Inhibition of hepatitis C replicon RNA synthesis by β-D-2′-deoxy-2′-fluoro-2′-C-methylcytidine: a specific inhibitor of hepatitis C virus replication

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β-D-2′-Deoxy-2′-fluoro-2′-C-methylcytidine (PSI-6130) is a cytidine analogue with potent and selective anti-hepatitis C virus (HCV) activity in the subgenomic HCV replicon assay, 90% effective concentration (EC90)=4.6 ±2.0 µM. The spectrum of activity and cytotoxicity profile of PSI-6130 was evaluated against a diverse panel of viruses and cell types, and against two additional HCV-1b replicons. The S282T mutation, which confers resistance to 2′-C-methyl adenosine and other 2′-methylated nucleosides, showed only a 6.5-fold increase in EC90. When assayed for activity against bovine diarrhoea virus (BVDV), which is typically used as a surrogate assay to identify compounds active against HCV, PSI-6130 showed no anti-BVDV activity. Weak antiviral activity was noted against other flaviviruses, including West Nile virus, Dengue type 2, and yellow fever virus. These results indicate that PSI-6130 is a specific inhibitor of HCV. PSI-6130 showed little or no cytotoxicity against various cell types, including human peripheral blood mononuclear and human bone marrow progenitor cells. No mitochondrial toxicity was observed with PSI-6130. The reduced activity against the RdRp S282T mutant suggests that PSI-6130 is an inhibitor of replicon RNA synthesis. Finally, the no-effect dose for mice treated intraperitoneally with PSI-6130 for six consecutive days was ≥100 mg/kg per day.

Keywords: antiviral activity, HCV, PSI-6130

Introduction

Hepatitis C virus (HCV), an important member of the Flaviviridae, is the leading cause of liver transplantation in the United States. Nearly 2% of the U.S. population and an estimated 170 million people worldwide are HCV carriers (Poynard et al., 2000; Alter et al., 1999). The current standard of care is a combination of pegylated interferon and ribavirin (Di Bisceglie et al., 2002; Collier & Chapman, 2001; Alter et al., 1999). Because of the adverse effects associated with both interferon and ribavirin (Di Bisceglie et al., 2002; Collier & Chapman, 2001; Alter et al., 1999), there is a need for more potent anti-HCV compounds with fewer adverse effects.

The lack of cell-based assays for HCV has hindered the discovery and development of therapies to treat HCV infection. However, surrogate models such as the HCV RNA replicon that replicates in human hepatoma cells has facilitated the identification of candidate anti-HCV drugs (Lohmann et al., 1999; Blight et al., 2000). Nucleoside analogues, which inhibit viral encoded polymerases, have a proven track record as therapies for viral infections caused by herpes viruses, HIV and hepatitis B virus (De Clercq, 2004). The HCV RNA-dependent RNA polymerase NS5B protein (RdRp) is considered to be essential for HCV replication and therefore is an ideal
therapeutic target for nucleoside analogues (Yamashita et al., 1998; Lohmann et al., 1998; Lohmann et al., 1997; Ishii et al., 1999; Blight et al., 2000).

Recently, several 2'-modified nucleoside analogues with activity against HCV have been identified (Yamashita et al., 1998; Lohmann et al., 1998; Lohmann et al., 1997; Ishii et al., 1999; Blight et al., 2000). These compounds are phosphorylated to the corresponding 5'-triphosphate which in turn inhibits the HCV RdRp. Of these compounds the valine ester of β-d-2'-C-methylcytidine (NM283, valopicitabine) is currently undergoing Phase II clinical trials in HCV-infected individuals (Pierra et al., 2005). Here we describe the in vitro results of studies with β-d-2'-deoxy-2'-fluoro-2'-C-methylcytidine (PSI-6130; Figure 1), a new, potent and specific anti-HCV compound, which shows little or no toxicity in vitro and in vivo.

Materials and methods

Chemistry

PSI-6130 (Figure 1) was synthesized according to the methods of Clark et al. (2005). 2'-C-Methylcytidine and 2'-C-methyladenosine were synthesized in our laboratories following published procedures (Eldrup et al., 2004; Clark et al., 2005). Interferon-α2a (Roferon-A) was obtained from Hoffmann-La Roche Inc., Nutley, NJ, USA.

