

The novel nucleoside analog R1479, 4'-azidocytidine, is a potent inhibitor of NS5B dependent RNA synthesis and HCV replication in cell culture

Klaus Klumpp, Vincent Leveque, Sophie Le Pogam, Han Ma, Wen-Rong Jiang, Hyunsoon Kang, Caroline Granycome, Margaret Singer, Carl Laxton, Julie Qi Hang, Keshab Sarma, David B. Smith, Dieter Heindl¹, Chris J. Hobbs, John H. Merrett, Julian Symons, Nick Cammack, Joseph A. Martin, Rene Devos, and Isabel Nájera

Roche Palo Alto LLC, Palo Alto, CA, USA; ¹Roche Diagnostics GmbH, Penzberg, Germany
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Address correspondence to: Klaus Klumpp, Roche Palo Alto LLC, 3431 Hillview Av, Palo Alto, CA, 94304, Tel: 6508556028, Fax: 6503547554, E-mail: klaus.klumpp@roche.com

HCV polymerase 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 (IC₅₀ = 1.28 μM) with similar potency as compared to 2'-C-methyl-cytidine (IC₅₀ = 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 value of 40 nM. On an HCV RNA derived template substrate (cIRES) R1479-TP showed similar potency of NS5B inhibition as compared to 3'-deoxyCTP. R1479-triphosphate was incorporated into nascent RNA by HCV polymerase and reduced further elongation with similar efficiency as 3'-deoxy-CTP under the reaction conditions. The S282T point mutation in the coding sequence of NS5B confers resistance to inhibition by 2'-C-methyl-adenosine-TP and other 2'-methylated nucleotides. In contrast, the S282T mutation did not confer cross-resistance to R1479.

Hepatitis C virus (HCV¹) infection is a major cause of chronic liver disease, cirrhosis and hepatocellular carcinoma and currently the

leading cause of liver transplantation (1,2). Viral genome sequence analysis established six HCV genotype classes (HCV GT 1-6) with genotypes 1-3 most prevalent in the US, Europe and Japan. Current treatment options available to HCV infected persons are limited, and sustained virological response rates (SVR) are particularly low for HCV GT 1 infected patients. Only about 50 % of individuals infected with HCV GT 1 with serum viral titers of greater than 2 x 10⁶ copies/ml achieved SVR when treated with a combination of pegylated interferon alpha and ribavirin (3,4). Response rates are even lower in persons with HIV coinfection 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 positive strand RNA virus which enters host cells via receptor-mediated endocytosis and replicates in the host cell cytoplasm. A membrane associated replicase complex containing HCV genome encoded non-structural 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 protein contains the HCV polymerase active site within the replicase complex, a RNA dependent RNA polymerase (RdRp). 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 (NTP) analogs by cellular enzymes. NTP 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 (NM-107) has entered clinical development as a 3'-valinate prodrug (NM-283) (8). Additional nucleoside analogs carrying the 2'-beta 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 paper 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 IRES, contains the renilla luciferase gene in fusion with the neomycin phosphotransferase gene (NPTII) and the second open reading frame, driven by EMCV IRES, contains the HCV non-structural genes NS3, NS4a, NS4b, NS5A and NS5B derived from the NK5.1 replicon backbone, originally described by Krieger et al. (12). Thus, the replicon cell line 2209-23 is resistant to geneticin (G418) and expresses the reporter renilla luciferase gene as a marker of HCV RNA replication. Cells were cultured in

DMEM supplemented with Glutamax and 100 mg/ml sodium pyruvate (Gibco). 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 / 95% air atmosphere. The selection and characterization of replicon clones carrying a point mutation S282T in the NS5B coding sequence will be described elsewhere². Nucleoside analogs were synthesized at Roche and dissolved in 100 % DMSO, then diluted in DMEM with 5 % (v/v) fetal calf serum before addition to cells. The final concentration of DMSO was 1 % (v/v) in all experiments. Quantification of renilla luciferase activity was performed using the Renilla Luciferase Assay Kit (Promega) according to manufacturer's instructions. The WST-1 cell proliferation assay (Roche Diagnostics) was used to measure cell viability. Direct quantification of HCV RNA levels was performed using quantitative kinetic RT-PCR (TaqMan) using endogenous β-actin mRNA as a control. Primers used were Forward (5'-GCTGCTATTGGGCGAAGTG-3'), Reverse (5'-GCCGCCGCATTGCA-3') and Probe (5'-TCCTGCCGAGAAAGTATCCATCATGGC T-3'). Reactions were analyzed on a 7700 Sequence Detector (PE Biosystems). The GT-1b Con1 adapted transient replicon (rep PI-luc/ET) was obtained from R. Bartenschlager (13). The transient Con1-S282T replicon was constructed and assays were performed as described².

