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Antiviral Efficacy upon Administration of a HepDirect Prodrug of 2′-*C*-Methylcytidine to Hepatitis C Virus-Infected Chimpanzees▽

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Hepatitis C virus (HCV) infects an estimated 170 million individuals worldwide, and the current standard of care, a combination of pegylated interferon alpha and ribavirin, is efficacious in achieving sustained viral response in ~50% of treated patients. Novel therapies under investigation include the use of nucleoside analog inhibitors of the viral RNA-dependent RNA polymerase. NM283, a 3′-valyl ester prodrug of 2′-*C*-methylcytidine, has demonstrated antiviral efficacy in HCV-infected patients (N. Afdhal et al., J. Hepatol. 46[Suppl. 1]:S5, 2007; N. Afdhal et al., J. Hepatol. 44[Suppl. 2]:S19, 2006). One approach to increase the antiviral efficacy of 2′-*C*-methylcytidine is to increase the concentration of the active inhibitory species, the 5′-triphosphate, in infected hepatocytes. HepDirect prodrug technology can increase intracellular concentrations of a nucleoside triphosphate in hepatocytes by introducing the nucleoside monophosphate into the cell, bypassing the initial kinase step that is often rate limiting. Screening for 2′-*C*-methylcytidine triphosphate levels in rat liver after oral dosing identified 1-[3,5-difluorophenyl]-1,3-propandiol as an efficient prodrug modification. To determine antiviral efficacy *in vivo*, the prodrug was administered separately via oral and intravenous dosing to two HCV-infected chimpanzees. Circulating viral loads declined by ~1.4 $\log_{10}$ IU/ml and by >3.6 $\log_{10}$ IU/ml after oral and intravenous dosing, respectively. The viral loads rebounded after the end of dosing to predose levels. The results indicate that a robust antiviral response can be achieved upon administration of the prodrug.

Chronic hepatitis C virus (HCV) infection is associated with increased incidence of liver fibrosis, cirrhosis, and hepatocellular carcinoma and is the leading cause of liver transplantation in the United States (13). The current standard for treatment is a combination of pegylated interferon alpha and ribavirin, which results in sustained viral response (SVR) (undetectable virus 6 months after the end of treatment) in 50% or 80% of treated patients harboring a genotype 1 or a genotype 2 or 3 infection, respectively (25). Thus, novel therapies resulting in higher SVR rates, particularly in the treatment of genotype 1 infections, are needed.

Nucleoside analogs inhibiting the HCV RNA polymerase have been investigated as potential therapeutics to treat HCV infections. 2′-*C*-Methylcytidine inhibits HCV RNA replication in the replicon assay (consensus 1 50% effective concentration [EC_{50}], ~1 μM) via inhibition of the HCV RNA polymerase, which is inhibited by the 2′-*C*-methylcytidine triphosphate *in vitro* in cell-free biochemical assays (50% inhibitory concentration [IC_{50}], ~0.2 μM) (15). NM283, the 3′-*O*-valinyl ester prodrug of 2′-*C*-methylcytidine, has improved oral bioavailability compared to 2′-*C*-methylcytidine (21) and has been investigated in HCV-infected patients in clinical studies. In a phase IIb study, patients receiving NM283 in combination with pegylated interferon alpha and ribavirin at 24 weeks of therapy experienced mean viral load reductions of up to 3.3 $\log_{10}$ IU/ml compared to 2.3 $\log_{10}$ IU/ml reductions for patients receiving pegylated interferon-ribavirin alone (2). Due to the high incidence of gastrointestinal side effects, which required reduction of the highest daily dose of 800 mg to 200 or 400 mg, further development of NM283 was placed on hold.

The HepDirect prodrug modifications of nucleoside 5′-monophosphates (NMP) are substituted cyclic 1,3-propanyl esters that offer an opportunity to bypass the initial phosphorylation step that is often rate limiting for the formation of nucleoside 5′-triphosphates (reviewed in references 5, 6, and 7). Prodrug activation is accomplished via cytochrome P450-catalyzed oxidation, resulting in the intracellular generation of nucleoside 5′-monophosphate. The two subsequent phosphorylations that yield the 5′-triphosphate are catalyzed by endogenous kinase activities. Liver tissue contains higher levels of P450 activity than other tissues; thus, tissue specificity for the activation and triphosphate formation is observed.

HepDirect prodrugs of 2′-*C*-methylcytidine were investigated for conversion to the triphosphate *in vivo* in rats and rhesus macaques. A 1-[3,5-difluorophenyl]-1,3-propanediol modification was determined to yield the highest levels of triphosphate in rat liver after oral (p.o.) dosing. *In vivo* efficacy was observed in the chimpanzee model of chronic HCV infection upon oral and intravenous dosing of the compound.

