Inhibition of Hepatitis C Virus Replication by GS-6620, A Potent C-Nucleoside Monophosphate Prodrug

Running Title: In Vitro Pharmacology of GS-6620

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Abstract

As a class, nucleotide inhibitors (NIs) of the hepatitis C virus (HCV) non-structural protein 5B (NS5B) RNA-dependent RNA polymerase offer advantages over other direct acting antivirals, with properties including pan-genotype activity, a high barrier to resistance and reduced potential for drug-drug interactions. We studied the in vitro pharmacology of a novel C-nucleoside adenosine analog monophosphate prodrug, GS-6620. It was found to be a potent and selective HCV inhibitor against HCV replicons of genotypes 1-6 and against an infectious genotype 2a virus (EC_{50} 0.048-0.68 µM). GS-6620 showed limited activity against other viruses, only maintaining some of its activity against the closely related bovine viral diarrhea virus (EC_{50} 1.5 µM). The active 5’-triphosphate metabolite of GS-6620 is a chain-terminator of viral RNA synthesis and a competitive inhibitor of NS5B-catalyzed ATP incorporation with K_i/K_m values of 0.23 and 0.18 for genotype 1b and 2a HCV NS5B, respectively. With its unique dual substitutions of 1’-CN and 2’-CMe on the ribose ring, the active triphosphate metabolite was found to have enhanced selectivity for the HCV NS5B polymerase over host RNA polymerases. GS-6620 demonstrated a high barrier to resistance in vitro. Prolonged passaging resulted in selection of the S282T mutation in NS5B that was found to be resistant in both cellular and enzymatic assays (>30-fold). Consistent with its in vitro profile, GS-6620 exhibited the potential for potent anti-HCV activity in a proof of concept clinical trial but its utility was limited by the requirement for high dose levels and pharmacokinetic and pharmacodynamic variability.
Introduction

Hepatitis C virus (HCV) is a global health problem with an estimated 180 million individuals chronically infected with the virus (1). The infection is the leading cause of end-stage liver disease and liver cancer in North America and Europe (2). It is estimated that HCV surpassed the human immunodeficiency virus (HIV) as a cause of death in 2007 and the disease burden has continued to grow as the duration of infection has increased for those initially infected (3).

The recent regulatory approval of two HCV NS3/4A protease inhibitors, telaprevir and boceprevir, has led to increased treatment response rates when given in combination with pegylated interferon (IFN) and ribavirin (RBV) for those with HCV genotype 1 infection. However, these regimens are limited by emergence of viral resistance, increased adverse events, inability to treat patients intolerant or contraindicated to IFN, and decreased efficacy in many patient populations most in need of therapy, including those with advanced liver diseases and those infected with other HCV genotypes (4). Nucleotide inhibitors (NI) have demonstrated great promise as direct acting antivirals with broad genotype coverage, lack of pre-existing variants with reduced susceptibility, a high barrier to resistance, and the ability to produce potent and durable antiviral responses (5-7). Following intrahepatic activation involving nucleotide kinases, the active 5’-triphosphates of NIs target the HCV non-structural protein 5B (NS5B) RNA-dependent RNA polymerase by serving as alternative substrates and non-obligate chain-terminators of viral RNA synthesis. The initial HCV NIs to enter clinical trials were N-nucleosides, containing the natural C-N glycosidic linkage, with either 2’-C-Me or 4’-azido substituted ribose sugars (5, 8).
Recently, we reported the discovery of a C-nucleoside HCV polymerase inhibitor GS-6620 (9) (chemical structure shown in Fig. 1). GS-6620 is a pan-genotype inhibitor with additive to synergistic effects when combined with other classes of HCV antivirals in vitro. The pharmacologically active metabolite of GS-6620, 1’-CN-2’-C-Me-4-aza-7,9-dideaza A-TP (GS-441326), is a potent inhibitor of NS5B and is not a substrate for the mitochondria RNA polymerase, a potential off-target that may lead to toxicity observed for some of the HCV NIs (9, 10). Cross-resistance studies showed that GS-6620 had reduced activity against genotype 1b replicons carrying the S282T NS5B mutation. In this study, we fully characterized the pharmacology of GS-6620 in vitro. Reported studies include the activity of GS-6620 against HCV genotype 1-6 replicons and infectious genotype 2a virus, antiviral activity against a panel of RNA and DNA viruses, and detailed kinetic characterization of the interaction of GS-441326 with the NS5B polymerase. We also evaluated the potential for general and mitochondrial cytotoxicity of GS-6620 using cell- and enzyme-based assays. The potential for cross-resistance with other NIs and additional HCV inhibitor classes was assessed using resistant mutants selected by NS5B non-nucleoside inhibitors (NNIs), protease NS3/4A inhibitors (PIs), and NS5A inhibitors. Resistance selections with GS-6620 were also conducted using both the infectious genotype 2a virus and the genotype 1b replicon.

Materials and Methods

Compounds. GS-6620 [(S)-isopropyl 2-(((S)-((2R,3R,4R,5R)-3-isobutyroxy-5-(4-aminopyrrolo[1,2-f][1,2,4]triazin-7-yl)-5-cyano-4-hydroxy-4-methyltetrahydrofuran-2-yl)methoxy)(phenoxy)phosphorylamino)propanoate] and other NIs were synthesized by Gilead Sciences, Inc. unless mentioned otherwise.. The NS3 protease inhibitor BILN-
2061 [14-cyclopentyloxy carbonylamino-18[2-(2-isopropylaminothiazol-4-yl)-7-
methoxy-quinolin-4-yloxy]-2,15-dioxo-3,16-diaza-tricyclo(14.3.0.0)nonadec-7-ene-4-
carboxylic acid] and the nucleoside 2'-C-methyl adenosine (2'CMeA) [2-(6-amino-purin-
9-yl)-5-hydroxymethyl-3-methyl-tetrahydro-furan-3,4-diol] were purchased from Acme
Bioscience (Palo Alto, CA). All natural dNTPs and NTPs were from GE Healthcare
(Piscataway, NJ). [α-33P] dNTPs or NTPs were purchased from PerkinElmer (Waltham,
MA). 3’-deoxy NTPs were from TriLink BioTechnologies (San Diego, CA). Aphidicolin,
α-amanitin, and IFN-α were purchased from Sigma (St. Louis, MO).

**DNA and RNA oligonucleotides.** A 244 nucleotide heteropolymeric RNA lacking
secondary structure (sshRNA) with the sequence 5'-3' (UCAG)20(UCCAAG)14(UCAG)20
was used as the template in the NS5B enzymatic assay and was designed and prepared as
described previously (11). An RNA dimer GpC [guanylyl(3’→5’)cytidine] was
purchased from Sigma-Aldrich and was used as the primer for RNA synthesis. GpC was
5',32P-labeled by incubating in a 25 μL mixture containing 10 μL of 8 mM GpC, 10.5 μL
of γ-32P-ATP (3000 Ci/mmol), 20 units T4-kinase (New England Biolabs, Ipswich, MA),
and 2.5 μL T4-kinase buffer at 37°C for 30 min. The mixture was heated at 90°C for 2
min and subsequently diluted with 187.5 μL of water to reach a final concentration of 0.2
mM labeled 32P-GpC. This final mixture was used directly in the RNA elongation assays.
The trace amount of residual γ-32P-ATP had no impact on the assay (data not shown). A
16-nt RNA template (R16) (Table S1) was used in the NTP analog incorporation and
chain-termination assays. It was synthesized and PAGE purified by Thermo
Scientific/Dharmacon (Lafayette, CO).
Cells. The differentiated hepatocellular carcinoma Huh-7 cell line was obtained from JCRB Cell Bank (Japan). The HTLV-1 transformed human T lymphoblastoid cell line MT-4 was obtained from the NIH AIDS Research and Reference Reagent Program (Germantown, MD). The hepatoblastoma cell line HepG2 and the prostate metastatic carcinoma cell line PC-3 were obtained from ATCC (Manassas, VA). Human peripheral blood mononuclear cells (PBMCs) were isolated from human buffy coats obtained from normal healthy volunteers (Stanford Medical School Blood Center, Palo Alto, CA). For quiescent PBMCs cultures, total cells were seeded in 96-well plates followed by immediate drug treatment. Stimulated PBMCs were cultured in the present of 10 units per mL of recombinant human interleukin-2 (Roche, Indianapolis, IN) and 1 µg per mL phytohemagglutinin-P (Sigma-Aldrich) for 48 hours prior to drug treatment. Human primary hepatocytes were obtained from either Celsis In Vitro Technologies (Baltimore, Maryland) or Invitrogen (Carlsbad, CA). Human primary bone marrow (BM) light density cells from three different lots were obtained from AllCells (Emeryville, CA) or Lonza (Walkersville, MD) and the cytotoxicity studies were conducted by STEMCELL Technologies (Vancouver, Canada).

