INX-08189, a Phosphoramidate Prodrug of 6-O-Methyl-2’-C-Methyl Guanosine, Is a Potent Inhibitor of Hepatitis C Virus Replication with Excellent Pharmacokinetic and Pharmacodynamic Properties

John H. Vernachio,1* Blair Bleiman,1 K. Dawn Bryant,1 Stanley Chamberlain,1 Damound Hunley,1 Jeff Hutchins,1 Brenda Ames,1 Elena Gorovits,1 Babita Ganguly,1 Andrea Hall,1 Alexander Kolykhalov,1 Yule Liu,1 Jerry Muhammad,1 Nicholas Raja,1 C. Robin Walters,1 Jin Wang,1 Karen Williams,1 Joseph M. Patti,1 Geoffrey Henson,1 Karolina Madela,2 Mohamed Aljarah,2 Arnaud Gilles,2 and Christopher McGuigan2

Received 29 September 2010/Returned for modification 4 January 2011/Accepted 19 February 2011

INX-08189 is an aryl-phosphoramidate of 6-O-methyl-2’-C-methyl guanosine. INX-08189 was highly potent in replicon assays, with a 50% effective concentration of 10 ± 6 nM against hepatitis C genotype 1b at 72 h. The inhibitory effect on viral replication was rapid, with a 50% effective concentration (EC90) of 35 ± 8 nM at 24 h. An intracellular 2’-C-methyl guanosine triphosphate (2’-C-MeGTP) concentration of 2.43 ± 0.42 pmol/10^6 cells was sufficient to achieve 90% inhibition of viral replication. In vitro resistance studies confirmed that the S282T mutation in the NS5b gene conferred an approximately 10-fold reduction in sensitivity to INX-08189. However, the complete inhibition of S282T mutant replicons still could be achieved with an EC90 of 344 ± 170 nM. Drug combination studies of INX-08189 and ribavirin indicated significant synergy in antiviral potency in both wild-type and S282T-expressing replicons. Genotype 1b replicons could be cleared after 14 days of culture when exposed to as little as 20 nM INX-08189. No evidence of mitochondrial toxicity was observed after 14 days of INX-08189 exposure in both HepG2 and CEM human cell lines. In vivo studies of rats and cynomolgus monkeys demonstrated that 2’-C-MeGTP concentrations in liver equivalent to the EC90 could be attained after a single oral dose of INX-08189. Rat liver 2’-C-MeGTP concentrations were proportional to dose, sustained for greater than 24 h, and correlated with plasma concentrations of the nucleoside metabolite 2’-C-methyl guanosine. The characteristics displayed by INX-08189 support its continued development as a clinical candidate for the treatment of chronic HCV infection.

Hepatitis C virus (HCV) is one of the most important causes of chronic liver disease worldwide (36). In the United States alone, an estimated 3.9 million people are infected with HCV (16), and an estimated 10,000 to 12,000 HCV-related deaths occur annually (http://digestive.niddk.nih.gov/dddiseases/pubs/chronichepc/).

The HCV inhibitory activity of 2’-C-modified nucleosides has been well studied and has been shown to specifically inhibit HCV RNA replication both in biochemical assays and in cell-based replicon assays (6). The corresponding intracellular triphosphates of these 2’-substituted nucleosides were potent, competitive inhibitors of NS5B-catalyzed reactions in vitro. The incorporation of the 2’-modified monophosphates onto the 3’ end of the RNA strand resulted in the efficient termination of the elongation of the growing RNA chain. Accordingly, 2’-substituted nucleosides have been shown to function as nonobligate chain terminators (5). Despite the potential of 2’-C-modified nucleosides in the inhibition of RNA-dependent RNA polymerase (RdRp) activity, they have failed to progress as drug candidates due to one or more of the following: lack of oral bioavailability, poor pharmacokinetic characteristics, lack of cell penetration, and inefficient intracellular conversion to the active triphosphate (6, 12, 28). For example, the triphosphate of 2’-C-MeGuanosine (2’-C-MeGTP) has been shown to be a highly potent inhibitor of RdRp activity in biochemical assays, and the nucleoside analog 2’-C-methylguanosine (2’-C-MeG) had high oral bioavailability in rats, but it lacked potency in cell-based subgenomic replicon assays (12, 28).

In an effort to unlock the potential of nucleoside NS5B inhibitors, we have employed a phosphoramidate prodrug approach to improve upon the characteristics of cellular uptake and intracellular activation. This approach is designed to bypass the rate-limiting initial phosphorylation step of activation by delivering the monophosphate form of the nucleoside analog to the liver, where it can be efficiently converted to the active triphosphate (24). INX-08189 is a phosphoramidate of 6-O-methyl-2’-C-methyl guanosine (Fig. 1) (26). This compound was selected from a number of phosphoramidate candidates because of its significant potency in replicon assays and its ability to efficiently generate intracellular triphosphate in primary human hepatocytes (26).