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MATERIALS AND METHODS

Nucleoside triphosphate (NTP) generation in rat hepatocytes. Hepatocytes were prepared from fed Sprague-Dawley rats (250 to 300 g) according to a published procedure (3) as modified (9). Hepatocytes (20 mg/ml wet weight;

>85% trypan blue viability) were incubated at 37°C in 2 ml of Krebs-bicarbonate buffer containing 20 mM glucose and 1 mg/ml bovine serum albumin (BSA) for 2 h in the presence of 1 to 250 μM nucleoside or prodrug (from 25 mM stock solutions in dimethyl sulfoxide [DMSO]). Following the incubation, a 1,600-μl aliquot of the cell suspension was centrifuged, and 300 μl of acetonitrile was added to the pellet. The resulting mixture was vortexed and sonicated until the pellet was dispersed. Then, a volume of 200 μl of water was added to make a 60% acetonitrile solution. After 10 min of centrifugation at 14,000 rpm, the resulting supernatant was transferred to a new vial and evaporated to near dryness in a Savant SpeedVac Plus at room temperature. The dried residue was reconstituted with 200 μl of water, and the mixture was centrifuged for 10 min at 14,000 rpm. A mixture of a 35-μl aliquot of supernatant and 35 μl of mobile phase A (20 mM *N*,*N*-dimethylhexylamine and 10 mM propionic acid in 20% methanol) was analyzed by the liquid chromatography-tandem mass spectrometry (LC-MS/MS) method described below.

Evaluation of liver NTP levels in rat. Nucleoside analogs and their prodrugs were administered to Sprague-Dawley rats via oral gavage, intravenous (i.v.) bolus, or intraperitoneal injection. At prespecified times following drug administration, animals were anesthetized with halothane, and liver samples (~1 g) were collected, snap-frozen, and homogenized in 3 volumes of ice-cold 70% methanol containing 20 mM EDTA/EGTA. Following centrifugation to clarify the homogenate, aliquots of the supernatants (100 μl) were evaporated to dryness in a Savant Speed-Vac Plus (1 h; room temperature). The resulting dried residue was reconstituted with 100 μl of mobile phase and then analyzed for nucleotides by a modification (8) of an LC-MS/MS method as described below.

The reconstituted extracts in mobile phase A (20 mM *N*,*N*-dimethylhexylamine and 10 mM propionic acid in 20% methanol) were analyzed by LC-MS/MS (Applied Biosystems; API 4000) with a mass spectrometer equipped with an Agilent 1100 binary pump and a Leap injector. A volume of 10 μl of sample was injected onto an Xterra MS C_{18} column (3.5 μm; 2.1 by 50 mm; Waters Corp.) with a SecurityGuard C_{18} guard column (5 μm; 4.0 by 3.0 mm; Phenomenex) and eluted with a gradient mobile phase A and B (20 mM *N*,*N*-dimethylhexylamine and 10 mM propionic acid in 80% methanol) at a flow rate of 0.3 ml/min (0 min, 0% B; 0 to 1 min, 0 to 50% B; 1 to 3 min, 50 to 100% B; 3 to 6 min, 100% B; 6 to 6.1 min, 100 to 0% B; 6.1 to 9 min, 0% B). NTP was detected by using MS/MS mode (M^-/78.8). The quantitative analysis of liver NTP was calculated based on a calibration curve generated using an authentic standard of 2′-*C*-methylcytidine triphosphate (0.01 to 30 μM).

Oral multiple-dose study in rhesus macaques. Rhesus macaques were administered compound 7 via oral gavage once daily for 7 consecutive days at a dose level of 50 mg/kg of body weight/day. Plasma samples (1, 2, 4, 8, and 24 h postdose) were obtained on days 1 and 7. Plasma concentrations of compound 7 and 2′-*C*-methylcytidine were determined by LC-MS/MS. For all determinations of analyte concentrations in rhesus and chimpanzee liver tissue and plasma, quality control (QC) samples that spanned the entire concentration range of calibration standards were run. These QC samples were prepared by spiking blank matrix (plasma or liver) with each analyte at 5 to 7 concentration levels (6 replicates per level). The criterion for acceptance of analysis results was <15% relative standard deviation (RSD) on QC replicates (and ±20% accuracy) at each concentration level (6 replicates per concentration level). The analytical accuracy and reproducibility of each reported value are thus ±20%.

Intravenous multiple-dose study in rhesus macaques. Compound 7 was administered to rhesus macaques at a dose level of 20 mg/kg/day once daily for 7 consecutive days via i.v. bolus injection. Plasma samples (5 min to 4 h postdosing) were obtained on days 1 and 7. The plasma concentrations of compound 7 and 2′-*C*-methylcytidine were determined by LC-MS/MS.