Replicon cell proliferation assay – The effect of compounds on the incorporation of tritiated thymidine into cellular DNA was measured using the SPA [³H]-thymidine incorporation assay system from Amersham Biosciences. MTT 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 µM 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 a further 15 days in culture medium containing 0.25 mg/ml of neomycin to allow replication of residual replicon RNA molecules remaining after treatment. Levels of HCV RNA were determined by quantitative kinetic RT-PCR using TaqMan technology as described above and are expressed as log change compared to the HCV replicon RNA level in untreated cells at day 0.

HCV replicase assay – The membrane associated, native HCV replicase complex was isolated from 2209-23 HCV replicon cells and a derived cell line carrying HCV replicon RNA with a S282T mutation in the NS5B coding sequence according to previously published procedures (14). The in vitro replicase assay contained 10 µl cytoplasmic membrane fraction, 50 mM Hepes, pH 7.5, 10 mM KCl, 10 mM DTT, 5 mM MgCl₂, 20 µg/ml actinomycin D, 1 mM ATP, GTP and UTP, 30 µCi [α -³³P]-CTP (3000 Ci/mmol, 10 mCi/ml), 1 U/µl SUPERase.In (Ambion), 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 polymerase assay – The enzymatic activity of NS5B570-BK and NS5B570-Con1 and NS5B570-S282T-Con1 proteins was measured as incorporation of radiolabeled NMP into acid-insoluble RNA products as previously described (14). Briefly, HCV polymerase reactions contained 10 µg/ml cIRES or 3'-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 tritiated UTP or CTP (1-5 µCi), 1 µM ATP, CTP and GTP (with cIRES and 3'-UTR RNA templates), 40 mM Tris-HCl, pH 8.0, 4 mM DTT and 4 mM MgCl₂. NS5B570 proteins contain a C-terminal deletion of 21 amino acids, which removes a trans-membrane domain and increases solubility of the protein (14,15). HCV RNA templates used were: cIRES RNA, corresponding to 377 nucleotides from the 3'-end of HCV negative strand RNA (14), base content A (21%), U (23%), C (28%),

G (28%), and 3'-UTR RNA, corresponding to 389 nucleotides from the 3'-end of HCV positive strand RNA, base content A (15%), U (38%), C (26%), G (21%). RNA was transcribed in vitro using a T7 transcription kit (Ambion) and purified using either phenol chloroform extraction or the Qiagen RNeasy Maxi kit, with similar results. Poly-A RNA was from Amersham Pharmacia Biotech Inc. and poly-I RNA from Yamasa Corp. Data were analyzed with GraphPad® Prism® and/or Microsoft® Excel®. The apparent Michaelis constant ($K_{m(app)}$) of CTP for NS5B570-BK was calculated by non-linear fitting using equation (1),
$$Y = \frac{(V_{max(app)})X}{K_{m(app)} + X} \quad (1),$$

where “Y” corresponds to the rate of RNA synthesis by NS5B570-BK (in CPM/min.), “ $V_{max(app)}$ ” is the maximum rate at saturating substrate concentration, and “X” corresponds to CTP concentration. The compound concentration at which the enzyme-catalyzed rate is reduced by 50 % (IC_{50}) was calculated by fitting the data to equation (2),
$$Y = \%Min + \frac{(\%Max - \%Min)}{1 + \frac{X}{(IC_{50})}} \quad (2),$$

where “Y” corresponds to the relative enzyme activity (in %), “%Min” is the residual relative activity at saturating compound concentration, “%Max” is the relative maximum enzymatic activity, and X corresponds to the compound concentration. The mean IC_{50} value was derived from the mean of several independent experiments. The standard deviation from the mean (Stdev) was calculated from the nonbiased method using equation (3),

$$Stdev = \sqrt{\frac{n \sum (IC_{50})^2 - (\sum IC_{50})^2}{n(n-1)}} \quad (3)$$

The $K_i(CTP)$ of RO1048297-TP was derived by fitting the data to the Cheng-Prusoff

equation (4), assuming competitive inhibition relative to CMP incorporation:

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

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

Gel-based nucleotide incorporation assay

– The RNA template-directed incorporation and extension of the nucleotide and nucleotide analogs by HCV NS5B was performed with a 19 nucleotide (nt) RNA oligo (5'-AUGUAUAAUUAUUGUAGCC-3') and 5' end-labeled GG primer (Dharmacon). The RNA template is predicted to form a single stem-loop structure with an unpaired 3-nt sequence at the 3'end. The 5' end-labeling of GG primer was performed with [γ -³³P]-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 DTT, 2 μ M NS5B570-BK, 5 μ M RNA template, 0.15 μ M end-labeled GG primer, and nucleoside triphosphates of the indicated concentrations in a volume of 10 μ l. The reactions were incubated at 30°C for 60 minutes and stopped by the addition of 10 μ l formamide gel loading buffer II (Ambion). After denaturing at 95°C for 3 minutes, the RNA template, primer and extended primer were separated in a 20% acrylamide TBE-urea gel. The dried gel was exposed to storage phosphor screen and analyzed with a phosphorimager (Amersham Biosciences).