Enzymes. The coding sequences of NS5B polymerase from genotype 1b (Con-1 strain) and 2a (JFH-1 strain) were PCR amplified from plasmids encoding the I389luc-ubi-neo/NS3-3’/ET replicon and pJFH1, respectively. The 3’-PCR primers were designed to encode a construct excluding the C-terminal 21 (or 55) amino acids of full length NS5B and including a C-terminal hexa-histidine tag. The resulting PCR fragments from Con-1 and JFH-1 strains were cloned into pET21a and pET30a protein expression vectors (Invitrogen), respectively, yielding plasmids pET21-NS5B(1b)Δ21(or Δ55)C6His and
pET30-NS5B(2a)Δ21C6H. Mutations were introduced using the QuikChange® II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). Wild-type and mutant NS5B proteins were over-expressed in E. coli and purified following published protocols (12). Recombinant human DNA polymerases α and β were gifts from Dr. Robert Kuchta at the University of Colorado and Dr. Zucai Suo at the Ohio State University, respectively. Recombinant human DNA polymerase γ (including both the large subunit and the small subunit) was cloned, over-expressed, and purified based on published methods (13, 14). RNA polymerase II was purchased as part of the “HeLaScribe® Nuclear Extract in vitro Transcription System” kit from Promega (Madison, WI). The recombinant human mitochondrial RNA polymerase was purchased from Enzymax (Lexington, KY).

**HCV subgenomic replicons.** Genotypes 1a-H77 and 1b-con1 subgenomic replicons were generated as described previously (15, 16). Stable genotype 2a JFH-1 Rluc subgenomic HCV replicon cells were created using plasmid pRLucNeo2a that encodes a bicistronic genotype 2a subgenomic replicon with the renilla luciferase reporter in the first cistron. Plasmid pRLucNeo2a was derived from the plasmid pLucNeo2a containing the nonstructural genes of genotype 2a JFH-1 strain and a firefly luciferase reporter (17). The firefly luciferase/neo gene of pLucNeo2a was replaced by the renilla luciferase/neo gene in the plasmid pRLucNeo2a as described previously (15). Briefly, the fragment encoding the renilla luciferase/neo reporter gene was amplified by PCR from the plasmid pF9A (Promega) using primers with the addition of AfeI and NotI restriction sites (underlined) at the ends (AfeIRlucNeoF 5′-ATA GCG CTA TGG CTT CCA AGG-3′ and RlucneoNotIR 5′-AAT GCG GCC GCT CAG AAG AAC-3′) and subsequently cloned.
into a pTA TOPO vector (Invitrogen). The plasmid pRLucNeo2a was then generated by ligation of the TOPO cloned AfeI-NotI fragment containing *renilla* luciferase/neomycin into the plasmid vector pLucNeo2a digested with the same enzymes. The final sequence of plasmid pRLucNeo2a was confirmed by DNA sequencing. In vitro-transcribed replicon RNA (12 μg) of pRLucNeo2a was transfected into HuH7-Lunet cells by electroporation and clonal cell line 2a Rluc-15 was selected for antiviral assays as described previously (15).

Stable genotype 3a Rluc subgenomic HCV replicon cells were established with a plasmid (pGT3aS52NeoSG) that encodes a subgenomic genotype 3a replicon based on the S52 infectious clone (GenBank accession #GU814263) as published previously (18). Stable genotype 4a (ED43 strain) Rluc subgenomic HCV replicon cells were established as described previously (19). A genotype 5a chimeric replicon was generated with a consensus NS5B sequence of genotype 5 derived from the European HCV database as described earlier (20, 21).

Stable genotype 6a Rluc subgenomic HCV replicon cells were established with a plasmid pGT6aNeoSG that encodes a bicistronic genotype 6a replicon based on the consensus sequence of 16 genotype 6a genomes available in the European HCV database and the 2a JFH-1 3′UTR (230 nt). pGT6aNeoSG was prepared by DNA synthesis and cloning (Genscript). Plasmid pGT6aRLucNeoSG was generated from the pGT6aNeoSG plasmid as described above for plasmid pRLucNeo2a. The stable genotype 6a subgenomic replicon cell line was established in the same way as the above stable genotype 3a subgenomic replicon.
Establishment of genotype 2a infectious HCV. Cell culture adapted genotype 2a-JFH-1 virus was established in Lunet-CD81 cells as described previously (22).

Antiviral assays against genotypes 1-6 subgenomic HCV replicon cells. Genotype 1a-H77, 1b-con-1, 2a-JFH-1, 3a-S52, 4a-ED43, and 6a-HK HCV replicon cells were seeded into 384-well plates at a density of 2,000 cells/well as described previously (23). Genotype 5a NS5B chimeric replicon cells were seeded into 96-well plates at 4000 cells/well (21). Assay plates were incubated for 72 h at 37°C with 5% CO₂ and 85% humidity, after which cell culture medium was removed and cells were assayed for luciferase activity as markers for replicon levels. Luciferase activity was quantified by using a renilla Luciferase Assay system (Promega). Concentrations inhibiting replicon replication by 50% (EC₅₀) and concentrations inhibiting cell viability by 50% (CC₅₀) were reported.

Antiviral assays against genotype 2a infectious HCV. Lunet-CD81 cells were seeded in 96-well plates at a density of 4 × 10³ cells per well in 100 µL of complete DMEM medium. Cells were allowed to attach overnight and then compounds were added in a volume of 50 µL medium. Compounds were serially diluted in 100% DMSO in 3-fold dilution series and then added at a 1:50 dilution to complete DMEM before being transferred to the cell culture wells. Immediately following the compound addition, virus was added to the cells in a volume of 50 µL and at a multiplicity of infection (m.o.i.) of 0.5. The final compound dilution was therefore 1:200 with a final DMSO concentration of 0.5%. Cell plates were incubated at 37°C for three days, after which culture medium was removed and cells were assayed for viral replication levels. The proteolytic activity of viral-expressing NS3 protease was measured as a marker for viral replication using an...
europium-labeled NS3 peptide substrate and EC$_{50}$ values were determined as described previously (17).

Antiviral screening against other viruses. The antiviral activity of GS-6620 against a panel of non-HCV viruses including bovine diarrhea virus (BVDV), West Nile virus, Dengue virus, Yellow Fever virus, human rhinovirus (HRV), coxsackie virus, respiratory syncytial virus (RSV), parainfluenza virus, influenza virus, vaccinia, human immunodeficiency virus (HIV), and hepatitis B virus (HBV). Detailed information regarding virus strains, cell lines, and incubation duration is shown on Table S2. In general, virus and cells were mixed in the presence of test compound, incubated for 4-10 days, and measured for the reduction in viral cytopathic effects (CPE). The antiviral effect of GS-6620 against HBV was monitored by HBV DNA quantification using real-time PCR (24). Whenever applicable, viruses were pre-titered such that control wells exhibit 85-95% loss of cell viability. Concentrations inhibiting virus replication by 50% (EC$_{50}$) and concentration inhibiting cell viability (CC$_{50}$) were reported.

Cytotoxicity. Huh7, HepG2, PC-3, MT-4, primary hepatocytes, quiescent PBMCs, and stimulated PBMCs were treated with GS-6620 for 5 days and the cell viability was measured using intracellular ATP level (CellTiter Glo, Promega) and the luminescence signal was quantified using an Envision Luminescence plate reader (PerkinElmer). The CC$_{50}$ value was defined as the concentration at which there was a measured 50% decrease in cell viability. Data was analyzed using Pipeline Pilot software (Accelrys, San Diego, CA) or GraphPad Prism 5.0 (La Jolla, CA). Unless otherwise mentioned, CC$_{50}$ values were calculated by non-linear regression analysis using a sigmoidal dose-response (variable slope) equation (four parameter logistic equation):
Y = Bottom + (Top-Bottom)/(1+10^{((\text{LogCC50}-X)\cdot\text{HillSlope})}), where the Bottom and Top values were fixed at 0 and 100, respectively. X is log of the concentration of the test compound and Y is the response.

