Antiviral activity and mode of action of TMC647078, a novel nucleoside inhibitor of the HCV NS5B polymerase

Running title: Characterization of HCV nucleoside inhibitor TMC647078

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Abstract

Chronic infection with hepatitis C virus (HCV) is a major global health burden and is associated with increased risk of liver cirrhosis and hepatocellular carcinoma. Current therapy for HCV infection has limited efficacy, in particular against the genotype 1 virus, and is hampered by a range of adverse effects. Therefore, there is a clear unmet medical need for efficacious and safe direct antiviral drugs for use in combination with current treatments to increase cure rates and shorten treatment times. The broad genotypic coverage achievable with nucleos(t)ides and the high genetic barrier to resistance of these compounds observed in vitro and in vivo, suggest that this class of inhibitors could be a valuable component of future therapeutic regimens. Here, we report the in vitro inhibitory activity and mode of action of 2’-deoxy-2’-spirocyclopropylcytidine (TMC647078), a novel and potent nucleoside inhibitor of the HCV NS5B RNA-dependent RNA polymerase that causes chain termination of the nascent HCV RNA chain. In vitro combination studies with a protease inhibitor resulted in additive efficacy in suppression of HCV RNA replication, highlighting the potential for combination of these two classes in treatment of chronic HCV infection. No cytotoxic effects were observed in various cell lines. Biochemical studies indicated that TMC647078 is mainly phosphorylated by dCK without inhibiting phosphorylation of the natural substrate, and high levels of triphosphate were observed in Huh7 cells and in primary hepatocytes in vitro. TMC647078 is a potent novel nucleoside inhibitor of HCV replication with a promising in vitro virology and biology profile.
Introduction

Infection with hepatitis C virus (HCV), the causative agent of hepatitis C, is an important global health burden with an estimated 120 to 170 million persons chronically infected (7, 38). Chronic HCV infection can lead to liver cirrhosis and hepatocellular carcinoma, and is the leading cause of liver transplantation (10). The virus is mainly transmitted via blood-blood contact, and spontaneous virus clearance is estimated to be achieved in 26% of infected subjects (29).

HCV is a member of the *Flaviviridae* family of viruses in the *Hepacivirus* genus, comprising of at least six major genotypes and multiple subtypes. The 9.6-kb positive-sense, single-stranded RNA genome of HCV encodes four structural proteins (the core protein, envelope glycoproteins E1 and E2, and p7) and six non-structural proteins responsible for viral replication (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The non-structural protein NS5B is an RNA-dependent RNA polymerase (RdRp) responsible for the amplification of the HCV RNA and assembly of the replicase complex at the endoplasmic reticulum membrane (3, 25).

Currently, HCV patients are treated with a combination of weekly pegylated interferon-α (IFN-α) injections and twice-daily ribavirin, which has considerable tolerability issues (28), and results in a sustained viral response in only 40 to 50% of patients infected with genotype 1 (37), the predominant genotype in Europe, North America, Japan, and China (36). Therefore, new drugs to treat HCV infection are needed, and research and development efforts are focusing on small-molecule drugs targeting essential viral proteins. It is expected that combinations of such specifically targeted drugs with or without the current standard of care, will lead to improved therapeutic outcomes (6, 28). Several direct antiviral agents (DAAs) are currently in clinical
development, where the most advanced candidates are the protease inhibitors telaprevir and
boceprevir, currently filed for regulatory approval.

Both non-nucleoside inhibitors (NNIs) and nucleoside inhibitors (NIs) of NS5B have been
described (15). NNIs are chemically diverse and bind to one of the four sites on the enzyme
(NNI-1, NNI-2, NNI-3 and NNI-4) (34). NIs are nucleoside analogs that, after conversion to
their active triphosphates (TPs) by cellular kinases, are accepted as substrates by the RdRp and
incorporated into the nascent RNA chain thereby blocking elongation and RNA replication. The
rate-limiting step in the NI activation is intracellular phosphorylation where the initial step is
catalyzed by the (deoxy)nucleoside kinases present in the cytosol (thymidine kinase 1 (TK1) and
deoxyctydine kinase (dCK)), and in the mitochondria (thymidine kinase 2 (TK2) and
deoxyguanosine kinase (dGK)) (9). In addition pyrimidine ribonucleoside analogs can be
phosphorylated constitutively by cytosolic uridine-cytidine kinase 1 and 2 enzymes (39). The
role and effect of the various cellular kinases is of pivotal importance in the evaluation of the
metabolic pathway leading to triphosphate analogs with regard to the antiviral activity and also
side effects.

Several NIs with 2’- or 4’-modified sugar moieties have shown efficacy in clinical trials, with
R7128 – the prodrug of PSI-6130 – being the most advanced in Phase IIb clinical development.
In contrast to the rapid selection of resistant viruses in monotherapy proof of concept trials with
PIs or NNIs, stable resistant variants have not been selected during monotherapy studies with
investigational NIs (2, 20).
Here, we report the *in vitro* antiviral activity, selectivity and mode of action of a novel NI, 2′-deoxy-2′-spirocyclopropylcytidine (TMC647078). Special attention is paid to its genotypic coverage, the effects of known resistance mutations on its activity, and its potency - alone or in combination with the potent HCV PI TMC435 (23) - in suppressing the formation of resistant replicon colonies and clearance of HCV from replicon cells. In addition, biochemical studies were conducted investigating the phosphorylation of TMC647078 into its active triphosphate, its incorporation efficiency into a nascent RNA chain, and its mechanism of action.
Materials and Methods

Compound synthesis

TMC647078 and TMC435 were synthesized as described elsewhere (13, 35).