Results

Nucleoside analogs have proven to be highly successful agents for the treatment of viral infections including HIV, HBV and herpes virus infections. 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, which contains the HCV 5'-nontranslated region directing the translation of renilla luciferase and the selectable marker neomycin phosphotransferase. The EMCV IRES directs the translation of HCV proteins NS3 to NS5B (12,17). The renilla replicon system was validated using a range of different types of HCV replication inhibitors including interferon alpha 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, which inhibited HCV subgenomic RNA replication without interfering with cell viability and cell proliferation. After further optimization, 4'-azidocytidine (R1479; 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 (4'-azidocytidine) inhibited HCV RNA replication with a mean IC_{50} value of 1.28 μ M, 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-Me-cytidine (2'-C-Me-C) with a mean IC_{50} value of 1.13 μ M. Similar potencies for both

compounds were also obtained from the measurement of the dose-dependent 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 μ M. R1479 showed only low cytotoxicity in human hepatoma cell derived replicon cells. Cell viability was monitored by measuring intracellular ATP concentration or cellular MTT 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 non-specific manner, as exemplified by the inosine monophosphate dehydrogenase (IMPDH) inhibitor mycophenolic acid (MPA). MPA is a potent inhibitor of cell proliferation, as measured by the inhibition of tritiated thymidine incorporation into cellular DNA (IC_{50} = 0.6 μ M), without affecting cell viability over a 72 h incubation period at concentrations up to 100 μ M (Table 1). The apparent IC_{50} value for the inhibition of HCV replicon RNA replication for MPA was similar to the IC_{50} value of thymidine incorporation into cellular DNA, suggesting an indirect cell culture specific effect of HCV replicon RNA reduction by MPA. In contrast, R1479 was not cytostatic and did not inhibit tritiated thymidine incorporation at concentrations up to 1 mM, suggesting that R1479 inhibited HCV replication by a direct antiviral mechanism.

Previously, the point mutation S282T in the NS5B coding sequence has been associated with resistance to nucleoside analogs carrying a 2'-beta-methyl moiety, as shown using WT 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 sequence². In contrast, prolonged incubation of HCV replicon cells with R1479 resulted in a continued decrease of HCV RNA to undetectable levels (Fig. 2). To determine the kinetics of the subgenomic HCV replicon inhibition by R1479, HCV replicon cells were cultured in the presence of inhibitor at 6 μ M and 12 μ M 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 were cultured for a further 30 days in culture medium containing 0.25 mg/ml of 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 three days by quantitative kinetic RT-PCR as described in Materials and Methods. In the untreated cells, HCV RNA levels remained constant throughout the viral clearance and the rebound phases in the untreated cells. In the presence of R1479 at 6 μ M and 12 μ M (approximately 5x IC_{50} ~ 1x IC_{90} and 10x IC_{50} respectively) the HCV RNA was reduced by more than 5 logs 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_{90} , was able to completely clear the HCV subgenomic replicon RNA from the replicon cells within a 15 day incubation period.

R1479-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 triphosphate. NS5B likely forms large multiprotein – RNA complexes with the other HCV encoded non-structural proteins and the genomic HCV 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 to assess direct effects of R1479-TP on HCV RNA synthesis (14). R1479-TP inhibited RNA synthesis by native HCV replicase in vitro with high potency (IC_{50} = 0.79 μ M) (Table 3; Fig. 3). The IC_{50} of R1479-TP for the inhibition of HCV RNA synthesis by the native HCV replicase was similar to that measured with the reference inhibitor 3'-deoxy-CTP and 3-fold lower than that reported for 2'-C-Me-adenosine (Table 3, (19)) and 2'-O-Me-cytidine (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'-C-methyl nucleosides, consistent with the resistance observed in cell culture (10,19)². 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 NS5B in vitro

RNA synthesis activity by recombinant NS5B protein was measured as 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 (3'-UTR and cIRES). Two additional templates were homopolymers of adenosine-monophosphate (poly-A) and inosine-monophosphate (poly-I). Assay conditions were optimized for each RNA template such that the rate of labeled nucleotide incorporation was constant for at least 3 hours and dose dependent inhibition by

nucleotide analogs was determined after a 2 h incubation period. R1479-TP inhibited RNA synthesis by NS5B on all templates tested in a template sequence dependent manner (Table 4). The lowest IC_{50} values were obtained with poly-I template RNA, which directs the synthesis of poly-C and the highest IC_{50} values were obtained with poly-A template RNA, which directs 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 derived from the HCV negative strand RNA (cIRES) and UTP as a labeled substrate, R1479-TP inhibited RNA synthesis with an IC_{50} value of 0.29 μ M (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-fold 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 dependency of inhibitory potency suggested specific inhibition of CMP incorporation by R1479-TP, consistent with the requirement of base-specific hydrogen bond formation to 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 K_i value for R1479-TP was determined from three independent experiments at eight different CTP concentrations (mean $K_i = 40 \pm 25$ nM) based on $K_{m(app)}(CTP) = 81.4 \pm 25$ nM (Fig. 4A).