The current study reports a detailed characterization of INX-08189, including a description of its potency against mul-

* Corresponding author. Mailing address: Inhibitex, Inc., 9005 Westside Parkway, Alpharetta, Georgia 30009, Phone: (678) 746-1100. Fax: (678) 746-1299. E-mail: jvernachio@inhibitex.com.

† Published ahead of print on 28 February 2011.
tiple HCV genotypes, the relationship between potency and intracellular 2′-C-MeGTP production, the resistance geno-
types selected by the compound, and its pharmacokinetic and pharmacodynamic properties in rats and primates.

MATERIALS AND METHODS
INX-0819. INX-0819 was synthesized in the laboratory of Christopher McGuigan at the Welsh School of Pharmacy, Cardiff University, as described previously (26).

Replicon assays. The HCV inhibitory activity of INX-0819 was evaluated in replicon cell culture systems (Apartment, LLC, Brooklyn, NY). For HCV genotype 1b (Con1), a Huh-7 cell line (29) expressing a stable, bicistronic subgenomic replicon encoding the Renilla luciferase reporter gene was utilized (2). The HCV genotype 1a (H77), a stable, full-length, bicistronic replicon cell line was used (3). Genotype 1a HCV RNA replication was monitored in this cell line using a quantitative reverse transcriptase PCR assay (qRT-PCR; TaqMan) as follows. Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA). The RNA preparations were quantified using the Ribogreen RNA quantitation kit (Invitrogen) and were used as the template in a qRT-PCR mixture containing 1× EZ buffer, 1× Mn acetate, 300 nM deoxynucleotide triphosphates (dNTPs), 1 μl (2.5 U) rTh, 300 nM primers specific for the HCV 3′-untranslated region (UTR) (forward, 5′-GGCTCCATCTTAGCCCTAGTC-3′; HCV-R, reverse, 5′-AGTA TGGCAGCCTCTTCTCAGT-3′; and 150 nM TaqMan probe (6-carboxyfluorescein [FAM]-ATGGCCTGACGAC) (MGB probe). Amplification was performed using an ABI 7500 real-time PCR system and the following thermal cycling program: 95°C for 15 min, 45 cycles of 95°C for 15 s, 60°C for 1 min. The HCV RNA copy number was calculated from a standard curve generated with synthetic HCV RNA standards of known concentra-
tions.

Generation of NS5B mutant replicons. Mutations in the NS5B gene were generated in the HCV genotype 1b (Con1) subgenomic replicon. To introduce the S282T mutation in NS5B, a plasmid containing a restriction fragment incorporating the mutation (pT7GG-1b-S282T) was synthesized (Geneart Inc., Bur-
ingame, CA), and the restriction fragment was purified and used to replace the corresponding fragment in the wild-type replicon. NS5B mutations I585T and S96T were generated using the QuikChange site-di-
rected mutagenesis kit (Stratagene, La Jolla, CA). Briefly, an Xhol/Spel fragment from the HCV genotype 1b replicon plasmid was cloned into pBluescript SKI and used as the template for mutagenesis using the primers 5′-GGATCCGGTACCCTACTCCCAAC-3′ and 5′-GGTTGGGAGTGA TAGTTGCTACCCCTACAGA-3′ for I585T and 5′-AACCTGCTCCATCAA CGCGTCGTCGCCAGG-3′ and 5′-CTGGAGCAGCCGCTGATTGGA AGTT-3′ for S96T. The Xhol/Spel fragments containing the mutations then were cloned into the HCV genotype 1b replicon plasmid. To generate the HCV genotype 1b replicon carrying the NS5B double mutation S282T+I585T, an SfiI restriction fragment containing the S282T mutation was used to replace the SfiI fragment in the replicon containing the I585T mutation. The replicon carrying NS5B mutations S96T and N142T was generated as follows. The S96T mutation was generated by overlap extension PCR. In the first step, two overlapping PCR fragments were amplified from the wild-type replicon using primers Xhol-F1 (5′-GAA ATT CCC TCG AGC GAT GC-3′), S96T-R1 (5′-TTA GAT CTG GCC GTA GGG GGC GT-3′), S96T-F2 (5′-ACG CCC CCA CAT gCG GCC AGA TCT AA-3′), and MfeI-R2 (5′-CAT GAT GTG GGT GTG AA TGG TG-3′) (underlining indicates the introduced restriction enzyme sites and lowercase lettering indicates point mutations introduced by the oligonucleo-
tsides). In the second step, the aforementioned overlapping PCR fragments were used as the template in a PCR mixture containing primers Xhol-F1 (5′-GAA ATT CCC TCG AGC GAT GC-3′) and MfeI-R2 (5′-CAT GAT GTG GGT GTG AA TGG TG-3′). The N142T mutation was generated by PCR using primers N142T-MfeI-F3 (5′-ACC AAT TGA CAC CAC CAT GAT CGC AAA AA>GTA GAT TTT CTG CG-3′) and SfiI-R3 (5′-TCC ACA GGC CGC AGG GCC GTT-3′). The two PCR fragments independently carrying S96T and N142T were cloned into the genotype 1b replicon. All replicons containing altered NS5B genes were confirmed by sequencing (SeqWright, Houston, TX).

