RNA polymerases effectively discriminate against deoxyribo-nucleotides and specifically recognize ribonucleotide substrates most likely through direct hydrogen bonding interaction with the 2′-α-hydroxy moieties of ribonucleosides. Therefore, ribonucleoside analogs as inhibitors of viral RNA polymerases have mostly been designed to retain hydrogen bonding potential at this site for optimal inhibitory potency. Here, two novel nucleoside analogs as inhibitors of viral RNA polymerases have been fully progressed into clinical development. Most antiviral candidates are currently being evaluated in clinical studies, the majority targeting either the HCV protease or HCV polymerase enzymes, which are essential for viral replication. The HCV RNA-dependent RNA polymerase, NS5B, contains the active site responsible for viral RNA synthesis and functions as part of a membrane-associated replicase complex. Nucleoside and non-nucleoside inhibitors of HCV polymerase have successfully progressed into clinical development. Most antiviral nucleoside analogs are further metabolized to the corresponding nucleoside 5′-triphosphate analogs by cellular enzymes. Nucleoside 5′-triphosphate analogs then function as alternative substrates for the viral polymerase, competitively inhibit viral nucleic acid synthesis, and can terminate nucleic acid synthesis after incorporation.

Previously, R1479 was identified as a potent and selective inhibitor of HCV replication in cell culture (6, 7). R1626, a prodrug of 4′-azidocytidine, is presently in phase II clinical development. In a multiple ascending dose phase I study, R1626 showed dose proportional pharmacokinetics and robust antiviral effects in HCV-infected persons. When administered for 14 days, R1626 achieved mean HCV viral load reductions of 1.2,
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2.6, and 3.7 log IU/ml at dose levels of 1500, 3000, and 4500 mg, twice a day, respectively (8). R1626 therefore provided clinical proof-of-concept for 4'-modified ribonucleoside analogs as inhibitors of HCV replication. Further studies were aimed at increasing the antiviral potency of 4'-modified nucleosides as potential second generation inhibitors with improved efficacy for the treatment of HCV infection.

Antiviral potency of nucleosides is often limited by phosphorylation efficiency, as modified analogs can be poor substrates for human nucleoside kinases (9). To identify novel nucleosides with increased antiviral potency, the focus of optimization was therefore on increasing phosphorylation efficiency by human kinases as well as on increasing intrinsic incorporation efficiency by the recombinant NS5B enzyme. RNA polymerases are believed to discriminate against 2'-deoxyribonucleotides by forming a hydrogen bond interaction with the 2'-α-hydroxyl group that differentiates ribonucleotides from deoxyribonucleotides (10–12). Structural analysis of HCV polymerase in complex with ribonucleotides suggested that the conserved Asp-225 of HCV NS5B functions in this manner to confer ribonucleotide specificity (13). We tested the hypothesis that the addition of a 4'-substituent could provide novel interactions in the HCV polymerase active site to compensate for the absence of a 2'-α-hydroxy group. 2'-Deoxy-2'-β-fluoro-4'-azidocytidine (RO-0622) and 2'-deoxy-2'-β-hydroxy-4'-azidocytidine (RO-9187) were found to be surprisingly potent inhibitors of HCV replication with antiviral potencies up to 50-fold higher than that of R1479, despite the lack of hydrogen bonding potential with the conserved Asp-225 of NS5B.

EXPERIMENTAL PROCEDURES

Human Nucleoside Kinase Assays—Recombinant human deoxycytidine kinase (dCK), thymidine kinases 1 and 2 (TK1 and TK2), and uridine-cytidine kinases 1 and 2 (UCK-1 and UCK-2) were expressed and purified using a bacterial vector system Escherichia coli BL21 (DE3) pLysS (14–18). The proteins were purified by metal chelate affinity chromatography using a Ni2+-nitrilotriacetic acid–agarose resin. The His tag was removed from the purified proteins with thrombin. Kinase activity was measured in an adenosine 5'-triphosphate transfer assay performed with 0.05 μM [γ-32P]ATP (10 μCi/μl), 100 μM ATP, 50 mM Tris-HCl, pH 7.6, 5 mM MgCl2, 100 mM KCl, 0.5 mg/ml bovine serum albumin, 10 mM dithiothreitol, and different concentrations of nucleoside analogs. The reaction was initiated by adding 50 ng of the enzyme followed by incubation at 37 °C for 25 min and terminated by boiling. 2 ml of the supernatant was applied to polyethyleneimine cellulose F TLC plates. Chromatography was performed for 8–12 h with isobutyric acid/NH4OH/H2O (66:1:33) (v/v/v) as the mobile phase. The products of the kinase reaction were detected by autoradiography. The spots were excised and eluted with 0.5 ml of 0.2 M KCl, 0.1 M HCl (1:1, v/v) and quantified by liquid scintillation counting or by the Fuji BAS 2500/LAS 1000 PhosphorImaging system, with an Image Reader version 1.7E. Kinetic parameters were determined using nonlinear regression analysis with SigmaPlot 2001 software and Michaelis-Menten equation.