R1479-TP inhibited recombinant NS5B enzymes from HCV strains BK and Con1 with similar potency (Table 5). IC_{50} values were also similar, within 2-fold, to those obtained with 3'-deoxy-CTP. 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 a HCV polymerase assay using a 19-nucleotide RNA template (Figure 5A). Nucleotide incorporation was initiated using labeled GG dinucleotide primer and either CTP, R1479-TP or 3'-deoxy-CTP as substrates. As shown in Figure 5B, CTP could be used as a substrate by HCV polymerase to incorporate CMP into nascent RNA (Figure 5B, lane 4) and the product could be further elongated with UTP as the next nucleoside triphosphate substrate (Figure 5B, lanes 5-6). R1479-TP and 3'-deoxy-CTP could also serve as substrates for incorporation (Figure 5B, lanes 7 and 10), but further elongation with UTP was blocked (Figure 5B, lanes 8, 9 and 11, 12). 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'-deoxy-CTP. Control reactions with UTP alone in 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 is the only nucleoside analog used for the treatment of HCV infection, in combination with interferon alpha. However, ribavirin is a non-selective 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 have been described, which appear to provide some improvement of potency and/or selectivity against HCV NS5B. 2'-deoxy-2'-alpha-fluoro-cytidine-TP was shown to be a potent inhibitor of NS5B (25). However, this and other nucleosides carrying the 2'-deoxy-2'-alpha 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'-alpha-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'-beta-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-methyl-cytidine (2'-C-Me-C) is currently in Phase 2 clinical development for the treatment of HCV infection (8). In vitro resistance selection experiments in the HCV replicon system demonstrated the selection of a point mutation in the coding sequence of NS5B, S282T, which conferred resistance to inhibition by a number of nucleosides carrying the 2'-C-methyl moiety

(10,19,28)². 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-Me-C showed similar potency in the replicon system, when assessed with multiple batches of compounds and more than 100 experiments over a time period of more than one year. R1479-TP is also a potent inhibitor of the 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 IC₅₀ value derived with poly-A RNA template was 8650-fold higher than with poly-I RNA template, whereas intermediate values were obtained from heteropolymeric templates. The triphosphate potency of R1479-TP was higher than that published for 2'-C-Me-ATP and 2'-O-Me-CTP, and similar to that of 3'-deoxy-CTP and 2'-C-Me-CTP². These results suggest high intrinsic potency of R1479-TP for inhibiting the HCV polymerase activity, consistent with high potency in cell culture. Similar to 3'-deoxy-CTP, 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, 3'-deoxy-cytidine was not active as an inhibitor of HCV replication in cell culture, despite the intrinsic high potency of 3'-deoxy-CTP against HCV replicase and HCV polymerase, suggesting inefficient triphosphate formation from 3'-deoxy-cytidine or metabolic instability of 3'-deoxy-cytidine in replicon cells (29). The potency of R1479 in HCV replicon cells suggests the formation and stability of its active triphosphate metabolite in Huh7 cells. Further studies were aimed at establishing the intracellular metabolism of R1479 and will be published in due course.

Importantly, 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-beta-methylated nucleosides like 2'-C-Me-adenosine and 7-deaza-2'-C-Me-adenosine. In the replicon system the S282T point mutation has been described to decrease susceptibility to inhibition by 2'-beta-methyl nucleosides, however, the potency of HCV replication inhibition was not affected by this mutation. The results from the effect of R1479 on HCV replicon, native replicase and recombinant NS5B protein were all highly consistent, confirming 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-Me nucleosides are warranted.

Taken together, 4'-azidocytidine (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 site directed mutant S282T suggest the absence of cross-resistance with the class of 2'-C-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.