Cells used in replicon experiments

HCV genotype 1b replicon-containing Huh7-Luc cells, Huh7-CMV-Luc cells used in the
counterscreening assays, and Huh7-lunet cells used in the transient replicon assay were kindly
provided by R. Bartenschlager, University Heidelberg, Germany (24, 26). HCV genotype 1b
replicon-containing Huh7-Con1b cells, HCV genotype 1a replicon-containing Huh7-SG1a cells,
and Huh7.5 cells were obtained from Apath LLC (St Louis, US) (4, 5). The MT4-LTR-Luc cell
line was described previously (34).

Replicon-containing cells were maintained in Dulbecco’s modified Eagles’ medium (DMEM)
(Sigma D-5546 medium, supplemented with 10% fetal calf serum [FCS], 1% L-glutamine,
0.04% gentamycin [50 mg/ml]) containing 500-750 μg/ml G418. Parent (containing no replicon)
cells were maintained in DMEM.

HCV replication assays

The luciferase reporter replicon assay and the replicon assays with a quantitative real-time PCR
read-out were performed as described previously (23). Briefly, Huh7-Luc replicon-containing
cells were incubated with serially diluted test compound, after which the luciferase activity was
determined or HCV RNA levels were measured and normalized to a cellular reference mRNA.
Quantification on of phenotypic changes due to mutations

The transient replicon assay, replicon mutants and chimeras were described in detail elsewhere (22, 32). In brief, replicon plasmid DNA was prepared and in vitro transcribed to yield replicon RNA. Purified replicon RNA was electroporated into permissive Huh7-lunet cells. After 48 hours of incubation with test compound, luciferase activity was measured and fold changes (FC) in EC$_{50}$ compared with the EC$_{50}$ of the wild type ET replicon were calculated.

Selectivity of TMC647078 to HCV

Antiviral activities against HIV (IIB, MT-4 cells), hepatitis B virus (HBV; HepG2.2.15 cells), yellow fever virus (YFV-17D; VeroE6 cells), influenza virus (influenza A Virginia/88; MDCK cells), herpes simplex virus (HSV) type 2 (G strain; E6 Vero cells), human cytomegalovirus (HCMV AD169 GFP, Hel299 cells), adenovirus (A549 EGFP cells) and vaccinia virus (WR strain, Vero cells) were determined as described previously (11, 12, 17).

Human and HIV-1 RT polymerase assay

DNA synthesis by HIV-1 reverse transcriptase (RT) (10 nM), human polymerase α (20 nM), β (20 nM), and γ (10 nM) was monitored in the presence of 50μM of the four dNTPs and increasing concentration of TMC647078-triphosphate (TMC647078-TP). The reaction was carried out using a template-primer complex with a 5'-end Cy3 labeled primer annealed to a 31-mer template. Inhibition of primer extension was measured using gel electrophoresis.

Phosphorylation kinetics of TMC647078

The kinetic parameters of phosphorylation of TMC647078 were determined using phosphoryltransfer assays as described previously (14) using 0.05 μM [$^32$P] ATP (10 μCi/μl) (PerkinElmer...
Sverige AB), 100 µM ATP, 20 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, 100 mM KCl, 0.5 mg/ml bovine serum albumin (BSA), 5 mM dithiothreitol (DTT) and different concentrations of the nucleoside analog. The reaction was initiated by adding the enzyme followed by incubation at 37°C, and terminated after 25 min by heating to 100°C. Following termination of the reaction, 4 µl of the supernatant was applied to PEI-cellulose FTLC plates (Merck KgaA). Chromatography was performed and analyzed as described (14). The $K_m$ and $V_{max}$ values were determined using the Michaelis-Menten equation and non-linear regression analysis with the KaleidaGraph™ program, version 3.52 (Synergy Software). Kinetic values were obtained from several experiments, which have been repeated at least twice with very similar results.

**Quantification of triphosphate metabolites of [³H]-TMC647078 in Huh7 cells and in primary human hepatocytes**

Primary human hepatocytes (12-well plate, 8 x $10^5$ cells/well) and Huh7 cells (12-well plate, 8 x $10^4$ cells/well) were incubated for 3 days with [³H]-TMC647078 at concentrations of 1, 4 and 16 µM. After incubation, cells were washed with phosphate buffered saline (PBS) and cells were sonicated in a solution of 70% methanol, 20 mM EDTA, 20 mM EGTA (0.4 ml/well). Radioactivity was determined by liquid scintillation counting. Based on the radioactivity levels, samples were evaporated before injection in liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) for triphosphate quantification.

**Multiple site nucleotide incorporation assay**

Incorporation of the nucleotide analog at multiple positions was investigated using the RNA template 5’-AACAGUGUGCUUUUCUCC-3’ (sites for the incorporation of the inhibitor are underlined). The RNA was purified on a 12% polyacrylamide-7 M urea gel containing 50 mM...
Tris-borate pH 8 and 1 mM EDTA, and then eluted overnight from gel slices in a buffer containing 500 mM NH₄Ac and 0.1% SDS. A 5′-GG-3′ dinucleotide (Trilink) was used as a primer. 5′-end labeling of the primer with [γ-³²P] ATP was carried out using T4 polynucleotide kinase (Invitrogen). Nucleotides were added to a concentration of 10 µM, with the exception of cytidine triphosphate (CTP), which was added at 100 nM. NS5B was at a concentration of 1 µM. Reactions were all carried out in a buffer containing 40 mM Hepes, pH 8, 1 mM DTT, 15 mM NaCl, 0.5 mM EDTA and started with 6 mM MgCl₂. After 40-minutes incubation at room temperature, the reaction was stopped by the addition of formamide. Samples were resolved on a 20% polyacrylamide-7M urea gel.