**Mitochondrial DNA (mtDNA) content determination.** The assay was modified based on a previously published method (25). HepG2 cells were seeded into 12-well plates at a density of $2 \times 10^5$ cells per well and allowed to attach overnight. Following attachment, the medium was replaced with 1.0 mL of fresh medium containing tested compounds and incubated for 10 days. The medium was replaced with fresh medium and compounds every 3 to 4 days. The DMSO concentration was normalized to 1.0% for all treatments. Following the incubation, the cells were washed once with PBS and the total DNA was extracted from the cells using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Real-time PCR reactions were performed using TaqMan universal mastermix (Applied Biosystems/Life Technologies, Grand Island, NY) in an ABI Prism 7900HT Fast Real-Time PCR System (Applied Biosystems).

Quantification of mtDNA was achieved by amplification of a fragment of the mitochondrial specific cytochrome b gene described in detail in Supplementary Information. Chromosomal DNA was quantified by the amplification of a fragment of the β-actin gene using a β-actin Assay-on-Demand kit (Applied Biosystems). The final results are presented as the mean % of mtDNA (± SD) relative to chromosomal DNA from 3 independent experiments, each performed in triplicate.

**Mitochondrial biogenesis assay.** Compounds were tested starting at a concentration close to the CC$_{50}$ value of the compound from a 5-day treatment. For compounds with CC$_{50}$ ≥ 100 μM, the starting concentration was 100 μM. PC-3 cells were plated at a
density of $2.5 \times 10^3$ cells per well in a final assay volume of 100 μL per well with a constant amount of DMSO equal to 0.5%. After a 5-day incubation, the cells were analyzed with the MitoTox™ MitoBiogenesis™ In-Cell ELISA Kit (MitoSciences/Abcam, Eugene, Oregon), which uses quantitative immunocytochemistry to measure protein levels of a mitochondrial DNA-encoded protein [cytochrome c oxidase 1 (COX1)] and a nuclear DNA-encoded protein [succinate dehydrogenase (SDH-A)] in cultured cells. The cells were fixed in 96-well plates and target proteins were detected with highly specific and well-characterized monoclonal antibodies. Protein levels were quantified with IRDye®-labeled Secondary Antibodies. IR imaging and quantitation were performed using a LI-COR® Odyssey instrument (LI-COR, Lincoln, Nebraska). All ratios were expressed as a percentage of the 0.5% DMSO control. In cases where cell viability was severely affected, the data for mitochondrial biogenesis were not included for analysis due to significant errors associated with low signals.

Chloramphenicol was used as a positive control for the assay.

Cross-resistance studies. For the genotype 1b or 2a NS5B mutant HCV replicons and genotype 1b NS3/4A and NS5A mutant replicons, mutations were introduced into the genotype 1b or 2a parental replicons by using a QuikChange II XL mutagenesis kit (Agilent Technologies) according to the manufacturer’s instructions. After confirming the mutations by DNA sequencing, replicon RNA was transcribed in vitro from replicon-encoding plasmids using a MEGAscript T7 kit (Life Technologies, Grand Island, NY). Mutation replicon RNAs were transfected into Huh7-Lunet cells and were determined for their susceptibility to GS-6620 as described previously (16, 23).
Selection of GS-6620-resistant J6/JFH-1 viruses. Naïve Lunet-CD81 cells were infected with the cell culture-adapted J6/JFH-1 virus at an m.o.i of 3.0 as described previously (22). The infected cells were maintained in the presence of GS-6620 or other nucleos(t)ide analog HCV inhibitors, starting at a concentration of 1x EC₅₀. Infected cells were passaged every 3-4 days until viral CPE was observed. Viral supernatants were then passaged over naïve Lunet-CD81 cells at escalating drug concentrations for continuous selection as previously described (17). Virus titer determination (TCID₅₀ assay) is described in Supplementary Information.

Selection of GS-6620-resistant genotype 1b HCV replicon. Genotype 1b-Rluc-2 cells (6 x 10⁵) were seeded in a 150 mm cell culture dish. After overnight culture, cells were treated with GS-6620 at a concentration of 1.5 µM (5 x EC₅₀ ) in the presence of 0.5 mg/mL G-418. Cell culture medium was refreshed every two days with fresh compounds until massive cell death occurred. When the cell colonies grew and reached 90% confluency the cells were split 1:3 and the GS-6620 concentration was increased by 2-fold. The GS-6620 concentration was progressively increased until the cell growth became stable in the presence of 50 µM GS-6620. Cell pellets were collected and stored for genotypic analysis as described in Supplementary Information.

Resistance barrier assessment by replicon colony reduction assay. Huh-9-13 replicon cells were cultured in 6-well plates at a density of 1 x 10⁵ cells per well. Twenty-four hours post-seeding, the cells cultured in the presence of 0.5 mg/mL of G-418 were treated with either GS-6620, 2’CMeA, HCV-796 (HCV NNI), or lomibuvir at 1, 10, or 15 times EC₅₀ concentrations using the pre-determined EC₅₀ values of 500 nM, 300 nM, 3 nM, and 4 nM for GS-6620, 2’CMeA, HCV-796, and lomibuvir, respectively. Culture medium
was replaced with freshly prepared compound-containing medium every 72 hours. After 21 days in culture, the medium was removed and the cells were fixed in 20% methanol solution and stained with crystal violet.

**Biochemical assays.** All of the biochemical assays used radio-labeled dNTP or NTP to track DNA or RNA product formation. The products were analyzed using affinity filter-binding or electrophoresis systems and quantified using a Typhoon Trio Imager and Image Quant TL Software (GE Healthcare). All concentrations refer to the final concentrations unless mentioned otherwise. Product formation in the presence of the inhibitors was expressed as a percentage of the product in water-treated controls (defined as 100%). The IC$_{50}$ value was defined as the concentration at which there was a 50% decrease in product formation. Data were analyzed using GraphPad Prism 5.0. IC$_{50}$ values were calculated as an average of at least three independent experiments.

**Inhibition of NS5B.** A reaction mixture containing 50 mM Tris-HCl (pH 7.5), 10 mM KCl, 1 mM DTT, 0.1 mg/mL BSA, 0.2 unit/µL RNasin Plus RNase Inhibitor (Promega), 4 ng/µL sshRNA, 5 mM MgCl$_2$, and 70-150 nM wild-type or mutant HCV NS5B was pre-incubated with NTP analogs for 5 min at room temperature. The reaction was initiated by the addition of a mixture containing 2.5 µM ATP, 2.5 µM CTP, 2.5 µM UTP, 1.25 µM GTP, and 0.06 µCi/µL of α-$^{33}$P-GTP (3000 mCi/mol). Reactions were allowed to proceed for 90 min at 30°C. After 90 min, 10 µL of the reaction mixture was spotted on DE81 anion exchange paper (Whatman, UK), which was then washed with 3x Na$_2$HPO$_4$ (125 mM, pH 9), 1x water, and 1x EtOH. The filter paper was air-dried and exposed to the phosphor imager screen, and the amount of synthesized RNA was quantified as described earlier.
Mode of Inhibition and Ki measurements for inhibitors. The assay setup was similar to the IC$_{50}$ assay described above except that the inhibition of NS5B by the analogs was measured under various concentrations of ATP (2.5, 5, and 10 µM). Reactions were allowed to proceed for 70 min at 30°C, and 2 µL of the reaction mixture was spotted on DE81 anion exchange paper (Whatman, UK) that was washed with 3x Na$_2$HPO$_4$ (125 mM, pH 9), 1x water, and 1x EtOH. The filter paper was air-dried and exposed to a phosphor imager screen. RNA product formation was plotted as a function of the natural NTP concentration under each different NTP analog concentration. The $K_i$ values were calculated by global fitting the data using a mixed inhibition model with the following equations: 

\[ V_{\text{max App}} = \frac{V_{\text{max}}}{1 + I/(\alpha \cdot K_i)} \]
\[ K_{m\text{App}} = \frac{K_m \cdot (1 + I/K_i)}{1 + I/(\alpha \cdot K_i)} \]
\[ Y = \frac{V_{\text{max App}} \cdot X}{K_{m\text{App}} + X} \]

where X and I are the concentrations of the natural NTP and its analog, respectively (GraphPad Prism 5.0). The value of $\alpha$ indicates the mode of inhibition. When $\alpha = 1$, noncompetitive inhibition is indicated; when $\alpha >> 1$, competitive inhibition is indicated; and when $0 < \alpha < 1$, uncompetitive inhibition is indicated (26).