REFERENCES

1. Thomson, B. J., and Finch, R. G. (2005) *Clin. Microbiol. Infect.* **11**, 86-94
2. Centers of Disease Control and Prevention. (1998) *MMWR* **47(RR19)**, 1-39
3. Fried, M. W., Shiffman, M. L., Reddy, K. R., Smith, C., Marinos, G., Goncalves, F. L., Jr., Haussinger, D., Diago, M., Carosi, G., Dhumeaux, D., Craxi, A., Lin, A., Hoffman, J., and Yu, J. (2002) *N. Engl. J. Med.* **347**, 975-982.
4. Manns, M. P., McHutchison, J. G., Gordon, S. C., Rustgi, V. K., Shiffman, M., Reindollar, R., Goodman, Z. D., Koury, K., Ling, M., and Albrecht, J. K. (2001) *Lancet* **358**, 958-965.
5. Pawlotsky, J.-M. (2004) *N. Engl. J. Med.* **351**, 422-423
6. Torriani, F. J., Rodriguez-Torres, M., Rockstroh, J. K., Lissen, E., Gonzalez-Garcia, J., Lazzarin, A., Carosi, G., Sasadeusz, J., Katlama, C., Montaner, J., Sette, H. J., Pante, S., De Pamphilis, J., Duff, F., Schrenk, U., and Dieterich, D. T. (2004) *N. Engl. J. Med.* **351**, 438-450
7. Chung, R. T., Andersen, J., Volberding, P., Robbins, G. K., Liu, T., Sherman, K. E., Peters, M. G., Koziel, M. J., Bhan, A. K., Alston, B., Colquhoun, D., Nevin, T., Harb, G., and van der Horst, C. (2004) *N. Engl. J. Med.* **351**, 451-459
8. Zhou, X. J., Afdahl, N., Godofsky, E., Dienstag, J., Rustgi, V., Schick, L., McInery, D., Fielman, B. A., and Brown, N. A. (2005), Abstract S926. Digestive Disease Week. May 914-919, 2005. Chicago, IL
9. Carroll, S. S., Tomassini, J. E., Bosserman, M., Getty, K., Stahlhut, M. W., Eldrup, A. B., Bhat, B., Hall, D., Simcoe, A. L., LaFemina, R., Rutkowski, C. A.,

- Wolanski, B., Yang, Z., Migliaccio, G., De Francesco, R., Kuo, L. C., MacCoss, M., and Olsen, D. B. (2003) *J. Biol. Chem.* **278**, 11979-11984
10. Olsen, D. B., Eldrup, A. B., Bartholomew, L., Bhat, B., Bosserman, M. R., Ceccacci, A., Colwell, L. F., Fay, J. F., Flores, O. A., Getty, K. L., Grobler, J. A., LaFemina, R. L., Markel, E. J., Migliaccio, G., Prhac, M., Stahlhut, M. W., Tomassini, J. E., MacCoss, M., Hazuda, D. J., and Carroll, S. S. (2004) *Antimicrob. Agents Chemother.* **48**, 3944-3953.
 11. Eldrup, A. B., Prhac, M., Brooks, J., Bhat, B., Prakash, T. P., Song, Q., Bera, S., Bhat, N., Dande, P., Cook, P. D., Bennett, C. F., Carroll, S. S., Ball, R. G., Bosserman, M., Burlein, C., Colwell, L. F., Fay, J. F., Flores, O. A., Getty, K., LaFemina, R. L., Leone, J., MacCoss, M., McMasters, D. R., Tomassini, J. E., Von Langen, D., Wolanski, B., and Olsen, D. B. (2004) *J. Med. Chem.* **47**, 5284-5297.
 12. Krieger, N., Lohmann, V., and Bartenschlager, R. (2001) *J. Virol.* **75**, 4614-4624
 13. Lohmann, V., Hoffmann, S., Herian, U., Penin, F., and Bartenschlager, R. (2003) *J. Virol.* **77**, 3007-3019.
 14. Ma, H., Leveque, V., De Witte, A., Li, W., Hendricks, T., Clausen, S. M., Cammack, N., and Klumpp, K. (2005) *Virology* **332**, 8-15.
 15. Ferrari, E., Wright-Minogue, J., Fang, J. W., Baroudy, B. M., Lau, J. Y., and Hong, Z. (1999) *J. Virol.* **73**, 1649-1654
 16. Frese, M., Schwarzle, V., Barth, K., Krieger, N., Lohmann, V., Mihm, S., Haller, O., and Bartenschlager, R. (2002) *Hepatology* **35**, 694-703.
 17. Vrolijk, J. M., Kaul, A., Hansen, B. E., Lohmann, V., Haagmans, B. L., Schalm, S. W., and Bartenschlager, R. (2003) *J. Virol. Methods* **110**, 201-209.
 18. Pietschmann, T., Lohmann, V., Rutter, G., Kurpanek, K., and Bartenschlager, R. (2001) *J Virol* **75**, 1252-1264
 19. Migliaccio, G., Tomassini, J. E., Carroll, S. S., Tomei, L., Altamura, S., Bhat, B., Bartholomew, L., Bosserman, M. R., Ceccacci, A., Colwell, L. F., Cortese, R., De Francesco, R., Eldrup, A. B., Getty, K. L., Hou, X. S., LaFemina, R. L., Ludmerer, S. W., MacCoss, M., McMasters, D. R., Stahlhut, M. W., Olsen, D. B., Hazuda, D. J., and Flores, O. A. (2003) *J. Biol. Chem.* **278**, 49164-49170
 20. Moradpour, D., Gosert, R., Egger, D., Penin, F., Blum, H. E., and Bienz, K. (2003) *Antiviral Res.* **60**, 103-109.
 21. Sidwell, R. W., Huffmann, J. H., Khare, G. P., Allen, L. B., Witkowski, J. T., and Robins, R. K. (1972) *Science* **177**, 705-706
 22. Witkowski, J. T., Robins, R. K., Sidwell, R. W., and Simon, L. N. (1972) *J. Med. Chem.* **15**, 1150-1154
 23. Lau, J. Y., Tam, R. C., Liang, T. J., and Hong, Z. (2002) *Hepatology* **35**, 1002-1009
 24. Crotty, S., Maag, D., Arnold, J. J., Zhong, W., Lau, J. Y., Hong, Z., Andino, R., and Cameron, C. E. (2000) *Nat. Med.* **6**, 1375-1379
 25. Stuyver, L. J., McBrayer, T. R., Whitaker, T., Tharnish, P. M., Ramesh, M., Lostia, S., Cartee, L., Shi, J., Hobbs, A., Schinazi, R. F., Watanabe, K. A., and Otto, M. J. (2004) *Antimicrob. Agents Chemother.* **48**, 651-654.
 26. Tisdale, M., Ellis, M., Klumpp, K., Court, S., and Ford, M. (1995) *Antimicrob. Agents Chemother.* **39**, 2454-2458.