Virology

Viruses and cells. The HCV subgenomic replicon RNA-containing Huh 7 cells (Clone A cells; Apath, LLC, St. Louis, MO, USA) and the full length HCV replicon RNA-containing Huh 7 cells, 21-5, kindly provided by Dr Ralf Bartenschlager (Johannes-Gutenberg University Mainz, Mainz, Germany), were maintained in exponential growth in Dulbecco’s modified Eagle’s medium (high glucose and no pyruvate) containing 10% fetal bovine serum, 1x nonessential amino acids, 100 U/ml of penicillin, 100 µg/ml of streptomycin, 0.292 mg/ml of glutamine and 500 µg/ml of G418. Madin–Darby bovine kidney (MDBK) cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% horse serum and 100 µg/ml of penicillin-streptomycin. HepAD38 cells (a gift from Dr Brent Korba) were maintained in Dulbecco’s modified Eagle’s/F12 medium (DMEM/F12; GIBCO/Invitrogen Technologies, Carlsbad, CA, USA) supplemented with 10% heat inactivated fetal bovine serum, 50 µg/ml of penicillin, 50 µg/ml of streptomycin, 100 µg/ml of kanamycin and 0.3 µg/ml of tetracycline in a humidified 5% CO₂ atmosphere at 37°C. The cytopathic NADL strain of bovine diarrhoea virus (BVDV) was kindly provided by Dr Ruben Donis, University of Nebraska. The New Guinea strain of Dengue type 2 virus (DV) and the New York strain of the West Nile virus (WNV) were provided by Drs N Karabatsos and R Lanciotti, respectively, of the Centers for Disease Control and Prevention, Atlanta, GA USA. The 17D strain of yellow fever virus (YFV), CEM and HepG2 cells (HB-8065) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Generation of NS5B S282T mutant replicon. Clone A cells were seeded into six-well plates at 2.4x10⁴ cells/well in the presence of 1 mg/ml G418 and 5 µM of 2'-C-Me-adenosine in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1x nonessential amino acids, 100 U/ml of penicillin, 100 µg/ml of streptomycin and 0.292 mg/ml of glutamine. After 10 days, cells became confluent. Cultures were then split with a one to five dilution into fresh medium and the concentration of compound was increased to 10 µM. On day 21, the concentration was increased to 20 µM. On day 34, cell death was first noted, and small colonies of cells resistant to the inhibitor and the antibiotic became visible. The medium was renewed as needed. and on day 47, resistant colonies were isolated and transferred to a 24-well plate. Resistant colonies were then expanded and characterized. RNA was isolated from a representative clone using the RNeasy 96 kit (Qiagen, Valencia, CA, USA), reverse transcribed and amplified. The resulting DNA was sequenced using primers specific for NS5B to identify any mutations present in the NS5B polymerase gene. The only mutation found in the NS5B of the resistant clone was
the substitution of serine 282 with threonine (S282T), consistent with Migliaccio et al. (2003).

**HCV replicon assay.** The HCV replicon assay was performed as previously described by Stuyver et al. (2003b). Briefly, clone A cells were added to a 96-well plate at 1,000 cells/well in 50 µl of medium without G418. Test compounds in 50 µl (two-fold serial dilutions) were added immediately after seeding. Plates were incubated at 37°C in a 5% CO₂ atmosphere for 4 days. Replicon RNA was extracted and amplified in a single-step multiplex RT-PCR protocol as described (Stuyver et al. 2003b). Antiviral activity was determined by subtracting the average threshold RT-PCR cycle of the test compound from the average threshold RT-PCR cycle of the no-drug control (ΔCtHCV). A ΔCt of 3.3 equals a 1-log reduction (equal to the 90% effective concentration [EC₉₀]) in replicon RNA levels. Cytotoxicity of test compounds was also determined by calculating the ΔCt for ribosomal RNA (ΔCtRNA).