Liver tissue samples were obtained on day 8, ~24 h after the final intravenous dose of compound 7. Liver samples designated for measurement of total 2′-*C*-methylcytidine were frozen on dry ice after sacrifice. Levels of 2′-*C*-methylcytidine in homogenates of these liver samples were measured by LC-MS/MS after complete hydrolysis of 2′-methylcytidine-related nucleotide anabolites back to 2′-*C*-methylcytidine with acid phosphatase treatment. To minimize nucleotide degradation, liver samples for measurement of 2′-*C*-methylcytidine triphosphate were obtained by an alternate surgical *in situ* freeze clamp (FC) procedure prior to animal sacrifice. The concentration of 2′-*C*-methylcytidine triphosphate was determined using LC-MS/MS after homogenization of FC liver samples on ice in 70/30 methanol/20 mM aqueous EDTA/EGTA, pH 7.4, and solid-phase extraction (SPE).

Chimpanzees. The housing, maintenance, and care of the chimpanzees (*Pan troglodytes*) used in the study were in compliance with all relevant requirements at Merck Research Laboratories and at New Iberia Research Center (University of Louisiana at Lafayette). The study protocols were reviewed and approved by the Institutional Animal Care and Use Committees at both sites. The HCV genotype infecting the chimpanzees was determined by a line probe assay (Versant HCV genotype assay, LiPa; Bayer Diagnostics/Innogenetics) and confirmed by reverse transcription (RT)-PCR rescue of HCV genetic material and DNA sequencing. Chimpanzees X1 and X2 were infected with HCV genotype 1a, H77 isolate.

Administration of compound 7 to HCV-infected chimpanzees. Doses for i.v. administration of compound 7 were formulated in Captisol/phosphate and stored at 4°C prior to administration. The HCV-infected chimpanzees were sedated using a minimal amount of tiletamine/zolazepam or ketamine prior to i.v. administration of the compound and dosed at a level of 4 mg/kg once daily for 6 consecutive days. Blood samples for viral load determinations were removed just prior to the administration of the next dose of compound. Blood samples for determination of compound concentrations during the i.v. dosing phase were removed just prior to and just after the administration of each dose to represent 24-h and 10-min time points, respectively. Blood was processed into plasma within 1 h of collection. Plasma samples were aliquoted and stored frozen at −70°C.

The compound was also dosed orally as a solution in Tang to conscious HCV-infected chimpanzees. Doses were administered once daily for seven consecutive days at a dose level of 10 mg/kg. Plasma samples for determination of viral load and compound concentration were collected under ketamine sedation 7 h after dosing on selected days. Plasma samples were collected weekly for 4 weeks after the last dose was administered for both the i.v. and oral dosing phases to monitor the rebound of the viral load.

The dose levels for the chimpanzee experiments were chosen to deliver the highest dose that could be administered based on the solubility of compound 7 in the vehicles and practical limitations of the dosing volume.

Viral load determinations. Viral load determinations were performed on plasma samples using the HCV TaqMan assay (Analyte Specific Reagent version; Roche) for quantitative analysis.

RESULTS

Synthesis of prodrugs. The synthesis of prodrugs of 2′-*C*-methylcytidine was carried out as depicted in Fig. 1 (6, 23). The nucleoside was protected as its 2′,3′-isopropylidene ketal and converted to dimethylformamidine 2. Phosphorylation at 5′ was accomplished with *trans-p*-nitrophenylphosphate reagents 3a to 3h, affording protected derivatives 4a to 4h. Removal of the protecting groups gave the desired *cis*-prodrugs 5a to 5h. In most cases, the phosphorylating reagents 3a to 3h were prepared in racemic form, and therefore, the prodrugs 5a to 5h are a mixture of two *cis*-diastereomers; a single *cis*-diastereomer was prepared in the cases of 3-chlorophenyl prodrug 5b and 4-pyridyl prodrug 5g, since the corresponding phosphorylation reagents, 3b and 3g, were available as single enantiomers from other projects (7). In this study, as in others, the corresponding *cis*-diastereomer prodrugs derived from the (*R*)-diol precursors to 3b and 3g were efficiently activated in rat hepatocytes, although to a lesser extent (up to 2-fold) than those derived from the (*S*)-diol precursors (data not shown). Substituents on the phenyl group were limited to halogens, as these analogs had previously shown the highest levels of activation (10).

Activation in rat hepatocytes. Activation of prodrugs 5a to 5h to the corresponding NTPs, which requires prodrug cleavage as well as phosphorylation catalyzed by cellular nucleotide kinases, was evaluated in rat hepatocytes (Table 1). In this assay, nucleoside 1 showed no conversion to triphosphate. All of the prodrugs were activated to the NMP and converted to NTP, with the halosubstituted phenyl analogs giving the highest levels.