HCV Replicon Assays—HCV replicon assays were performed using either the 2209-23 cell line or the transient replicon system in cured Huh-7 cells as described (6, 19). Nucleoside analogs were synthesized at Medivir, dissolved in Me2SO, and then diluted in Dulbecco’s modified Eagle’s medium with 5% (v/v) fetal bovine serum before addition to cells. The final concentration of Me2SO was 1% in all experiments. Quantification of Renilla and firefly luciferase activities was performed using luciferase kits from Promega according to the manufacturer’s instructions. The WST-1 cell proliferation assay (Roche, Palo Alto, CA) was used to measure cell viability. The effect of compounds on the incorporation of tritiated thymidine into cellular DNA was measured using the [3H]thymidine incorporation scintillation proximity assay system from Amer sham Biosciences.

HCV Polymerase Assay—The HCV polymerase activity was measured as the incorporation of radiolabeled nucleotide monophosphates into acid-insoluble RNA products using NS5B570-BK protein and cIRES RNA template as described previously (6) with the following modifications. HCV polymerase reactions contained 200 nM cIRES, 1 μM tritiated UTP (42 Ci/mmol), 1 μM ATP, 1 μM CTP, 1 μM GTP, 40 mM Tris-HCl (pH 8.0), 40 mM NaCl, 4 mM dithiothreitol, 4 mM MgCl2, serial diluted inhibitor, and 200 nM NS5B570-BK enzyme in a total volume of 50 μl. Reactions were incubated for 2 h at 30°C and stopped by the addition of 10% (v/v) trichloroacetic acid.

Gel-based Nucleotide Incorporation Assay—The RNA template-directed incorporation of the nucleotide and nucleotide analogs by HCV NS5B were performed with a 19-nucleotide RNA oligonucleotide (5’-AUGUAAUAUUAAU-UGUAGCC-3’) and a 5’-end-labeled GG primer (Dharmacon, Inc.). The RNA template was predicted to form a single stem-loop with an unpaired 3-nucleotide sequence at the 3’-end. The nucleotide incorporation reactions and RNA product separation on denaturing polyacrylamide gel were conducted as described (6), except that the reactions were carried out with 0.25 μM GG primer and nucleotide triphosphates at the indicated concentrations for 30 min. The kinetics of the single nucleotide incorporation of CTP and CTP analogs were determined with the same RNA oligonucleotide template and primer pair for 25 min at the following assay conditions: 7.5 μM 19-nucleotide RNA template, 10 μM unlabeled GG primer, 0.075 μM 5’-end-labeled GG primer, 3 μM NS5B, and serial dilutions of CTP or each CTP analog. The GG primer input and the GGC RNA product were quantified using a Typhoon9400 PhosphorImager scanner and ImageQuant software (GE Healthcare). The reaction velocity was plotted as a function of the nucleotide concentration, and the single nucleotide incorporation kinetic parameters were derived by fitting the data to the Michaelis-Menten equation.

Analysis of Phosphorylation in Primary Human Hepatocytes—Nucleoside phosphorylation efficiency and the intracellular half-life of the 5’-triphosphate of RO-9187 were determined by incubating fresh primary human hepatocytes with tritium-labeled RO-9187 as described (20) with the following modifications of the cell extraction procedure. The soluble content of the primary hepatocytes was incubated with 500 μl of 1.5 M perchloric acid for 30 min on ice, followed by neutralization with 300 μl of 2.5 N potassium hydroxide. The supernatant was collected after centrifugation at 10,000 × g for 10 min to
remove the cell debris and precipitated macromolecules. The pH of the supernatants was adjusted by the addition of 200 mM Tris-HCl, pH 7.5. The phosphates of RO-9187 were separated by ion exchange high performance liquid chromatography as described (20).

**Compound Crystallography**—Suitable crystals of NM107 were obtained by evaporation of a saturated solution with methanol as solvent. Crystals were mounted in loops and cooled to 100 K in a nitrogen stream. Diffraction data were collected at the Swiss light source beamline X10SA using a MAR CCD225 detector with synchrotron radiation (0.70 Å), and data were processed with the program XDS. The crystal structures were solved and refined with ShelXTL (Bruker AXS, Karlsruhe, Germany). Crystals of RO-9187 were also obtained from methanol evaporation, mounted in loops, and cooled to 89 K. Data from one crystal were collected on a STOE imaging plate diffraction system (STOE & Cie GmbH, Darmstadt, Germany) with molybdenum radiation (0.71 Å), and data were processed with STOE imaging plate diffraction system software.

**Pharmacokinetic Studies**—Pharmacokinetic studies were performed in Hanover-Wistar rats (Charles River Laboratories, Inc., Hollister, CA), beagle dogs, and cynomolgus monkeys. Three animals per dose regime were administered either single 10, 200, or 2000 mg/kg oral solution using an aqueous 10 mg/kg intravenous bolus doses (50% cyclodextran/water) or 3 mg/kg intravenous bolus doses (50% 2-hydroxypropyl-beta-cyclodextrin). Plasma concentrations of test compounds were determined by liquid chromatography and tandem mass spectroscopy with a lower limit of quantification of 5 ng/ml.

