Antiviral Activity and Mode of Action of TMC647078, a Novel Nucleoside Inhibitor of the Hepatitis C Virus NS5B Polymerase†‡

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Chronic infection with hepatitis C virus (HCV) is a major global health burden and is associated with an increased risk of liver cirrhosis and hepatocellular carcinoma. Current therapy for HCV infection has limited efficacy, particularly against 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 nucleosides or nucleotides 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 the suppression of HCV RNA replication, highlighting the potential for the combination of these two classes in the treatment of chronic HCV infection. No cytotoxic effects were observed in various cell lines. Biochemical studies indicated that TMC647078 is phosphorylated mainly by deoxycytidine kinase (dCK) without inhibiting the 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.

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, 11). Chronic HCV infection can lead to liver cirrhosis and hepatocellular carcinoma and is the leading cause of liver transplantation (10). The virus is transmitted mainly via blood-blood contact, and spontaneous virus clearance has been estimated to be achieved for 26% of infected subjects (29).

HCV is a member of the Flaviviridae family of viruses in the genus Hepacivirus, comprising 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, the envelope glycoproteins E1 and E2, and p7) and six nonstructural proteins responsible for viral replication (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The nonstructural protein NS5B is an RNA-dependent RNA polymerase (RdRp) responsible for the amplification of the HCV RNA and the assembly of the replicase complex at the endoplasmic reticulum membrane (3, 25).

Currently, HCV patients are treated with a combination of weekly pegylated alpha interferon (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 nucleoside inhibitors (NNIs) and 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 NI activation is intracellular phosphorylation, where the
initial step is catalyzed by the (deoxy)nucleoside kinases pres-
ent in the cytosol (thymidine kinase 1 [TK1]) and deoxyocty-
idine [dCdY] kinase [dCK]). In the mitochondria (thymi-
dine kinase 2 [TK2] and deoxyguanosine [dGuo] kinase
[dGK]) (9). In addition, pyrimidine ribonucleoside analogs can be phosphorylated constitutively by cytosolic uridine (Urd)-
cytidine kinase 1 and 2 enzymes (38). The role and effect of the various cellular kinases are of pivotal importance in the eval-
uation of the metabolic pathway leading to triphosphate ana-
logs with regard to 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 of clinical
development. In contrast to the rapid selection of resistant viruses in monotherapy proof-of-concept trials with protease
inhibitors (PIs) or NNIs, stable resistant variants have not been
selected during monotherapy studies with investigational NIs
(2, 22).

Here, we report the in vitro antiviral activity, selectivity, and
mode of action of a novel NI, 2'-deoxy-2'-spirocycloprenopyridine
(TM647078). Special attention is paid to its genotypic
coverage, the effects of known resistance mutations on its ac-
tivity, and its potency, alone or in combination with the potent
HCV PI TMC435 (23), in suppressing the formation of resis-
tant replicon colonies and the clearance of HCV from replicon
cells. In addition, biochemical studies were conducted to in-
vestigate the phosphorylation of TM647078 into its active
triphasophate, its incorporation efficiency into a nascent RNA
chain, and its mechanism of action.

MATERIALS AND METHODS

Compound synthesis. TM647078 and TMC435 were synthesized as de-
scribed elsewhere (14, 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, 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, MO) (4, 5). The MT4-LTR-Luc cell line was described previously
(23). Briefly, Huh7-Luc replicon-containing cells were incu-
bated with Dulbecco’s modified Eagle’s
medium (DMEM) (Sigma D5546 medium supplemented with 10% fetal cell
serum [FCS], 1% L-glutamine, and 0.04% gentamicin [50 mg/ml]) containing 500
mg/ml to 750 µg/ml G418. Parent cells (containing no replicon) were maintained
in DMEM.

HCV replication assays. The luciferase reporter replicon assay and the repli-
con assays with a quantitative real-time PCR readout were performed as de-
scribed previously (23). Briefly, Huh7-Luc replicon-containing cells were in-
ubated with a 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 of phenotypic changes due to mutations. The transient replicon
assay, replicon mutants, and chimeras were described in detail elsewhere (20, 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 h of incubation with the test compound, the luciferase
activity was measured, and fold changes (FCs) in the 50% effective concentra-
tions (EC50) compared with the EC50 of the wild-type ET replicon were cal-
culated.