Single nucleoside incorporation and chain-termination of RNA synthesis. The incorporation of NTP analogs was studied using a 5'-$	ext{32P}$-labled GpC primer and an R16 template. As shown in Table S1, the first position for an ATP or ATP analog incorporation is +8 nt beyond the GpC primer. A reaction mixture containing 50 mM HEPES (pH 7.3), 10 mM DTT, 20 µM R16, 20 µM GpC, 4.7 µM NS5B (con1, Δ55, C6His), and 5 mM MgCl$_2$ was pre-incubated at room temperature for 5 min. The reaction was started by adding 100 µM CTP and 100 µM UTP and the reaction was allowed to proceed for 15 min at 25°C, followed by the addition of a mixture containing 10 µM ATP analog in the presence or absence of 100 µM GTP. After incubation at 30°C for 30 min,
15 µL of the reaction mixture was quenched with 5 µL 0.25 M EDTA in a gel loading buffer (50% formamide and bromophenol blue) to reach a final concentration of 35 µM EDTA. The samples were heated at 65°C for 5 min, run on a 25% polyacrylamide gel (8 M urea), and exposed to a phosphor imager screen prior to quantification and analysis.

**Inhibition of Human DNA and RNA polymerases.** All reaction mixtures contained 50 mM Tris-HCl buffer (pH 8.0), 0.2 mg/mL BSA, 2 mM DTT, 0.05 mg/mL activated fish sperm DNA, 10 mM MgCl2, 1.3 µCi α-33P-dTTP (3000 Ci/mmol), and 2 µM of each of dATP, dGTP, and TTP. The optimal enzyme concentrations were chosen to be in the linear range of [E] vs. activity and the reaction time was selected to ensure less than 10% of the substrate was consumed. All reactions were run at 37 °C. The amounts of enzyme used in each assay were 160 nM and 30 nM for polymerase α and β, respectively. For polymerase γ holoenzyme, 1.2 nM of the large catalytic subunit and 3.6 nM of the small accessory subunit were used in the assay. The incubation times were 30 min, 10 min, and 60 min for polymerase α, β, and γ, respectively. The reactions were started by addition of a mixture of the above mentioned natural NTPs and MgCl2 into a pre-incubated mixture containing enzyme and inhibitors. At the end of the incubation, 5 µL of the reaction mixture was removed and spotted on DE81 anion exchange paper (Whatman, UK) which was washed with 3x Na2HPO4 (125 mM, pH 9), 1x water, and 1x EtOH. The filter paper was air-dried and exposed to the phosphor imager screen prior to quantification and analysis.

Human RNA Pol II reactions were conducted by pre-incubating 7.5 µL 1x transcription buffer [20 mM HEPES (pH 7.2-7.5), 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol], 3 mM MgCl2, 100 ng CMV-(+) control DNA (Promega), a mixture
of natural NTPs, and various concentrations of the inhibitors at 30 °C for 5 min. The 
mixture of four natural NTPs contained 5 µCi of the competing NTP with the 
concentration set at $K_m$ and 400 µM of the three non-competing NTPs. The reaction was 
started by addition of 3.5 µL of HelaExtract to a final 25 µL reaction. After 1 hr 
incubation at 30 °C, the polymerase reaction was stopped by addition of 10.6 µL of 
Proteinase K mixture which contained final concentrations of 2.5 µg/µL Proteinase K 
(New England Biolabs), 5% SDS, and 25 mM EDTA. After incubation at 37 °C for 3-12 
hrs, 10 µL of the reaction mixture was mixed with 10 µL of the loading dye (98% 
formamide, 0.1% xylene cyanol and 0.1% bromophenol blue), heated at 75 °C for 5 min, 
and loaded onto a 6% polyacrylamide gel (8 M urea). The gel was dried for 45 min at 70 
°C and the full length product (363 nt runoff RNA) was quantified.

The inhibition of POLRMT was evaluated using 20 nM POLRMT pre-incubated 
with 20 nM template plasmid (pUC18-LSP) containing POLRMT light strand promoter 
region and transcription factors mtTFA (100 nM) and mtTFB2 (20 nM) in buffer 
containing 10 mM HEPES (pH 7.5), 20 mM NaCl, 10 mM DTT, 0.1 mg/mL BSA, and 
10 mM MgCl₂ (27). Reactions were heated to 32 °C and initiated by addition of 2.5 µM 
of each of the four natural NTPs and 1.5 µCi of $^{33}$P-GTP. After incubation for 30 min at 
32 °C, reactions were spotted on DE81 paper before processed for quantification.

**Modeling of GS-441326 in the NS5B active site.** We constructed a model of elongating 
genotype 1b NS5B based on the NS5B 2a Δ21-Δ8 primer-template bound structure 
published by Mosley et al. (4E7A) (28). Analysis of this structure suggested it had some 
elements necessary for binding dsRNA, however the RNA observed in the structure was 
not properly positioned to be catalytically competent. We thus modified the structure
based on structures of poliovirus (3OL7) (29) and Norwalk virus (3BSO) (30) polymerases. The poliovirus polymerase and the NS5B structures were overlaid based on Cα superposition of active site residues. Primer/template, metals, and substrate from the poliovirus structure were then merged into the NS5B structure. Some side chain minimization surrounding the RNA was required, but otherwise this RNA was a good fit. Metals were modified from Mn$^{2+}$ to Mg$^{2+}$. Active site side chains (particularly D225 and S282) were manipulated into conformations consistent with the poliovirus and Norwalk structures and subsequently minimized, constraining the coordination of the metals using Macromodel OPLS-AA/GB/SA (Suite 2012, version 9.9, Schrödinger, New York).

Additional side chains were conformationally sampled using Prime (Suite 2012, version 3.1, Schrödinger). The β-loop was manually incorporated and optimized with Macromodel dynamics. Finally, the structure was mutated to conform with other genotype sequences (including 1b Con1) and side chains were re-optimized with Prime. In brief, the structural changes required to bind the RNA product are relatively minimal in our model. With respect to apo structures, the palm domain remains essentially unchanged (RMSD ~ 0.5 Å) while the fingers move ~1 Å and the thumb moves by ~2 Å. In particular, the largest structural rearrangements occur at the interface of the C-terminal tail/thumb domain and the β-loop/C-terminal tail, which form the moving parts that rearrange to accommodate RNA product. The final genotype 1b Con1 NS5B structure is shown in Figure 2.

Results
Anti-HCV activity and specificity of GS-6620. The anti-HCV activity of GS-6620 was evaluated using a panel of genotypes 1a, 1b, 2a, 3a, 4a, 5a, and 6a subgenomic replicons. Different from the genotype 3, 4, and 6 NS5B chimeric replicons used in our earlier report (9), all of the replicons used in this study are subgenomic replicons except genotype 5a which is a chimeric replicon with GT5a NS5B sequence engineered into a genotype 1b backbone. GS-6620 showed potent activities against replicons of all six genotypes with EC_{50} values ranging from 0.05 to 0.68 µM (Table 1). It is also active against the infectious genotype 2a J6/JFH-1 virus with an EC_{50} value of 0.25 µM. GS-6620 showed no cytotoxicity toward any of the replicon cells or Clone-5 cells at the highest concentration tested (90 or 50 µM). It is worth mentioning the recent reclassification of HCV genotypes presents the need to study the efficacy of future HCV direct acting antivirals on genotype 7, a currently poorly understood genotype (31).

To establish the antiviral specificity of GS-6620, the compound was tested against a panel of RNA and DNA viruses (Table 2). Among the closely related Flaviviridae family, only the BVDV replicon showed appreciable inhibition by GS-6620, with an EC_{50} value of 1.5 µM, while BVDV infectious virus, West Nile, and Yellow Fever viruses were not inhibited by GS-6620. Weak activity was found against different strains of human rhinovirus (HRV) (EC_{50} 19-78 µM) but not against coxsackie virus in the Picornaviridae family. Similarly, GS-6620 showed no inhibition of a group of (-) RNA viruses including human respiratory syncytial virus (RSV), parainfluenza, and influenza virus or reverse transcriptase-containing viruses such as human immunodeficiency virus (HIV) and hepatitis B virus (HBV) at the highest concentration tested. Interestingly, GS-6620 was moderately active against vaccinia virus, a complex double-stranded DNA.
virus in the *Poxviridae* family, with an EC$_{50}$ of 8 µM that was also confirmed with an alternative prodrug closely related to GS-6620 (data not shown).

**Inhibition of NS5B and mechanism of action for GS-441326**

GS-441326, the pharmacologically active 5’-triphosphate metabolite of GS-6620 (chemical structure shown in Fig. 1) was tested as an inhibitor for NS5B-catalyzed RNA synthesis using recombinant NS5B polymerase derived from genotype 1b (Con1) and genotype 2a (JFH-1) replicon sequences. The IC$_{50}$ values and $K_i/K_m$ values are summarized in Table 3. GS-441326 was a potent inhibitor of HCV NS5B polymerase with IC$_{50}$ value of 0.39 and 1.3 µM and $K_i/K_m$ values of 0.23 and 0.18 for genotype 1b and genotype 2a NS5B, respectively. It was 12-14 fold more potent than 2’CMeATP based on the $K_i/K_m$ values.