**RESULTS**

Deoxyribonucleosides and Dideoxyribonucleosides Are Poor Substrates and Inhibitors of HCV Polymerase—RNA polymerases synthesize RNA molecules by polymerization of ribonucleotide triphosphate substrates through a divalent metal ion-catalyzed reaction mechanism. Discrimination against 2’-α-deoxyribonucleotide triphosphates is believed to be achieved through conserved amino acid residues in the active site of RNA polymerases, which form hydrogen bonds with the 2’-α-hydroxy group of ribonucleotides (10, 11). To determine the substrate specificity of nucleotide incorporation by HCV polymerase, RNA synthesis reactions were performed in the presence of either CTP, 2’-dCTP, 3’-dCTP or 2’,3’-ddCTP substrates, and the CTP analogs were also characterized as inhibitors of RNA synthesis by HCV polymerase. The chemical structures of nucleosides used in this study are shown in Fig. 1. CTP and 3’-dCTP were efficiently incorporated into nascent RNA. 3’-dCTP prevented further chain elongation as an obligatory chain terminator. As compared with the natural substrate CTP, the incorporation efficiency of 3’-dCTP was 14-fold lower, mostly driven by an increased $K_m$ value for 3’-dCTP (Table 1 and Fig. 2). 3’-dCTP was also a potent inhibitor of RNA synthesis by HCV polymerase in vitro (Table 2). In contrast, 2’-dCTP was not efficiently incorporated and did not inhibit RNA synthesis from cIRES RNA template (Table 2 and Fig. 2). Similarly, 2’,3’-ddCTP did not inhibit RNA synthesis by HCV polymerase (Table 2). The comparative results between CTP and 3’-deoxy-CTP on one hand and 2’,3’-deoxy-CTP on the other hand indicated a significant contribution of the 2’-α-hydroxy group on binding and incorporation by HCV polymerase. These results are consistent with the model of 2’-deoxyribonucleo-

**TABLE 1**

Incorporation efficiency of nucleotide triphosphate analogs by HCV NS5B

| Compound       | $K_m$ $^a$ (μM) | $V_{max}$ (μM min$^{-1}$) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ (min$^{-1}$ μM$^{-1}$) | Relative incorporation efficiency $^b$ (%) |
|----------------|-----------------|--------------------------|------------------------|-----------------------------------|-----------------------------------------|
| CTP            | 10.6 ± 2.93     | 0.29 ± 0.048             | 0.096 ± 0.016          | 9.1                               | 100                                     |
| 2’-dCTP        | NA              | NA                       | NA                     | NA                                | NA                                      |
| 3’-dCTP        | 101 ± 7.66      | 0.20 ± 0.023             | 0.065 ± 0.008          | 0.64                              | 7.0                                     |
| R1479-TP       | 39.1 ± 5.16     | 0.25 ± 0.043             | 0.084 ± 0.014          | 2.1                               | 23                                      |
| RO-9187-TP     | 107 ± 6.73      | 0.17 ± 0.039             | 0.055 ± 0.013          | 0.51                              | 5.6                                     |
| RO-0622-TP     | 54 ± 4.43       | 0.20 ± 0.031             | 0.066 ± 0.010          | 1.2                               | 13                                      |
| Ara-CTP        | 307 ± 100       | 0.061 ± 0.002            | 0.020 ± 0.001          | 0.065                             | 0.71                                    |

$^a$ Mean values and standard deviations for kinetic constants were determined from $n = 12$ (CTP) and $n = 3$ (other CTP analogs) experiments.

$^b$ Relative incorporation efficiency is as follows: $100 \times (k_{cat}/K_m)_{analog}/(k_{cat}/K_m)_{CTP}$

* NA means not applicable.
performed as described under “Experimental Procedures.” The migration of 4′-substituents in the RNA polymerase active site, with 4′-ido being the most effective to allow productive binding to both HCV polymerase and human nucleoside kinases (6, 7, 9). The addition of a 4′-ido moiety to the ara-CTP molecule resulted in an unexpected 15–30-fold increase in inhibitory potency of 2′-α-deoxy-2′-β-hydroxy-4′-ido-CTP (RO-9187-TP) as compared with 2′-α-deoxy-ara-CTP. With this, the overall inhibitory potency of RO-9187-TP was similar to that of the ribonucleoside analog 4′-ido-CTP (R1479-TP) in the cIRES RNA-dependent RNA polymerase assay (Table 2). The β-hydroxy moiety could be replaced with a β-fluoro moiety with similar inhibitory potency in the RNA synthesis assay (2′-α-deoxy-2′-β-fluoro-4′-ido-CTP; RO-0622-TP). Both RO-9187-TP and RO-0622-TP were used as substrates for incorporation into nascent RNA by HCV NS5B and prevented further chain elongation, similar to the obligatory chain terminator 3′-dCTP (Fig. 2). 4′-Substitution improved incorporation efficiencies of RO-9187-TP and RO-0622-TP ~8- and 18-fold as compared with ara-CTP, respectively (Table 1).