Selectivity of TM647078 for HCV. Antiviral activities against HIV (HIB-
MT-4 cells), hepatitis B virus (HBV) (HepG2.2.15 cells), yellow fever virus
(YFV) (YFV-17D; E6 Vero cells), influenza virus (influenza A Virginia88;
MDCK cells), herpes simplex virus (HSV) type 2 (G strain; E6 Vero cells),
human cytomegalovirus (HCMV) (AD169 strain expressing green fluorescent
protein; HeLa299 cells), adenosine (A549 cells expressing enhanced green fluo-
rescent protein), and vaccinia virus (WR strain; Vero cells) were determined as
described previously (12, 13, 18).

Human and HIV-1 RT polymerase assay. DNA synthesis by HIV-1 reverse
transcriptase (RT) (10 nM), human polymerase α (20 nM), human polymerase β
(20 nM), and human polymerase γ (10 nM) was monitored in the presence of 50
µM the four deoxynucleoside triphosphates (dNTPs) and increasing concentra-
tions of TM647078-triphosphate (TMC647078-TP). The reaction was carried out
by use of a template-primer complex with a 5'-end Cy3-labeled primer
annealed to a 31-mer template. The inhibition of primer extension was measured by
gel electrophoresis.

Phosphorylation kinetics of TMC647078. The kinetic parameters of the phos-
phorylation of TM647078 were determined by using phosphoryl transfer assays
as described previously (15), using a mixture containing 0.05 µM [γ-32P]ATP (10
µCi/µl) (Perkin-Elmer Sweden AB), 100 µM ATP, 20 mM Tris-HCl (pH 7.6), 5
mM MgCl2, 100 mM KCl, 0.5 mM MgCl2, 100 mM KCl, 0.5 mM ml bovine serum albumin (BSA), 5 mM dithiothreitol (DTT), and different concentrations of the nucleoside analog. The reaction was initiated by the addition of the enzyme to the mixture followed
by incubation at 37°C and was terminated at 25 min by heating to 100°C.
Following the termination of the reaction, 4 µl of the supernatant was applied onto
polyethyleneimine (PEI)-cellulose F thin-layer chromatography plates (Merck
Kga), Chromatography was performed and analyzed as described previously
(15). The Kd and fmax, values were determined by using the Michaelis-Menten
equation and nonlinear regression analysis with the KaleidaGraph program,
version 3.52 (Synergy Software). Kinetic values were obtained from several
experiments, which were repeated at least twice, with very similar results.

Quantification of triphosphate metabolites of [1H]TM647078 in Huh7 cells
and in primary human hepatocytes. Primary human hepatocytes (PHH) (12-16
primary plates; x 106 cells/well) and Huh7 cells (12-well plates; x 105 cells/well)
were incubated for 3 days with [1H]TM647078 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 EGTA, 20 mM
EGTA (0.4 ml/well). Radioactivity was determined by liquid scintillation count-
ing. Based on the radioactivity levels, samples were evaporated before injection
for liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS)
for triphosphate quantification.

Multiple-site nucleotide incorporation assay. The incorporation of the nucleo-
tide analog at multiple positions was investigated by using the RNA template
5'-AACAGUGUCUUUUCUCCC-3' (sites for the incorporation of the inhi-
TABLE 1. In vitro activity and cytotoxicity of TMC647078

<table>
<thead>
<tr>
<th>Replicon and cell line(s)</th>
<th>Median EC&lt;sub&gt;50&lt;/sub&gt; (IQR) (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 (Huh7-Luc)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1b SG-Con1b</td>
<td>7.2 (5.7–9.3)</td>
<td></td>
</tr>
<tr>
<td>1a Huh7-SG1a</td>
<td>5.4 (3.9–9.8)</td>
<td></td>
</tr>
<tr>
<td></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,</td>
<td>&gt;400</td>
<td></td>
</tr>
<tr>
<td>VeroE6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Huh7, MT4, He1299</td>
<td>&gt;98</td>
<td></td>
</tr>
</tbody>
</table>

* IQR, interquartile range.