Transient transfection of NS5B mutant replicons. Replicon RNA for transfection was prepared as follows. Replicon plasmid DNA was linearized with ScaI (Fermentas, Glen Burnie, MD) and used in in vitro reverse transcription using the T7 Megascript kit (Ambion, Austin, TX). The DNA template was removed by digestion with Turbo DNase, and the RNA was precipitated with 2.5 M LiCl. RNA was quantified using the Quant-iT Ribogreen RNA kit (Molecular Probes, Eugene, OR). In preparation for transfection, Huh-7 cells were cultured in the presence of INX-0819 for 6 h after seeding at the following final concentrations: 0 nM (control), 5, 10, 20, 40, and 80 nM. The medium was changed daily, and the cells were subcultured on days 5 and 10. On days 0, 5, 8, 10, 12, and 14, the cell cultures were analyzed for HCV genome-encoded Renilla luciferase expression with the Renilla luciferase assay kit (Promega, Madison, WI) using a Veritas luminometer (Turner Biosystems, Sunnyvale, CA). On days 5, 10, and 14, a portion of the INX-0819-treated and control cell cultures were seeded into T-75 tissue culture flasks and incubated without INX-0819 but in the presence of 0.5 μg/ml GenomicINX-0819 (Invitrogen, Carlsbad, CA). As these secondary cultures grew, individual flasks were fixed and stained with crystal violet. For cultures where there were no visible surviving colonies, the flasks were stained after 5 weeks of G418 selection.

Measurement of intracellular 2′-C-methyl-GTP in vitro. Genotype 1b replicon cells were seeded into six-well plates at 1 × 105 cells/well without the selective antibiotic G418. INX-0819 was added to cell cultures 4 h after seeding at the following final concentrations: 0 nM (control), 5, 10, 20, 40, and 80 nM. The medium was changed daily, and the cells were subcultured on days 5 and 10. On days 0, 5, 8, 10, 12, and 14, the cell cultures were analyzed for HCV genome-encoded Renilla luciferase expression with the Renilla luciferase assay kit (Promega, Madison, WI) using a Veritas luminometer (Turner Biosystems, Sunnyvale, CA). On days 5, 10, and 14, a portion of the INX-0819-treated and control cell cultures were seeded into T-75 tissue culture flasks and incubated without INX-0819 but in the presence of 0.5 μg/ml of the selective antibiotic G418 (Invitrogen, Carlsbad, CA). As these secondary cultures grew, individual flasks were fixed and stained with crystal violet. For cultures where there were no visible surviving colonies, the flasks were stained after 5 weeks of G418 selection.

Drug combination studies in genotype 1b replicon assay. Compounds were tested both as single agents and in the following combinations: INX-0819 with IFN-α-2B (ProspecBio, Rehovot, Israel) and INX-0819 with ribavirin (Rbv; Research Products, Mt. Prospect, IL). Inhibition data from the replicon cultures was analyzed for drug interactions using a three-dimensional surface model based on the Bliss independence effects definition for additivity (MacSynergy II: obtained from M. N. Prichard, K. R. Aseltine, and C. Shipman, University of Michigan) (32). As suggested by the software authors and as utilized by others, volumes of synergy or antagonism greater than 25 μM2% were considered minor

but significant, volumes greater than 50 μM% were considered moderate and potentially important in vivo, and values greater than 100 μM% indicate strong synergy and probable importance in vivo (10).

Pharmacokinetic studies of rats. Rat studies were conducted at Inhibitex, Inc., in accordance with NIH guidelines and by following protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Inhibitex. Inc. INX-08189 was formulated in 95% Capmul MCM (Abitec Corp., Janesville, WI)-5% Tween 80 (Sigma, St. Louis, MO) and administered by oral gavage to male Sprague-Dawley rats (Taconic Farms, Germantown, NY). Blood and tissue samples were collected after oral administration as described previously (24). The concentrations of INX-08189 in plasma samples collected from rats were measured by LC-MS/MS as follows. Fifty μl of each test sample was analyzed by LC-MS/MS to measure 2'-C-MeGTP concentrations at both the time to maximum concentration of drug in serum (T_{max}) and at the trough (24 h). To study the relationship between liver 2'-C-MeGTP levels and plasma 2'-C-MeG concentrations, the oral dose range was expanded to 300 mg/kg, approximating equivalent doses of 30 to 3,000 mg. Plasma samples were collected at 1, 2, 4, 8, 16, 24, 72, 96, and 168 h postadministration. Time points were selected to measure 2'-C-MeGTP concentrations at both the time to maximum concentration of drug in serum (T_{max}) and at the trough (24 h).