The mode of inhibition of GS-441326 was studied in the presence of increasing concentrations of ATP and the data were fit with a non-biased mixed inhibition model that yielded a $\alpha$ factor of 140 with global fit $R^2$ of 0.998, indicating that GS-441326 is a competitive inhibitor of NS5B-catalyzed ATP incorporation (Fig. 3A). Furthermore, a detailed gel-based single nucleotide incorporation assay demonstrated that, once incorporated, GS-441326 serves as a chain-terminator similar to 2’CMeATP and 3’-deoxy ATP (Fig. 3B).

**Evaluation of GS-6620 for general cytotoxicity, mitochondrial toxicity, and off-target effects on host polymerases.** GS-6620 and the major circulating metabolite observed after administration of GS-6620, the nucleoside analog GS-441285 (structure shown in Fig. 1) (32) were investigated for their cytotoxicity in a panel of human cell lines derived from different tissues, including hepatic (Huh-7, HepG2), T-cell (MT-4),
and prostate (PC-3), and primary cells including peripheral blood mononuclear cells (PBMC, un-stimulated and stimulated cultures), primary hepatocytes, and human bone marrow derived hematopoietic progenitor cells (erythroid and myeloid). As summarized in Table 4, GS-6620 showed minimal cytotoxicity in Huh-7, HepG2, and PC-3 cells with CC50 values of 40-67 µM. No cytotoxicity was observed in quiescent PBMC, stimulated PBMC, or primary hepatocytes at the highest concentration tested (100 µM). GS-6620 showed complex, multi-phasic effects on the MT-4 T-cell line and myeloid bone marrow progenitor cells and detectable toxicity in erythroid bone marrow progenitor cells (CC50 15 µM). GS-441285 showed no effect on all cell types at the highest concentration tested (100 µM).

The potential risk of mitochondrial toxicity of GS-6620 was evaluated by measuring mitochondria DNA level and protein synthesis in treated HepG2 and PC-3 cells, respectively. After a 10-day incubation of HepG2 cells with GS-6620, the level of mitochondrial DNA (mtDNA) was measured by real-time PCR and compared to levels of chromosomal DNA. As shown in Fig. 4A, GS-6620 showed no inhibition of mtDNA level at the highest concentration tested (20 µM) in comparison to a >97% decrease by dideoxycytidine (ddC), a HIV antiviral known as an inhibitor of human mtDNA polymerase γ.

The effect of GS-6620 on mitochondrial protein synthesis was evaluated in PC-3 cells using an in-cell ELISA-based MitoTox™ MitoBiogenesis™ assay. The cellular levels of a mitochondrial DNA-encoded COX1 and a nuclear DNA-encoded SDH-A proteins were monitored after 5 day incubation with GS-6620. GS-6620 showed no specific inhibition of mitochondrial biogenesis (Fig. 4B). In contrast, ddC and
chloramphenicol, an inhibitor of mitochondrial protein synthesis showed selective inhibition of mitochondrial protein synthesis.

The inhibitory effect of GS-441326 was tested against a panel of human DNA and RNA polymerases including DNA polymerases α, β, γ, and RNA polymerases II and mitochondrial RNA polymerase (POLRMT). Inhibition for the incorporation of radio-labeled dNTP or NTP (concentration = $K_m$) was studied with heteropolymeric primer/templates using filter-binding or gel-based assays. GS-441326 showed no inhibition at the highest concentrations tested (200 or 500 µM) for any of the human DNA and RNA polymerases tested (Table 5).

**Cross-resistance studies.** The cross-resistance profile of GS-6620 against known HCV drug-resistant mutants was studied using transient replicon assays. The panel of resistance mutations includes NS5B NI mutations (S282T and N142T), NS5B NNI mutations (M423T and Y448H/Y452H), protease NS3/4A inhibitor mutations (T54A, R155K, A156T, and D168V), and NS5A inhibitor mutations (L31V and Y93H). As summarized in Table 6, GS-6620 retained full activity against all of the mutations except S282T, which showed 39-fold resistance. All of the positive control compounds showed fold resistance consistent with historical values.

**Resistance selection using genotype 2a infectious J6/JFH-1 virus.** Selection of infectious viruses resistant to GS-6620 was performed with step-wise escalating drug concentrations as previously described (17). To determine the scope of drug resistance mutations, four independent selections were performed with GS-6620 (supplementary Table S6) in parallel with NIs 2’CMeA and 2’CMeC as controls. The selection of drug-resistant viruses started at a drug concentration of 1x EC$_{50}$ (0.25, 0.38, and 1.1 µM for
After 48 to 78 days of selection with escalating drug concentrations, infectious viruses with significantly reduced susceptibility were successfully selected at 60x, 6x, and 10x EC$_{50}$ for GS-6620, 2’CMeA, and 2’CMeC, respectively. Selection charts for viruses resistant to GS-6620 (two representative experiments) and 2’CMeA are summarized in Figure 5. The selection chart for the 2’CMeC-resistant virus was very similar to that for 2’CMeA-resistant virus and is not shown in the figure.

Genotypic changes in the NS5B gene were determined throughout the resistance selection process by population sequencing. Emerging amino acid changes are listed in Table S6. At low drug concentrations (e.g. 3x EC$_{50}$), although viruses were able to overcome drug pressure, no amino acid changes were observed in NS5B gene from any of the selection experiments (data not shown). These results were confirmed by phenotypic analyses that showed no change in inhibitor susceptibility for these viruses. When the viruses were continuously selected at a higher drug concentrations (6x EC$_{50}$), a single substitution of serine to threonine at amino acid 282 (S282T) was identified in viral supernatants from 2’CMeA and 2’CMeC selections. This finding is consistent with the knowledge that S282T in NS5B is the signature resistance-associated variant to 2’-C-Me nucleoside inhibitors. Interestingly, genotypic analyses of the viruses selected at 10x EC$_{50}$ of GS-6620 identified substitutions of M289V in three independent selection and M289L in the fourth selection (Table S6). At higher drug concentrations (60x EC$_{50}$), population sequencing of the NS5B gene revealed that the resistant viruses retained the M289V/L mutation and acquired the S282T mutation (Table S6). Substitution V421A in
combination with M289V/L and S282T was also observed but appeared only once in the various selections.

All of the viruses shown in Table S6 with genotypic changes in the NS5B gene were analyzed for phenotypic changes in drug susceptibility (Table 7). These analyses indicated that single substitutions in NS5B (M289V or M289L) were sufficient to confer low-level resistance to GS-6620 with an average of 4.4-fold increases in EC\textsubscript{50} values. These two mutants were cross-resistant to 2'CMeA, conferring an average 3.4-fold loss in susceptibility, but not to 2'CMeC (< 1.6-fold). In contrast, the S282T mutant selected by 2'CMeA or 2'CMeC was highly resistant to both inhibitors, with average susceptibility losses of 33- and 19-fold, respectively. S282T alone also conferred significant resistance to GS-6620 with an average of 33-fold decrease in susceptibility.

Viruses with a combination of either M289V or M289L and S282T had high level resistance to GS-6620 (25- to 48-fold). However, this level of resistance was comparable to that conferred by S282T alone (within 2-fold). Not surprisingly, all the selected viruses remained fully susceptible to the NS3 protease inhibitor BILN-2061. These results indicate that in genotype 2a J6/JFH-1 virus, S282T is the primary resistance mutation (33-fold resistance) for GS-6620 while substitutions of M289L/V cause low-levels of resistance to the compound (3- to 5-fold).

**Resistance selection of GS-6620 in HCV genotype 1b replicon.** Genotype 1b HCV replicon cells (1b-Rluc-2) were treated with progressively increasing concentrations of GS-6620 for 106 days, which resulted in the selection of a drug-resistant population. The resistant cells were expanded and assayed for susceptibility to GS-6620 and other related NIs. As shown in Table 8, GS-6620 lost 51-fold in potency against the resistant replicon.
cells which is similar to the 39-fold resistance found in the phenotypic analyses of the
S282T mutant in the transient replicon replication assay using genotype 1b replicon
(Table 6). Significant reductions in antiviral activity were also observed for 2'-CMeA
and 2'CMeC. Genotypic analysis by population sequencing of the resistant replicons
revealed a single amino acid substitution, S282T, within the NS5B polymerase gene. No
other mutations were detected.

