The Novel Nucleoside Analog R1479 (4’-Azidocytidine) Is a Potent Inhibitor of NS5B-dependent RNA Synthesis and Hepatitis C Virus Replication in Cell Culture*

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Hepatitis C virus (HCV) replication activity is essential for HCV replication. Targeted screening of nucleoside analogs identified R1479 (4’-azidocytidine) as a specific inhibitor of HCV replication in the HCV subgenomic replicon system (IC_{50} = 1.28 μM) with similar potency compared with 2’-C-methylcytidine (IC_{50} = 1.13 μM). R1479 showed no effect on cell viability or proliferation of HCV replicon or Huh-7 cells at concentrations up to 2 mM. HCV replicon RNA could be fully cleared from replicon cells after prolonged incubation with R1479. The corresponding 5’-triphosphate derivative (R1479-TP) is a potent inhibitor of native HCV replicase isolated from replicon cells and of recombinant HCV polymerase (NS5B)-mediated RNA synthesis activity. R1479-TP inhibited RNA synthesis as a CTP-competitive inhibitor with a K_i of 40 nM. On an HCV RNA-derived template substrate (complementary internal ribosome entry site entry), R1479-TP showed similar potency of NS5B inhibition compared with 3’-dCTP. R1479-TP was incorporated into nascent RNA by HCV polymerase and reduced further elongation with similar efficiency compared with 3’-dCTP under the reaction conditions. The S282T point mutation in the coding sequence of NS5B confers resistance to inhibition by 2’-C-MeATP and other 2’-methyl-nucleotides. In contrast, the S282T mutation did not confer cross-resistance to R1479.

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease, cirrhosis, and hepatocellular carcinoma and is currently the leading cause of liver transplantation (1, 2). Viral genome sequence analysis established six HCV genotype classes (HCV genotypes 1–6), with genotypes 1–3 being the most prevalent in the United States, Europe, and Japan. Current treatment options available to HCV-infected persons are limited, and sustained virological response rates are particularly low for HCV genotype 1-infected patients. Only ~50% of individuals infected with HCV genotype 1 with serum viral titers of >2 × 10^5 copies/ml achieved sustained virological response rates when treated with a combination of pegylated interferon-α and ribavirin (3, 4). Response rates are even lower in persons with HIV co-infection or cirrhosis and also decrease with age (1, 5–7). Urgently required improvements in anti-HCV therapy will depend on the development of novel therapeutic approaches, especially in difficult to treat populations.

HCV is an enveloped (+)-strand RNA virus that enters host cells via receptor-mediated endocytosis and replicates in the host cell cytoplasm. A membrane-associated replicase complex containing HCV genome-encoded nonstructural proteins and HCV genomic RNA in a tight complex is responsible for the formation of viral RNA for packaging into new virus particles during the HCV replication process. The viral NS5B polymerase contains the HCV replicase active site within the replicase complex, an RNA-dependent RNA polymerase. The concept of polymerase inhibition to attain antiviral efficacy has been successfully established in other viral infections (human immunodeficiency virus, hepatitis B virus, and herpes viruses). Polymerase inhibitors are the largest class of approved antiviral drugs, and nucleosides are the largest chemical class therein. The majority of 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. A novel ribonucleoside analog, 2’-C-methylcytidine (NM107), has entered clinical development as a 2’-methyl-5’-methyl prodrug (NM283). Additional nucleoside analogs carrying the 2’β-methyl moiety were also found to specifically inhibit HCV RNA replication in cell culture (9–11).

Targeted screening of a nucleoside analog library combined with rational lead optimization at Roche identified R1479 (4’-azidocytidine) as a specific inhibitor of HCV replication in the HCV subgenomic replicon system. This study describes the profile of potency and selectivity of 4’-azidocytidine as an antiviral agent in cell culture and the characterization of 4’-azidocytidine triphosphate as an inhibitor of native HCV replicase and recombinant HCV polymerase (NS5B). Based on its promising preclinical profile, R1479 is currently under evaluation in clinical trials as a drug candidate for the treatment of HCV infection.