27. Beaulieu, P. L., and Tsantrizos, Y. S. (2004) *Curr. Opin. Investig. Drugs* **5**, 838-850
28. Najera, I., and others. manuscript in preparation
29. Shim, J., Larson, G., Lai, V., Naim, S., and Wu, J. Z. (2003) *Antiviral Res.* **58**, 243-251.

Footnotes

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¹, Abbreviations: HCV, hepatitis C virus; R1479, 4'-azidocytidine; TP, 5'-triphosphate; IRES, internal ribosome entry signal; BVDV, bovine viral diarrhea virus; GT, genotype; SVR, sustained viral response; NTP, nucleoside triphosphate; EMCV, encephalomyocarditis virus; DMEM, Dulbecco's modified eagle medium; SPA, scintillation proximity assay; MDCK, Madin-Darby canine kidney cell line;

², Nájera, I et al., manuscript in preparation.

Figure Legends

Fig. 1. Chemical structures of A) R1479, B) 3'-deoxy-CTP, C) 2'-C-Me-cytidine and D) 2'-C-Me-adenosine.

Fig. 2. Clearance of HCV RNA from replicon cells by treatment with R1479. HCV replicon cells were treated with 6 μM (black triangles) and 12 μM (black squares) of R1479 in the absence of neomycin selection for 15 days (replicon clearance). An untreated control was set up in parallel (white squares). During the rebound phase, the inhibitor was removed from the culture medium and cells were cultured in the presence of 0.25 mg/ml of neomycin. Levels of HCV replicon RNA in treated cells are expressed as a log change compared to the HCV RNA level in untreated cells at day 0.

Fig. 3. Inhibition of wild-type (WT) and S282T native HCV replicase by R1479-TP. Representative denaturing analytical agarose gel electrophoresis for the quantification of full length replicon RNA synthesized by HCV replicase in vitro. Replicase reactions were performed as described in Experimental Procedures in the absence of added compound (lane C), or in the presence of 0.03 – 25 μM R1479-TP (lanes 2-8) in a 3-fold dilution series. IC_{50} values shown in table 3.

Fig. 4. Effect of CTP concentration on the inhibition of HCV polymerase activity by R1479-TP. A) dependence of HCV polymerase activity on the concentration of CTP. Mean $K_{m(\text{app})} = 81.4 \pm 10.5$ nM (n= 3). B) dose response curves of HCV polymerase inhibition by R1479-TP were generated in the presence of 0.5 (open triangles), 1 (diamonds), 5 (filled triangles), 10 (circles) or 50 (squares) μM CTP. Mean $K_i = 40 \pm 25$ nM (n=3). C) dose response curves in the presence of 0.5, 5, 10 or 50 μM ATP.

