EC_{50}) PSI-7851 or benzothiadiazine NNI showed a slight decrease in HCV RNA with a 0.58-log reduction for PSI-7851 and a 0.69-log reduction for the NNI (Fig. 4A and B). The level of HCV RNA was dramatically reduced at the higher concentrations of PSI-7851 (1 and 2 μM). By day 10 there was an approximately 4-log decrease in HCV replicon RNA at the 1 μM dose, and at 2 μM the reduction in HCV RNA was >5 logs. After 3 weeks of treatment there was about a 5-log reduction at both the 1 μM and 2 μM concentrations compared to the DMSO control cells. Increasing the concentration of the NNI also caused a dose-

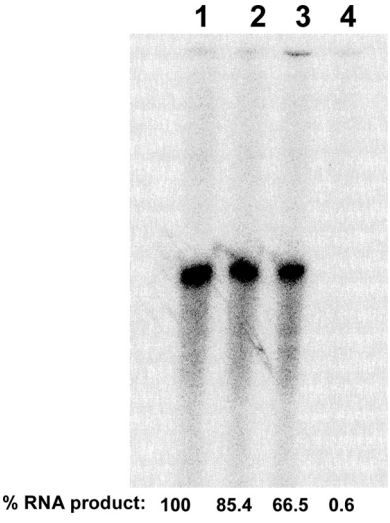


FIG. 3. Inhibition of human RNA polymerase II activity by the no-compound control (lane 1), 0.5 mM PSI-7409 (lane 2), 0.5 mM 3′-dCTP (lane 3), or 1 μM α-amanitin (lane 4). Synthesized RNA products from *in vitro* transcription using HeLa nuclear extract were analyzed by using a 6% polyacrylamide sequencing gel and phosphorimaging.

TABLE 4. Cross-resistance studies using replicon mutants (S282T and S96T/N142T) resistant to modified nucleoside inhibitors[a]

Compound	Clone A cells			ET-lunet cells		
	EC_{90} (μM)		EC_{90} fold increase	EC_{90} (μM)		EC_{90} fold increase
	WT	S282T	S282T vs WT	WT	S96T	S96T vs WT
PSI-7851	0.45 ± 0.19	7.4 ± 1.0	16.4	0.52 ± 0.25	0.39 ± 0.19	0.66
4′-Azidocytidine	18.6 ± 7.5	24.9 ± 23.5	1.3	17.0 ± 3.6	80.1 ± 6.3	4.7

[a] Increasing concentrations of PSI-7851 or 4′-azidocytidine were added to wild-type (WT), S282T, or S96T/N142T replicon cells, and the EC_{90} was calculated. The fold change in the EC_{90} of the inhibitor against the S282T or S96T/N142T mutant replicon versus that of the wild-type replicon was determined. At least three independent experiments were performed for the compounds in each cell line, and data are averages ± standard deviations.