**Single nucleotide incorporation assay**

The dinucleotide primer was extended to the position preceding the unique site for incorporation (underlined) on the template 5′-AACAGUUUCUUCUCUCC-3′ with the addition of 1.5 µM of GTP and ATP, and using 1 µM NS5B. Rapid addition of either the nucleotide or nucleotide analog in the presence of a heparin trap followed and reactions were quenched with the addition of 0.5M EDTA. Products were resolved on a 20% polyacrylamide gel. Time-dependent changes in product formation at various concentrations of the nucleotide or nucleotide analog were fitted to a single exponential to obtain observed rate constants (k_{obs}). The observed single-turnover rates were plotted against nucleotide concentration and data fit to the hyperbolic equation $Y = \frac{k_{pol}*X}{K_D+X}$ to obtain maximum rate of incorporation ($k_{pol}$) and the dissociation constant ($K_D$).

**Chain-termination assay.**
The dinucleotide primer was extended in the presence of 5 µM ATP and GTP to position +15 for 40 minutes at room temperature using 1 µM NS5B. This was followed by the addition of 10 µM of the inhibitor for 10 minutes. Finally, addition of the next nucleotide followed at concentrations up to 250 µM for 10 minutes. Reactions were stopped with formamide and the products were resolved on a polyacrylamide gel.

Excision and rescue of RNA synthesis

Extension of the dinucleotide primer to the position preceding the unique site for incorporation (+15) occurred with the addition of 5 µM GTP and ATP in the presence of 1 µM NS5B for 40 minutes at room temperature. TMC647078 triphosphate at a concentration leading to 100% incorporation at position +16 (100 µM) was added. After 10 minutes of incubation increasing concentrations of pyrophosphate were added in the presence or absence of 100 µM CTP and 10 µM uridine triphosphate (UTP). The addition of pyrophosphate can lead to excision of the chain terminator. High concentrations of the natural nucleotide (CTP) are required to compete for incorporation at position +16 with the chain terminator, while the addition of UTP allows for further extension of the primer to position +20.
Results

In vitro activity, cytotoxicity and selectivity of TMC647078.

TMC647078 inhibited the replication of a genotype 1b ET transient replicon with an EC$_{50}$ of 3.2 µM (Table 1). To address the activity of TMC647078 on genotype 1a, a genotype 1a H77 transient replicon was used and comparable activity was observed with an EC$_{50}$ of 5.1 µM (Table 1). Next, the activity of TMC647078 was tested in two stable replicon-containing cell lines. The compound inhibited the replication of a genotype 1b ET replicon in the Huh7-Luc cell line with an EC$_{50}$ of 7.2 µM (Table 1), and suppressed replicon RNA levels in the genotype 1b SG-Con1b cell line with an EC$_{50}$ of 5.4 µM (Table 1). In the genotype 1a Huh7-SG1a stable replicon cell line, the EC$_{50}$ for reduction of replicon RNA was > 31.5 µM (Table 1). A possible explanation for the variation in EC$_{50}$ of TMC647078 in these assay systems could be differences in the phosphorylation of the compound in these cell lines influencing the formation of the TMC647078 active triphosphate. Although the phosphorylation level of TMC647078 in Huh7-SG1a cells was not determined, the reduced activity of other HCV NS5B nucleoside inhibitors that were tested in parallel supports the assumption (data not shown). The addition of up to 40% human serum to the Huh7-Luc stable replicon cell line did not lead to an increase in the EC$_{50}$ of TMC647078 (40% HS: EC$_{50}$ = 3.6 µM, interquartile range: 2.2 µM – 5.8 µM).

TMC647078 demonstrated specific inhibition of HCV replication as no inhibitory effect was found for other RNA and DNA viruses such as HIV, HSV, human CMV, influenza virus, adenovirus, vaccinia virus, YFV (EC$_{50}$ all > 100 µM), or HBV replication (EC$_{50}$ > 50 µM). In biochemical experiments, no inhibition of human polymerases α, β and γ, the HIV reverse transcriptase or the Klenow fragment of E. coli DNA polymerase I was observed with
TMC647078-TP up to a concentration of 1000 µM. The CC$_{50}$ of TMC647078 was > 400 µM in the human HepG2, MRC-5, and HEK-293T cell lines and in VeroE6 cells and > 98 µM in the human Huh7, MT4 and Hel299 cell lines (Table 1).

**Broad genotypic coverage of TMC647078**

The genotypic coverage of TMC647078 was investigated using chimeric replicons based on the genotype 1b ET replicon in which the C-terminal part of NS5A (amino acids 440-447) and the full length NS5B (amino acids 1-591) was replaced by the corresponding sequence of a clinical isolate of genotype 1a (n=7), 1b (n=10), 2b (n=2), 3a (n=2), 4a (n=1) or 6a (n=2). For genotype 2a, the NS5B sequence of JFH-1 was used. The genotype 1a and 1b chimeric replicons were generated from either one single clone per sample or a pool of clones per sample. For the non-genotype 1 chimeric replicons, only one single clone per sample was used. Profiling the activity of TMC647078 on chimeric replicons generated from a total of ten genotype 1b and seven genotype 1a samples confirmed that the compound has similar activity on genotype 1b (median EC$_{50}$ = 4.0 µM) and genotype 1a (median EC$_{50}$ = 3.1 µM;) clinical isolates. In addition, for the non-genotype 1 chimeric replicons, TMC647078 activity was in the same range as for the reference genotype 1b ET replicon.