EXPERIMENTAL PROCEDURES

HCV Replicon Assays—The 2209-23 cell line was established from Huh-7 cells by stable transfection with a bicistronic replicon (genotype 1b), of which the first open reading frame (driven by the HCV internal ribosome entry site (IRES)) contains the Renilla luciferase gene fused with the neomycin phosphotransferase gene (nptII) and the second open reading frame (driven by the ecephalomyocarditis virus IRES)
contains the HCV nonstructural genes NS3, NS4a, NS4b, NS5A, and NS5B derived from the NS5A1 replicon backbone, originally described by Krieger et al. (12). Thus, the replicon cell line 2209-23 is resistant to Genetecin (G418) and expresses the Renilla luciferase reporter gene as a marker of HCV RNA replication. Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with Glutamax and 100 mg/ml sodium pyruvate (Invitrogen). The medium was further supplemented with 10% (v/v) fetal calf serum, 1% (v/v) penicillin/streptomycin, and 500 μg/ml G418. Cells were maintained at 37 °C in a humidified 5% CO2 and 95% air atmosphere. The selection and characterization of replicon clones carrying the point mutation S282T in the NS5B coding sequence will be described elsewhere. Nucleoside analogs were synthesized at Roche, dissolved in 100% Me2SO, and then diluted in Dulbecco’s modified Eagle’s medium with 5% (v/v) fetal calf serum before addition to cells. The final concentration of Me2SO was 1% (v/v) in all experiments. Quantification of Renilla luciferase activity was performed using the Renilla luciferase assay kit (Promega) according to the manufacturer’s instructions. The WST-1 cell proliferation assay (Roche Diagnostics) was used to measure cell viability. The ATP bioluminescence assay kit HSII (Roche Diagnostics) was used to measure intracellular ATP levels. The WST-1 cell proliferation assay (Roche Diagnostics) was used to measure cell viability. Direct quantification of HCV RNA levels was performed by quantitative kinetic reverse transcription (RT)-PCR (TaqMan) using endogenous β-actin mRNA as a control. The primers used were 5′-GCTGTAATTGCGGCAAGTG-3′ (forward), 5′-GCCGCCGGATTGCA-3′ (reverse), and 5′-TCTTGCAGGAAA-GTATCCATCGGCT-3′ (probe). Reactions were analyzed on a Model 7700 sequence detector (PE Biosystems). The genotype 1b Con1-adapted transient replicon (pKFlucPI-luc/ET) was obtained from Dr. R. Bartenschlager (13). The Con1-S282T transient replicon was constructed, and assays were performed as described. Replicon Cell Proliferation Assay—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 Amersham Biosciences. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and WST-1 assay systems (Roche Diagnostics) were used to measure cell viability. The ATP bioluminescence assay kit HSII (Roche Diagnostics) was used to measure intracellular ATP levels.

HCV RNA Clearance Assay—HCV replicon cells were cultured with 6 and 12 μM R1479 in the absence of neomycin selection for 15 days (replicon clearance phase). Cells were passaged at a 1:2 ratio when they reached confluency, and samples were taken for HCV replicon RNA level determination. After 15 days, the inhibitor was removed, and cells were cultured for an additional 15 days in culture medium containing 0.25 mg/ml neomycin to allow replication of residual replicon RNA molecules remaining after treatment. The levels of HCV RNA were determined by quantitative kinetic RT-PCR using TaqMan technology (Ambion, Inc.), 10 mM creatine phosphate, and 200 μg/ml creatine phosphokinase in a final volume of 25 μl. Inhibition by nucleotide analogs was determined as described (14).