**BVDV assay.** Cells were seeded in a 96-well plate at 5×10⁴ cells/well and incubated for 72 h at 37°C in a humidified 5% CO₂ atmosphere. The cells were then infected with the cytopathic NADL strain of BVDV at a virus dilution of 10⁻² and incubated for 45 min. Cell monolayers were washed three times with medium. Fresh medium containing serial dilutions of test compounds or ribavirin (positive control) was added to cultures and medium containing no drug was added to the no-drug controls. After 72 h incubation, supernatant was collected and viral RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen). Viral load was determined by quantitative RT-PCR using primers specific for the NADL strain of BVDV (Stuyver et al., 2003b).

**DV, WNV and YFV assays.** Antiviral activity against DV, WNV and YFV was determined using the neutral red dye uptake assay described by McManus (1976). A known positive control compound was included in each assay. Ribavirin was used as the positive control in the DV virus assays, and 6-azauridine was the positive control for the WNV and YFV assays.

**HIV assay.** The assay was performed using a modification of the assay described by Schinazi et al. (1990 & 1992). Briefly, primary human peripheral blood mononuclear (PBM) cells were isolated from sero-negative donors and activated with phytohemagglutinin A (1 mg/ml). Cells were infected with HIV-1 at (Centers for Disease Control and Prevention) at a multiplicity of infection of 0.1. At 1 hr post-infection, compounds were added in duplicate at concentrations of 0.1, 1.0, 10 and 100 µM. 3′-Azido-3′-deoxythymidine (AZT) was used as a positive control. After incubating for 6 days at 37°C in a humidified 5% CO₂ atmosphere, 1 ml of culture supernatant was centrifuged and the virus pellet resuspended in 100 µl of a buffer containing 0.05 M Tris, pH 7.8, 0.5% Triton X-100, 0.8 M NaCl, 0.5 mM phenylmethylsulfonyl fluoride, and 20% glycerol. Ten microlitres of solubilized virus were added to 75 µl of reverse transcriptase reaction mixture (0.06 M Tris at pH 7.8, 0.012 M MgCl₂, 0.006 M dithiothreitol, 0.006 mg/ml poly rA-oligo dT₁₂₋₁₈ [Amersham Bioscience, Piscataway, NJ, USA], 96 µg/ml dATP [Sigma-Aldrich, St. Louis, MO, USA] and 1 µM [³²P]-thymidine-5′-triphosphate [87.0 Ci/m mole; Perkin Elmer, Boston, MA, USA]) and incubated at 37°C for 2 h. The reaction was stopped and the reaction product precipitated by the addition of 10% trichloroacetic acid (100 µl) containing 0.05% sodium pyrophosphate. The precipitate was collected using a Packard FilterMate Cell Harvester (Packard, Meriden, CT, USA) and counted in a Packard Direct Beta Counter. The 50% effective concentration was determined using the method of Belen’kii and Schinazi (1994).

**HBV assay.** The HBV quantitative-PCR assay with HepAD38 cells was performed as previously described (Stuyver et al., 2002; Hassan et al., 2003). HepAD38 cells replicate HBV under conditions that can be regulated with tetracycline (Ladner et al., 1997). HepAD38 cells were seeded into 96-well plates at 5×10⁴ cells/well in 200 µl of medium and incubated at 37°C in a humidified 5% CO₂ atmosphere. On day two, medium was removed and the cells were washed with PBS. Compounds and controls were prepared in medium without tetracycline and added at 10 µM (final concentration) in duplicate. On day seven, HepAD38 cell supernatant was collected and stored for analysis. Supernatant containing extracellular HBV was extracted using DNeasy® 96 Tissue Kit (Qiagen, catalog #69582) in a 96-well format. DNA was eluted in 100 µl total volume and 5 µl was used for real time PCR in a 25 µl reaction. HBV primers were used at 22.5 pmol/reaction and probe was used at 5 pmol/reaction (Operon, Huntsville, AL, USA/Qiagen). Taqman® Universal PCR Master Mix was added at twice the concentration (Applied Biosystems, Foster City, CA, USA/Roche, Pleasanton, CA, USA).