Evaluation of liver NTP levels in rat. Levels of NTP generated in the liver following administration of compound 1 and its prodrugs 5a to 5h were evaluated in the rat. Animals were

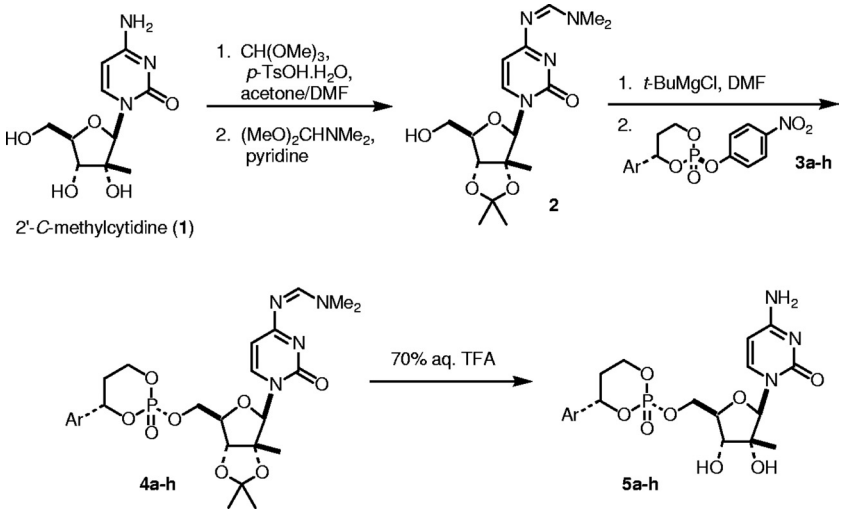

FIG. 1. Synthesis of HepDirect prodrugs of 1. aq. TFA, aqueous trifluoroacetic acid.

sacrificed at 1 h and 3 h following intraperitoneal (i.p.) dosing and at 3 h and 5 h following p.o. dosing. The higher of the two values is reported in Table 1. With i.p. dosing, nucleoside 1 affords low but measurable levels of NTP in the liver. It is not known why the triphosphate of compound 1 was detectable in rat liver after i.p. dosing but not after incubation with rat hepatocytes (Table 1). However, the levels of the triphosphate of compound 1 were relatively low even in rat liver tissue after i.p. dosing. With the exception of 2,3-difluorophenyl prodrug 8h, all of the HepDirect prodrugs gave liver levels at least 10-fold higher than those achieved with compound 1. The halophenyl prodrugs gave higher levels than did the pyridyl prodrugs 5f and 5g, and among these, the 3,5-difluorophenyl prodrug 5d was superior at 192 nmol/g. With oral administration, NTP levels were considerably lower with all compounds; the highest value was again with the 3,5-difluorophenyl prodrug 5d, at 15.1 nmol/g.

Full PK evaluation of compound 5d in rat. Based on the superior performance of 3,5-difluorophenyl analog 5d in the oral NTP screen, a more complete temporal profile of liver NTP levels after intravenous and oral administration was determined (Fig. 2). Upon intravenous administration (5.2 mg/kg nucleoside equivalents [n.e.]), conversion to the triphosphate is rapid, achieving a maximum concentration of drug in serum (C_{max}) of >80 nmol/g within 1 h. The half-life is 14.8 h, and NTP levels remain above 30 nmol/g for 24 h. The initial observation of low oral bioavailability in the screening study was confirmed in this more thorough pharmacokinetic (PK) evaluation. The maximum liver NTP concentration was 3.8 nmol/g at the 5-h time point following intravenous administration. Oral bioavailability, based on the area under the concentration-time curve from 0 to 24 h ($AUC_{0\text{-}24}$), was less than 5%.

Synthesis and evaluation of individual diastereomers comprising compound 5d. As noted above, compound 5d is a mixture of two *cis*-diastereomers as a result of being derived from racemic phosphorylating agent 3d. We sought to identify the preferred diastereomer of the two prior to initiating advanced evaluation. Thus, the enantiomers of 3d were prepared in optically pure form from the corresponding (*R*)- and (*S*)-1-[3,5-difluorophenyl]-1,3-propanediols, which were synthesized

TABLE 1. Levels of NTP in rat hepatocytes and after administration of prodrugs to rats[a]

Compound	Aryl	Rat hepatocyte [NTP] at 2 h (25 μM) (nmol/g)	Rat liver [NTP] (5 mg/kg[b] i.p.) (nmol/g)	Rat liver [NTP] (10 mg/kg[b] p.o.) (nmol/g)
1		0	1.9	<1
5a	3-F-phenyl	226	50	1.6
5b	(*S*)-3-Cl-phenyl	310		4.9
5c	3-Br-phenyl	148	149	7.5
5d	3,5-F_2-phenyl	134	192	15.1
5e	3,5-Cl_2-phenyl	168	71	1.9
5f	3-Pyridyl	31	31	1.2
5g	(*S*)-4-Pyridyl	26	26	1.2
5h	2,3-F_2-phenyl	71	2.5	<1

[a] Data are from single determinations.
[b] Nucleoside equivalents; dose normalized to 5 mg/kg i.p. and 10 mg/kg p.o.