Min of incubation, increasing concentrations of pyrophosphate (PP<sub>i</sub>) were added in the presence or absence of 100 µM CTP and 10 µM UTP. The addition of pyrophosphate can lead to the excision of the chain terminator. High concentrations of the natural nucleotide (CTP) are required to compete for incorporation.

RESULTS

In vitro activity, cytotoxicity, and selectivity of TMC647078. TMC647078 inhibited the replication of a genotype 1b ET transient replicon with an EC<sub>50</sub> of 3.2 µM (Table 1). To address the activity of TMC647078 against genotype 1a, a genotype 1a H77 transient replicon was used, and a comparable activity was observed, with an EC<sub>50</sub> of 5.1 µM (Table 1). Next, the activity of TMC647078 was tested with 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<sub>50</sub> of 7.2 µM (Table 1) and suppressed replicon RNA levels in the genotype 1b SG-Con1b cell line with an EC<sub>50</sub> of 5.4 µM (Table 1). In the genotype 1a Huh7-SG1a stable replicon cell line, the EC<sub>50</sub> for the reduction of replicon RNA was >31.5 µM (Table 1). A possible explanation for the variation in the EC<sub>50</sub>s of TMC647078 in these assay systems could be differences in the phosphorylations 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 (HS) to the Huh7-Luc stable replicon cell line did not lead to an increase in the EC<sub>50</sub> of TMC647078 (EC<sub>50</sub> of 3.6 µM with 40% HS; interquartile range, 2.2 µM to 5.8 µM).

TMC647078 demonstrated a specific inhibition of HCV replication, as no inhibitory effect on replication was found for other RNA and DNA viruses such as HIV, HSV, HCMV, influenza virus, adenovirus, vaccinia virus, YFV (EC<sub>50</sub>s were all >100 µM), or HBV (EC<sub>50</sub> of >50 µM). In biochemical experiments, no inhibition of human polymerases α, β, and γ; HIV reverse transcriptase; or the Klenow fragment of Escherichia coli DNA polymerase I was observed with TMC647078-TP up to a concentration of 1,000 µM. The 50% cytotoxic concentrations (CC<sub>50</sub>) of TMC647078 were >400 µM in the human HepG2, MRC-5, and HEK-293T cell lines and in Vero E6 cells and >98 µM in the human Huh7, MT4, and He1299 cell lines (Table 1).

Broad genotypic coverage of TMC647078. The genotypic coverage of TMC647078 was investigated by using chimeric replicons based on the genotype 1b ET replicon in which the C-terminal part of NS5A (amino acids 440 to 447) and full-length NS5B (amino acids 1 to 591) were replaced by the corresponding sequence of clinical isolates of genotype 1a (n = 7), 1b (n = 10), 2b (n = 2), 3a (n = 2), 4a (n = 1), and 6a (n = 2). For genotype 2a, the NS5B sequence of JFH1-I 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 of the activity of TMC647078 on chimeric replicons generated from a total of 10 genotype 1b and 7 genotype 1a samples confirmed that the compound has a similar activity on genotype 1b (median EC<sub>50</sub> of 3.2 µM) and suppressed replicon RNA levels compared with that of the reference ET replicon was found for the genotype 1a chimeric replicons.