GS-6620 has a high barrier to the emergence of resistance in vitro. Genotype 1b
replicon cells were treated with either GS-6620, 2’-CMeA, HCV-796, or lomibuvir at 1x,
10x or 15x EC50 concentrations. After 21 days of treatment, the cell culture plates were
stained for emergence of drug-resistant colonies. As shown in Figure 6, GS-6620
completely suppressed the emergence of resistant colonies at 10x and 15x EC50, matching
the results of 2’-CMeA. In contrast, the NNI, HCV-796 and lomibuvir, permitted
resistant colonies to emerge under both 10x and 15x EC50 concentrations, reflecting their
lower resistance barriers.

Effects of NS5B mutations S282T, M289L, and M289V on GS-441326 activity in
biochemical assays. To evaluate the impact of S282T, M289L, and M289V mutations at
the enzymatic level, the inhibitory activity of GS-441326 and triphosphates of other NIs
were tested against genotypes 1b and 2a recombinant NS5B polymerases carrying the
S282T mutation, and genotype 2a-specific mutations M289L, M289V, and the double
mutation S282T/M289L (Table 9). Consistent with the resistance data from cell-based
assays (Table 7), S282T NS5B showed 91- and 65-fold resistance to GS-441326 and
2’CMeATP, respectively. Either M289L or M289V alone conferred little resistance to

25
GS-441326 or 2’CMeATP, however, the double mutation S282T/M289L increased the resistance 2-fold over S282T for GS-441326 and 4-6 fold for 2’CMeATP.

**Binding model of GS-441326 with HCV NS5B.** Based on an analysis of available X-ray crystal structures, sequences deposited in the European HCV database (33) and modeling of the elongating form of NS5B complex, all key residues that shape the polymerase active site are conserved across genotypes 1a, 1b, 2a, 2b, 3a, 4a, and 5a. These residues include R48, K51, K155, R158, D220, T221, F224, D225, R280, S282, T287, N291, G317, D318, and D319. Further analysis of 793 sequences deposited in the European HCV database (including 209 1a and 425 1b genotypes) indicates the two key residues forming the 1’ pocket are highly conserved (Fig. 7A). N291 is 100% conserved and T287 is 99.9% conserved, with one instance of S287 in genotype 1b. Additional residues that may influence the 1’ pocket are also highly conserved. G317 is 99.9% conserved and S282 is 99.5% conserved. The particular favorability of 1’CN in this pocket is most likely due to a direct interaction with N291.

With respect to resistance, based on the Norwalk (30) and poliovirus structures polymerase structures (29), we believe that residue S282 changes its conformation relative to its apo state when binding the substrate. Specifically, it flips into the active site after substrate binding to hydrogen bond with the substrate 2’OH whereas at the apo state it is seen to hydrogen bond with V161. Concomitant with this conformational change, D225 flips out of the active site. The ribose is thus recognized by hydrogen bonds to 2’OH through S282 and N291 and to 3’OH through D225. In the case of S282T mutation, this residue conformation would put the threonine methyl group in close proximity to the inhibitor 2’C-methyl, conveying resistance.
The slight resistance observed with M289V/L mutation is likely due to subtle shifts in positioning of the substrate in the pocket. The residue lies in proximity to the 1’ pocket, but does not directly shape it (Fig. 7B). Notably, this residue is not well conserved across genotypes. It is M for genotypes 2a, 2b, 5a and 6a, C for genotypes 1a and 1b, and F for genotypes 3a and 4a.

Discussion

GS-6620 is a nucleotide prodrug of a 1’-CN-2’-C-Me C-nucleoside analog and was the first adenosine analog tested clinically in HCV infected patients (9). This study presents a detailed characterization of the pharmacology of GS-6620. GS-6620 was found to be a potent and highly selective HCV antiviral: (1) it showed potent pan-genotype activity against HCV replicons and infectious virus; (2) it is a specific antiviral against HCV when tested against a broad panel of RNA and DNA viruses; (3) it showed minimal cytotoxicity in cell lines and primary cells, no inhibition of mitochondrial DNA content, no specific inhibition of mitochondria protein synthesis, and no inhibition of host DNA or RNA polymerases at the highest concentration tested (200-500 µM); (4) the NS5B S282T mutation was selected by GS-6620 in both GT2a infectious virus and GT1b replicon after approximately 60 days and 106 days of in vitro selection, and conferred resistance to GS-6620 in both cell-based and biochemical assays; (5) GS-6620 demonstrated a high barrier to the emergence of resistance in vitro. In addition, a previous study showed GS-6620 was additive to synergistic in combination with other classes of direct antivirals including NS5B NNI, HCV NS 3/4A protease inhibitors, and NS5A inhibitors (9).

2’CMeA was one of the earliest adenosine analogs reported with potent in vitro anti-HCV activity, but it is rapidly deactivated in vivo due to deamination by adenosine.
deaminase (34, 35). An adenosine deaminase-resistant analog 2’-C-Me-7-deazaA (MK-608) overcame the metabolic liability and produced an impressive 4.6 log₁₀ reduction in HCV viral load after 37-day oral dosing in chimpanzees (36). However MK-608 was not tested in HCV-infected human subjects. GS-6620 is a monophosphate prodrug of an adenosine deaminase-resistant C-nucleoside adenosine analog (GS-441285) that efficiently delivers a bioactive triphosphate (GS-441326) with similar intrinsic potency to that formed by MK608. Thus it was hoped that potent antiviral activity would be observed for GS-6620 in clinical studies. The safety, tolerability, pharmacokinetics, and anti-HCV activity of GS-6620 was assessed in genotype 1 treatment-naïve subjects over 5 days in a first in human clinical study (37). While demonstrating the potential for potent antiviral activity (viral load reductions in excess of 4 logs in a few patients), high doses of up to 900 mg BID were required for efficacy and substantial intra and inter subject pharmacokinetic and pharmacodynamic variability were observed. The clinical findings with GS-6620 validated the findings of potent inhibition observed in vitro but clinical efficacy appeared to be limited by the performance of the prodrug following oral administration in humans (32).

The nucleoside analog present in GS-6620 has a unique structural feature including 1’-CN and a modified 4-aza-7,9-dideaza adenosine base. Biochemical assays clearly demonstrated the active triphosphate GS-441326 is a potent, competitive inhibitor for NS5B-catalyzed ATP incorporation with activities similar to MK-608-TP and 12-14-fold more potent than 2’CMeATP (9). Also like 2’CMeATP, the incorporated GS-441326 served as a chain-terminator for RNA synthesis. All of these results suggest that
neither 1’-CN modification nor the carbon substitution at the N9 position alter the base pairing property or the substrate recognition at the NS5B active site.