**Cytotoxicity assay.** Human PBM cells (5×10⁴ cells/well), CEM cells (2.5×10⁵ cells/well), HepG2 (5×10⁵ cells/well), Huh-7 (5×10⁴ cells/well) and Clone A cells (5×10⁵ cells/well) were seeded in 96-well plates in the presence of increasing concentrations of test compound and incubated at 37°C in a humidified 5% CO₂ atmosphere for 3–5 days. For each assay, 50 µl of twofold serial dilutions of test compound...
were added in to each well of a 96-well plate. Final concentrations of PSI-6130 ranged from 1 to 100 μM. A “no drug” (medium only) control and a “cells plus medium only” control were included. After 5 days incubation for PBM cells, 3 days incubation for CEM cells or 4 days incubation for all others, cell viability was determined using the CellTiter 96 AQ™. One Solution cell proliferation colorimetric assay (Promega, Madison, WI, USA). The absorbance (490 nm) was then read on an ELISA plate reader using the ‘no drug’ wells as blanks. Cytotoxicity was expressed as the concentration of test compound that inhibited cell growth by 50% (CC₅₀).

**Human bone marrow cytotoxicity assay.** Primary human bone marrow mononuclear cells were obtained from Cambrex Bioscience (Walkersville, MD, USA). CFU-GM assays were performed using a bilayer soft agar in the presence of 1 unit/ml erythropoietin (Sommadossi & Carlisle, 1987). Cells were incubated in the presence of the compound for 14–18 days at 37°C with 5% CO₂. Colonies of greater than 50 cells were counted using an inverted microscope to determine 50% inhibition concentration (Sommadossi et al., 1992). Each experiment was performed in duplicate using cells from three different donors. 3′-Azido-3′-deoxycytidine (AZT) was used as a positive control.

**Mitochondrial toxicity assays.** HepG2 cells (5,000 cells/well) were seeded in 96-well, collagen-coated plates. Test compound was added to the medium at selected concentrations and the plates were incubated at 37°C in a humidified 5% CO₂ atmosphere for 14 days. After incubation, the supernatant was removed and cellular nucleic acids were extracted using a RNeasy 96 kit (Qiagen). The mitochondrial cytochrome C oxidase subunit II (cox2) gene and ribosomal DNA (rDNA) were amplified from a 5 μL sample using a multiplex quantitative PCR protocol (Stuyver et al., 2002) and the ΔCt (mitochondrial DNA) and ΔCt (rDNA) for each sample were determined. The fold difference in mitochondrial DNA normalized for rDNA relative to control was calculated.

Lactic acid quantification was performed using the D-lactic acid/ L-lactic acid test kit (Boehringer Mannheim, Indianapolis, IN, USA / R-Biopharm, South Marshall, MI, USA / Roche). The total amount of lactic acid produced for each sample was determined as well as the fold change in lactic acid production (% of lactic acid % of rDNA), following a 7 day incubation in the presence of various concentrations of PSI-6130, as described in the manufacturer’s instructions.

**Evaluation of toxicity in mice.** Five groups of five six-week-old female Swiss mice (SWR/J; Charles River Laboratory, Wilmington, MA, USA) were dosed intraperitoneally (i.p.) with 0, 3.3, 10, 33 or 100 mg/kg per day of PSI-6130 dissolved in pyrogen-free, sterile saline (0.85% NaCl, Sigma-Aldrich, St. Louis MO, USA). Animals were monitored daily for weight changes, general appearance and mortality up to 24 days post-treatment. The statistical significance of changes in animal weight was evaluated by one-way analysis of variance. A P-value of <0.05 was deemed statistically significant. These studies were conducted under the approval of the Institutional Animal Care and Use Committee (IACUC) of the Department of Veteran Affairs, Atlanta, GA, USA.