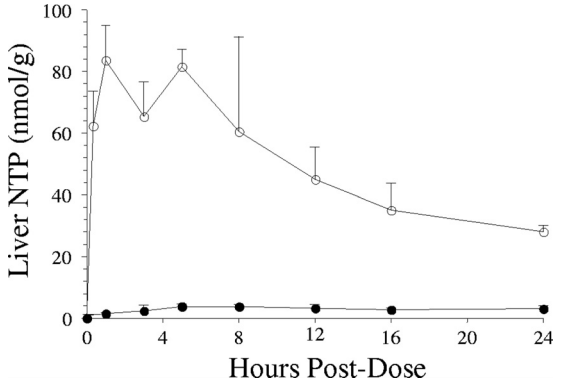

FIG. 2. Mean (plus standard deviation [SD]) liver concentration-time profile of NTP following an i.v. bolus (○) and oral administration ([circf]) of 2.6 and 5.2 mg/kg nucleoside equivalents of compound 5d, respectively, to male Sprague-Dawley rats. The values represent the means of three replicates.

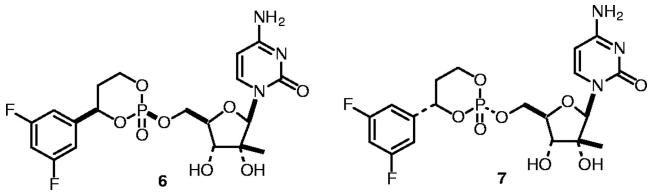

FIG. 3. Structures of (*R*)- and (*S*)-diastereomers of compounds 6 and 7 comprising mixture 5d.

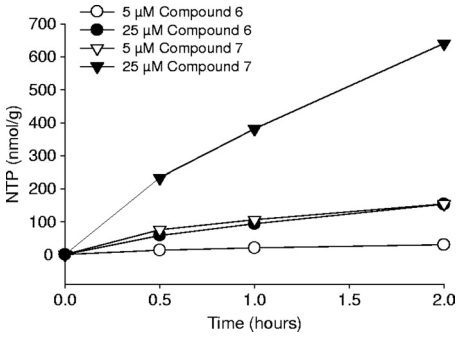

FIG. 4. Time course of NTP formation from compounds 6 and 7 in rat hepatocytes. The error in the determination of the triphosphate concentration was typically 10 to 15%.

analogously to the corresponding 3-chlorophenyl analog (24). Application of the synthetic sequence in Fig. 1 produced (*R*)-diastereomer 6 and (*S*)-diastereomer 7 (Fig. 3).

The time course of NTP formation in rat hepatocytes following exposure to 5 and 25 μM compound 6 and compound 7 is shown in Fig. 4. The rate of NTP formation from prodrug 7 is approximately 4-fold greater than that from 6 at both concentrations tested. This increased rate of activation is seen *in vivo*, as well; diastereomer 7 affords significantly higher levels of NTP in liver than 6 (Fig. 5). Based on these results, compound 7 was selected as the preferred diastereomer for further evaluation.

Pharmacokinetic parameters after oral dosing of compound 7 in rhesus macaques. The mean plasma pharmacokinetic parameters ($AUC_{0\text{-}24}$, C_{max}, and time to maximum concentration of drug in serum [T_{max}]) are tabulated in Table 2. The low plasma exposure observed is suggestive of poor oral absorption and/or high hepatic extraction of compound 7 after oral administration to the rhesus macaques. Plasma concentrations of 2′-*C*-methylcytidine at 24 h after administration of the dose averaged 0.03 μM.

Intravenous multiple-dose study in rhesus macaques. Compound 7 was administered intravenously to rhesus macaques at a dose level of 20 mg/kg/day once daily for 7 consecutive days. Compound 7 exhibited a short plasma half-life (~1 h), a moderate volume of distribution (0.8 liters/kg), and a plasma $AUC_{0\text{-}4}$ of 20 μM · h. Plasma clearance of the prodrug was high, on the order of hepatic blood flow in monkey (~45 ml/min/kg). The mean plasma pharmacokinetic parameters on day 7 for the 2′-*C*-methylcytidine metabolite were as follows: C_{max}, ~1.3 μM; T_{max}, ~0.75 h; $AUC_{0\text{-}4}$, ~3.7 μM · h. Thus, 2′-*C*-methylcytidine plasma exposure was approximately 17 to 33% that of the prodrug AUC.

The mean concentration of 2′-*C*-methylcytidine in liver tissue samples taken 24 h after the final intravenous dose of compound 7 and treated with phosphatase to convert phosphorylated anabolites to the nucleoside was 27 μM, which represents the total concentration of 2′-*C*-methylcytidine-related material in the liver. Liver tissue samples were also obtained 24 h after the final intravenous dose of compound 7 using a freeze-clamping technique to minimize the dephosphorylation of 2′-*C*-methylcytidine triphosphate as a result of phosphatase activity in liver tissue. The mean liver concentration of 2′-*C*-methylcytidine triphosphate was 17 μM. Similar concentrations of 2′-*C*-methylcytidine after acid phosphatase treatment and 2′-*C*-methylcytidine triphosphate measured in FC liver samples suggest that the majority (>60%) of nucleoside-related material remaining in the liver 24 h after i.v. administration of compound 7 to the rhesus monkey is present in the form of the 5′-triphosphate.