Toxicity often associated with mitochondrial toxicity caused by inhibition of the mtDNA Pol γ or POLRMT has been reported for some nucleotide analogs (10, 38, 39). GS-6620 and its metabolites were extensively studied in both cell and enzyme-based toxicity and off-target screening assays. GS-6620 showed minimal toxicity in multiple cell lines and primary cells. The only results suggesting some meaningful cytotoxicity potential was the observation of complex concentration-dependent effects on MT4 cells and erythroid bone marrow progenitor cells. However, the effects on MT4 cells, a T-cell line, did not correlate with the observation of toxicity in either stimulated or unstimulated PBMCs and, therefore, these effects were deemed of low relevance to the toxicity potential of the compound. A key motivation for the selection of GS-6620 was the observation that the combination of 1’- and 2’- substitutions in the context of C nucleoside base resulted in reduced incorporation by POLRMT (9). Consistent with the prior results showing a lack of incorporation by POLRMT, results presented here illustrated that GS-441326 did not inhibit POLRMT in biochemical assays. In accord with the lack of effects of its active triphosphate on mt DNA POL γ or POLRMT, GS-6620 was found to neither deplete mtDNA nor inhibit protein production of a mitochondrial gene product. Another potential off-target for a ribonucleotide analog RNA POL II was also not inhibited. Consistent with the low potential for toxicity observed in vitro, the no-observed-adverse-effect levels (NOAEL) for GS-6620 were the highest doses tested following repeated oral administration for 26 weeks in rats and 39 weeks in dogs (1,000 and 60 mg/kg/day, respectively).
Cross-resistance studies using a panel of replicons encoding mutations selected by NS5B polymerase NI and NNI, NS3/4A protease inhibitors, and NS5A inhibitors showed that GS-6620 remained fully active against all of the mutations tested with the exception of NS5B S282T mutant. The resistance selection of GS-6620 yielded S282T in both genotype 2a infectious virus and genotype 1b replicon cells. No other mutations were detected in genotype 1b replicon cells. However in genotype 2a virus, mutations M289V or M289L emerged prior to the detection of S282T. Interestingly, the resistant viruses carrying both M289V and S282T, replicate efficiently at a level comparable to wild type virus but approximate 3-fold higher than the viruses carrying S282T alone, suggesting mutations at residue M289 may function as an compensatory mutation to S282T mutation. However, further studies are needed to determine the viral fitness of these resistance mutations individually or in combination. The amino acid at residue 289 is variable among different genotypes: C289 in genotypes 1a and 1b, M289 in genotypes 2a, 5a, 6a, and F289 in genotypes 3a and 4a (33). M289V and M289L alone conferred low levels of resistance (an average of 4.4-fold) to GS-6620 and slightly (< 2-fold) augmented GS-6620 resistance when combined with S282T in 2a virus assays. Similarly, biochemical studies showed that genotype 2a NS5B carrying M289V or M289L mutations alone didn’t show significant change in sensitivity to the inhibition by 2'CMe,1'CN,4-aza-7,9-dideazaA-TP but when combined with S282T, a 5-fold increase in resistance was observed for NS5B with S282T/M289L double mutation. In addition, GS-6620 showed a high barrier to resistance, a key feature and advantage of HCV NI over other classes of direct antivirals.
In conclusion, GS-6620 is a specific and potent HCV antiviral with pan-genotype coverage. With its unique dual substitution at 1’-CN and 2’-CMe, this novel C-nucleotide analog significantly enhanced the selectivity of the active triphosphate metabolite for HCV NS5B polymerase over host RNA polymerases. The pharmacology presented here translated into GS-6620 becoming the first adenosine analog to obtain clinical proof-of-concept, including the potential for potent anti-HCV activity. However, GS-6620 has not progressed further in clinical development due to the observation of unacceptably high variability and the poor oral pharmacokinetics of the prodrug in humans.

Acknowledgments

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References


of Mitochondrial Transcription and Resistance of RNA Polymerase II Dependent Nuclear Transcription to Antiviral Ribonucleosides. PLoS pathogens 8:e1003030.


resistant viruses using the genotype 2a hepatitis C virus infection system.


Table 1. Antiviral Activity of GS-6620 against HCV replicon and infectious viruses

<table>
<thead>
<tr>
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<th>GS-6620</th>
<th>2'-CMeA</th>
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<tbody>
<tr>
<td></td>
<td>EC₅₀</td>
<td>CC₅₀</td>
</tr>
<tr>
<td></td>
<td>(µM)</td>
<td>(µM)</td>
</tr>
<tr>
<td>HCV repliconᵇ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GT1a</td>
<td>0.18 ± 0.12</td>
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</tr>
<tr>
<td>GT1b</td>
<td>0.46 ± 0.28</td>
<td>&gt;90</td>
</tr>
<tr>
<td>GT2a</td>
<td>0.68 ± 0.31</td>
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</tr>
<tr>
<td>GT3a</td>
<td>0.048 ± 0.001</td>
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</tr>
<tr>
<td>GT4a</td>
<td>0.11 ± 0.06</td>
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</tr>
<tr>
<td>GT5a</td>
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</tr>
<tr>
<td>GT6a</td>
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<td>&gt;90</td>
</tr>
<tr>
<td>HCV infectious virusᶜ</td>
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<td></td>
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<tr>
<td>GT2a J6/JFH-1</td>
<td>0.25 ± 0.08</td>
<td>&gt;50</td>
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</table>

ᵃ Valued based on average of at least three independent experiments. GT = genotype.
b All replicons are full-length Rluc replicons except genotype 5a which is a chimeric replicon with genotype 5a NS5B sequence engineered into a genotype 1b backbone. Part of the data has been published (9).
c Clone-5 cells obtained from cured Huh-7 genotype 1b replicon clone were used in this study.
Table 2. Antiviral Activity of GS-6620 against other viruses

<table>
<thead>
<tr>
<th>(+) Strand RNA virus</th>
<th>GS-6620</th>
<th>Positive Controls</th>
</tr>
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<tbody>
<tr>
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<tr>
<td></td>
<td>CC₅₀</td>
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<td></td>
<td></td>
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<td>HRV 10</td>
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</tr>
<tr>
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<td>Influenza A (H1N1)</td>
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<td>&gt;100</td>
</tr>
<tr>
<td>ds DNA virus</td>
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<td></td>
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<tr>
<td>Virus</td>
<td>IC₅₀ Value</td>
<td>RT-Containing Virus²&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>---------</td>
<td>-----------</td>
<td>---------------------------------</td>
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<tr>
<td>Vaccinia</td>
<td>&gt;100</td>
<td>Rifampicin 25 µM &gt;500 µM</td>
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<tr>
<td>HBV</td>
<td>&gt;100</td>
<td>TFV 9.0 µM &gt;50 µM</td>
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<sup>a</sup> Valued based on average of at least three independent experiments.

<sup>b</sup> A GS-6620-containing diastereomeric mixture was used in the assay.
Table 3. Inhibition of genotypes 1b and 2a NS5B polymerases by GS-441326

<table>
<thead>
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<th>Triphosphate</th>
<th>Genotype 1b</th>
<th>Genotype 2a</th>
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<tr>
<td></td>
<td>IC₅₀ (µM)</td>
<td>Kᵢ/Kₘᵇ</td>
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<tr>
<td>GS-441326</td>
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<td>2′CMeA-TP</td>
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</tbody>
</table>

⁴ IC₅₀ values were reported as an average ± STD of at least three independent experiments. Genotype 1b-Conc1 and genotype 2a-JFH1 sequences were used for the NS5B cloning.

⁵ Kᵢ value for ATP with genotype 1b-Conc NS5B was 0.09 µM.

⁶ Kᵢ values for ATP with genotype 2a-JFH1 NS5B was 0.96 µM.
Table 4. *In vitro* cytotoxicity of GS-6620 in human cell lines and primary cells

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Hepatic</th>
<th>Prostate</th>
<th>T-Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Huh7</td>
<td>HepG2b</td>
<td>PC-3</td>
</tr>
<tr>
<td>GS-6620</td>
<td>67 ± 13</td>
<td>66 ± 13</td>
<td>40 ± 0.7</td>
</tr>
<tr>
<td>GS-441285</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Puromycin</td>
<td>0.52 ± 0.19</td>
<td>0.65 ± 0.18</td>
<td>0.22 ± 0.07</td>
</tr>
</tbody>
</table>

Primary cells (CC₅₀, µM)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Hepatic</th>
<th>PBMC</th>
<th>Bone Marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primary</td>
<td>quiescent</td>
<td>stimulated</td>
</tr>
<tr>
<td>GS-6620</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>GS-441285</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Puromycin</td>
<td>1.9 ± 0.8</td>
<td>4.5 ± 1.9</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>5-FU</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

* CC₅₀ values represent the average of at least three independent experiments. Puromycin or 5-FU were used as positive control in all assays. Depending on the cell line, 90 µM or 100 µM was the highest concentration tested for each compound. All cells were treated...
for 5 days except the bone marrow derived progenitor cells which were treated for 14 days.

b Galactose-adapted HepG2 cells were used in the study.