**Results**

**Inhibition of HCV RNA in replicon cells**

The results from the subgenomic HCV replicon assay with PSI-6130 are presented as EC₉₀ values in Table 1. An EC₉₀ value of 4.6 ± 2.0 μM was determined for PSI-6130. Comparing the activity of PSI-6130 with that of 2′-C-methylcytidine (2′-C-MeC), 2′-C-methyladenosine (2′-C-MeA) and 2′-deoxy-2′-fluorocytidine (2′-F-C), we found that PSI-6130 was greater than fourfold more potent than 2′-C-MeC, half as active as 2′-C-MeA and showed similar activity to 2′-F-C (Table 1). The activity of PSI-6130 was also compared with that of 2′-C-MeC and 2′-C-MeA using the full length replicon 21–5. The EC₉₀ values were lower for each of the compounds, but the relative potency was similar to what was seen with the Clone A subgenomic replicon (Table 1).

It has been demonstrated that candidate antiviral agents can indirectly alter replicon RNA levels by affecting cell growth rates (Stuyver et al., 2003a). To address this issue, we followed the level of HCV replicon RNA on a per cell basis over the course of 7 days in cells

<table>
<thead>
<tr>
<th>Table 1. In vitro activity of PSI-6130 compared with other nucleoside analogues in the Clone A replicon, and Bovine diarrhea virus</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>90% Effective concentration ± SD, μM</strong></td>
</tr>
<tr>
<td><strong>Clone A</strong></td>
</tr>
<tr>
<td><strong>Compound</strong></td>
</tr>
<tr>
<td>PSI-6130</td>
</tr>
<tr>
<td>2′-C-MeC</td>
</tr>
<tr>
<td>2′-C-MeA</td>
</tr>
<tr>
<td>2′-F-Cytidine</td>
</tr>
</tbody>
</table>

* Single assay performed in duplicate. ND, not determined.
treated with PSI-6130. Cells were seeded in the presence or absence of PSI-6130 (5 µM and 25 µM) and incubated at 37°C. On days 3–7, cells were harvested and counted using the trypan blue exclusion method, followed by total cellular RNA isolation and quantification of replicon RNA. When the log_{10} change in HCV replicon RNA copy number was determined per cell, cells treated with PSI-6130 showed a significant and steady decrease in replicon copy number per cell compared to untreated control cells, which showed a slight increase in replicon copy number (Figure 2A). Interferon-α2a and ribavirin was used as a positive and negative control, respectively (Figure 2B). Compared to the “no drug” control, interferon-α2a significantly reduced the HCV replicon RNA copy numbers per cell (Figure 2B), whereas ribavirin reduced the replicon RNA copy number per cell only minimally (Figure 2B). These results indicate that PSI-6130 selectively inhibited replication of the HCV replicon.

Migliaccio et al. (2003) previously isolated a resistant replicon by passaging in the presence of 2′-C-MeA and identified a serine to threonine mutation at position 282 of the HCV RdRp that conferred a loss of sensitivity to 2′-C-MeA. In contrast to 2′-C-MeA and 2′-C-MeC, which were inactive against the S282T mutant, PSI-6130 showed only a 6.5-fold increase in EC_{90} (30.7 ±11.7 µM) with the S282T mutant replicon (Table 1).

Prevention of PSI-6130 inhibition of HCV replicon replication

Using a real-time RT-PCR assay (Stuyver et al., 2003b), the ability of natural nucleosides to prevent the anti-HCV activity of PSI-6130 was explored to gain some insight as to the mechanism by which PSI-6130 is phosphorylated in replicon cells. These reversal studies were performed with exogenously added natural nucleosides. In these studies, 5 µM of PSI-6130 (the concentration of PSI-6130 that approximates the EC_{90} value) was incubated with natural ribo- or 2′-deoxyribonucleosides at a concentration of 50 µM (approximately 10 times the EC_{90} of PSI-6130). Cells were incubated at 37°C in a humidified 5% CO_{2} atmosphere for 4 days and antiviral activity was determined by real time PCR as described in the Materials and methods. Of the natural nucleoside analogues tested, only 2′-deoxy-2′-C-methylcytidine completely inhibited the antiviral activity of PSI-6130 (Table 2). Exogenous cytidine caused a partial reversal of antiviral activity whereas none of the other ribo- or

![Figure 2. Effect of PSI-6130 (A), Ribavirin or Interferon (B) on HCV replicon RNA per cell](image)