Pharmacokinetic studies of cynomolgus monkeys. Primate studies were conducted at MPI Research Inc., of Mattawan, MI, in accordance with protocols approved by the IACUC of MPI Research Inc. INX-08189 was formulated in 5% (vol/vol) dimethylacetamide–20% (vol/vol) Solutol HS 15–20% (vol/vol) polyethylene glycol 400–55% (vol/vol) 50 mM sodium acetate, pH 4.0, and was administered as a single oral gavage dose. The dose level of 25 mg/kg was chosen for comparison to the equivalent dose of 50 mg/kg in rats. Liver biopsy samples (four to six samples/animal) were collected from two animals/group/time point at 3 and 8 h postdose in an effort to measure 2'-C-MeGTP levels near the T_{max}. Anesthesia was induced and maintained, and pre- and postoperative procedures were performed according to MPI Research standard operating procedures. The liver biopsy samples were pooled for each animal. Blood samples (approximately 1.2 to 2.0 ml) were collected into tubes containing sodium heparin from the femoral artery/vein or from the portal vein cannula at 0.5, 1, 2, 3, 4, 8, 12, and 24 h postadministration. Time points were selected to measure 2'-C-MeG concentrations at both the T_{max} and at the trough (24 h). Plasma was separated by centrifugation and stored frozen until analysis.

Bioanalysis of pharmacokinetic samples. The concentration of 2'-C-MeGTP in liver samples and the concentration of 2'-C-MeG in plasma samples from rats and primates was performed by LC-MS/MS as described previously (24). The assay measuring 2'-C-MeGTP in rat or primate liver was linear (r^2 = 0.99) in the concentration range of 100 to 4,000 ng per g of tissue, with %CV ≤ 10% and CV ≤ 5%. The assay measuring 2'-C-MeG in rat or primate plasma was linear (r^2 = 0.99) in the concentration range of 2 to 1,250 ng/ml, with %CV ≤ 10% and CV ≤ 5%. The concentrations of INX-08189 in plasma samples collected from primates were measured by LC-MS/MS as follows. Fifty μl of each test sample was added to 200 μl of acetonitrile containing an internal standard. The samples were centrifuged at 1,300 × g at 4°C for 20 min, and 50 μl of supernatant from each sample was diluted with 50 μl H2O. Samples were covered, mixed by vortexing, and incubated at 2 to 8°C before and during analysis. Calibration curves were generated by spiking various concentrations of INX-08189 into control plasma samples. Fifteen μl of each test sample was analyzed by LC-MS/MS. Liquid chromatography was performed with an Agilent 1100 series HPLC system equipped with a Synergi 4-μm Polar-RP 30- by 2.0-mm column (Phenomenex, Torrance, CA). The HPLC system was coupled to an API 4000 triple-quadrupole mass spectrometer (Applied Biosystems, Framingham, MA). Mass spectrometry was performed in positive-ion mode, and data were analyzed using Analyst 1.4.2 software (Applied Biosystems, Framingham, MA). The assay was linear (r^2 = 0.99) in the concentration range of 10 to 1,000 ng/ml, with %CV ≤ 10% and CV ≤ 10%.

Pharmacokinetic analysis. Noncompartmental pharmacokinetic analyses were performed on the plasma and liver concentration data for the analytes using WinNonlin v5.2 software (Pharsight, St. Louis, MO). The extravascular dosing pharmacokinetic model was used to calculate the pharmacokinetic parameters, which included maximum observed concentration (C_{max}), time to maximum concentration (T_{max}), terminal half-life (t_{1/2}), and trapezoidal area determined from the plasma concentration time data from zero to last observed concentration (AUC_{0-}), from time 0 to 24 h (AUC_{24}), and from time 0 extrapolated to infinity (AUC_{∞}). Estimates for the terminal half lives were obtained using regression analysis. Values that were below the lower limit of quantitation were assigned the value of zero for the analyses.