**Effect on mitochondrial DNA and mRNA**

Mitochondrial DNA and mRNA levels, normalized to nuclear DNA and mRNA levels, were quantified in HepG2 cells treated for 14 days with TMC647078 and compared with corresponding levels in non-treated control cells. Zalcitabine (ddC) and didanosine (ddI) were included as positive controls. When treated with ddC, the normalized mitochondrial DNA and mRNA levels were significantly reduced in HepG2 cells, and a CC$_{50}$ of 0.4 µM for mtDNA and
1.9 µM for mtRNA was determined; ddI also caused a significant reduction in mitochondrial DNA levels (CC<sub>50</sub> of 55.3 µM) but did not seem to affect the mitochondrial mRNA levels at the highest test concentration tested (202 µM). Treatment with TMC647078 did not result in significant changes in normalized mitochondrial DNA and mRNA levels with a CC<sub>50</sub> > 202 µM.

**Effect of polymerase mutations in a genotype 1b replicon on TMC647078 susceptibility**

The presence of a 2’-C-cyclopropyl modification in TMC647078 suggested a reduced affinity against S282T-variant HCV strains based on resistance selection data generated with a 2’ substituted nucleoside (NM-107) (19). Indeed, a 39-fold reduction in EC<sub>50</sub> compared with the reference ET replicon was found for the S282T mutant replicon in a transient replicon assay. Despite the sensitivity of TMC647078 to the S282T change, resistance selection experiments (n=16) failed to generate this mutation. TMC647078 maintained full activity against the mutants associated with reduced sensitivity to the 4’-azido nucleoside R-1479 (S96T, N142T, S96T+N142T) (Table 2).

The activity of TMC647078 was further assessed on mutations that confer resistance to HCV NNI polymerase and protease inhibitors. The major mutants associated with reduced sensitivity to NNI-1, NNI-2, NNI-3, NNI-4, 4’-azido nucleoside polymerase and protease inhibitors (16), were all shown to be as sensitive to TMC647078 as the reference ET replicon (Table 2).

**Resistant colony formation with TMC647078.**

The frequency of resistant colony formation was determined in a colony formation assay using Huh7-Luc replicon cells incubated in the presence of TMC647078 or the HCV PI TMC435, or a combination of both compounds.
The untreated Huh7-Luc control culture elicited many colonies whereas a dose-dependent reduction of colony formation was observed in the TMC647078- and TMC435-treated dishes. TMC435 alone at 80 nM (10× EC_{50}) or 200 nM (25× EC_{50}) did not completely inhibit colony formation resulting in 45 and 20 colonies respectively. TMC647078 at a concentration of 25 µM (~5× EC_{50}) reduced colony formation to eight colonies and 50 µM (~10× EC_{50}) prevented formation of resistant colonies completely. Interestingly, TMC647078 at 25 µM in combination with 80 nM TMC435 resulted in complete suppression of colony formation, pointing towards an additive effect on suppression of HCV RNA replication. Colony formation was completely prevented when TMC647078 was combined with 200 nM TMC435 as well. No toxicity on Huh7-Luc replicon cells was observed for the combination of 50 µM TMC647078 and 200 nM TMC435 (data not shown).

**In vitro clearance of HCV genotype 1b replicon RNA**

The *in vitro* clearance of HCV-genotype-1b replicon RNA, monitored by quantitative real-time PCR, was determined in a clearance rebound assay using Huh7-Luc replicon cells incubated in the presence of different concentrations of TMC647078 alone or in combination with the HCV PI TMC435 (Figure 1). In the untreated control cells, the HCV replicon RNA content was relatively constant over the duration of the experiment with a maximum fluctuation of ~0.5 log_{10}. In the cells treated with 25 µM and 50 µM TMC647078 (~5× and 10× EC_{50}, respectively) a rapid dose-dependent reduction of HCV RNA was detected within the first 3 days during the clearance phase with continued reduction until day 11 to a maximum decline of 1.8 log_{10} for the 25-µM-treated cells and 2.6 log_{10} for the 50-µM-treated cells. During the remainder of the clearance phase the low level of HCV RNA remained relatively constant and no rebound was observed in presence of the compound over 2 weeks. However, TMC647078 alone was not sufficient to clear
the replicon completely from the cells as observed by a rebound of the HCV RNA when cells were cultured in the absence of compound; rebound of the HCV RNA was concentration dependent and a return to baseline levels was observed at day 28 in the case of the 25-µM-treated cells, and at day 35 in the case of the 50-µM-treated cells. Interestingly, the combination of 25 µM TMC647078 with 100 nM TMC435 resulted in a steeper decline of HCV RNA within the first 3 days of treatment followed by a continued decline to below the level of quantification of the assay at day 7 leading to a maximum reduction of 4.0 log_{10}. The HCV RNA content remained below the level of quantification during the rest of the clearance phase, suggesting that occurrence of resistance was efficiently suppressed. More importantly, combination of the two compounds prevented rebound of HCV RNA during the rebound phase, indicating “cure” of the replicon from the cells.

**Kinetic determination of TMC647078 as a substrate for human dCK**

To form the active triphosphate nucleotide from the parent nucleoside, the first step of phosphorylation (i.e., monophosphate formation) by a cellular kinase is critical. To identify the kinases involved in the phosphorylation of TMC647078 and measure the respective phosphorylation capacity of TMC647078, six nucleoside kinases were tested: deoxycytidine kinase (dCK), cytosolic thymidine kinase 1 (TK1), mitochondrial thymidine kinase 2 (TK2), mitochondrial deoxyguanosine kinase (dGK), uridine-cytidine kinase 1 (UCK1) and uridine-cytidine kinase 2 (UCK2) (9, 14, 39-41).