RO-9187 and RO-0622 Are Phosphorylated by Human Deoxycytidine Kinase—To achieve antiviral potency as competitive inhibitors of HCV polymerase, nucleoside analogs need to be phosphorylated to their 5′-triphosphate analogs by human nucleoside kinases. RO-9187 and RO-0622 together with control compounds 4′-ido-cytidine (R1479), 2′-β-methylcytidine (NM107), ara-C, dC, dT, and C were tested as substrates of recombinant human dCK, thymidine kinases 1 and 2 (TK1 and TK2), and uridine-cytidine kinases 1 and 2 (UCK-1 and UCK-2). In an initial screen, nucleoside analogs were incubated with human kinases at a single concentration of 100 μM, and the phosphorylation products were quantified by thin layer chromatography. Kinetic parameters were then determined for the kinase, which provided sufficient product formation for each nucleoside. None of the compounds tested were quantifiable substrates of TK1 or UCK-2 under the reaction conditions. Nucleoside analogs R1479, RO-9187, RO-0622, and ara-C were substrates for human dCK. Ara-C and RO-0622 were also substrates for human TK2. R1479 and RO-9187 were phosphorylated with lower efficiency as compared with the natural substrate 2′-deoxycytidine (dC), whereas RO-0622 and ara-C were phosphorylated with 3- and 2-fold increased efficiency relative to deoxycytidine, respectively (Table 3). With this, RO-0622 was phosphorylated with 11-fold increased efficiency as compared with R1479. Interestingly, 2′-β-methylcytidine (NM107) was not phosphorylated by dCK but was a substrate of UCK-1.

RO-9187 and RO-0622 Are Potent Inhibitors of HCV Replication—The antiviral potencies of the novel nucleoside analogs RO-9187 and RO-0622 were determined in the HCV genotype 1b (Con1) subgenomic replicon cell line 2209-23 and in the transient replicon system as described previously (6, 19). Both compounds were potent inhibitors of HCV replication in these systems (Table 4). RO-9187 had similar or 5–10-fold higher antiviral potency as compared with R1479 and NM107. RO-0622 was 50–100-fold more potent as compared with R1479 and NM107 (Table 4). RO-9187, RO-0622, as well as R1479 and NM107 inhibited HCV genotype 1a- and genotype 2′-α-hydroxyl group. Earlier, we discovered that 4′-substituents on ribonucleoside analogs may pick up additional binding interactions in the HCV polymerase active site, with 4′-ido being the most effective to allow productive binding to both HCV polymerase and human nucleoside kinases (6, 7, 9). The addition of a 4′-ido moiety to the ara-CTP molecule resulted in an unexpected 15–30-fold increase in inhibitory potency of 2′-α-deoxy-2′-β-hydroxy-4′-ido-CTP (RO-9187-TP) as compared with 2′-α-deoxy-ara-CTP. With this, the overall inhibitory potency of RO-9187-TP was similar to that of the ribonucleoside analog 4′-ido-CTP (R1479-TP) in the cIRES RNA-dependent RNA polymerase assay (Table 2). The β-hydroxy moiety could be replaced with a β-fluoro moiety with similar inhibitory potency in the RNA synthesis assay (2′-α-deoxy-2′-β-fluoro-4′-ido-CTP; RO-0622-TP). Both RO-9187-TP and RO-0622-TP were used as substrates for incorporation into nascent RNA by HCV NS5B and prevented further chain elongation, similar to the obligatory chain terminator 3′-dCTP (Fig. 2). 4′-Substitution improved incorporation efficiencies of RO-9187-TP and RO-0622-TP ~8- and 18-fold as compared with ara-CTP, respectively (Table 1).

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![A diagram showing nucleotide incorporation](image)

**TABLE 2**  Inhibition of cIRES RNA-dependent RNA synthesis of HCV NS5B

| Compound | Con1-IC₅₀ μM | BK-IC₅₀ μM |
|----------|-------------|-----------|
| 2′′-dCTP | >100        | >100      |
| 3′-dCTP  | 0.25 ± 0.09 | 0.15 ± 0.06 |
| 2′,3′-dCTP | >100        | >100      |
| Ara-CTP  | 3.8 ± 1.1   | 5.8 ± 1.2 |
| R1479-TP | 0.32 ± 0.04 | 0.32 ± 0.13 |
| RO-9187-TP | 0.24 ± 0.13 | 0.19 ± 0.05 |
| RO-0622-TP | 0.51 ± 0.07 | 0.39 ± 0.10 |

- HCV polymerase activity was measured using 200 nM NS5B570-BK or NS5B570-Con1 enzymes, 200 nM cIRES RNA, and 1 μM NTP substrates. Mean values and standard deviations for kinetic constants were determined from at least three experiments.

**FIGURE 2.** RO-9187-TP and RO-0622-TP are substrates of HCV polymerase and block further elongation after incorporation. A, sequence and structure of the RNA template and primer. B, nucleotide incorporation assay was performed as described under “Experimental Procedures.” The migration positions of the GP primer and the RNA elongation products on the denaturing polyacrylamide gel are shown on the left of the gel; FL, full-length product. Reactions with different nucleotides were performed in sets of three: lane 1, CTP or CTP analog only at 100 μM (single nucleotide incorporation); lane 2, CTP or CTP analog at 100 μM and UTP at 20 μM (chain extension); lane 3, CTP or CTP analog at 100 μM, UTP at 20 μM, and ATP at 100 μM (full-length product formation). a, CTP; b, 3′-dCTP; c, 2′,3′-dCTP; d, RO-9187-TP; e, RO-0622-TP.

Side discrimination through the lack of hydrogen bonding interaction in the RNA polymerase active site.