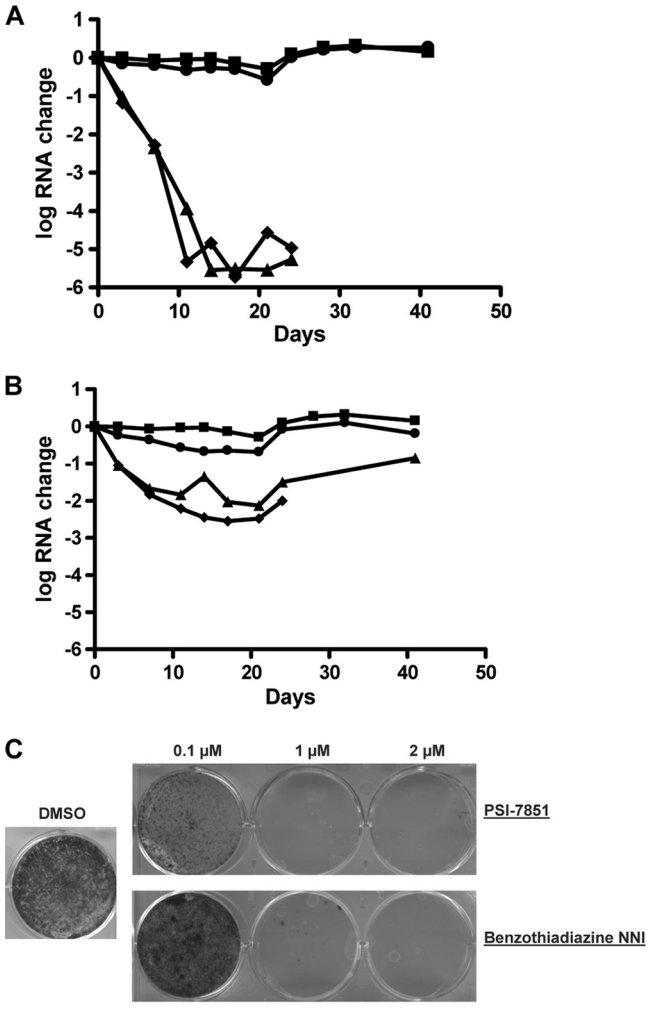


FIG. 4. HCV replicon rebound and clearance study. Replicon cells were treated with either PSI-7851 or a benzothiadiazine NNI for 3 weeks in the absence of G418 with fresh replenishment of each compound at every passage followed by removal of compounds and G418 selection for an additional 3 weeks. An aliquot of cells at each passage was harvested for RNA analysis. Cells remaining at the end of the experiment were stained with crystal violet. (A) Level of HCV replicon RNA change in the presence of DMSO (■) or 0.1 μM (●), 1 μM (▲), or 2 μM (◆) PSI-7851. (B) Level of HCV replicon RNA change in the presence of DMSO (■) or 0.1 μM (●), 1 μM (▲), or 2 μM (◆) benzothiadiazine NNI. (C) Colony formation assays in the presence of DMSO, PSI-7851, or a benzothiadiazine NNI (0.1, 1, or 2 μM).

and time-dependent reduction of HCV RNA (Fig. 4B). However, the overall rate of HCV RNA reduction was slower in the presence of the NNI compared to PSI-7851, and the maximal reductions caused by the NNI were only 2.13 and 2.55 logs at 1 and 2 μM, respectively.

In order to determine whether the cells treated with HCV inhibitors were cleared of the HCV replicon, both compounds were withdrawn after 3 weeks and cells were cultured for an additional 3 weeks in the presence of G418. Cells that retained the HCV replicon would be able to grow in the presence of G418. On the other hand, cells that cleared HCV RNA would not survive the G418 treatment. Results indicated PSI-7851 prevented viral rebound both at 1 and 2 μM, suggesting the HCV replicon was cleared (Fig. 4A). In contrast, replicons in cells incubated with both 0.1 or 1 μM NNI rebounded to levels similar to the no-drug control cells by the end of the experiment (Fig. 4B). Prevention of viral rebound by the NNI was only observed at the highest concentration (2 μM). Results from the RT-PCR HCV RNA assay corroborated the results from the colony formation staining assays. As shown in Fig. 4C, cells incubated with 0.1 μM PSI-7851 or NNI propagated similarly as the DMSO control. No staining of cells was observed at either 1 or 2 μM PSI-7851, indicating clearance of HCV replicon. Single colonies were obtained when cells were treated with 1 μM NNI, but cells did not recover in the presence of 2 μM NNI.

DISCUSSION

The triphosphate form of a nucleoside analog acts as an alternative substrate and inhibits the target polymerase. Among the various metabolic steps involved in the generation of the active 5′-triphosphate, the first phosphorylation step producing the monophosphate has often been found to be rate limiting (14). In the case of PSI-6206, 2′-F-2′-*C*-methyluridine, the nucleoside cannot be converted to its monophosphate form because it is not a substrate for nucleoside kinases, including uridine-cytidine kinase, deoxycytidine kinase, and thymidine kinase 1 or 2 (42). To circumvent this limitation, phosphoramidate prodrugs of PSI-6206 monophosphate were synthesized. Of the phosporamidates evaluated, PSI-7851 was selected for development because of its overall anti-HCV activity, pharmacokinetic properties, and safety profile. The present study characterizes the antiviral activity of PSI-7851 and its safety profile *in vitro*. Our overall results indicate (i) PSI-7851 is a highly effective pan-genotype HCV inhibitor, (ii) PSI-7851 and its active metabolite PSI-7409 are not associated

with any observable cytotoxicity or mitochondrial toxicity at the concentrations tested, and (iii) PSI-7851 facilitates a rapid clearance of HCV RNA from replicon cells which results in prevention of viral rebound.