The relative phosphorylation rates comparing 100 µM of TMC647078 with 100 µM of the natural substrates (deoxycytidine (dCyd) for dCK, deoxythymine (dTThd) for TK1 and TK2, uridine (Urd) for UCK1 and UCK2, deoxyguanosine (dGuo) for dGK) were determined. These
experiments were repeated three times with similar results. The standard error of measurement was +/- 10%. dCK efficiently phosphorylated TMC647078 with relative activity more than three-fold greater than dCyd. TK2 phosphorylated TMC647078 with about 12% relative activity and dGK, TK1 and UCK showed no detectable phosphorylation of TMC647078.

The kinetic determination of monophosphate formation was performed using 1.5 to 100 μM concentrations of dCyd and 3 to 100 μM TMC647078 and the results are presented in Table 3. TMC647078 showed five-fold greater \( V_{\text{max}} \) (312.6) and 10 times higher \( K_m \) (41.9) values compared with dCyd (\( V_{\text{max}} \) 62.9 and \( K_m \) 4.7). The phosphorylation efficiency (\( k_{\text{cat}}/K_m \)) of TMC647078 as a dCK substrate was thus two-fold lower relative to that of dCyd.

As dCyd and TMC647078 are substrates of dCK, the inhibitory effect of dCyd on the phosphorylation of TMC647078 and vice versa could have important implications for the efficacy and safety of the compound in a cellular context. Therefore, the inhibitory effect of dCyd on the phosphorylation of TMC647078 by dCK was determined using two constant concentrations of dCyd (2 μM and 10 μM) with 0.75 to 100 μM concentrations of TMC647078. The \( K_i \) value for dCyd as inhibitor of TMC647078 phosphorylation was 2.8 μM.

At a concentration of 100 μM the percentage of TMC647078 monophosphorylated by dCK in the presence of 2 μM (ratio 1:50) and 10 μM (ratio 1:10) dCyd was 73% and 24% of that formed in the absence of dCyd, respectively. Therefore it can be concluded that, although the presence of dCyd resulted in a reduced efficiency of TMC647078 phosphorylation, a considerable amount of this analog was phosphorylated by dCK. On the other hand, 100 and 200 μM TMC647078 did not have an inhibitory effect on the monophosphorylation of the natural dCK substrate dCyd at
concentrations of 25, 12.5, 6, 3 and 1.5 µM. There was no significant decrease in intensity of the dCMP spots in these experiments (data not shown).

Quantification of triphosphate metabolites of [\(^3\)H]-TMC647078 in Huh7 cells and primary human hepatocytes

The levels of triphosphate of TMC647078 and the uridine-metabolite of TMC647078 were measured in primary human hepatocytes (PHH) and in Huh7 cells after 3 days of incubation with TMC647078 at concentrations of 1 µM, 4 µM and 16 µM. Overall, in both cell types a concentration-dependent increase in triphosphate formation of both the parent compound and the uridine-metabolite was observed. TMC647078-triphosphate (TMC647078-TP) formation was significantly higher in Huh7 cells than in PHH. In PHH, higher triphosphate levels of the uridine-metabolite than of the parent compound were found, where the reverse was observed in Huh7 (Table 4).

Efficiency of incorporation of TMC647078-monophosphate (MP) during RNA strand synthesis, termination of elongation and excision of incorporated TMC647078-MP in the presence of pyrophosphate (PPI)

Multiple- and single-site incorporation experiments were performed to determine the efficiency of incorporation of TMC647078-MP during RNA strand synthesis. In a multiple incorporation experiment with opportunities for TMC647078-TP to bind to positions +12, +14 and +16, increasing concentrations of the triphosphate were used to monitor formation of the final product +20 and premature termination. A concentration-dependent increase in specific, premature termination at position +12, +14 and +16 was observed with TMC647078-MP and an obligate chain terminator (Figure 2). We employed pre-steady state kinetics to determine kinetic...
parameters $K_D$ and $k_{pol}$. The $K_D$ value for TMC647078-TP ($K_D$ 1057 µM) was 8-fold higher than that for CTP ($K_D$ 135 µM). The maximum rate of incorporation ($k_{pol}$) was 0.055 sec$^{-1}$, which is 405-fold lower than for its natural counterpart CTP ($k_{pol}$ 25 sec$^{-1}$). This translated to a 3500-fold lower efficiency of incorporation ($k_{pol}/k_D$) of the inhibitor.

Next, the effect of incorporated TMC647078-MP on RNA chain elongation was determined. For this purpose, 10 µM TMC647078-TP was used to establish incorporation in the RNA chain after which elongation was measured for increasing concentrations of the next nucleotide that would allow chain extension (UTP). Once incorporated, TMC647078-MP caused chain-termination; increasing concentrations of UTP up to 250 µM did not extend the primer (Figure 2).

As it has previously been reported that cellular pyrophosphate (PPI) can remove incorporated nucleoside reverse-transcriptase inhibitors (NRTIs), thereby restoring elongation of the nascent HIV DNA chain, we also examined whether increasing concentrations of PPI could excise the fraction of TMC647078-MP that was incorporated in the RNA chain at a concentration of 100 µM. The percentage of rescue of TMC647078-MP from the RNA chain, leading to restored RNA synthesis, was insignificant, even at the highest PPI concentration of 500 µM (Figure 2).
Discussion