**4′-Substitution Can Increase the Inhibitory Potency of 2′-α-Deoxyribonucleotides**—Interestingly, 2′-β-hydroxy-CTP (ara-CTP) showed a moderate inhibitory effect on RNA synthesis despite the lack of a 2′-α-hydroxyl group, suggesting that substituents in the β-configuration may allow additional binding interactions in the NS5B active site (Table 2). However, the incorporation efficiency of ara-CTP was 140-fold lower as compared with CTP, in line with an important interaction of the 2′-α-hydroxy group.
1b-driven replication with similar potency, as expected for nucleoside analogs (Table 4). Interestingly, RO-9187 showed 4–5-fold higher antiviral potency in the stable 2209-23 cell line as compared with the transient replicon system, whereas all other compounds showed similar potencies between the stable and transient systems (Table 4).

RO-9187 and RO-0622 were not cytotoxic and did not inhibit tritiated thymidine incorporation in 2209-23 cells, i.e., these compounds did not inhibit cell proliferation (Table 4). In contrast, ara-C was potently cytostatic and inhibited 2209-23 cell proliferation with an IC_{50} of 29 nM, which prevented the assessment of its potential antiviral effect in cell culture. Similarly, 2’-β-fluorocytidine (RO-8013) was potently cytostatic in 2209-23 cells.

Interestingly, RO-0622 and RO-9187 were also potent inhibitors of replicon variants carrying S96T or S282T point mutations in NS5B. These mutations were previously shown to confer resistance to R1479 and NM107, respectively (19). Therefore, the combination of 4’- and 2’-β-substitution in the absence of a 2’-α-substituent resulted in two compounds, which show no apparent cross-resistance with 4’-azidocytidine and 2’-β-methylcytidine, respectively.

RO-9187 is Efficiently Phosphorylated in Primary Human Hepatocytes—The kinetics of RO-9187-triphosphate (RO-9187-TP) formation in human hepatocytes was determined using tritium-labeled RO-9187. As shown in Fig. 3, the formation of RO-9187-TP increased in a time- and dose-dependent manner. The maximal triphosphate concentration at 24 h was 87 pmol/10^6 cells with half-maximal triphosphate formation achieved at 12 μM RO-9187. 15.6 pmol/10^6 cells of RO-9187-TP were formed at 24 h from incubation of primary hepatocytes with 2 μM RO-9187. In the absence of extracellular RO-9187, intracellular RO-9187-TP disappeared in a biphasic decay pattern with an effective half-life (50% reduction of initial TP concentration) of 5.4 h and a terminal half-life of 28 h (Fig. 3C). At 24 h after removal of extracellular RO-9187, the intracellular RO-9187-TP concentrations were still well above 4 pmol/10^6 (1.3 μM) cells, and thus above the antiviral IC_{50} assuming a 3-μl volume of normal human liver parenchymal cells (20, 21), reflective of very robust phosphorylation efficiency of RO-9187 in primary human hepatocytes.

Table 4

| Compound | GT-1b replicon (2209-23 cells) | GT-1a replicon (2209-23 cells) | S96T replicon (transient) | S282T replicon (transient) | Cytotoxicity (2209-23 cells) | Proliferation |
|----------|-------------------------------|-------------------------------|--------------------------|--------------------------|-----------------------------|--------------|
|          | IC_{50} (μM)                  | IC_{50} (μM)                  | IC_{50} (μM)              | IC_{50} (μM)              | CC_{50} (μM)                | [^{3}H]Thy incorporation |
| R1479    | 1280 ± 80                     | 1960 ± 140                    | 1900 ± 260               | 530 ± 100                | 8600 ± 660                  | >1000         |
| NM107    | 1130 ± 30                     | 1050 ± 90                     | 500 ± 220                | 28500 ± 2950             | 1280 ± 210                 | >1000         |
| RO-9187  | 171 ± 12                      | 800 ± 270                     | 906 ± 380                | 1164 ± 314               | 619 ± 262                  | >1000         |
| RO-0622  | 24 ± 3                        | 19 ± 6                        | 14 ± 10                  | 79 ± 94                  | 31 ± 12                    | >1000         |
| Ara-C    | 20 ± 7                        | ND                            | ND                       | ND                       | ND                          | 100           |
| RO-8013  | 114 ± 50                      | ND                            | ND                       | ND                       | ND                          | 0.029         |

*ND means not determined.
form RO-9187 therefore did not affect the 2’-endo ribose conformation preference of ara-C typical of B-form DNA (22, 23).

RO-9187 Shows Good Oral Bioavailability in Hanover-Wistar Rats and Beagle Dogs and No Toxicologically Significant Findings in a 2-Week Range Finding Toxicity Study in Hanover-Wistar Rats—The pharmacokinetics profile of RO-9187 was assessed in Hanover-Wistar rats and beagle dogs (Table 5). Plasma exposures of RO-9187 in rats increased in a dose-dependent manner between 10 and 2000 mg/kg after oral dosing.