Effect on mitochondrial DNA and mRNA. Mitochondrial DNA (mtDNA) and mitochondrial mRNA (mtRNA) levels, normalized to nuclear DNA and mRNA levels, were quantified in HepG2 cells treated for 14 days with TMC647078 and compared with the corresponding levels in nontreated control cells (see the supplemental material). Zalcitabine (ddC) and didanosine (ddI) also caused a significant reduction in mitochondrial mRNA levels at the highest test concentration tested (202 µM). Treatment with ddC, the normalized mitochondrial DNA and mRNA levels were significantly reduced in HepG2 cells, and CC<sub>50</sub> of 0.4 µM for mtDNA and 1.9 µM for mtRNA were 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> of >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 for S282T-variant HCV strains based on resistance selection data generated with a 2'-substituted nucleoside (NM-107) (21). Indeed, a 39-fold reduction in the EC<sub>50</sub> of TMC647078 compared with that of 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 muta-
TMC647078 maintained full activity against the mutants associated with reduced susceptibility to this class of inhibitors when engineered into the genotype 1b ET replicon. The nucleoside inhibitor; NNI, nonnucleoside inhibitor; PI, protease inhibitor. The results were concentration dependent, and a return to baseline levels was observed at day 28 in the case of cells treated with 25 μM and at day 35 in the case of cells treated with 50 μM. Interestingly, the combination of 25 μM TMC647078 with 100 nM TMC435 resulted in a steeper decline of HCV RNA levels 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 the occurrence of resistance was efficiently suppressed. More importantly, the combination of the two compounds prevented the rebound of HCV RNA levels during the rebound phase, indicating a "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 [MP] formation) by a cellular kinase is critical. To identify the kinases involved in the phosphorylation of TMC647078 and measure the respective phosphorylation capacities 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, 15, 38–40). The relative phosphorylation rates comparing 100 μM TMC647078 and 100 μM the natural substrates (deoxycytidine [dCyd] for dCK, deoxothymine [dTmd] for TK1 and TK2, uridine [Urd] for UCK1 and UCK2, and deoxyguanosine [dGuo] for dGK) were determined. These experiments were repeated three times, with similar results. The standard error of the measurement was ±10%. dCK efficiently phosphorylated TMC647078 with a relative activity more than 3-fold greater than that of dCyd. TK2 phosphorylated TMC647078 with about 12% relative activity, and dGK, TK1, and UCK showed no detectable phosphorylation of TMC647078.

### Table 2. Effects of polymerase and protease mutations in the reference genotype 1b replicon ET on TMC647078 susceptibility

<table>
<thead>
<tr>
<th>Inhibitor class</th>
<th>Mutation(s)</th>
<th>Median EC_{50} (IQR)</th>
<th>Median FC (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference ET replicon</td>
<td>S222T</td>
<td>3.2 (2.2–4.5)</td>
<td>-</td>
</tr>
<tr>
<td>2'-substituted N1</td>
<td>S96T</td>
<td>48.2 (43.0–51.0)</td>
<td>-</td>
</tr>
<tr>
<td>4'-substituted N1</td>
<td>S96T</td>
<td>2.3 (1.8–5.4)</td>
<td>0.8 (0.6–1.1)</td>
</tr>
<tr>
<td>4'-substituted N1</td>
<td>N142T</td>
<td>2.9 (2.0–4.3)</td>
<td>0.8 (0.6–1.1)</td>
</tr>
<tr>
<td>4'-substituted N1</td>
<td>N142T + S96T</td>
<td>3.0 (2.0–5.1)</td>
<td>0.8 (0.6–1.1)</td>
</tr>
<tr>
<td>NNN-1</td>
<td>F495L</td>
<td>4.7 (3.0–5.9)</td>
<td>2.4 (1.4–5.5)</td>
</tr>
<tr>
<td>NNN-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>MKI</td>
<td>Q80R</td>
<td>3.7</td>
<td>2.1</td>
</tr>
<tr>
<td>MKI</td>
<td>Q80K</td>
<td>2.6</td>
<td>1.4</td>
</tr>
<tr>
<td>MKI</td>
<td>D168A</td>
<td>2.8</td>
<td>1.5</td>
</tr>
<tr>
<td>MKI</td>
<td>D168E</td>
<td>3.6</td>
<td>2.0</td>
</tr>
<tr>
<td>MKI</td>
<td>D168V</td>
<td>2.5</td>
<td>1.4</td>
</tr>
<tr>
<td>Linear ketoamide</td>
<td>R155K</td>
<td>3.2</td>
<td>1.8</td>
</tr>
<tr>
<td>Linear ketoamide</td>
<td>A156T</td>
<td>2.8</td>
<td>1.6</td>
</tr>
<tr>
<td>Linear ketoamide</td>
<td>A156V</td>
<td>2.4</td>
<td>1.3</td>
</tr>
</tbody>
</table>

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**For each inhibitor class, the major mutations associated with reduced susceptibility to this class of inhibitors were engineered into the genotype 1b ET replicon. N1, nucleoside inhibitor; NNI, nonnucleoside inhibitor; PI, protease inhibitor.**