Nucleoside analogs form the cornerstone of antiviral therapy for several indications including HIV, HBV, herpes simplex virus and human CMV infections. Furthermore, several modified nucleoside analogs and non-nucleoside inhibitors with potent inhibitory activity against NS5B have been reported as potential therapeutics for HCV (for review see (21)). The majority of nucleoside analogs active against HCV can be divided into two classes: compounds harboring 2’-modifications (e.g. NM-107, PSI-6130) or 4’-modifications (e.g. R-1479) of the ribose ring. Prodrugs of nucleoside inhibitors NM-107 (NM-283), R-1479 (R-1626) and PSI-6130 (R7128) and of nucleotide inhibitors (IDX-184, PSI-7977, PSI-938) have been or are being evaluated in clinical trials and have proven efficacious in vivo (for review see (21)). With broad genotypic coverage and high barrier for development of resistance based on their mechanism of activity, potency in suppressing viral replication and lower replication fitness of drug resistant virus, nucleos(t)ide inhibitors may represent an important pillar of HCV antiviral therapy. While clinical development was halted for some compounds due to adverse effects (R-1626: neutropenia, NM-283: gastrointestinal side effects), R-7128, an early nucleoside inhibitor, and the nucleotide analogs IDX-184, PSI-7977 and PSI-938 remain in clinical development. Novel inhibitors with potent antiviral activity and fewer side effects clearly remain an urgent medical need for treatment of HCV.

We recently described the synthesis and anti-HCV activity of TMC647078, a 2’-deoxy-2’-spirocyclopynyl cytidine (13). Computational modeling studies suggested structural and electronic similarities between TMC647078 and related HCV-inhibiting nucleosides (NM-107 and PSI-6130). In the present study, TMC647078 demonstrated potent inhibition of HCV
replication in a genotype 1b subgenomic replicon-based assay, with an EC₅₀ of 7.8 µM. The potency of TMC647078 was not affected by the presence of human serum proteins, suggesting low protein binding properties. In addition, in biochemical assays the triphosphate of the compound did not inhibit the activity of human polymerases α, β or γ up to a concentration of 1000 µM. This is an important observation when addressing the different mechanisms of potential in vitro toxicity of a nucleoside polymerase inhibitor. The CC₅₀ was found to be higher than 400 µM in several human cell lines. We further analyzed the effect on mitochondrial DNA and mRNA levels. Some HIV NRTIs (zalcitabine, ddC; didanosine, ddI) have been shown to cause a concentration-dependent depletion of mitochondrial DNA levels which was associated with mitochondrial toxicity during therapy and was related to adverse events such as hepatic steatosis, severe hyperlactatemia, and polyneuropathy. For TMC647078, on the other hand, a CC₅₀ for mtDNA and mtRNA of > 202 µM was observed, suggesting that up to this concentration the compound does not have an effect on mitochondrial DNA and mRNA levels in a cellular assay. In addition, in assays of 3-day duration, no effects were observed on cell proliferation or on the cell cycle phase at concentrations up to 200 µM and 100 µM, respectively (data not shown).

An advantage of nucleosides in antiviral therapy is broad HCV genotypic coverage, as opposed to PIs and NNIs for which some HCV genotypes have greatly reduced susceptibility due to the variation in the sequence of the viral genome. Broad genotypic coverage was demonstrated for TMC647078 against chimeric replicons containing the polymerase sequences from genotype 1a, 1b, 2a, 2b, 3a, 4a and 6a clinical isolates.

TMC647078 remained active against HCV mutants associated with reduced susceptibility to PI, NNI 1-4 and 4’-substituted NI nucleoside inhibitors, which supports combination studies with
these compound classes. This is an important finding, as it is likely that HCV treatment regimens
will employ a combination of drug classes to achieve higher SVR rates and shorter treatment
duration to reduce side effects. The effect of TMC647078 on the suppression of drug-resistant
colonies and clearance of replicon RNA was addressed in colony formation and clearance
rebound assays in combination with the protease inhibitor TMC435. A robust suppression of
colony formation and reduction of HCV replicon RNA was observed for TMC647078.
Combination of the two compounds at low concentrations resulted in complete suppression of
colony formation without rebound, indicating that the replicon was efficiently cleared from the
cells and that combination of two direct antivirals with a different mechanism of action is
favored over a single compound treatment in terms of resistance. Moreover, no rebound of the
HCV RNA was observed during the treatment phase, suggesting an efficient suppression of
resistance by TMC647078 in vitro.

HCV replicon selection experiments with valopicitabine, 2’-C-methyl-7’-deaza-adenosine (MK-
0608) (33), and PSI-6130 resulted in the selection of the S282T mutation in NS5B. This amino
acid residue is part of the active site of the polymerase, located near the catalytic aspartate
residues (1, 18, 30). It was reported that this mutation confers resistance from a combination of
reduced affinity of the mutant polymerase for the drug and an increased ability to extend the
incorporated nucleoside analog (30). R-1479 shows no reduced in vitro activity against the
S282T mutant but selects S96T and S96T+N142T in vitro which confer a relatively small
decrease in susceptibility (18, 30).
Although several resistance selection experiments with TMC647078 did not reveal any of the described nucleoside inhibitor resistance mutations or other mutations in the viral polymerase, the compound showed a 39-fold change in susceptibility against the NI S282T mutant in a transient replicon assay. The lack of S282T detection during resistance selection experiments was surprising given the 2’ cyclopropyl modification of the sugar moiety of TMC647078 and the selection of the S282T resistance mutation in control resistance selection experiments with NM-107. One possible explanation why the mutation was detected might be the lack of sensitivity of the population sequencing approach for the detection of S282T variants that might have been present as a minority species. Another explanation might be the reduced replicative fitness of the S282T mutant as observed in our transient replicon assay (12% of the wild-type ET replicon) and by others (30) as it may impact the ability to select this mutant under the same G418 concentrations used for the parental replicon. The reduced susceptibility of the RdRp S282T mutant replicon confirmed the assumption that TMC647078 targets the active site of the RdRp, leading to inhibition of HCV RNA synthesis. Additional studies will be required to address if TMC647078 does indeed select for this mutation.