HCV Replication Inhibitor R1479

HCV Polymerase Assay—The enzyme activities of NS5B570-BK, NS5B570-Con1, and NS5B570-S282T-Con1 proteins was measured as incorporation of radiolabeled NMP into acid-insoluble RNA products as described previously (14). Briefly, HCV polymerase reactions contained 10 μg/ml complementary IRES (cIRES) or 3′-untranslated region (UTR) RNA template, 8.4 μg/ml poly(A) oligo(U) template-primer or 1.6 μg/ml poly(I) oligo(C) template-primer, 1 μM triitated UTP or CTP (1–5 μCi), 1 μM ATP, 1 μM CTP, 1 μM GTP (with cIRES and 3′-UTR RNA templates), 40 mM Tris-HCl (pH 8.0), 4 mM dithiothreitol, and 4 mM MgCl2. NS5B570 proteins contain a C-terminal deletion of 21 amino acids, which removes a transmembrane domain and increases solubility of the protein (14, 15). The HCV RNA templates used were as follows: cIRES RNA, corresponding to 377 nucleotides from the 3′-end of HCV (−) strand RNA (14), with a base content of 21% Ade, 23% Ura, 28% Cyt, and 28% Guo; and 3′-UTR RNA, corresponding to 389 nucleotides from the 3′-end of HCV (+) strand RNA, with a base content of 15% Ade, 38% Ura, 26% Cyt, and 21% Guo. RNA was transcribed in vitro using a T7 transcription kit (Ambion, Inc.) and purified either by phenol-chloroform extraction or using the Qiagen RNeasy maxi kit, with similar results. Poly(A) RNA was from Amersham Biosciences, and poly(I) RNA was from Yamasa Corp. Data were analyzed with GraphPad Prism® and/or Microsoft® Excel®. The apparent Michaelis constant (Km(app)) of CTP for NS5B570-BK was calculated by nonlinear fitting using Equation 1,

\[
Y = \frac{(V_{\text{max(app)}})X}{K_{\text{m(app)}} + X} \quad (\text{Eq. 1})
\]

where Y corresponds to the rate of RNA synthesis by NS5B570-BK (in cpm/min), V_{\text{max(app)}} is the maximum rate at saturating substrate concentration, and X corresponds to CTP concentration. The compound concentration at which the enzyme-catalyzed rate was reduced by 50% (IC50) was calculated by fitting the data to Equation 2,

\[
Y = \% \text{Min} + \frac{(\% \text{Max} - \% \text{Min})}{1 + \left(\frac{X}{(IC50)}\right)} \quad (\text{Eq. 2})
\]

where Y corresponds to the percent relative enzyme activity, % Min is the residual relative activity at saturating compound concentration, % Max is the relative maximum enzyme activity, and X corresponds to the compound concentration. The mean IC50 value was derived from the mean of several independent experiments. The S.D. was calculated from the nonbiased method using Equation 3.

\[
\text{S.D.} = \sqrt{\frac{n\sum(X_{50}^2) - (\sum X_{50})^2}{n(n-1)}} \quad (\text{Eq. 3})
\]

The K_{i(CTP)} for Ro10482975 γ-triphosphate was derived by fitting the data to the Cheng-Prusoff equation, assuming competitive inhibition relative to CMP incorporation (Equation 4),

\[
K_{i(CTP)} = \frac{IC_{50}}{1 + \left(\frac{[CTP]}{K_{\text{m(app)}}}\right)} \quad (\text{Eq. 4})
\]

where [CTP] is the initial concentration of CTP and K_{m(app)} is the apparent Km for CTP.

Gel-based Nucleotide Incorporation Assay—The RNA template-directed incorporation and extension of the nucleotide and nucleotide

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analogs by HCV NS5B were performed with a 19-nucleotide RNA oligonucleotide (5′-AUGUAUAAAUAUUGUAGCC-3′) and a 5′-end-labeled GG primer (Dharmacon, Inc.). The RNA template is predicted to form a single stem-loop structure with an unpaired 3′-nucleotide sequence at the 3′-end. The 5′-end labeling of the GG primer was performed with [γ-32P]ATP and T4 polynucleotide kinase (Roche Applied Science). The nucleotide incorporation reactions contained 40 mM Tris-HCl (pH 8.0), 20 mM KCl, 2 mM MgCl2, 5 mM dithiothreitol, 2 μM NS5B70-BK, 5 μM RNA template, 0.15 μM end-labeled GG primer, and nucleoside triphosphates at the indicated concentrations in a volume of 10 μl. The reactions were incubated at 30 °C for 60 min and stopped by the addition of 10 μl of formamide gel loading buffer II (Ambion, Inc.). After denaturing at 95 °C for 3 min, the RNA template, primer, and extended primer were separated on a Tris borate/EDTA-urea-20% acrylamide gel. The dried gel was exposed to a storage phosphor screen and analyzed using a PhosphorImager (Amersham Biosciences).