- EC_{50} are represented as the median values of 2 to 45 experiments. Interquartile ranges (IQR) are shown in parentheses for mutant replicons tested in more than 2 experiments.
- FC, fold change in EC_{50} compared to the reference ET replicon. An FC was calculated for every single experiment, and the medians of these values from 2 to 45 experiments are shown. Interquartile ranges (IQR) are shown in parentheses for mutant replicons tested in more than 2 experiments.
The kinetic determination of monophosphate formation was performed by using 1.5 to 100 μM dCyd and 3 to 100 μM TMC647078, and the results are presented in Table 3. TMC647078 showed 5-fold-greater $V_{\text{max}}$ (312.6) and 10-times-higher $K_m$ (41.9) values than those of dCyd ($V_{\text{max}}$, 62.9; $K_m$, 4.7). The phosphorylation efficiency ($k_{\text{cat}}/K_m$) of TMC647078 as a dCK substrate was thus 2-fold lower than 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 by using two constant concentrations of dCyd (2 μM and 10 μM) with 0.75 to 100 μM TMC647078. The $K_i$ value for dCyd as an inhibitor of TMC647078 phosphorylation was 2.8 μM. At a concentration of 100 μM the percentages of TMC647078 monophosphorylated by dCK in the presence of 2 μM (ratio, 1:50) and 10 μM (ratio, 1:10) dCyd were 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 the intensity of the dCMP spots in these experiments (data not shown).

Quantification of triphosphate metabolites of [3H]TMC647078 in Huh7 cells and primary human hepatocytes. The levels of triphosphate of TMC647078 and the uridine metabolite of TMC647078 (TMC647078-U-TP) 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 the level of triphosphate formation of both the parent compound and the uridine metabolite was observed. The level of TMC647078-triphosphate (TMC647078-TP) formation was significantly higher in Huh7 cells than in PHH. In PHH, higher triphosphate levels of the uridine metabolite than those of the parent compound were found, whereas the reverse was observed for Huh7 cells (Table 4).

Efficiency of incorporation of TMC647078-MP during RNA strand synthesis, termination of elongation, and exci-
sion of incorporated TMC647078-MP in the presence of PPi. Multiple- and single-site incorporation experiments were performed to determine the efficiency of the incorporation of TMC647078-MP during RNA strand synthesis. In a multiple-incorporation experiment with opportunities for incorporation of TMC647078-MP during RNA strand synthesis. In a multiple-incorporation experiment with opportunities for incorporation of TMC647078-MP during RNA strand synthesis. In a multiple-incorporation experiment with opportunities for incorporation of TMC647078-MP during RNA strand synthesis. In a multiple-incorporation experiment with opportunities for incorporation of TMC647078-MP during RNA strand synthesis.

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 into the RNA chain, after which elongation was measured for increasing concentrations of the next nucleotide that would allow chain extension (UTP). Once incorporated, TMC647078-TP caused chain termination; increasing concentrations of UTP of up to 250 μM did not extend the primer (Fig. 2).

As it was previously reported that cellular pyrophosphate (PPi) can remove incorporated nucleoside reverse transcriptase inhibitors (NRTIs), thereby restoring the 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 into 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 (Fig. 2).

**TABLE 4. Formation of triphosphate in primary human hepatocytes and Huh7 cells after incubation with 1, 4, and 16 μM TMC647078**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Triphosphate</th>
<th>Triphosphate concn (ng/ml) at TMC647078 concn of:</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHH</td>
<td>TMC647078-TP</td>
<td>6.4  17.3  64.8</td>
</tr>
<tr>
<td></td>
<td>TMC648078-U-TP</td>
<td>26.3  62.9  173</td>
</tr>
<tr>
<td>Huh7 cells</td>
<td>TMC647078-TP</td>
<td>30.5  107  264</td>
</tr>
<tr>
<td></td>
<td>TMC648078-U-TP</td>
<td>18.3  47.3  66.3</td>
</tr>
</tbody>
</table>

FIG. 2. (a) Chain termination efficiency of TMC647078-TP. The incorporation of each chain terminator into the newly synthesized RNA was followed by the addition of UTP, the next required nucleotide. The 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. (b) Pyrophosphate (PPi)-dependent excision of TMC647078-TP and other nucleoside inhibitor TPs and rescue of RNA synthesis. Incorporated chain terminators were subjected to increasing concentrations of PPi in the presence of CTP and UTP, and rescue of RNA synthesis after excision was monitored. NM-107-TP, which has previously been shown to be susceptible to PPi-mediated excision, and R-1479-TP, which does not prevent RNA synthesis after incorporation, were included for comparison.