Mitochondrial toxicity assay. Mitochondrial toxicity was measured by analyzing the ratio of the mitochondrial genome copy number to a nuclear gene copy number before and after drug treatment. The mitochondrial target sequence corresponded to the region between the genes TRNL1 encoding tRNA-leu, and ND1, encoding NADH dehydrogenase subunit 1, and the cellular genome comparator was the β-globin gene (7). Total DNA from untreated CEM cells was serially diluted and used to generate a standard curve for determining the absolute copy number of the gene targets. After drug treatment, total DNA was isolated from treated cells using the DNeasy tissue kit (Qiagen, Valencia, CA). The purified DNA was quantified by spectrophotometry using a Spectramax M2 plate reader ( Molecular Devices, Sunnyvale, CA). Duplex quantitative PCR was performed in an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA) using a 25 μl reaction volume containing 1× QuantiTect multiplex PCR (with β-carboxy-X-rhodamine mix) 250 nM mitochondrial-spec primer 5'-CCACCCAAAAGACGGTGTGTT-3' (forward) and 5'-TAAAGAGAGAGAAA CTTGAACTCTGACTG-3' (reverse); 250 nM mitochondrial-specific probe FAM-5'-TAAAGATGGCACAGGCCTGTA-3'-minor groove binding nonfluorescent quencher (MGBNFQ); 500 nM β-globin specific primers 5'-TTGGAATGGTGTGGTGGAGGAACTTTGAACTCTGACTG-3' (reverse) and 5'-CTCCACATGCCAGTTT TATTG-3' (forward). 250 nM globin-specific probe VIC-5'-CCTGGACAGGT TGCCA-3'-MGBNFQ (Integrated DNA Technologies, Coralville, IA); and genomic DNA template. Amplification was carried out using the following thermal cycling program: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and then 60°C for 1 min. The ratios between mitochondrial and cellular genome copy numbers were derived for each sample and compared to those of control cultures. Changes in mitochondrial copy number were expressed as a percent difference from the control value.

RESULTS

Activity of INX-0819 against wild-type HCV replicons. The HCV inhibitory activity of INX-0819 was evaluated in replicons expressing HCV genomes from genotypes 1a, 1b, and 2a. INX-0819 was found to be a highly potent inhibitor of HCV replication, with EC_{50} of 10 nM against genotype 1b, 12 nM against genotype 1a, and 0.9 nM against genotype 2a after 72 h of exposure (Table 1). Following 72 h of exposure, the concentration resulting in 50% cellular cytotoxicity (CC_{50}) in cultured Huh-7 cells was 7.01 μM, resulting in therapeutic indices ranging from 584 to 7,778.

To determine the magnitude of HCV inhibitory activity that could be achieved with less than 72 h of incubation, genotype 1b replicon cells were incubated with INX-0819 for 24 or 48 h, and the EC_{50} and EC_{90} were determined (Table 1). Similar levels of potency were observed when cells were incubated with INX-0819 for 48 or 72 h. If exposure time was reduced to only 24 h, the concentration required to achieve the EC_{50} level increased 3.5-fold; however, INX-0819 still was able to inhibit HCV replication by 90% (EC_{90} = 0.6 μM).

Intracellular metabolism in HCV replicon cells. The intracellular conversion of INX-0819 to 2'-C-MeGTP was deter-
mined in genotype 1b replicon cells. Replicon cells were incubated with concentrations of INX-08189 that produce 50% inhibition of HCV replication (EC₅₀ = 10 nM), 90% inhibition (EC₉₀ = 40 nM), and twice the EC₉₀ (2× EC₉₀ = 80 nM). Cells were harvested after 6 h of exposure to INX-08189, and the intracellular 2'-C-MeGTP concentrations were measured (Table 2). There was a linear relationship (R² = 0.91) between the measured intracellular 2'-C-MeGTP concentrations and the concentrations of INX-08189. At 40 nM INX-08189, the concentration of 2'-C-MeGTP in Huh-7 cells was 2.43 ± 0.42 pmol/10⁶ cells. Given an estimated liver cellularity of 1× 10⁶ cells per g (37), the intracellular concentration of 2'-C-MeGTP that would be expected to result in 90% viral inhibition in liver tissue was calculated to be 243 pmol/g of tissue. This value was useful for interpreting the relevance of liver 2'-C-MeGTP levels measured in subsequent in vivo pharmacokinetic studies.

To determine the intracellular half-life of 2'-C-MeGTP, actively dividing genotype 1b replicon cells were incubated with 1μM INX-08189 for 8 h, at which time the drug was removed. Intracellular 2'-C-MeGTP levels were measured at 12, 24, 32, 48, and 56 h after the drug was initially added (Fig. 2). Based on these data, the half-life of intracellular 2'-C-MeGTP in Huh-7 cells was calculated to be approximately 24 h.

**Selection and characterization of mutants resistant to INX-08189.** To determine the resistance mutations selected by INX-08189, long-term cultures of genotype 1b and 1a replicons were performed in the presence of G418 and with concentrations of INX-08189 that were increased up to eight times the EC₅₀ (80 nM) in the 1b replicon and 4× the EC₅₀ (40 nM) in the 1a replicon. The establishment of stable INX-08189 escape mutants was significantly delayed, requiring selection during a 6- to 9-week span in culture. In total, five single-colony-derived resistant cell lines were selected in the genotype 1b background, and one was selected in the genotype 1a background. The nucleotide sequences of the NS5B genes from these clones were determined. Two consistent NS5B mutations, a codon 282 alteration from serine to threonine (S282T) and a change in amino acid position 540 from isoleucine to threonine (I540T), were identified in drug-resistant clones from the genotype 1b background. The S282T substitution had been identified previously as a resistance mutant for 2'-C-Me-GTP (28).