Plasma concentrations of 1.4 and 26 μM (390 and 7454 ng/ml) were achieved in rats and dogs at the 10 mg/kg dose level, respectively. Plasma concentrations up to 57 μM were achieved in rats dosed with 2000 mg/kg/day (Table 5). A 2-week oral range finding toxicity study was performed with RO-9187 and ribavirin in Hanover-Wistar rats. Five male and five female rats in each of five treatment groups were administered once daily doses of vehicle, 200, 600, or 2000 mg/kg RO-9187 or 200 mg/kg ribavirin by oral gavage for 14 days. There were no treatment-related findings in hematology, clinical chemistry, and

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**FIGURE 3.** RO-9187-TP formation in primary human hepatocytes incubated with tritium-labeled RO-9187. A, time course of RO-9187-TP formation in hepatocytes incubated with 2 μM RO-9187. B, dose response of RO-9187-TP formation after 24 h of incubation with RO-9187. Data were fitted to the Michaelis-Menten equation. Maximal triphosphate concentration was 87 pmol/million cells, and half-maximal concentration was achieved with 12 μM RO-9187. C, disappearance of intracellular RO-9187-TP after removal of extracellular RO-9187. Data were fitted to a biexponential equation with a terminal half-life of 28 h.

**FIGURE 4.** Crystal structures of cytidine analog NM107 and deoxycytidine analog RO-9187. NM107 has 3’-endo conformation and RO-9187 has 2’-endo conformation.

**TABLE 5**

| Compound | Species | Dose | AUC<sub>0–24h</sub> | C<sub>max</sub> | CL<sup>a</sup> |
|----------|---------|------|-----------------|-------------|-------------|
| RO-9187  | Rat     | 10<sup>b</sup> | 2568 ± 843  | 390 ± 77  | 11.9  |
| RO-9187  | Rat     | 200<sup>c</sup> | 25,300 ± 2121| 3195 ± 247| 777  |
| RO-9187  | Rat     | 2000<sup>c</sup>| 199,500 ± 7772| 16,150 ± 1767| 1167 |
| RO-9187  | Dog     | 10<sup>e</sup> | 43,529 ± 12,021| 7454 ± 1887| 3.2  |
| Ribavirin| Rat     | 200<sup>f</sup> | 24,450 ± 4313| 1725 ± 265| 276  |

<sup>a</sup> Data were determined from pharmacokinetics studies with 2 mg/kg RO-9187 dosed intravenously.

<sup>b</sup> Mean values ± S.D. of data obtained from a single dose per os pharmacokinetics study in three male Hanover-Wistar rats.

<sup>c</sup> Mean values ± S.D. of data obtained on day 0 of a 2-week per os toxicokinetics study in five male and five female Hanover-Wistar rats.

<sup>d</sup> Data are from day 13.

<sup>e</sup> Mean values ± S.D. determined from a single dose per os pharmacokinetics study in three male beagle dogs.

<sup>f</sup> Data are from day 0.
anatomic pathology in rats dosed with RO-9187, and the NOEL was 2000 mg/kg/day. In contrast, clinical observations in ribavirin-treated animals included pale appearance, labored respiration, and reduced body weight gain. Hematology findings in ribavirin-treated animals included granulocytopenia, erythrocytopenia, reduced mean hemoglobin, and an increase in ala nine transferase, and microscopio changes were noted in multiple organs, including bone marrow, liver, and lymphoid organs. The plasma exposures of ribavirin increased ~2-fold between day 0 and day 13 of the study period, were similar to those achieved with RO-9187 at 200 mg/kg, and significantly lower than those obtained with RO-9187 at 2000 mg/kg. Therefore, RO-9187 showed an improved safety profile in rats as compared with ribavirin, when administered orally for 2 weeks.

DISCUSSION

The development of novel antiviral agents targeting HCV replication is paramount to achieve improved response rates to therapy among HCV-infected persons, especially those infected with difficult to treat genotype 1 virus. Nucleoside analogs have provided key medicines to treat viral diseases, including treatment of infections by herpesviruses, human immunodeficiency virus, and hepatitis B virus. The majority of marketed antiviral nucleoside analogs target viral DNA polymerases. In contrast, no specific nucleoside inhibitors of RNA polymerases have been developed to date, although novel ribonucleoside analogs targeting the RNA-dependent RNA polymerase of HCV have recently achieved promising results in clinical studies, encouraging further exploration within this chemical class. NM283, the 3'-valinate prodrug of NM107 (2'-C-methylcytidine), has demonstrated clinically significant antiviral potency in HCV-infected patients (24), and R1626, the triisobutyrate prodrug of R1479 (4'-azidocytidine), has shown robust antiviral potency in HCV-infected patients with dose-dependent mean viral load reductions up to 3.7 log after 14 days of dosing in monotherapy (8), the largest antiviral effect seen to date with a polymerase inhibitor in monotherapy (25). Options to further increase the antiviral potency of nucleoside analogs as inhibitors of HCV replication include optimization of nucleotide analog incorporation efficiency by HCV NS5B, or optimization of phosphorylation efficiency to increase the intracellular concentration of the nucleoside triphosphate analog. In particular, the first step in the pathway to the triphosphate, the formation of nucleoside monophosphate, appears to be a limiting step for a majority of nucleoside analogs (9, 26, 27).