Oral administration of compound 7 to HCV-infected chimpanzees. Two HCV-infected chimpanzees received compound 7 administered via oral dosing at a dose level of 10 mg/kg once daily for 7 consecutive days. Plasma levels of compound 7 for drug days 0 to 21 in samples collected 7 h after daily dose administration are presented in Table 3.

Intravenous administration of compound 7 to HCV-infected chimpanzees. After a rest period to allow complete clearance of the compound and to allow circulating viral loads to rebound to baseline levels, the same two HCV-infected chimpanzees were administered compound 7 via bolus intravenous injection once daily for 6 consecutive days. The concentrations of compound 7 and the metabolite 2′-*C*-methylcytidine were determined in plasma samples obtained 7 h after dosing for select days during the study and are listed in Tables 4 and 5, respectively (note that plasma prodrug levels in Table 4 are listed in μM units, whereas 2′-*C*-methylcytidine units in Table 5 are nM).

Serum clinical chemistry, urinalysis, and electrocardiograms (ECGs) were monitored periodically during the studies. No significant changes in these parameters were observed during the oral-dosing study. An increase (50 to 100%) in aspartate transaminase (AST) values was observed during the intravenous-dosing study in both chimpanzees. The basis for the increase in AST is not certain. It may be associated with drug exposure, or it may be associated with the repeated sedations and intravenous injections that occurred during the study. The serum AST values returned to near baseline levels after dosing ended. No significant changes in the other monitored parameters were observed.

Circulating viral load in chimpanzees administered compound 7. Circulating viral load levels were determined in plasma samples collected from the two HCV-infected chimpanzees that received multiple doses of compound 7 via either oral administration or i.v. bolus injection in separate experiments. Plasma samples were collected periodically before, during, and after the duration of dosing. Oral administration of compound 7 at a dose level of 10 mg/kg once daily for 7 consecutive days resulted in maximal viral load decreases of ~1.3 and 1.5 $\log_{10}$ IU/ml in the two chimpanzees (Fig. 6A).

i.v. administration of compound 7 at a dose level of 4 mg/kg

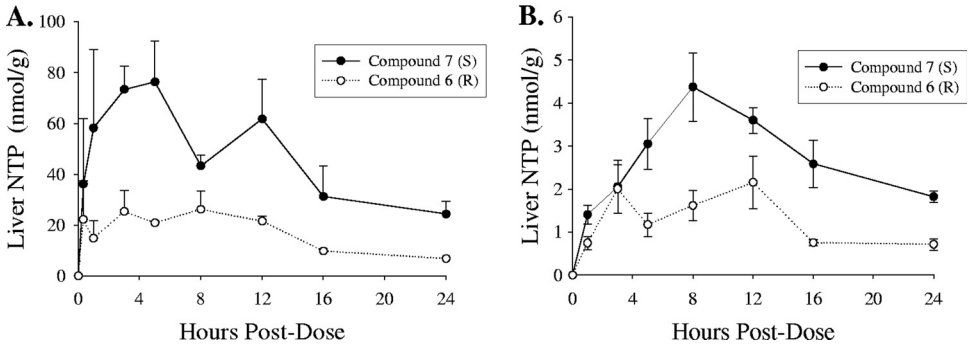

FIG. 5. Mean liver concentration-time profile of NTP following i.v. bolus (2.6 mg/kg; nucleoside equivalents) (A) and oral (5.2 mg/kg; nucleoside equivalents) (B) administration of compounds 6 and 7 to male Sprague-Dawley rats. The values represent the means of three replicates. The error bars represent the SDs.

once daily for 6 consecutive days resulted in a maximal viral load decline of 4.8 $\log_{10}$ IU/ml in chimpanzee X2. The viral load in chimpanzee X1 declined by 3.6 $\log_{10}$ IU/ml and reached the lower limit of the assay by day 2 of i.v. dosing. The viral load in chimpanzee X1 remained below the lower limit of quantitation throughout the duration of dosing and for at least 48 h after the administration of the last dose but became detectable by 5 days after the administration of the last dose (Fig. 6B).

DISCUSSION

Multiple antiviral targets have been investigated in the effort to develop novel therapies to improve the treatment of chronic HCV infection. Much of the effort has focused on inhibitors of the virally encoded NS3/4A protease and NS5B RNA polymerase. The administration of inhibitors of NS3/4A protease, such as telaprevir, boceprevir, TMC435, ITMN-191, or MK-7009, to HCV-infected patients has resulted in significant reductions in the level of circulating viral load (11, 25, 28).