The conversion of TMC647078 to the active triphosphate is a three-step process regulated by cellular kinases, of which the first phosphorylation event is the rate-limiting step. As for most other cytidine analogs, monophosphate formation of TMC647078 was (mainly) catalyzed by dCK. No clear inhibitory effect of high concentrations of TMC647078 on monophosphate formation of the natural substrate, dCyd, was observed, which is an important observation as an inhibition could have led to detrimental effects on the cellular (d)NTP homeostasis. However, dCyd was shown to efficiently inhibit TMC647078 phosphorylation. Nevertheless, significant TMC647078 phosphorylation occurred even at a high dCyd concentration. Subsequent
triphosphate formation was analyzed in primary human hepatocytes and Huh7 hepatoma cells after a 3-day incubation period with TMC647078 at different concentrations. In both cell systems, conversion into its triphosphate derivative and into the triphosphate of its uridine analog formed by base deamination, was demonstrated, similar to the findings for PSI-6130 (27). In primary human hepatocytes, the formation of both TMC647078-TP and TMC647078-U-TP increased linearly with the concentration of parent compound incubated with the cells. In addition, at the three TMC647078 concentrations tested, the triphosphate levels of the uridine metabolite exceeded those of the parent compound by 3- to 4-fold. Similar observations were reported for PSI-6130, in which higher levels of the triphosphate of its uridine metabolite were also found in primary human hepatocytes (27). In Huh7 cells, TMC647078-TP levels were 4-to 5-fold higher than in PHH. Triphosphate levels of the uridine metabolite were similar in both cell systems, except for the condition in which cells were incubated with 16 µM of parent compound, where a saturation plateau for uridine metabolite triphosphate formation was reached in Huh7. The formation of the triphosphate of the uridine analog would be beneficial when this metabolite also showed potent activity against HCV. Although no anti-HCV activity was observed when the uridine analog was tested in a replicon-containing cell line, several nucleotide prodrugs of the uridine analog showed EC$_{50}$ values similar to that of TMC647078 (data not shown). This suggests that the phosphorylation of the TMC647078 uridine analog occurred less efficiently in the replicon-containing cell line.

Incorporation of TMC647078-TP during RNA synthesis was shown to be approximately 3500 times less efficient than the natural substrate CTP. Once incorporated into the nascent RNA chain, TMC647078 acts as a non-obligate chain terminator, similar to the mechanism of action of PSI-6130 (8, 31). Interestingly, addition of PPI up to a concentration of 500 µM (which is
estimated to be multiple times higher than the cellular PPi concentration) resulted only in very low levels of rescue of RNA synthesis due to excision of TMC647078-TP, whereas the termination complexes of R-1479 and NM-107 were shown to be less stable.

In conclusion, TMC647078 is a potent and specific novel nucleoside inhibitor of the HCV NS5B RdRp with broad genotypic coverage and a high barrier to resistance. In addition, TMC647078 shows good in vitro phosphorylation properties and triphosphate formation, and demonstrated efficient in vitro inhibition of HCV through chain-termination.

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References


# TABLE 1. *In vitro* activity and cytotoxicity of TMC647078.

<table>
<thead>
<tr>
<th>TMC647078</th>
<th>Median EC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>Median CC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transient replicon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1b ET</td>
<td>3.2 (2.2 – 5.0)</td>
<td></td>
</tr>
<tr>
<td>1a H77</td>
<td>5.1 (3.1 – 6.5)</td>
<td></td>
</tr>
<tr>
<td>Stable replicon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1b ET (Huh7-Luc)</td>
<td>7.2 (5.7 – 9.3)</td>
<td></td>
</tr>
<tr>
<td>1b SG-Con1b</td>
<td>5.4 (3.9 – 9.8)</td>
<td></td>
</tr>
<tr>
<td>1a Huh7-SG1a</td>
<td>&gt;31.5</td>
<td></td>
</tr>
<tr>
<td>Cytotoxicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HepG2, MRC-5, HEK-293T, VeroE6</td>
<td>&gt;400</td>
<td></td>
</tr>
<tr>
<td>Huh7, MT4, Hel299</td>
<td>&gt;98</td>
<td></td>
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</tbody>
</table>
TABLE 2. Effect of polymerase and protease mutations in the reference genotype 1b replicon ET on TMC647078 susceptibility.