RESULTS

Nucleoside analogs have proven to be highly successful agents for the treatment of viral infections, including HIV, hepatitis B virus, and herpes viruses. In most cases, the nucleoside triphosphates are the biologically active metabolites, and nucleoside analogs are converted to their active triphosphate forms by cellular enzymes. The nucleoside triphosphate analogs then function as competitive substrate analogs of the viral polymerase and inhibit the synthesis of viral DNA and/or RNA molecules. The HCV subgenomic replicon provides a convenient cellular system for the assessment of nucleoside analogs as inhibitors of HCV replication (9, 16). A bicistronic HCV replicon was developed that contains the HCV 5′-UTR directing translation of Renilla luciferase and the selectable marker neomycin phosphotransferase. The encephalomyocarditis virus IRES directs the translation of HCV proteins NS3, NS4A, NS4B, NS5A, and NS5B (12, 17). The Renilla replicon system was validated using a range of different types of HCV replication inhibitors, including interferon-α, and established that the level of Renilla luciferase activity correlated with the level of HCV replicon RNA as determined by quantitative kinetic RT-PCR and Northern blot analysis. Screening of a nucleoside library identified a series of compounds that inhibited HCV subgenomic RNA replication without interfering with cell viability and cell proliferation. After further optimization, R1479 (4′-azidocytidine) (Fig. 1) was selected for further characterization based on an exceptionally high therapeutic window in HCV replicon cells.

Inhibition of HCV RNA Replication by R1479—R1479 inhibited HCV RNA replication with a mean IC50 value of 1.28 μM when measured as dose-dependent reduction of Renilla luciferase activity after a 72-h incubation of proliferating replicon cells (Table 1). The potency of R1479 in this cell line was similar to that obtained for 2′-C-methylcytidine with a mean IC50 value of 1.13 μM. Similar potencies for both compounds were also obtained from the measurement of the dose-dep-endent reduction of HCV replicon RNA by quantitative kinetic RT-PCR (Table 1). In contrast, the structurally related cytidine analog 3′-deoxycytidine did not inhibit HCV RNA replication in replicon cells at concentrations up to 100 μM. R1479 showed only low cytotoxicity in human hepatoma-derived replicon cells. Cell viability was monitored by measuring intracellular ATP concentration or cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or succinate-tetrazolium reductase activities. No reduction in cell viability was observed with R1479 at concentrations up to 2 mM using any of the three methods (Table 1).

It has been reported previously that ongoing cell proliferation appears to be important for HCV RNA replication in Huh-7-derived cell lines, whereas replicon RNA levels are reduced when cells reach confluency (18). Cytostatic compounds therefore reduce HCV RNA levels in replicon cells in a nonspecific manner, as exemplified by the IMP dehydrogenase inhibitor mycophenolic acid. Mycophenolic acid is a potent inhibitor of cell proliferation, as measured by the inhibition of tritiated thymidine incorporation into cellular DNA (IC50 = 0.6 μM) without affecting cell viability over a 72-h incubation period at concentrations up to 100 μM (Table 1). The apparent IC50 value for the inhibition of HCV replicon RNA replication by mycophenolic acid was similar to the IC50 value for thymidine incorporation into cellular DNA, suggesting an indirect cell culture-specific effect of HCV replicon RNA reduction by mycophenolic acid. In contrast, R1479 was not cytostatic and did not inhibit tritiated thymidine incorporation at concentrations up to 1 mM, suggesting that R1479 inhibits HCV replication by a direct antiviral mechanism.

The point mutation S282T in the NS5B coding sequence has been previously associated with resistance to nucleoside analogs carrying a 2′-β-methyl moiety, as shown using wild-type and mutant S282T transient replicons (10, 19). In contrast, the S282T mutation did not confer resistance to HCV replication inhibition by R1479. Both stable and transient mutant replicons carrying mutation S282T in the NS5B coding region were sensitive to inhibition by R1479, suggesting absence of cross-resistance between 2′-methyl-nucleosides and R1479 (Table 2).