**DISCUSSION**

Nucleoside analogs form the cornerstone of antiviral therapy for several indications, including HIV, HBV, herpes simplex virus, and HCMV infections. Furthermore, several modified nucleoside analogs and nonnucleoside inhibitors with potent inhibitory activities against NS5B have been reported as potential therapeutics for HCV (for a review, see reference 19). The majority of nucleoside analogs active against HCV can be divided into two classes: compounds harboring 2′ modifications (e.g., NM-107 and PSI-6130) or those harboring 4′ modifications (e.g., R-1479) of the ribose ring. Prodrugs of the nucleoside inhibitors NM-107 (NM-283), R-1479 (R-1626), and PSI-6130 (R7128) and of nucleotide inhibitors (IDX-184, PSI-7977, and PSI-938) have been or are being evaluated in clinical trials and have proven efficacious in vivo (for a review, see reference 19). With broad genotypic coverage and a high barrier to the development of resistance based on their mechanisms of activity, their potency in suppressing viral replication, and the lower replication fitness of drug-resistant viruses, nucleoside or nucleotide 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] and 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 the treatment of HCV.

We recently described the synthesis and anti-HCV activity of TMC647078, a 2′-deoxy-2′-spirocycloproplytidine (14).
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 a potent inhibition of HCV replication in a genotype 1b subgenomic replicon-based assay, with an EC$_{50}$ of 7.8 $\mu$M. 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 polymerase $\alpha$, $\beta$, or $\gamma$ up to a concentration of 1,000 $\mu$M. This is an important observation when addressing the different mechanisms of potential in vitro toxicity of a nucleoside polymerase inhibitor. The CC$_{50}$ was found to be higher than 400 $\mu$M in several human cell lines. We further analyzed the effects on mitochondrial DNA and mRNA levels. Some HIV NRTIs (zalcitabine [ddC] and 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 CC$_{50}$ for mtDNA and mtRNA of >202 $\mu$M 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 with a 3-day duration, no effects on cell proliferation or on the cell cycle phase at concentrations of up to 200 $\mu$M and 100 $\mu$M, respectively, were observed (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 to NNI-4, and 4'-substituted NIs, 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 sustained virologic response rates and shorter treatment durations to reduce side effects. The effect of TMC647078 on the suppression of drug-resistant colonies and the 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 a reduction of HCV replicon RNA levels were observed for TMC647078. The combination of the two compounds at low concentrations resulted in a complete suppression of colony formation without rebound, indicating that the replicon was efficiently cleared from the cells and that the combination of two direct antivirals with different mechanisms of action is favored over a single-compound treatment in terms of resistance. Moreover, no rebound of the HCV RNA level 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, 21, 30). It was reported previously that this mutation confers resistance from a combination of a 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 plus N142T in vitro, which confer a relatively small decrease in susceptibility (21, 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 to 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 for why the mutation was not 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 observed in our transient replicon assay (12% of the wild-type ET replicon) and by others previously (30), as it may impact the ability to select this mutant using 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 the inhibition of HCV RNA synthesis. Additional studies will be required to address whether 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, the monophosphate formation of TMC647078 was catalyzed (mainly) by dCK. No clear inhibitory effect of high concentrations of TMC647078 on the 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 with 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 findings reported previously for PSI-6130 (27). In primary human hepatocytes, the formation of both TMC647078-TP and TMC647078-U-TP increased linearly with the concentration of the 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 previously 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 those in PHH. Triphosphate levels of the uridine metabolite were similar in both cell systems, except for the condition under which cells were incubated with 16 μM parent compound, where a saturation plateau for uridine metabolite triphosphate formation was reached in Huh7 cells. 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 EC50s 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.


REFERENCES