<p>| Table 2. Prevention of the anti-HCV activity of PSI-6130 by exogenously added nucleosides |
|-----------------------------------------------|-----------------|-------------------|</p>
<table>
<thead>
<tr>
<th>Competing nucleoside (50 µM)</th>
<th>ΔCt ±SD</th>
<th>% Inhibition of HCV replication</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSI-6130 control</td>
<td>3.39 ±0.11</td>
<td>90.4</td>
</tr>
<tr>
<td>Cytidine</td>
<td>0.74 ±0.87</td>
<td>40.0</td>
</tr>
<tr>
<td>Uridine</td>
<td>3.52 ±0.61</td>
<td>91.2</td>
</tr>
<tr>
<td>Adenosine</td>
<td>2.82 ±0.53</td>
<td>85.8</td>
</tr>
<tr>
<td>Guanosine</td>
<td>2.90 ±0.06</td>
<td>86.5</td>
</tr>
<tr>
<td>2′-Deoxycytidine</td>
<td>0.00 ±0.14</td>
<td>0.0</td>
</tr>
<tr>
<td>2′-Deoxyuridine</td>
<td>3.38 ±0.01</td>
<td>90.3</td>
</tr>
<tr>
<td>Thymidine</td>
<td>4.59 ±0.14</td>
<td>95.8</td>
</tr>
<tr>
<td>2′-Deoxyadenosine</td>
<td>3.42 ±0.08</td>
<td>90.6</td>
</tr>
<tr>
<td>2′-Deoxyguanosine</td>
<td>3.42 ±0.17</td>
<td>89.3</td>
</tr>
</tbody>
</table>

A ΔCt (the average threshold RT-PCR cycle of the test compound subtracted from the average threshold RT-PCR cycle of the no-drug control) of 3.3 equals a 1-log reduction or 90% inhibition.
2′-deoxycytidinonucleoside analogues were effective (Table 2). These results were quite different from those obtained with 2′-C-MeC where cytidine completely reversed the anti-HCV activity of the compound (data not shown). These results suggest that there are differences in the metabolic pathways of PSI-6130 and 2′-C-MeC even though both compounds are cytidine analogues.

**Activity of PSI-6130 against other viruses**

Like HCV, BVDV, WNV, YFV and DV are members of the *Flaviviridae* family of viruses. To demonstrate the specificity of PSI-6130 for HCV, we tested the compound for activity against these other flaviviruses. BVDV is typically used as an HCV surrogate to assay for compounds for potential activity against HCV. Interestingly, unlike 2′-C-MeC and 2′-C-MeA that were active against the NADL strain of BVDV giving EC90 values of 2 µM and 1.5 µM, respectively; PSI-6130 was not active against this virus (EC90>100µM; Table 1). PSI-6130 had little or no activity against WNV (EC90=46.3 µM), YFV (in two separate experiments EC90=46.3 µM and 100 µM) and DV (EC90>100 µM). PSI-6130 was also found to be inactive against HIV (EC90>100µM) and HBV (EC90>10 µM).

**Cytotoxicity and mitochondrial toxicity of PSI-6130**

In standard 3-, 4- or 5-day cytotoxicity assays with Huh7, Clone A replicon cells, HepG2 cells, CEM cells and human PBM cells, PSI-6130 did not show significant toxicity in the MTT assay at concentrations up to 100 µM (Table 3). Bone marrow toxicity is the principal dose-limiting toxicity associated with a number of nucleoside antiviral drugs (Sommadossi et al. 1992; Sommadossi and Carlisle, 1987). Therefore, candidate antiviral nucleosides are typically evaluated *in vitro* for their haematopoietic toxicity potential. PSI-6130 showed inhibition of BFU-E and CFU-GM growth at concentrations >80 µM, whereas 2′-C-methylcytidine inhibited these cells at twofold lower concentrations (Table 4). The AZT control was toxic and gave values similar to published results (Table 4).

As mitochondrial toxicity has been associated with several nucleoside analogues, the effect of PSI-6130 on mitochondrial DNA content was determined using HepG2 cells. In a 14-day mitochondrial toxicity assay, no significant effect on mitochondrial DNA content was observed when PSI-6130 was evaluated up to 100 µM (Table 5). In contrast, the positive control, 2′,3′-dideoxycytidine, was toxic at a concentration less than 10 µM. In addition, the effect of PSI-6130 on lactate production, another measure of mitochondrial toxicity, was assessed. In a 7-day assay, no increase in lactic acid was noted at concentrations up to 33 µM, the highest concentration tested (data not shown).