<table>
<thead>
<tr>
<th>Inhibitor Class&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mutation</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
<th>FC&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference ET replicon</td>
<td>/</td>
<td>3.2 (2.2 – 5.0)</td>
<td>-</td>
</tr>
<tr>
<td>2'-substituted NI</td>
<td>S282T</td>
<td>48.2 (41.8 – 63.0)</td>
<td>38.7 (29.3 – 48.5)</td>
</tr>
<tr>
<td>4'-substituted NI</td>
<td>S96T</td>
<td>2.7 (1.8 – 5.4)</td>
<td>0.8 (0.6 – 1.1)</td>
</tr>
<tr>
<td>4'-substituted NI</td>
<td>N142T</td>
<td>2.9 (2.1 – 3.5)</td>
<td>0.8 (0.6 – 1.1)</td>
</tr>
<tr>
<td>4'-substituted NI</td>
<td>N142T+S96T</td>
<td>4.6 (2.9 – 10.0)</td>
<td>1.0 (0.8 – 1.5)</td>
</tr>
<tr>
<td>NNI-1</td>
<td>P495L</td>
<td>4.7 (3.0 – 10.9)</td>
<td>2.4 (1.4 – 5.5)</td>
</tr>
<tr>
<td>NNI-2</td>
<td>M423T</td>
<td>2.7 (0.9 – 3.0)</td>
<td>0.8 (0.7 – 1.5)</td>
</tr>
<tr>
<td>NNI-3</td>
<td>M414Q</td>
<td>2.2</td>
<td>1.1</td>
</tr>
<tr>
<td>NNI-4</td>
<td>C316Y</td>
<td>2.5 (1.9 – 4.4)</td>
<td>1.6 (0.8 – 2.2)</td>
</tr>
<tr>
<td>macrocyclic PI</td>
<td>Q80R</td>
<td>3.7</td>
<td>2.1</td>
</tr>
<tr>
<td>macrocyclic PI</td>
<td>Q80K</td>
<td>2.6</td>
<td>1.4</td>
</tr>
<tr>
<td>macrocyclic PI</td>
<td>D168A</td>
<td>2.8</td>
<td>1.5</td>
</tr>
<tr>
<td>macrocyclic PI</td>
<td>D168E</td>
<td>3.6</td>
<td>2.0</td>
</tr>
<tr>
<td>macrocyclic PI</td>
<td>D168V</td>
<td>2.5</td>
<td>1.4</td>
</tr>
<tr>
<td>linear ketoamide &amp; macrocyclic PI</td>
<td>R155K</td>
<td>3.2</td>
<td>1.8</td>
</tr>
<tr>
<td>linear ketoamide &amp; macrocyclic PI</td>
<td>A156T</td>
<td>2.8</td>
<td>1.6</td>
</tr>
<tr>
<td>linear ketoamide &amp; macrocyclic PI</td>
<td>A156V</td>
<td>2.4</td>
<td>1.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> For each inhibitor class, the major mutations associated with reduced susceptibility to this class of inhibitors were engineered into the genotype 1b ET replicon. NI, nucleoside inhibitor; NNI, non-nucleoside inhibitors; PI, protease inhibitor.

<sup>b</sup> EC<sub>50</sub> values are represented as the median values of 2-45 experiments; Interquartile ranges (Q1 – Q3) are shown in between brackets for mutant replicons tested in >2 experiments.

<sup>c</sup> Fold change (FC): change in EC<sub>50</sub> values as compared to reference ET replicon; a FC was calculated for every single experiment and the median of these values from 2-45 experiments is shown; Interquartile ranges (Q1 – Q3) are shown in between brackets for mutant replicons tested in >2 experiments.
TABLE 3. The kinetic parameters for phosphorylation of TMMC647078 and dCyd with dCK.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km (µM)</th>
<th>Vmax (nmol min(^{-1}) mg(^{-1}))</th>
<th>Vmax/Km</th>
<th>Kcat(^b) (s(^{-1}))</th>
<th>Kcat/Km (µM(^{-1}) s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>dCyd</td>
<td>4.7±0.5</td>
<td>62.9±1.6</td>
<td>13.3</td>
<td>0.0315</td>
<td>6.64 x 10(^{-3})</td>
</tr>
<tr>
<td>TMC647078</td>
<td>41.9±6.4</td>
<td>312.6±17.5</td>
<td>7.4</td>
<td>0.1565</td>
<td>3.73 x 10(^{-3})</td>
</tr>
</tbody>
</table>

\(^{a}\) The values are means ±SD of the three determinations.

\(^{b}\) The Kcat values were calculated based on a dCK subunit molecular weight of 30 kDa.
TABLE 4. Formation of triphosphate in primary human hepatocytes (PHH) and Huh7 cells after incubation with 1, 4 and 16 µM TMC647078.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Triphosphate (TP)</th>
<th>TMC647078</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 µM</td>
<td>4 µM</td>
</tr>
<tr>
<td>PHH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMC647078-TP (ng/ml)</td>
<td>6.4</td>
<td>17.3</td>
</tr>
<tr>
<td>TMC648078-U-TP (ng/ml)</td>
<td>26.3</td>
<td>62.9</td>
</tr>
<tr>
<td>Huh7 cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMC647078-TP (ng/ml)</td>
<td>30.5</td>
<td>107</td>
</tr>
<tr>
<td>TMC648078-U-TP (ng/ml)</td>
<td>18.3</td>
<td>47.3</td>
</tr>
</tbody>
</table>
**Figure Legends**

**Figure 1.** Rebound of HCV replicon RNA after a 14-day treatment with the nucleoside inhibitor TMC647078 alone or in combination with the HCV protease inhibitor TMC435 in the absence of G418. After 14 days of treatment, the compounds were withdrawn and 250 µg of G418 per ml was added to enrich the remaining HCV replicon-positive cells that are capable of growing in the presence of G418 (rebound). The cultures were monitored for another 21 days in the presence of G418, and cell samples were collected whenever the cells were split. The level of HCV RNA in the cells was determined by a quantitative real-time PCR assay.

**Figure 2.** a) Incorporation efficiency of TMC647078-TP during RNA synthesis determined in a multiple incorporation experiment. The template contained 3 positions (+12, +14, +16) for incorporation of CTP or analogues. Efficient incorporation combined with inhibition of chain elongation resulted in a decrease of generation of full length RNA chain (+20). 3’dCTP was used as a control inhibitor. b) Chain termination efficiency of TMC647078-TP. Incorporation of each chain terminator into the newly synthesized RNA was followed by the addition of UTP, the next required nucleotide. Extension of the primer past the position of chain termination was then monitored. Two compounds were tested for comparative purposes; R-1479-TP which does not act as a chain terminator during elongation and NM-107-TP which has been confirmed to act as a chain terminator.