Fig. 5. R1479-TP is a substrate for HCV polymerase and can reduce further elongation after incorporation. **A**, Sequence and structure of RNA template and primer. **B**, Nucleotide incorporation assay was performed as described in Experimental Procedures. The nucleoside triphosphates included in the reactions were: 2 μM UTP (lane 2), 10 μM UTP (lane 3); 10 μM CTP with either no UTP (lane 4), or 2 μM UTP (lane 5), or 10 μM UTP (lane 6); 10 μM R1479-TP with either no UTP (lane 7), or 2 μM UTP (lane 8), or 10 μM UTP (lane 9); 10 μM 3' dCTP with either no UTP (lane 10), or 2 μM UTP (lane 11), or 10 μM UTP (12). Lane 1 was a no enzyme control reaction with 10 μM CTP and 10 μM UTP. Product size markers are shown on the left.

Table 1. Inhibition of HCV RNA replication in HCV replicon cells

Compound	IC ₅₀ [μM] Luc ^a	IC ₅₀ [μM] Taq ^b	CC ₅₀ [μM] WST-1 ^c	IC ₅₀ [μM] [³ H]-Thy ^d	IC ₅₀ [μM] ATP ^e
4'-azidocytidine (R1479)	1.28 ± 0.08	0.67 ± 0.13	> 2000	> 100	> 2000
2'-C-methyl- cytidine	1.13 ± 0.03	0.76 ± 0.01	> 100	> 100	nd
3'-deoxy- cytidine	> 100	nd	>100	nd	nd
Mycophenolic acid (MPA)	0.32 ± 0.07	nd	> 100	0.60 ± 0.02	nd

^a Inhibition of HCV replicon encoded renilla luciferase reporter activity; mean IC₅₀ value and standard error of mean, determined from n= 101 (R1479) and n= 354 (2'-C-methyl-cytidine) experiments.

^b Inhibition of HCV RNA determined by quantitative kinetic RT-PCR; mean IC₅₀ value and standard error of mean calculated from at least n = 4 experiments.

^c cell viability, determined from at least 4 experiments.

^d Inhibition of tritiated thymidine incorporation into cellular DNA, determined from at least 4 experiments.

^e Reduction of cellular ATP levels, determined from n = 4 experiments.

^f nd, not determined.

Table 2: Inhibition of GT 1b Con1-WT and Con1-S282T stable and transient replicons replication

Compound	Stable replicons IC ₅₀ [μM]			Transient replicons IC ₅₀ [μM]	
	Wildtype ^a	S282T ^b		Wildtype ^c	S282T ^d
		Cell line 16	Cell line C		
R1479	0.67 ± 0.13	0.3	0.27	1.9 ± 0.26	0.53 ± 0.1

^a Inhibition of HCV RNA replication of wildtype GT 1b Con1 stable replicon.

^b Inhibition of HCV RNA determined by quantitative kinetic RT-PCR; IC₅₀ value determined from two different stable replicon cell lines containing the S282T mutation (cell line “clone 16” and cell line “clone C”).

^{c, d} Inhibition of HCV RNA replication; mean IC₅₀ value and standard error of mean, determined from at least 5 independent experiments by quantification of replicon encoded luciferase activity.

Table 3. Inhibition of wild-type and S282T HCV replicases by nucleotide analogs

Replicon	Native HCV Replicase IC ₅₀ , μM		
	2'-C-methyl-ATP	3'-dCTP	R1479-TP
NS5B-S282T	nd	0.76 ± 0.12 ^a	0.71 ± 0.15 ^b
Wild-type	2.5 ^c	0.78 ± 0.5 ^b	0.79 ± 0.12 ^a
Fold of shift	nd	0.97	0.89

^a: n = 3, ^b n = 2, ^c from (19)

Table 4. Inhibition of NS5B570-BK RNA synthesis activity by R1479-TP

Template RNA	Nucleotide ^a	Label	IC ₅₀ (μM) ± SD ^b
HCV RNA, 3'-UTR	A, C, G, U	[³ H]-UTP	0.67 ± 0.21 (3)
HCV RNA, 3'-UTR	A, C, G, U	[³ H]-ATP	1.43 ± 0.37 (3)
HCV RNA, cIRES	A, C, G, U	[³ H]-UTP	0.29 ± 0.13 (2)
HCV RNA, cIRES	A, C, G, U	[³ H]-ATP	0.21 ± 0.12 (2)
Poly-I	C	[³ H]-CTP	0.02 ± 0.002 (3)
Poly-A	U	[³ H]-UTP	173 ± 25 (3)

^a Denotes nucleotide present in RNA synthesis reaction (A=ATP, C=CTP, G=GTP, U=UTP).

^b IC₅₀ values in μM shown with standard deviations; number of independent experiments in parentheses. NS5B protein was from HCV strain BK, genotype 1b.