**Evaluation of toxicity in mice**

Swiss mice (five animals/dose) were given single i.p. injections of PSI-6130 for six consecutive days (that is, days 0–5). The doses tested were 0, 3, 10, 33.3 and 100 mg/kg administered in 500 µl of sterile saline solution. Mortality and overall appearance (that is, ruffled fur, dehydration, etc.) of the mice were monitored daily and individual animal weights were determined on days 0, 1, 2, 3, 4, 5, 7, 9, 11, 14, 18, 21, 25, 28 and 30. As shown in Figure 3, there were no significant differences in the weight gain among the mice at any of the doses tested.

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### Table 3. Cytotoxicity of PSI-6130 compared with other nucleoside analogues with anti-HCV activity

<table>
<thead>
<tr>
<th>Compound</th>
<th>Clone A</th>
<th>Huh7</th>
<th>HepG2</th>
<th>CEM</th>
<th>PBM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSI-6130</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2′-C-MeC</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>29.4</td>
<td>24.5</td>
</tr>
<tr>
<td>2′-C-MeA</td>
<td>30.5</td>
<td>50.2</td>
<td>31.2</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2′-F-Cytidine</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

All experiments were performed in duplicate. CC90 concentration of compound that inhibits cell growth by 50%; ND, not determined; PBM, peripheral blood mononuclear.

### Table 4. Effect of PSI-6130 and 2′-C-Methylcytidine on human bone marrow progenitor cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>BFU-E</th>
<th>CFU-GM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSI-6130</td>
<td>83.6±4.8</td>
<td>86.5±6.4</td>
</tr>
<tr>
<td>2′-C-Methylcytidine</td>
<td>36.1±6.8</td>
<td>33.7±2.8</td>
</tr>
<tr>
<td>AZT</td>
<td>0.09±0.01</td>
<td>2.9±1.2</td>
</tr>
</tbody>
</table>

BFU-E, erythroid blast forming unit; CC90, concentration of compound that inhibits cell growth by 50%; CFU-GM, granulocyte-macrophage colony forming unit.

### Table 5. Fourteen day mitochondrial toxicity assay comparing PSI-6130 with 2′-C-Methyladenosine and 2′-F-Cytidine

<table>
<thead>
<tr>
<th>Compound</th>
<th>MitCoxII</th>
<th>rDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dideoxycytidine</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>PSI-6130</td>
<td>&gt;100</td>
<td>71.80±33.6</td>
</tr>
<tr>
<td>2′-C-Methylcytidine</td>
<td>32.5±11.7</td>
<td>43.5±9.5</td>
</tr>
<tr>
<td>2′-F-Cytidine</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

rDNA, ribosomal DNA; mitCoxII, mitochondrial Cox2 DNA.
Discussion

Recently, several modified nucleoside analogues with potent inhibitory activity against the HCV NS5B polymerase have been described (Walker & Hong, 2002; Shim et al., 2003; Migliaccio et al., 2003; Lai et al., 2003; Eldrup et al., 2004; Devos, 2002; Carroll et al., 2003). These analogues can be divided into the following three classes: 2′-modifications of the ribose ring (methyl or O-methyl; Walker & Hong, 2002; Migliaccio et al., 2003; Eldrup et al., 2004; Carroll et al., 2003); 3′-modifications — mainly 3′-deoxy (Migliaccio et al., 2003; Lai et al., 2003) and 4′-modifications (Devos, 2002). Among the most potent compounds are β-D-2′-C-methyl-cytidine and 2′-C-methyl-adenosine. In a recent publication, the synthesis and anti-HCV activity of PSI-6130 was described (Clark et al., 2005). Because of the similar size and electronegativity of fluorine and oxygen, and because the hydrogen bonding characteristics of fluorine are similar to those of a hydroxy group, substituting fluorine would be expected to allow the molecule to have biological activity. In addition, the presence of a 2′-fluoro group should stabilize the glycosidic bond (Watanabe et al., 1983; Watanabe et al., 1979).