HCV is a positive strand RNA virus encoding an RNA-dependent RNA polymerase essential for the replication of viral RNA in the cytoplasm of infected cells. RNA polymerases are generally highly selective for incorporation of ribonucleotides into nascent RNA molecules and are believed to discriminate against 2'-deoxynucleotide triphosphates substrates by direct interaction with the 2'-hydroxy moiety of ribonucleotides (10, 11). Therefore, efforts into the design of nucleoside inhibitors against RNA polymerase targets have generally focused on satisfying this interaction by providing either α-hydroxy or α-fluoro substituents. The role of hydrogen bonding potential of fluorine on ribose molecules has been questioned, however, and a conformational impact of 2’-α-substituents may also be of biological relevance. In particular, 2’-α-substitution is conducive to the formation of the RNA-specific 2’-endo conformation, whereas 2’-α-deoxyribonucleotides form preferentially the 3’-endo conformation typical of B-type DNA (22, 23, 28–30). Previous studies with recombinant HCV polymerase (NS5B) suggested high selectivity of HCV NS5B for RNA templates and ribonucleotide triphosphate substrates, although oligodeoxyribonucleotides could be used as primers with homopolymeric RNA templates (31, 32). The crystal structure of an NS5B-UTP complex suggested that the conserved amino acid Asp-225 in the NS5B active site forms a hydrogen bond with the 2’-α-hydroxyl group of UTP and could therefore function as the key residue conferring NTP substrate selectivity over dNTPs by HCV NS5B (13).

Consistent with the high ribonucleotide substrate binding selectivity of HCV NS5B, we detected only low level incorporation of 2’-dCTP into nascent RNA in a gel-based single nucleotide incorporation assay and no inhibition in an in vitro RNA synthesis assay using recombinant NS5B and HCV cIRES RNA template. These results demonstrate that CTP competitive binding of 2’-dCTP to HCV NS5B was negligible under the assay conditions, consistent with high level active site binding discrimination against 2’-deoxy-CTP.

Interestingly, moderate inhibition of RNA synthesis by NS5B was observed with ara-CTP, suggesting that new interactions of β-substituents in the HCV polymerase active site may partially compensate for the loss of 2’-α-hydroxy hydrogen bonding. Similarly, 2’-β-hydroxy-GTP was described as a moderate inhibitor of HCV NS5B, but 2’-β-hydroxy-ATP was inactive (33). The increased base pairing stability of G:C base pairs may therefore be required to allow sufficient binding affinity of these compounds in the NS5B active site to confer measurable inhibitory potency. Based on the discovery that 4’-substitutions on ribonucleosides could provide potent and selective inhibitors of HCV RNA synthesis (6, 7) and assuming that additional interactions with the 4’-substituent could further compensate for the loss of α-hydroxy interaction, the effect of such substitutions on deoxyribonucleosides was assessed. The results were unexpected in their magnitude.

The synthesis of 2’-α-deoxy-2’-β-hydroxy-4’-azido-CTP (4’-azido-ara-CTP, RO-9187-TP) provided a novel inhibitor of HCV NS5B with inhibitory potency in the cIRES RNA-dependent RNA polymerase assay similar or slightly improved to that of 4’-azido-CTP (R1479-TP). RO-9187-TP was incorporated into nascent RNA by NS5B and prevented further RNA elongation as an effective chain terminator in the HCV transcription initiation assay. Interestingly, the incorporation efficiency measured in a gel-based RNA synthesis initiation assay was 7.8-fold higher than that of ara-CTP but 4-fold lower than that of R1479-TP. Effects on both $K_m$ and $V_{max}$ values contributed to this difference in incorporation efficiency. The inhibitory potency of RO-9187-TP was similar to or slightly higher in the cIRES-based RNA synthesis assay as compared with R1479-TP, despite the lower incorporation efficiency during RNA synthesis initiation. It is possible that RO-9187-TP and R1479-TP incorporation efficiencies are different during initiation and elongation phases of RNA synthesis by NS5B and that differences in incorporation efficiency during RNA elongation may
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dependent to the overall inhibitory potency of both compounds in the cIRES-RNA-dependent NS5B RNA synthesis assay. Studies to quantify incorporation during elongation are currently in progress and will help to clarify this potential difference between these two nucleoside analogs.

The β-hydroxy group of RO-9187-TP could be replaced by a β-fluoro moiety with only minor effects of reduced inhibitory potency in the RNA synthesis assay. RO-0622-TP was also efficiently incorporated into nascent RNA by NS5B and was an effective chain terminator at transcription initiation. Fluoro substitution may provide hydrogen bond acceptor properties as a replacement of hydroxyl moieties, but the resulting hydrogen bond strength is expected to be substantially lower as compared with a hydroxyl group (34). However, it has been shown that under certain circumstances the 2'-hydroxy group may be replaced by a fluoro moiety without major reduction of inhibitory potency against certain RNA polymerases. 2'-α-Fluoro-NTPs showed antiviral potencies against influenza virus and were substrates for the RNA-dependent RNA polymerase of influenza virus (35, 36). 2'-α-Fluorocytidine was also assessed as an inhibitor of HCV replication but was a potent inhibitor of cell proliferation in the HCV replicon system (7, 37). The addition of a 2'-β-methyl substituent resulted in the discovery of PSI-6130 (2'-α-fluoro-2'-β-methylcytidine), a potent inhibitor of HCV replication (38). Interestingly, PSI-6130 showed high selectivity against the closely related positive strand RNA virus bovine viral diarrhea virus, whereas the 2'-α-hydroxy analog 2'-C-methylcytidine (NM107) inhibited both HCV and bovine viral diarrhea virus replication in cell culture (38). Fluoro-nucleosides may therefore provide considerable hydrogen bond acceptor functionality in the RNA polymerase active site (34), although such substitutions may also function by facilitating the formation of an RNA-like 3'-endo conformation in the RNA polymerase active site (39) or by accelerating the chemical reaction through electronic stabilization of the nucleophilic substitution reaction in the polymerase active site (40). Consistent with the conformational effect of the 2'-α-fluoro substituent, PSI-6130 crystallized in the RNA typical 3'-endo conformation (38) similar to NM107 (Fig. 4) and other ribonucleosides and 2'-α-fluoro-nucleosides (22, 23). In contrast, the 2'-α-deoxyribonucleoside RO-9187 crystallized in the DNA-like 2'-endo-conformation, similar to other 2'-deoxyribonucleosides.