Clearance of HCV RNA from Replicon Cells—Prolonged incubation of replicon cells with HCV polymerase inhibitors can result in the selection of inhibitor-resistant replicon variants with point mutations in the NS5B protein coding sequence. In contrast, prolonged incubation of HCV replicon cells with R1479 resulted in a continued decrease in HCV RNA to undetectable levels (Fig. 2). To determine the kinetics of the HCV subgenomic replicon inhibition by R1479, HCV replicon cells were cultured in the presence of inhibitor at 6 and 12 μM and in the absence of neomycin selection for 15 days (replicon clearance phase). During this phase in the absence of neomycin, cells were able to proliferate even in the absence of active replicon replication. After this time and during the rebound phase, the inhibitor was removed, and cells

![Figure 1. Chemical structures of R1479 (A), 3′-dCTP (B), 2′-C-methylcytidine (C), and 2′-C-methyladenosine (D).](image-url)
were cultured for an additional 30 days in culture medium containing 0.25 mg/ml neomycin (15 days for the untreated control). In the presence of neomycin, only cells harboring active replicating replicons are able to proliferate. HCV RNA levels were monitored every 3 days by quantitative kinetic RT-PCR as described under “Experimental Procedures.” HCV RNA levels remained constant throughout the viral clearance and the rebound phases in the untreated cells. In the presence of Mycophenolic acid (0.32 ± 0.07 μM), the HCV RNA was reduced by 5 log units and became undetectable by RT-PCR after the 15-day incubation period. In addition, cells had lost the ability to grow in the presence of neomycin during the rebound phase (Fig. 2). Therefore, R1479 at concentrations of approximately its IC_{50} was able to completely clear the HCV subgenomic replicon RNA from the replicon cells within a 15-day incubation period.

**R14795’-Triphosphate (TP) Inhibits Native HCV Replicase in Vitro**—The highly specific inhibition of HCV RNA replication by R1479 suggested HCV polymerase NS5B as a possible target of the corresponding R1479-TP. NS5B likely forms large multiprotein-RNA complexes with the other HCV-encoded nonstructural proteins and the HCV genomic RNA during the assembly of the functional HCV replicase (20). Membrane-associated, native HCV replicase complexes were isolated from HCV replicon cells according to published procedures (14) to assess the direct effects of R1479-TP on HCV RNA synthesis. R1479-TP inhibited RNA synthesis by native HCV replicase in vitro with high potency (IC_{50} = 0.79 μM) (Fig. 3 and Table 3). The IC_{50} value of R1479-TP for the inhibition of HCV RNA synthesis by native HCV replicase was similar to that measured with the reference inhibitor 3’-dCTP and 3-fold lower than those reported for 2’-O-methyladenosine (Table 3) (19) and 2’-O-methylcytidine (19), indicating high intrinsic potency of R1479-TP. Native HCV replicase isolated from HCV replicon cells carrying the S282T mutation in NS5B was resistant to inhibition by 2’-O-methyl-nucleosides, consistent with the resistance observed in cell culture (10, 19). In contrast, the inhibitory potencies of 3’-dCTP and R1479-TP were not affected by the S282T mutation (Table 3).

**R1479-TP Inhibits RNA Synthesis by NSSB in Vitro**—The RNA synthesis activity of recombinant NSSB protein was measured as the primer-independent incorporation of labeled nucleotides into acid-insoluble RNA molecules using four different RNA templates. Two heteropolymeric templates were derived from the HCV genomic RNA (5’-UTR and cIRES). Two additional templates were homopolymers of AMP (poly(A)) and IMP (poly(I)). Assay conditions were optimized for each RNA template such that the rate of labeled nucleotide incorporation was constant for at least 3 h, and dose-dependent inhibition by nucleotide analogs was determined after a 2-h incubation period. R1479-TP inhibited RNA synthesis by NSSB5 on all templates tested in a template sequence-dependent manner (Table 4). The lowest IC_{50} values were obtained with poly(I) RNA template, which directs the synthesis of poly(C), and the highest IC_{50} values were obtained with poly(A) RNA template, which directs the synthesis of poly(U). Intermediate inhibitory potencies were obtained for R1479-TP when the heteropolymeric templates were used in the reactions (Table 4). Using an RNA template

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**TABLE 1**

Inhibition of HCV RNA replication in HCV replicon cells

| Compound                           | IC_{50} μM | IC_{50} μM | IC_{50} μM | IC_{50} μM | IC_{50} μM |
|------------------------------------|------------|------------|------------|------------|------------|
| 4’-Azidocytidine (R1479)           | 1.28 ± 0.08| 0.67 ± 0.13| >2000      | >100       | >2000      |
| 2’-C-Methylcytidine                | 1.13 ± 0.03| 0.76 ± 0.01| >100       | >100       | ND         |
| 3’-Deoxycytidine                   | >100       | ND         | >100       | ND         | ND         |
| Mycophenolic acid                  | 0.32 ± 0.07| ND         | >100       | 0.60 ± 0.02| ND         |