Complex, multi-phasic dose response was observed with an initial drop in viability of 50% observed at 3.3 µM followed by a rebound to over 50% at higher concentrations. Flow cytometry studies showed the compound effect on cells was cytostatic instead of cytotoxic. In addition, MT-4 cell viability remained unchanged after 6-week continuous culture with the compound.

d Complex, multi-phasic, behavior observed in myeloid cells with an initial drop in viability of 50% observed at the following concentration of 1.5, 1.8 and 6.2 µM, respectively, in cells from three different donors. The viability of the cells rebounded to 30-40% at the highest concentration tested (100 µM).

e ND = not determined
Table 5. Inhibition of human DNA and RNA polymerases by GS-441326

<table>
<thead>
<tr>
<th>Human Polymerases</th>
<th>GS-441326 IC₅₀ (µM)ᵃ</th>
<th>Positive Controls IC₅₀ (µM)</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Pol αᵇ</td>
<td>&gt;200</td>
<td>1.6 ± 1.1</td>
<td>Aphidicolin</td>
</tr>
<tr>
<td>DNA Pol βᵇ</td>
<td>&gt;200</td>
<td>1.9 ± 0.8</td>
<td>3’deoxy TTP</td>
</tr>
<tr>
<td>DNA Pol γᵇ</td>
<td>&gt;200</td>
<td>1.0 ± 0.6</td>
<td>3’deoxy TTP</td>
</tr>
<tr>
<td>RNA Pol IIᶜ</td>
<td>&gt;200</td>
<td>0.0035 ± 0.0014</td>
<td>α-amanitin</td>
</tr>
<tr>
<td>POLRMT</td>
<td>&gt;500</td>
<td>4.2 ± 1.4</td>
<td>3’deoxy GTPᵈ</td>
</tr>
</tbody>
</table>

ᵃ Values represent the average of at least three independent experiments.
ᵇ The concentrations for dATP, dGTP, dCTP, and TTP were kept at 2 µM in the assay.
ᶜ IC₅₀ values were measured under conditions where the competing NTP concentration was held at Kₘ: 25 µM for ATP, 5 µM for GTP, UTP, and CTP. The Kₘ values for each natural NTP were measured in the presence ³²P-labeled NTP of interest and 400 µM of the three other NTPs. The Kₘ values are consistent with published data (40).
ᵈ IC₅₀ values for 3’deoxy ATP, 3’deoxy CTP, and 3’deoxy UTP were 4.6, 1.4, and 4.7 µM, respectively.
Table 6. Cross-resistance of known resistance mutations associated with different classes of HCV antiviral to GS-6620

<table>
<thead>
<tr>
<th>Mutations&lt;sup&gt;a&lt;/sup&gt;</th>
<th>GS-6620</th>
<th>Positive Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fold Resistance&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Compound</td>
</tr>
<tr>
<td>NS5B NI mutations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S282T</td>
<td>39</td>
<td>2′CMeA</td>
</tr>
<tr>
<td>N142T</td>
<td>1.1</td>
<td>4′-azido cytidine</td>
</tr>
<tr>
<td>NS5B NNI mutations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M423T</td>
<td>0.76</td>
<td>VX-222</td>
</tr>
<tr>
<td>Y448H/Y452H</td>
<td>0.80</td>
<td>GS-9190</td>
</tr>
<tr>
<td>NS3/4A mutations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T54A</td>
<td>0.81</td>
<td>telaprevir</td>
</tr>
<tr>
<td>R155K</td>
<td>0.93</td>
<td>telaprevir</td>
</tr>
<tr>
<td>A156T</td>
<td>0.36</td>
<td>BILN-2061</td>
</tr>
<tr>
<td>D168V</td>
<td>0.61</td>
<td>BILN-2061</td>
</tr>
<tr>
<td>NS5A mutations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L31V</td>
<td>1.6</td>
<td>BMS-790052</td>
</tr>
<tr>
<td>Y93H</td>
<td>0.33</td>
<td>BMS-790052</td>
</tr>
</tbody>
</table>

<sup>a</sup> Genotype 1b replicons (wild type or encoding the mutations) were transiently transfected into 1C cells. GS-6620-containing diastereomeric mixture was used in these studies.
Fold resistance is calculated as the ratio of mutant EC$_{50}$ to wild-type EC$_{50}$. The values reported are averages from at least three independent experiments. The S96T mutation could not be studied due to its lack of fitness in transiently transfected replicons.
Table 7. Phenotypic analyses of combinational NS5B mutations against GS-6620 in infectious virus antiviral activity assay

<table>
<thead>
<tr>
<th>NI</th>
<th>EC₅₀ (µM)</th>
<th>EC₅₀ Fold Resistanceᵃᵇᶜ</th>
<th>GS-6620 Select #1 and #2</th>
<th>GS-6620 Select #3</th>
<th>GS-6620 Select #4</th>
<th>2′-CMeA 10x</th>
<th>2′-CMeC 10x</th>
</tr>
</thead>
<tbody>
<tr>
<td>2′-CMeA</td>
<td>0.22</td>
<td>3.3</td>
<td>21.9</td>
<td>2.4</td>
<td>22.7</td>
<td>4.2</td>
<td>23.3</td>
</tr>
<tr>
<td>2′-CMeC</td>
<td>1.0</td>
<td>1.4</td>
<td>18.0</td>
<td>1.2</td>
<td>20.1</td>
<td>1.6</td>
<td>19.1</td>
</tr>
</tbody>
</table>

ᵃ Values represent the average of two or more independent experiments.

ᵇ Fold resistance is calculated as the ratio of mutant virus EC₅₀ to wild-type virus EC₅₀.

ᶜ The viruses were selected at a drug concentration of either 6x, 10x, or 60x EC₅₀. Only the NS5B region was subjected to genotypic analysis.
Table 8. Phenotypic analysis of GS-6620 resistant genotype 1b replicon cells

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Wild-type EC$_{50}$ (µM)</th>
<th>Fold Resistance$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS-6620</td>
<td>0.25</td>
<td>51</td>
</tr>
<tr>
<td>2’CMeA</td>
<td>0.24</td>
<td>&gt; 206</td>
</tr>
<tr>
<td>2’CMeC</td>
<td>1.2</td>
<td>&gt; 40</td>
</tr>
</tbody>
</table>

$^a$ Fold resistance is calculated as the ratio of mutant replicon EC$_{50}$ to the wild-type replicon EC$_{50}$.
Table 9. Fold resistance of mutant genotypes 1b and 2a NS5B polymerases against GS-441326, the active triphosphate of GS-6620.

<table>
<thead>
<tr>
<th>Triphosphates</th>
<th>GT 1b</th>
<th>GT 2a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S282T</td>
<td>S282T</td>
</tr>
<tr>
<td>GS-441326</td>
<td>240</td>
<td>91</td>
</tr>
<tr>
<td>2’CMeATP</td>
<td>29</td>
<td>65</td>
</tr>
</tbody>
</table>

*Fold resistance was calculated using the ratio of mutant NS5B IC$_{50}$ to the wild-type NS5B IC$_{50}$. Values were averages from at least three measurements.*
Figure Legends

Figure 1. Chemical structures of GS-6620, its nucleoside metabolite GS-441285, and active triphosphate metabolite GS-441326.

Figure 2. Model of elongating NS5B, based on the structure of Mosley et al. (28) and structures of poliovirus (29) and Norwalk virus (30). The palm domain is shown in blue, the fingers red, the thumb green, and the beta loop yellow.

Figure 3. Inhibition of RNA synthesis by GS-441326 under different concentration of ATP (A) and incorporation and chain-termination by GS-441326 during NS5B-catalyzed RNA synthesis (B).

Figure 4. Effect of GS-6620 on the level of mitochondrial DNA in HepG2 cells (A) and on the level of mitochondrial protein synthesis in PC-3 cells (B). (A) HepG2 cells were with compounds treated for 10 days and compounds were refreshed every 3-4 days. The three concentrations used were 0.2 µM (white bar), 2 µM (hatched bar), and 20 µM (black bar). (B) PC-3 cells were incubated with GS-6620 (open symbols) and ddC (closed symbols) for 5 days prior to analysis. The levels of mitochondrial DNA-encoded protein (COX-1, circles and solid lines) and nuclear DNA-encoded protein (SDH-A, squares and dotted lines) were plotted relative to untreated control.

Figure 5. Representative resistance selection charts for GS-6620 and 2’CMeA resistant viruses. The selection of drug-resistant viruses started at a drug concentration of 1x EC_{50}. Infected cells were maintained in the presence of drug and were passaged every 3-4 days till viral CPE was observed. Viral supernatants were then passaged over naïve Lunet-CD81 cells at escalating drug concentrations for continuous selection. Each symbol represents a passage of viral supernatant onto naïve cells. The circles indicate the viral
supernatants that were selected for genotypic analyses. The selection charts for GS-6620 selection #1, #2, and 2’CMeA selection are shown in blue, green and red, respectively.

Figure 6. Nucleoside and non-nucleoside NS5B inhibitors show different barriers to resistance.

Figure 7. Model of GS-441326 in the active site of elongating NS5B. (A) S282 and D225 are proposed to change conformation relative to apo structures, with S282 forming a hydrogen bond to the 2’OH of the substrate or inhibitor. The 1’ pocket is formed by N291, T287 and G317 (not shown). (B) Position of mutations selected using the genotype 2a infectious HCV. S282 is in direct contact with GS-441326 and the S282T mutation is likely to produce a steric clash with the 2’CMe of the inhibitor. M289 is not in direct contact with the inhibitor and M289V/L mutations likely only subtly change the shape of the active site. Notably, this residue is not well conserved across genotypes.
Figure 1.

GS-6620    GS-441285

GS-441326
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.