Unexpectedly, the antiviral potency of RO-0622 increased more than 50-fold as compared with R1479 in HCV replicon cells, whereas RO-9187 was 7.5-fold more potent than R1479, even though all three compound triphosphates showed similar inhibitory potencies in the NS5B RNA synthesis assay in vitro, and even though R1479-TP was the most efficient substrate for incorporation by NS5B during transcription initiation. This increase in potency was most likely related to a substantially increased phosphorylation efficiency of the deoxynucleoside analogs in human cells. All three compounds (RO-0622, RO-9187, and R1479) were substrates of human dCK, but RO-0622 was phosphorylated with an unprecedented efficiency more than 13-fold higher than that of R1479 and 3-fold higher than that of 2'-deoxycytidine. In addition, RO-0622 was also a substrate of human thymidine kinase 2. RO-9187 was phosphorylated by human dCK in vitro with an efficiency similar to R1479. However, phosphorylation of RO-9187 in human hepatocytes was higher than that of previously studied ribonucleosides, achieving a mean triphosphate concentration of 15.6 pmol/million cells from 2 μM RO-9187 within 24 h, which is 3.3–20-fold higher than that reported for NM107 and 6.5-fold higher than that achieved from PSI-6130 under similar conditions (20, 27). It is therefore possible that additional enzymes are involved in the phosphorylation of RO-9187 or that phosphorylation of the intermediates RO-9187-MP or RO-9187-DP by the respective nucleoside monophosphate and diphosphate kinases could be more efficient than that of ribonucleoside-MP and -DP analogs.

The novel nucleoside analog RO-9187 was derived from ara-C (cytarabine), a cytotoxic nucleoside analog, which forms the backbone of induction therapy for the treatment of acute myelogenous lymphoma. Ara-C is a potent inhibitor of cell proliferation in cell culture and also inhibited proliferation of Huh-7 hepatoma cell-derived 2209-23 HCV replicon cells with an IC₅₀ of 29 nm, measured as inhibition of [³H]thymidine incorporation into cellular DNA. HCV replication in this cell line is dependent on cell proliferation, such that the antiviral effect of cytostatic agents cannot be assessed (41). The introduction of a 4'-azido group dramatically increased the selectivity of RO-9187 as compared with ara-C, with no inhibitory effect on [³H]thymidine incorporation measurable in HCV replicon cells at concentrations up to 1 nm. This correlates to an increase in antiviral selectivity over cell proliferation inhibition of more than 34,000-fold achieved through nucleoside 4'-substitution. Similarly, RO-8013 (2'-α-deoxy-2'-β-fluorocytidine) was a potent inhibitor of cell proliferation with an IC₅₀ of 174 nm. The introduction of a 4'-azido group to generate RO-0622 also substantially increased the antiviral selectivity. These results are consistent with previous findings that R1479 (4'-azidocytidine) showed high selectivity for the inhibition of HCV replication and R1479-TP did not inhibit the related RNA-dependent RNA polymerase of influenza virus.

RO-9187 and RO-0622 inhibited HCV replicon variants resistant to inhibition by R1479 (S96T) and 2'-C-methyl nucleosides (S282T) with similar potency as wild-type replicons. The absence of a 2'-α-substituent may therefore allow these compounds to bind to NS5B in a conformation not affected by the increased bulk of S282T in the NS5B active site. The mechanism of resistance to R1479 conferred by the S96T mutation in NS5B has not been determined yet, but the availability of novel 4'-substituted analogs with high inhibitory potency against S96T replicons may help to resolve the molecular mechanism of the S96T effect on R1479.

The safety profile of RO-9187 was assessed in a 2-week toxicity study in Hanover-Wistar rats, in comparison with ribavirin, a nonselective nucleoside analog, currently forming part of standard of care therapy for HCV-infected patients. There were no treatment related toxicity findings in rats treated with RO-9187 at dose levels up to 2000 mg/kg/day for 14 days, consistent with the apparent high selectivity ratio observed for RO-9187 in cell culture. Plasma concentrations in rats treated with RO-9187 reached levels in excess of 16 μg/ml (>50 μM), i.e., ~300-fold above the antiviral potency in HCV replicon cells. In contrast, treatment of rats with 200 mg/kg ribavirin for 14 days was associated with significant toxic changes in Hanover-Wistar rats affecting multiple organ systems, similar to pre-
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