* a Inhibition of HCV replicon-encoded Renilla luciferase reporter activity; mean IC_{50} value ± S.D. determined from 101 (R1479) and 354 (2’-C-methylcytidine) experiments.
* b Inhibition of HCV RNA determined by quantitative kinetic RT-PCR; mean IC_{50} value ± S.D. calculated from at least four experiments.
* c Cell viability determined from at least four experiments.
* d Inhibition of tritiated thymidine incorporation into cellular DNA determined from at least four experiments.
* e Reduction of cellular ATP levels determined from four experiments.
* f ND, not determined.

**TABLE 2**

Inhibition of genotype 1b wild-type Con1 and Con1-S282T stable and transient replicon replication

| Compound | Stable replicons | Transient replicons |
|----------|------------------|----------------------|
|          | Wild type | S282T | Wild type | S282T |
|          | IC_{50} μM | IC_{50} μM | IC_{50} μM | IC_{50} μM |
| R1479    | 0.67 ± 0.13 | 0.30 | 0.27 | 1.9 ± 0.26 | 0.53 ± 0.1 |

* a Inhibition of HCV RNA replication of the genotype 1b wild-type Con1 stable replicon.
* b Inhibition of HCV RNA determined by quantitative kinetic RT-PCR; IC_{50} value determined from two different stable replicon cell lines containing the S282T mutation (cell lines 16 and C).
* c Inhibition of HCV RNA replication; mean IC_{50} value ± S.D. determined from at least five independent experiments by quantification of replicon-encoded luciferase activity.
CTP (Fig. 4A).

R1479-TP inhibited recombinant NS5B enzymes from HCV strains BK and Con1 with similar potency (Table 5). IC50 values were also similar (within 2-fold) to those obtained with 3′-dCTP. In contrast, ribavirin triphosphate was only a very weak inhibitor of RNA synthesis by NS5B under similar conditions. Consistent with the replicase results, the S282T point mutation had no effect on NS5B inhibition by R1479-TP (Table 5).

**R1479-TP Can Be Incorporated into Nascent RNA by HCV Polymerase and Prevent Further Elongation**—The identification of R1479-TP as a competitive inhibitor of RNA synthesis by HCV polymerase suggested that this compound could serve as an alternative substrate for incorporation into nascent RNA. This hypothesis was tested in an HCV polymerase assay using a 19-nucleotide RNA template (Fig. 5A). Nucleotide incorporation was initiated using the labeled GG dinucleotide primer and CTP, R1479-TP, or 3′-dCTP as a substrate. As shown in Fig. 5B, CTP could be used as a substrate by HCV polymerase to incorporate CMP into nascent RNA (lane 4), and the product could be further elongated with UTP as the next nucleoside triphosphate substrate (lanes 5 and 6). R1479-TP and 3′-dCTP could also serve as substrates for incorporation (Fig. 5B, lanes 7 and 10, respectively), but further elongation with UTP was blocked (lanes 8 and 9 and lanes 11 and 12, respectively). Under the reaction conditions, the blockage of further nucleotide incorporation after incorporation of R1479-TP was similar to that obtained with the obligatory chain terminator 3′-dCTP. Control reactions with UTP alone (Fig. 5B, lanes 2 and 3) confirmed that nucleotide incorporation under the reaction conditions was template-directed and CTP-dependent and did not occur with UTP in the absence of CTP.

**DISCUSSION**

New therapy options are urgently required for the treatment of HCV infection, and nucleoside analogs hold great promise to deliver novel medicines with improved efficacy and tolerability profiles. Currently, ribavirin (in combination with interferon-α) is the only nucleoside analog used for the treatment of HCV infection. However, ribavirin is a nonselective agent with broad antiviral activity against a large number of unrelated RNA and DNA viruses (21, 22). Ribavirin triphosphate inhibits viral polymerases weakly, consistent with weak direct antiviral effects observed in vitro and in clinical studies. The mechanism of HCV inhibition for this compound remains unresolved and may involve immunomodulatory activities, template inactivation, or ‘error catastrophe’ after misincorporation of ribavirin monophosphate by HCV polymerase (23, 24). Improved anti-HCV nucleoside analogs should therefore demonstrate higher selectivity and higher intrinsic inhibitory potency against the molecular target, HCV polymerase.