In this present study, PSI-6130 was found to be both a potent and a selective inhibitor of HCV RNA replication in the HCV replicon assay system. Instead of using a surrogate virus for assaying compounds of anti-HCV activity, we assayed for anti-HCV activity using a subgenomic or a full length HCV replicon. EC90 values of 4.6 ± 2.0 µM and 1.6 ± 0.6 µM were obtained for PSI-6130 with the subgenomic and full length replicon, respectively. Interestingly, little or no antiviral activity was observed when PSI-6130 was tested for activity using other members of the Flaviviridae family. This modest or lack of activity seen with other flaviviruses, including BVDV, could be due to an inability of certain cells, for example, MDBK cells to phosphorylate PSI-6130. Alternatively, the RdRp of these viruses might be less...
susceptible to inhibition by the 5′-triphosphate of PSI-6130. Since the differential activity of PSI-6130 extends to a number of flaviviruses in different cell lines, it is more likely a result of target sensitivity brought about by the dual substitution of methyl and fluorine at the 2′ position than levels of phosphorylation.

To gain insight into the mechanism of action of PSI-6130, inhibition studies were performed using exogenously added natural ribo- and 2′-deoxyribonucleosides to determine which nucleoside could prevent the anti-HCV activity of PSI-6130. The antiviral effect was prevented strongly by 2′-deoxyctydine. This would suggest that the compound is primarily phosphorylated by the host cell’s deoxyxycytidine kinase and not by uridine–cytidine kinase. Although deoxycytosine and deoxyguanosine are substrates of cytosolic deoxycytidine kinase, PSI-6130 phosphorylation was not affected significantly by deoxycytosine or deoxyguanosine. This observation could be due to the poor binding affinity of deoxycytosine and deoxyguanosine for cytosolic deoxycytidine kinase. The weak inhibition of antiviral activity seen with cytidine could be the result of competition by cytidine which can be utilized as a weak substrate by deoxyxycytidine kinase (Sabini et al., 2003, Datta et al., 1989); competition of cytidine monophosphate or diphosphate with the corresponding phosphate derivatives of PSI-6130 with the cellular cytidylate kinase and/or nucleoside diphosphate kinase or competition between cytidine 5′-triphosphate and PSI-6130 triphosphate for binding to the HCV RdRp. The reduced activity of PSI-6130 seen when the compound was tested against a replicon, which carried the S282T mutation in the RdRp, is consistent with PSI-6130 being an inhibitor of the NS5B enzyme. The mechanism of action studies with purified HCV RdRp, which will be published elsewhere, indicate that the 5′-triphosphate of PSI-6130 is an alternative substrate inhibitor of the enzyme. To date, no mutations in NS5B have been selected in the in vitro passaging experiments with PSI-6130 (unpublished data) in the subgenomic replicon cells.

Studies were performed to assess the toxicity of PSI-6130 in vitro and in vivo. Cytotoxicity assays using several different cell types, including human bone marrow progenitor cells, indicated no toxicity associated with PSI-6130 at physiologically relevant concentrations. Mitochondria are often a target for nucleoside toxicity (Lewis & Dalakas, 1995). Mitochondrial toxicity can be determined by measuring the effect of a compound on mitochondrial DNA and the production of lactic acid in liver cells. In these studies, there was no detectable reduction in mitochondrial DNA or an increase in lactic acid production compared to untreated control cells, indicating that PSI-6130 did not produce any mitochondrial toxicity at the concentrations tested. Finally, the no-effect dose for mice treated i.p. with PSI-6130 was 100 mg/kg per day.

In summary, we describe the in vitro antiviral activity of the PSI-6130. PSI-6130 demonstrated potent and specific activity in the HCV replicon assay system. PSI-6130 showed little or no cytotoxicity and no mitochondrial toxicity. Prevention studies performed with natural nucleosides suggest that PSI-6130 is phosphorylated via the 2′-deoxycytidine salvage pathway. The details of the mechanism of action remain to be determined.

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