In the single clone derived from the genotype 1a replicon selection, the only alteration in the NS5B gene sequence was a codon change from alanine to threonine in amino acid position 540 (A540T in genotype 1a). To determine if these amino acid substitutions were sufficient to confer resistance to INX-08189, they were introduced singly or in combination into the genotype 1b replicon background, and potency was assessed in a transient replication assay. For comparative purposes, a replicon carrying the substitutions S96T and N142T that has been shown to confer resistance to 4'-azidothymidine (R1479) (20) was tested in parallel. INX-08189 potency against each of these mutant replicons and their relative replication competencies are summarized in Table 3. The presence of the S282T mutation resulted in drastically reduced replication efficiency, at approximately 4% of the rate of the wild-type replicon. Combining the I585T mutation with S282T improved replication efficiency to 8% of the wild-type level. INX-08189 potency against S282T mutant replicons was reduced approximately 10-fold, while INX-08189 potency against all other mutant replicons tested was unaffected, suggesting that S282T was sufficient to produce an INX-08189-resistant phenotype. Despite the shift in potency observed, exposure to INX-08189 still could significantly inhibit HCV replication in the S282T mutant replicons with an EC₉₀ of 344 ± 170 nM (Fig. 3).

**Effect of combining INX-08189 and Rbv.** In clinical trials, INX-08189 likely will be used in combination with standard therapy, which currently consists of pegylated interferon and Rbv. We therefore characterized the INX-08189 resistance mutant S282T as well as the other mutant replicons for their sensitivity to both Rbv and alpha interferon 2b (Table 3). Replicons expressing S282T proved to be more sensitive to the inhibitory activity of ribavirin, with an approximately 6-fold improvement in the EC₅₀, whereas the potency of IFN-α was equivalent across all mutants tested. The difference in ribavirin potency between wild-type and S282T mutants was significant (P = 0.0093, unpaired t test with Welch's correction.) To explore this effect further, drug combination studies of INX-08189 and Rbv were carried out in both wild-type and transiently expressed S282T replicons (Table 3). The combination of INX-08189 and Rbv was highly synergistic in the wild-type replicon, with a synergy volume of 808 μM²%. Likewise, INX-08189 combined with Rbv against replicons expressing the S282T mutation was found to be significantly synergistic (117 μM²%).

**Clearance of replicons.** To assess the antiviral effect of long-term treatment with INX-08189 on HCV replication, genotype...
1b replicon cells were cultured in the presence of 0 (control), 5, 10, 20, 40, or 80 nM INX-08189 for 14 days in the absence of G418 selection. At various time points during the culture period, cell samples were harvested and luciferase expression was determined as a measure of HCV replication activity. As summarized in Fig. 4A, luciferase activity in treated cultures decreased over time compared to that of the control, and inhibition was concentration dependent. Culturing in the presence of 10 nM INX-08189 resulted in a \( \frac{1}{2} \) log\(_{10} \) reduction in HCV replication activity. Culturing in the presence of 20 nM \( \frac{1}{2} \) EC\(_{50} \) resulted in a \( \frac{5}{2} \) log\(_{10} \) reduction in HCV replication activity after 12 days. After 14 days of culture at concentrations of \( \frac{1}{2} \) EC\(_{90} \), HCV replication activity was reduced to background levels. Samples of the treated cells were harvested at days 5, 10, and 14 and subcultured without INX-08189 but in the presence of G418. Any cells remaining in the cultures that retained the expression of the replicon genome would be resistant to G418 selection and after sufficient time in culture would be detected as visibly growing colonies (Fig. 4B). Cultures that were incubated in the presence of INX-08189 for only 5 days retained the expression of the replicon at all concentrations tested, although the frequency of expressing colonies diminished with increasing INX-08189 concentration. After 10 days of INX-08189 treatment, replicon-expressing cells were completely eliminated at concentrations of \( \geq 20 \) nM \( \frac{1}{2} \) EC\(_{50} \). After 14 days of INX-08189 treatment, replicon-expressing cells were completely eliminated from the culture at concentrations of \( \geq 20 \) nM \( \frac{1}{2} \) EC\(_{50} \).