The broad spectrum of activity for PSI-7851 against various HCV genotypes was demonstrated by using different replicon and infectious assay systems and by using purified recombinant NS5B polymerases from HCV genotypes 1 to 4. The activity of PSI-7851 was examined in four different genotype 1b replicon-containing cell lines. Clone A, ET-lunet, and Btat cells contained the HCV Con1 strain-derived replicons with various adaptive mutations, while the Ntat cells contained the N strain replicon. PSI-7851 inhibited these different genotype 1b replicons with similar efficiency, indicating its activity was not affected by modifications within the replicons, such as adaptive mutations, or by differences in HCV strains. In addition our results show that PSI-7851 inhibited HCV genotypes 1a and 2a in the replicon assay and the infectious assay with similar efficiencies. Biochemical studies using PSI-7409, the active 5′-triphosphate metabolite of PSI-7851, showed it inhibited recombinant NS5B polymerases from genotypes 1b, 2a, 3a, and 4a, suggesting that PSI-7851 would have broad genotypic activity. In contrast to its activity against HCV, PSI-7851 was inactive (EC_{50}, >100 μM) against a panel of closely related (WNV and YFV) and unrelated (IVA, HBV, and HIV) viruses. This lack of activity against other members of the flavivirus family, as well as HIV and HBV, suggests that PSI-7851, unlike 2′-*C*-MeC and 2′-*C*-MeA, is a specific inhibitor of HCV RNA replication. This specificity for HCV is likely due to the 2′-F-2′-*C*-methyl dual substitution, which was also observed with a related compound, PSI-6130 (2′-F-2′-*C*-MeC) (52). The lack of significant antiviral activity seen with other flaviviruses could be due to an inability of certain cells to metabolize PSI-7851. Alternatively, the RNA-dependent RNA polymerase (RdRp) of these viruses might be less susceptible to inhibition by the active 5′-triphosphate, PSI-7409. Since the differential activity of PSI-7851 extends to a number of flaviviruses in different cell lines, it is more likely a result of the inability of PSI-7409 to inhibit the RdRp of these viruses, brought about by the dual substitution of methyl and fluorine at the 2′ position, than levels of metabolism.

Studies were performed to assess the toxicity of PSI-7851 *in vitro*. Cytotoxicity assays using several different cell types, including human bone marrow progenitor cells, indicated no toxicity was associated with PSI-7851 at physiologically relevant concentrations. Mitochondria are often a target for nucleoside and nucleotide toxicity (6, 30, 39, 54). No mitochondrial toxicity was observed with PSI-7851 at concentrations up to 100 μM, the highest concentration tested. The lack of significant cytotoxicity and mitochondrial toxicity was further supported by biochemical studies using PSI-7409, which showed little or no inhibition of human DNA polymerases α, β, and γ and human RNA polymerase II.

Similar to other 2′-*C*-methyl-modified nucleoside analogs, PSI-7851 was less active against replicons that contained the S282T mutation but remained active against replicons containing the S96T/N142T mutation (29). The replication capacity of replicons containing the S282T or S96T mutation is significantly reduced, 4% for S96T, 5% for S96T/N142T, and 15% for S282T, compared to the wild-type replicon (1, 29). To date the S282T mutation has not been detected in patients undergoing clinical studies with RG7128 (28), the prodrug of PSI-6130, which has been previously shown to select the S282T mutation in the replicon system (1).

Among the current DAAs being investigated as drug candidates for treating hepatitis C, nucleoside analogs have been regarded by some as subordinate to NS3 protease inhibitors, NS5B NNIs, and NS5A inhibitors because of their apparently lower rate of reducing HCV RNA in clinical trials. However, our results indicate that PSI-7851 efficiently reduced and cleared HCV RNA: at 10 and 20 times the EC_{50} a maximum decrease in HCV replicon RNA of about 5 logs was achieved after 14 days and 11 days of treatment with PSI-7851, respectively, and these concentrations were found to have effectively cleared HCV replicon RNA. In contrast, the extent and rate of HCV RNA reduction by the NNI were significantly lower and clearance of HCV replicon RNA occurred only when cells were treated at a concentration that was 20 times the EC_{50} of the NNI. As a nucleotide prodrug, PSI-7851 bypasses the initial nonproductive step of being metabolized to the monophosphate intermediate. Upon cell entry, hydrolysis of PSI-7851 directly produces the monophosphate form, which is then phosphorylated to its di- and triphosphates by UMP-CMP kinase and nucleoside diphosphate kinase, respectively (42). The triphosphate of PSI-7851, PSI-7409, has a long intracellular half-life in primary human hepatocytes, approximately 38 h (34). The efficiency of metabolism and stability of its active 5′-triphosphate may have facilitated the rapid decrease of HCV RNA in the clearance and rebound study, in which the ability of PSI-7851 to eliminate HCV RNA was directly compared with a representative NNI. These data clearly demonstrated that PSI-7851 rapidly and significantly reduced the level of HCV RNA, which ultimately led to HCV replicon clearance and prevention of viral rebound.

Nucleoside analogs have long been the cornerstone of antiviral therapy for herpesvirus, hepatitis B virus, and HIV. Promising data are already accumulating for the most advanced HCV nucleoside analog, RG7128, which has shown a >4-$\log_{10}$ IU/ml reduction in viral load when combined with PegIFN-α and RBV or with RG7227, an NS3 protease inhibitor (10, 23). It has been projected that the rapidity of the viral load reduction by PSI-7851, based on 3-day monotherapy studies, will exceed that of RG7128 (25). RG7128 was estimated to produce a 1.07-$\log_{10}$ IU/ml reduction after 3-day monotherapy treatment at its highest dosage (1,500 mg twice a day) (44), while PSI-7851 produced a 2-$\log_{10}$ IU/ml reduction when administered at 400 mg once a day (25). Therefore, PSI-7851 represents a promising candidate among the various DAAs that are currently in clinical development.

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