Reductions in the circulating viral load have also been observed upon administration of nucleoside analog inhibitors of HCV RNA polymerase in clinical studies. Administration of R1626, an ester prodrug of 4′-azidocytidine, in combination with standard of care (SOC) (pegylated interferon alpha and ribavirin), resulted in a rapid viral response (RVR) (undetectable viral load at 4 weeks) rate of 81% of treated patients compared to 10% for standard of care alone (22). R7128, a prodrug of 2′-fluoro-2′-methylcytidine, administered in combination with SOC, resulted in an RVR rate of 85% at the highest dose, 1,500 mg twice daily (25). The development of viral resistance to inhibition by either R1626 or R7128 was not detected in these studies, consistent with the apparently higher barrier to the development of resistance observed with nucleoside analogs during *in vitro* experiments in the HCV replicon system (16).

NM283 has demonstrated antiviral efficacy upon oral administration in HCV-infected patients (1, 2, 14, 20). However, gastrointestinal side effects have limited the dose levels and resulted in the discontinuation of development of the compound. The administration of a novel prodrug of the monophosphate of 2′-*C*-methylcytidine offers the possibility of obviating the side effect profile and increasing the antiviral efficacy compared to NM283. Prodrugs of the monophosphates of other nucleoside analogs have shown improved potency *in vitro* in HCV replicon or viral replication assays compared to the parent nucleoside analog (12, 17–19, 26), and several are under clinical investigation.

In this work, HepDirect prodrugs of 2′-*C*-methylcytidine were evaluated. The potency of these HepDirect prodrugs in the HCV replicon assay was not assessed because the replicon cell line does not express sufficient levels of cytochrome P450 activity to activate the prodrugs. Screening of a small library of 1-aryl-1,3-propanediol derivatives identified the 3,5-difluorophenyl modification (5d) as resulting in the highest levels of 2′-*C*-methylcytidine 5′-triphosphate in livers of rats following oral dosing. The synthesis of the two *cis*-diastereomers of 5d

TABLE 2. Plasma pharmacokinetic parameters (day 1 and day 7) in rhesus macaques administered compound 7 orally once daily at 50 mg/kg/day

Subject no.	Study day[a]	Plasma $AUC_{0\rightarrow24}$ (μM · h)	Plasma C_{max} (μM)	Plasma T_{max} (h)
1	1	0.53	0.27	1
2	1	1.12	0.08	1
Mean	1	0.83	0.18	1
1	7	1.08	0.18	2
2	7	1.19	0.11	4
Mean	7	1.14	0.14	3

[a] Plasma samples were obtained on days 1 and 7 of the study (0 to 24 h).

TABLE 3. Concentrations of compound 7 in plasma 7 h after oral dosing at 10 mg/kg to HCV-infected chimpanzees X1 and X2 once daily for 7 days

Drug day	Plasma concn (μM)		
	X2 (male)	X1 (female)	Mean
0	0.01	0.03	0.02
2	0.01	0.06	0.04
5	0.03	0.12	0.08
7	0.01	0.01	0.01
10	<LOQ[a]	<LOQ	<LOQ
14	<LOQ	<LOQ	<LOQ
21	<LOQ	<LOQ	<LOQ

[a] LOQ, lower limit of quantitation (0.004 μM).

TABLE 4. Plasma concentrations of compound 7 in chimpanzees X1 and X2 dosed via i.v. administration at 4 mg/kg once daily for 6 days

Drug day[a]	Time	Plasma concn (μM)		
		X2 (Male)	X1 (Female)	Mean
Predose		<LOQ[a]	<LOQ	<LOQ
0	5 min	11.3	13.4	12.4
	24 h	<LOQ	<LOQ	<LOQ
1	5 min	12.8	17.6	15.2
	24 h	<LOQ	<LOQ	<LOQ
2	5 min	13.1	16.8	15.0
	24 h	<LOQ	<LOQ	<LOQ
3	5 min	11.7	15.9	13.8
	24 h	<LOQ	<LOQ	<LOQ
4	5 min	11.8	16.7	14.2
	24 h	<LOQ	<LOQ	<LOQ
5	5 min	13.0	18.3	15.6
	24 h	<LOQ	<LOQ	<LOQ

[a] Plasma concentrations for days 6 to 10 were less than the lower limit of quantitation (LOQ) (0.004 μM).

and further characterization identified the *S*-isomer, **7**, as resulting in higher triphosphate levels in rat liver.

Prior to administering compound 7 to HCV-infected chimpanzees, it was administered to rhesus macaques in order to assess acute toxicities and determine the plasma exposure and the concentration of 2′-*C*-methylcytidine-5′-triphosphate in liver tissue in a nonhuman primate species. The compound was administered either orally or via i.v. injection in a multiple-dose study in rhesus macaques. The results are consistent with excellent plasma exposure following i.v. dosing and relatively poor oral bioavailability of compound 7 in rhesus macaques. I.v. administration resulted in high levels of the active inhibitory species, the 5′-triphosphate, in rhesus macaque liver, in excess of 100 times the IC_{50} for inhibition of the HCV RNA polymerase in biochemical assays (16) 24 h after dosing. No signs of acute toxicity were observed.