Mitochondrial toxicity. Mitochondrial toxicity has been described as a manifestation of an adverse effect associated with the long-term use of certain nucleoside analogs (15). To evaluate...
The measurement of 2′-C-MeGTP in liver tissue is the most direct approach for determining the bioavailability of INX-08189; however, the use of this analytical tool is limited in higher species, as it can be employed only at a few time points and is impractical for pharmacokinetic studies of humans. Another approach is to monitor the generation of a metabolite of INX-08189 in the plasma that is proportional to the production of 2′-C-MeGTP in the liver. To this end, the plasma levels of 2′-C-MeG, a major metabolite of INX-08189, were measured in the rat. The plasma AUC₀₋₂₄ (in ng h/ml) values of 2′-C-MeG were compared to the 2′-C-MeGTP C₂₄ (ng/g) concentrations after the administration of oral doses ranging from 3 to 300 mg/kg (Table 5). At doses between 3 and 150 mg/kg, the 2′-C-MeG plasma AUCs (ng h/ml) and the 2′-C-MeGTP liver concentrations (ng/g) at 24 h were found to be dose proportional and linear, with R² values equal to 0.96 and 0.99, respectively. In addition, the 2′-C-MeG plasma AUCs and the 2′-C-MeGTP liver C₂₄ also were found to be highly correlative (R² = 0.97) at doses between 3 and 150 mg/kg. This correlation was not maintained at the 300-mg/kg dose. These data validate the utility of measuring 2′-C-MeG exposure in the plasma as a biomarker to monitor the liver pharmacokinetics of 2′-C-MeGTP in vivo.

<table>
<thead>
<tr>
<th>INX-08189 dose (mg/kg)</th>
<th>N</th>
<th>Liver 2′-C-MeGTP C₂₄ (ng/g)</th>
<th>Plasma 2′-C-MeG₉₀ AUC₀₋₂₄ (ng h/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>6</td>
<td>159</td>
<td>73.4</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>412</td>
<td>145.6</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>658</td>
<td>322.5</td>
</tr>
<tr>
<td>25</td>
<td>2</td>
<td>774</td>
<td>707.7</td>
</tr>
<tr>
<td>50</td>
<td>3</td>
<td>1,263</td>
<td>1,024.3</td>
</tr>
<tr>
<td>150</td>
<td>3</td>
<td>2,880</td>
<td>2,085.3</td>
</tr>
<tr>
<td>300</td>
<td>3</td>
<td>7,130</td>
<td>3,979.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4,309.3</td>
</tr>
</tbody>
</table>

* Mean concentrations for 2′-C-MeGTP measured at 24 h.  
* Area under the curve values covering 0 to 24 h (AUC₀₋₂₄) were calculated from average 2′-C-MeG plasma concentrations measured at six time points.
TABLE 6. Intracellular 2'-C-MeGTP concentration in liver samples from cynomolgus monkeys administered a 25-mg/kg oral dose of INX-08189

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>2'-C-MeGTP liver conc</th>
<th>pmol/g</th>
<th>ng/g</th>
<th>Avg ± SD (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>758</td>
<td>407</td>
<td>282 ± 177</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1,377</td>
<td>740</td>
<td>624 ± 165</td>
<td></td>
</tr>
</tbody>
</table>

Pharmacokinetics in cynomolgus monkeys. To determine the concentration of 2'-C-MeGTP in the liver upon INX-08189 oral dosing, four animals were administered 25 mg/kg, and surgical liver biopsy specimens were collected under anesthesia at 3 and 8 h postdose. The mean liver 2'-C-MeGTP concentration at 3 h was 282 ng/g and increased to 624 ng/g at 8 h (Table 6). The in vitro EC_{90} (130.5 ng/g) was exceeded by 2.16- and 4.78-fold at the respective sample time points.

In an effort to determine the efficiency of INX-08189 extraction by the liver, cynomolgus monkeys with surgically implanted portal vein cannulas were administered 25 mg/kg INX-08189 by oral gavage. INX-08189 concentrations were measured in plasma collected from either the portal vein or the systemic circulation (femoral vein). Following a single oral dose, INX-08189 was detected in the portal circulation within 30 min of dosing and continued to be measured for up to 4 h (Fig. 6). At this dose level, INX-08189 was not detected in the systemic circulation of this animal at any of the time points tested, whereas the metabolite 2'-C-MeG was detected in the systemic circulation beginning at 1 h postdose. These data suggest that INX-08189 is efficiently extracted from the portal circulation by the liver following oral administration, which results in the formation of the active HCV polymerase inhibitor 2'-C-MeGTP in liver tissue.

FIG. 6. Concentrations of INX-08189 and 2'-C-MeGMP guanosine in plasma after the oral dosing of INX-08189 in cynomolgus monkeys. Cynomolgus monkeys surgically fitted with portal vein cannulas were dosed orally with INX-08189 at 25 mg/kg. The analytes measured were 2'-C-MeGMP guanosine concentration in systemic plasma (○), INX-08189 in portal plasma (■), and INX-08189 in systemic plasma (●).
The ability to reduce viral loads rapidly and prevent the emergence of resistant clones during a lengthy treatment period is critical to establishing a robust sustained viral response in patients. We used the clearance of the genotype 1b replicon from Huh-7 cells as an in vitro surrogate to measure these characteristics of INX-08189. In the absence of G418 selection, concentrations as low as 10 nM INX-08189 (~1× EC_{50}) resulted in a ≥2 log_{10} decrease in luciferase activity, and at 40 nM INX-08189 (1× EC_{50}) a ≥7 log_{10} decrease was observed during the 14-day culture period. Culture in the presence of G418 demonstrated that replicon-expressing cells were completely eliminated after 14 days of treatment with as little as 20 nM INX-08189 (~2× EC_{50}). The ability to clear the replicon and eliminate the rebound of viral replication at such low concentrations of inhibitor is significant in light of the fact that other direct-acting antivirals, such as VX-950 (telaprevir) and Rbv, were unable to clear the genotype 1b subgenomic replicon with up to 15 times their respective EC_{50} to clear the genotype 1b replicon at day 21 (19).