In the last few years, a number of novel nucleoside analogs that appear to provide some improvement of potency and/or selectivity against HCV NS5B have been described. 2′-Deoxy-2′-α-fluoroctidylic triphosphate was shown to be a potent inhibitor of NS5B (25). However, this and other nucleosides carrying the 2′-deoxy-2′-α-fluoro moiety are known to have limited selectivity and can be substrates and chain-terminating inhibitors of viral and human polymerases (25, 26). Therefore, 2′-α-fluoro-nucleosides are unlikely to become useful drugs for the treatment of HCV infection due to a lack of polymerase selectivity.

More interestingly, several compounds carrying a 2′-β-methyl moiety have demonstrated potent inhibition of HCV polymerase in vitro and in cell culture (9, 10, 27). One member of this group of compounds, 2′-C-methylcytidine, is currently in Phase 2 clinical development for the treatment of HCV infection (3).

*In vitro* resistance selection experiments

![FIGURE 3. Inhibition of wild-type (WT) and S282T native HCV replicases by R1479-TP. Full-length replicon RNA synthesized by HCV replicase in vitro was quantified by representative denaturing analytical agarose gel electrophoresis. Replicate reactions were performed as described under 'Experimental Procedures' in the absence of added compound (lane C) or in the presence of 0.03–25 μM R1479-TP (second through eighth lanes) in a 3-fold dilution series. IC50 values are shown in Table 3.

**TABLE 3**

Inhibition of wild-type and S282T HCV replicases by nucleotide analogs

| Native HCV replicase | IC50 (μM) | CTP | dCTP | R1479-TP |
|----------------------|-----------|-----|------|----------|
| Replicon             |           |     |      |          |
| NS5B-S282T           | ND        | 0.76 ± 0.12 | 0.71 ± 0.15 |
| Wild-type            | 2.5       | 0.78 ± 0.5 | 0.79 ± 0.12 |

Fold of shift

| ND, not determined. |
|---------------------|
| n = 3.              |
| *n = 2.             |
| From Ref. 19.       |

**TABLE 4**

Inhibition of NS5B570-BK RNA synthesis activity by R1479-TP

| RNA template | Nucleotide | Label | IC50 ± S.D. (μM) |
|--------------|------------|-------|------------------|
| HCV 3′-UTR   | A,C,G,U    | [H]UTP | 0.67 ± 0.21 (3)  |
| HCV 3′-UTR   | A,C,G,U    | [H]ATP | 1.43 ± 0.37 (3)  |
| HCV cRES     | A,C,G,U    | [H]UTP | 0.29 ± 0.13 (2)  |
| HCV cRES     | A,C,G,U    | [H]ATP | 0.21 ± 0.12 (2)  |
| Poly(A)      | C          | [H]CTP | 0.02 ± 0.002 (3) |
| Poly(A)      | U          | [H]UTP | 173 ± 25 (3)     |

Denotes nucleotides present in the RNA synthesis reaction (A = ATP, C = CTP, G = GTP, and U = UTP).

Number of independent experiments indicated in parentheses.

Derived from HCV (−)-strand RNA (cRES) and UTP as a labeled substrate, R1479-TP inhibited RNA synthesis with an IC50 value of 0.29 μM (Table 4). Under the same conditions, the unphosphorylated nucleoside R1479 did not inhibit RNA synthesis at concentrations up to 1 mM. R1479 monophosphate and diphosphate showed weak inhibition of RNA synthesis with potencies 600- and 175-fold reduced relative to R1479-TP. Therefore, phosphorylation of R1479 to its 5′-triphosphate derivative was required for high potency inhibition of HCV polymerase. The template dependence of inhibitory potency suggested specific inhibition of CMP incorporation by R1479-TP, consistent with the requirement of base-specific hydrogen bond formation with the template by the CTP analog R1479-TP. The base-specific mode of inhibition was further analyzed using a competition assay with either CTP or ATP. Higher concentrations of R1479-TP were required to inhibit NS5B-dependent RNA synthesis in the presence of increasing concentrations of CTP (Fig. 4B), whereas the potency of NS5B inhibition was not affected by increasing concentrations of ATP (Fig. 4C). These results established R1479-TP as a competitive inhibitor of RNA template-directed CMP incorporation by NS5B. The Ki for R1479-TP was determined from three independent experiments at eight different CTP concentrations (mean Ki = 40 ± 25 nM) based on a Km(app) for CTP of 81.4 ± 25 nM (Fig. 4A).
in the HCV replicon system demonstrated the selection of a point mutation in the coding sequence of NS5B (S282T) that confers resistance to inhibition by a number of nucleosides carrying the 2′-C-methyl moiety (10, 19). As described here, R1479 (4′-azidocytidine) was identified as a specific inhibitor of HCV RNA replication in cell culture and has thus led to the discovery of a promising and completely novel group of 4′-substituted nucleoside analogs with potential for the treatment of HCV-infected patients.