TABLE 5. Plasma concentrations of 2′-*C*-methylcytidine in chimpanzees X1 and X2 intravenously administered 4 mg/kg of compound **7** once daily for 6 days

Drug day	Time	Plasma concn (nM)		
		X2 (male)	X1 (female)	Mean
Predose		<LOQ[a]	<LOQ	<LOQ
0	5 min	94	114	104
	24 h	19.6	55.2	37.4
1	5 min	114	141	128
	24 h	47.1	49.3	48.2
2	5 min	169	155	162
	24 h	51.1	73.9	62.5
3	5 min	167	118	142
	24 h	53.0	78.2	65.6
4	5 min	144	119	131
	24 h	46.4	65.3	55.9
5	5 min	120	134	127
	24 h	53.5	59.3	56.4
7		21.8	40.0	30.9
10		<LOQ	<LOQ	<LOQ

[a] LOQ, lower limit of quantitation (19.44 nM).

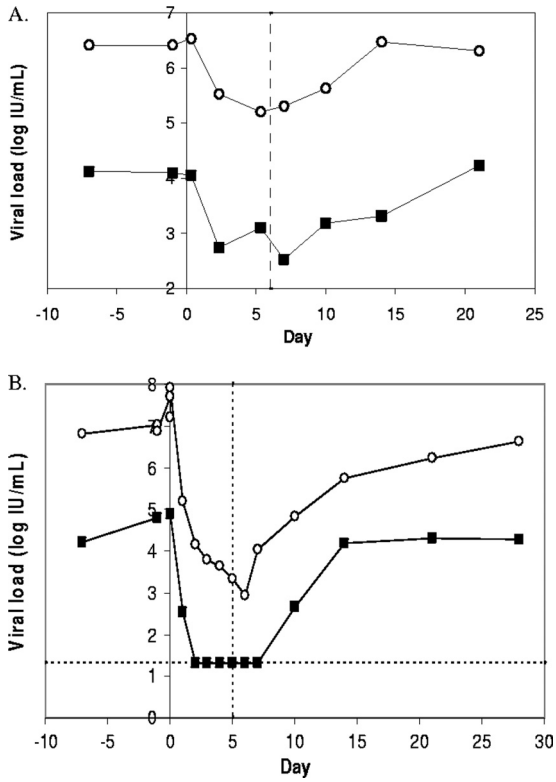

FIG. 6. Viral load in HCV-infected chimpanzees administered compound 7. The error in the determination of the viral load using the TaqMan assay when replicate determinations were conducted was typically ~0.3 $\log_{10}$ IU/ml. (A) Chimpanzees X1 (○) and X2 (■) were administered compound 7 via oral dosing at 10 mg/kg/day once daily for six consecutive days. The first dose was administered at time zero. Plasma samples were collected 7 h after the administration of the next dose on select days during therapy and at the same time of day pre- and posttherapy. (B) Chimpanzees X1 (○) and X2 (■) were administered compound 7 via intravenous dosing at 4 mg/kg per day. The first dose was administered at time zero. The viral load was determined by use of the HCV TaqMan assay (Roche). The horizontal dashed line represents the lower limit of quantitation of the HCV TaqMan assay (20 or 1.3 $\log_{10}$ IU/ml). The vertical dashed lines indicate the times of administration of the last doses.

The high concentration of 2′-*C*-methylcytidine-5′-triphosphate in liver tissue after administration to rhesus macaques relative to its inhibitory potency *in vitro* provided justification for investigating efficacy in the chimpanzee model of HCV infection. Oral administration of compound 7 at 10 mg/kg once daily for 7 days resulted in an approximate 1.4 $\log_{10}$ reduction in the viral load at the end of dosing. I.v. administration of the compound at 4 mg/kg once daily for 5 days resulted in 4.8 and

>3.6 $\log_{10}$ reductions in the viral load. In comparison, oral administration of NM283 to HCV-infected chimpanzees resulted in ~1.0 $\log_{10}$ reduction at a dose of 16 mg/kg and 0.8 $\log_{10}$ reduction at a dose of 8 mg/kg (27). Thus, oral administration of compound 7 did not result in substantially greater viral load reductions than oral administration of NM283 in the chimpanzee model. Furthermore, i.v. administration of 2′-*C*-methyl-7-deaza-adenosine (MK-0608) at a dose level of 2 mg/kg once daily resulted in a viral load reduction of >5.7 $\log_{10}$ after 7 days of dosing (4). Thus, i.v. administration of compound 7 gave viral load reductions comparable to those with i.v. dosing of MK-0608. The antiviral efficacy of compound 7 following oral administration is likely limited by poor oral bioavailability. Efforts to identify nucleoside analogs with robust efficacy following oral dosing and acceptable safety margins are continuing.

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