The S282T substitution in the HCV NS5B gene had been identified previously as a resistance mutation for 2′-C-MeGTP, which is present in the liver. Therefore, a surrogate marker of INX-08189 bioavailability was required to assess INX-08189 with regard to mitochondrial toxicity therefore was assessed in tissue culture studies with both a liver-derived cell line and a lymphocyte cell line. Incubation in the presence of INX-08189 for 3 or 14 days indicated no change in the ratio of mitochondrial genome copy number to cellular DNA. The results indicate a lack of mitochondrion-specific toxicity for INX-08189.

Mitochondrial toxicity has been observed with certain classes of nucleoside analog drugs that inhibit the activity of the mitochondrial DNA polymerase γ, and this effect has been well studied in the case of reverse transcriptase inhibitors used to treat HIV infection (1, 15). The potential liability of INX-08189 for 3 or 14 days indicated no change in the ratio of mitochondrial genome copy number to cellular DNA. The results indicate a lack of mitochondrion-specific toxicity for INX-08189.

The prodrug strategy embodied by INX-08189 complicates the analysis of pharmacokinetic and pharmacodynamic properties of the molecule. In rodents, the prodrug is short lived in the plasma and cannot be measured systemically. In cynomolgus monkeys, we have shown that at a dose of 25 mg/kg, the intact prodrug was present in the portal circulation for a short time postadministration but was not detected in the systemic circulation, suggesting that at this concentration of INX-08189 the molecule was efficiently extracted by the liver. Therefore, a surrogate marker of INX-08189 bioavailability was required to study its pharmacokinetic and pharmacodynamic properties. In clinical trials using a similar prodrug strategy to deliver 2′-C-MeGTP to the liver, the nucleoside metabolite 2′-C-MeG was utilized as a surrogate analyte to monitor pharmacokinetics in HCV patients (17, 18). To validate this approach for INX-08189, we measured the generation of 2′-C-MeGTP, the active inhibitor of NS5b RdRp, which is present in the liver, and 2′-C-MeG in the plasma. In rats where both metabolites could be measured in the same animals, the data clearly indicate a linear relationship between the 2′-C-MeG AU/C_{24} values and the concentration of 2′-C-MeGTP in the liver at 24 h. The data for cynomolgus monkeys, although more limited than those of the rodent studies, were consistent with this finding. The correlation relationship between the two metabolites suggests that 2′-C-MeG plasma AU/C_{24} values can be used to assess the delivery of 2′-C-MeGTP to the liver in vivo. The rodent data also showed very good dose proportionality for INX-08189, demonstrating a linear relationship in the exposure of both metabolites across a wide range of doses. Based on the measurements of 2′-C-MeGTP concentrations achieved in vivo, we evaluated if liver 2′-C-MeGTP concentrations achieved in vivo would be sufficient to inhibit INX-08189 with Rbv demonstrated a high degree of synergy against both the wild-type and S282T mutant replicons. This is unlike some other 2′-substituted nucleosides, which are additive or antagonistic when combined with ribavirin (8).
viral replication. In rats, the concentration of 2′‐C‐MeGTP in the liver 24 h postdose sufficient for 90% viral inhibition was achieved after a single oral dose of 3 mg/kg. Since this represents a trough level of 2′‐C‐MeGTP, multiple dosing would be expected to further improve triphosphate liver exposures. In monkeys, concentrations greater than the triphosphate EC₅₀ were measured 6 h after a single dose of 25 mg/kg. 2′‐C‐MeGTP in rodent liver was found to be long lived, with a half-life estimated to be greater than 24 h.

Taken together, the in vitro and in vivo data indicate that INX‐08189 is a highly potent inhibitor of HCV with a high barrier for resistance and good oral pharmacokinetic properties. The data support the continued advancement of INX‐08189 in clinical development for the treatment of chronic HCV infections.

ACKNOWLEDGMENTS

Work conducted at the University of Cardiff was supported by a grant from Inhibitex, Inc., to C.M. C.M. is a board member and shareholder of Inhibitex, Inc. We acknowledge Andrea Brancule, Nicola Zonta, and Sarah Jones for their assistance in modeling studies of the HCV RdRp.

REFERENCES