R1479 and 2′-C-methylcytidine showed similar potency in the replicon system when assessed with multiple batches of compounds in >100 experiments over a time period of >1 year. R1479-TP is also a potent inhibitor of native HCV replicase in vitro and a competitive inhibitor of CMP incorporation by recombinant NS5B. The CTP competitiveness of R1479-TP was first apparent as template-specific inhibition of NS5B activity (Table 3). The IC_{50} value derived with poly(A) RNA template was 8650-fold higher than that derived with poly(I) RNA template, whereas intermediate values were obtained from heteropolymeric templates. The triphosphate potency of R1479-TP was higher than those published for 2′-C-MeATP and 2′-O-MeCTP and similar to those of 3′-dCTP and 2′-C-MeCTP. These results suggest high intrinsic potency of R1479-TP in inhibiting the HCV polymerase activity, consistent with high potency in cell culture. Similar to 3′-dCTP, R1479-TP was a substrate for HCV polymerase and blocked further elongation of nascent RNA after incorporation. These results suggest that the presence of the azido moiety at the 4′-position of R1479 interferes with the chemical reaction at the 3′-position. In agreement with a previous report (8), 3′-deoxyctydine was not active as an inhibitor of HCV replication in cell culture, despite the intrinsic high potency of 3′-dCTP against HCV replicase and HCV polymerase, suggesting inefficient triphosphate formation from 3′-deoxyctydine or metabolic instability of 3′-deoxyctydine in replicon cells. The potency of R1479 in HCV replicon cells suggests the formation and stability of its active triphosphate metabolite in Huh-7 cells. Additional studies are aimed at establishing the intracellular metabolism of R1479 and will be published in due course.

Notably, prolonged incubation of HCV replicon cells with R1479 resulted in complete clearance of HCV replicon RNA without selection of resistance and without apparent effects on cell viability and proliferation. These results indicate persistently high potency of R1479 in cell culture and the ability of R1479 to cure cells from replicating HCV RNA. The inhibitory potency of R1479-TP in vitro was not affected by the S282T point mutation, indicating the absence of cross-resistance between R1479-TP and 2′-C-β-methyl-nucleosides such as 2′-C-methyladenosine and 7-deaza-2′-C-methyladenosine. In the replicon system, the S282T point mutation has been described to decrease susceptibility to inhibition by 2′-β-methyl-nucleosides; however, the potency of HCV replication inhibition was not affected by this mutation. The results from studies on the effect of R1479 on the HCV replicon, native replicase, and recombinant NS5B protein were all highly consistent, confirming the high potency and specificity of R1479-TP for direct HCV polymerase inhibition and no detrimental effect of the S282T mutation on the intrinsic anti-HCV potency of R1479. Therefore, further investigations into the possibility of combining R1479 with 2′-C-methyl-nucleosides are warranted.

R1479 has been identified as a novel, potent, and specific inhibitor of NS5B-directed HCV RNA replication in cell culture. In vitro selection experiments and assessment of the site-directed mutant S282T suggest the absence of cross-resistance with the class of 2′-C-β-methyl-nucleosides, which supports further investigations into opportunities for nucleoside drug combinations. Based on its promising preclinical profile, including low cytotoxicity, high metabolic stability, and a large therapeutic window in cell culture, R1479 has been selected for further clinical development as a potential medicine for the treatment of HCV infection.
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