Table 5. Inhibition of HCV polymerase activity

Enzyme	R1479-TP	3'-deoxy-CTP	Ribavirin-TP
	IC ₅₀ [μM] (n) ^a	IC ₅₀ [μM] (n)	IC ₅₀ [μM] (n)
NS5B570-Con1	0.32 ± 0.11 (9)	0.31 ± 0.04 (9)	nd
NS5B570_S282T-Con1	0.29 ± 0.09 (8)	0.55 ± 0.10 (9)	nd
NS5B570-BK	0.28 ± 0.12 (19)	0.17 ± 0.05 (11)	255 ± 59 (3)

^a mean IC₅₀ values and standard deviation determined from HCV polymerase reactions using HCV cIRES RNA template. Number of experiments in brackets.

Fig. 1.

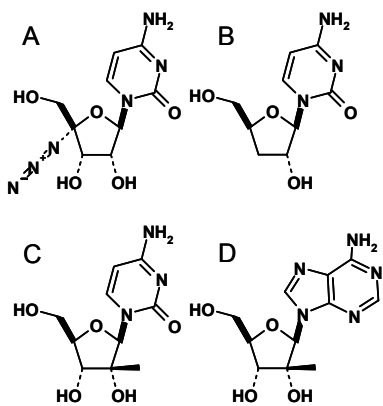


Fig. 2.

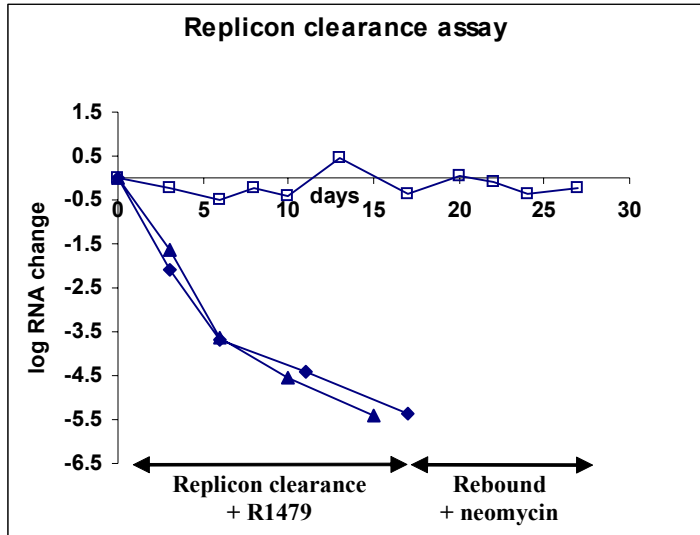


Fig. 3

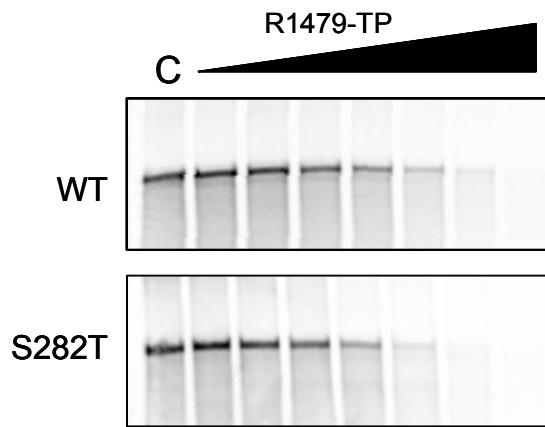


Fig. 4.

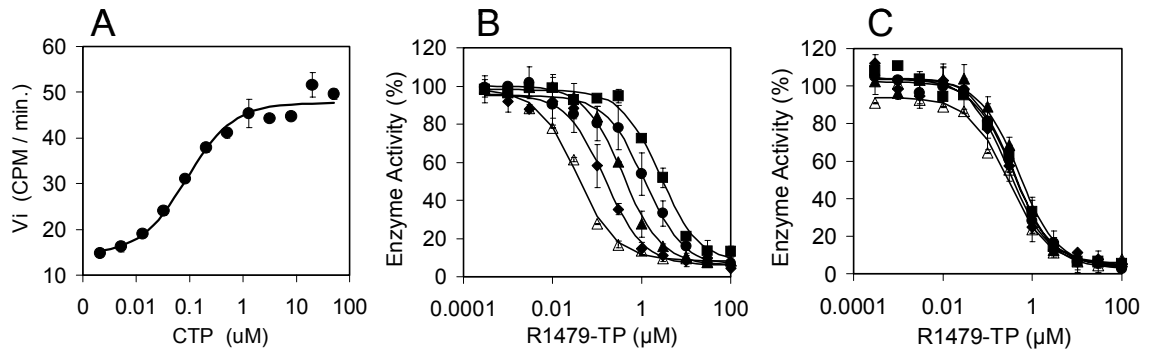


Fig. 5

