THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 41, pp. 29812–29820, October 12, 2007

Characterization of the Metabolic Activation of Hepatitis C Virus Nucleoside Inhibitor β-D-2′-Deoxy-2′-fluoro-2′-C-methylcytidine (PSI-6130) and Identification of a Novel Active 5′-Triphosphate Species*

Received for publication, June 27, 2007, and in revised form, August 6, 2007 Published, JBC Papers in Press, August 13, 2007, DOI 10.1074/jbc.M705274200

Han Ma[1], Wen-Rong Jiang, Nicole Robledo, Vincent Leveque, Samir Ali, Teresa Lara-Jaime, Mohammad Masjedizadeh, David B. Smith, Nick Cammack, Klaus Klumpp, and Julian Symons

From the Roche Palo Alto LLC, Palo Alto, California 94304

β-D-2′-Deoxy-2′-fluoro-2′-C-methylcytidine (PSI-6130) is a potent inhibitor of hepatitis C virus (HCV) replication in the subgenomic HCV replicon system, and its corresponding 5′-triphosphate is a potent inhibitor of the HCV RNA polymerase *in vitro*. In this study the formation of PSI-6130-triphosphate was characterized in primary human hepatocytes. PSI-6130 and its 5′-phosphorylated derivatives were identified, and the intracellular concentrations were determined. In addition, the deaminated derivative of PSI-6130, β-D-2′-deoxy-2′-fluoro-2′-C-methyluridine (RO2433, PSI-6026) and its corresponding phosphorylated metabolites were identified in human hepatocytes after incubation with PSI-6130. The formation of the 5′-triphosphate (TP) of PSI-6130 (PSI-6130-TP) and RO2433 (RO2433-TP) increased with time and reached steady state levels at 48 h. The formation of both PSI-6130-TP and RO2433-TP demonstrated a linear relationship with the extracellular concentrations of PSI-6130 up to 100 μM, suggesting a high capacity of human hepatocytes to generate the two triphosphates. The mean half-lives of PSI-6130-TP and RO2433-TP were 4.7 and 38 h, respectively. RO2433-TP also inhibited RNA synthesis by the native HCV replicase isolated from HCV replicon cells and the recombinant HCV polymerase NS5B with potencies comparable with those of PSI-6130-TP. Incorporation of RO2433-5′-monophosphate (MP) into nascent RNA by NS5B led to chain termination similar to that of PSI-6130-MP. These results demonstrate that PSI-6130 is metabolized to two pharmacologically active species in primary human hepatocytes.

Hepatitis C is a major health problem affecting ~170 million people worldwide of which around 3 million chronically infected patients reside within the United States (1). The current standard treatment for hepatitis C consisting of pegylated interferon-α and ribavirin only results in about a 50% sustained virological response in patients infected with genotype 1 hepatitis C virus (HCV),[2] the most predominant genotype in the United States and Europe (2–4). New treatment options with improved clinical efficacy and greater tolerability are urgently needed. Novel antiviral agents targeting essential processes of HCV replication as part of optimized combination regimens could achieve increased clinical efficacy and potentially improved adverse event profiles as well as shortened treatment duration as compared with the current standard of care.

HCV RNA replication is mediated by a membrane-associated multiprotein replication complex (5, 6). The HCV NS5B protein, the RNA-dependent RNA polymerase, is the catalytic subunit of the HCV replication complex and is responsible for the synthesis of the RNA progeny and, hence, is a prime target of anti-viral inhibition. Nucleoside analogs have been established as successful antiviral agents targeting the active site of DNA polymerases for the treatment of other viral diseases, including human immunodeficiency virus, hepatitis B virus, and herpes simplex virus (7). The majority of marketed antiviral nucleoside analogs need to be converted to the active 5′-triphosphate forms in the target cells. These nucleotide triphosphate analogs then serve as alternative substrates for the viral DNA polymerases and compete with the incorporation of the corresponding natural nucleotide triphosphates. Upon incorporation by the viral DNA polymerases, the lack of the 3′-hydroxyl group in the deoxyribose moiety leads to the termination of the nascent viral DNA (chain termination).

In the past few years a number of ribonucleoside analogs with 2′-C-methyl, 2′-O-methyl, or 4′-azido substituents on the ribose moiety have been reported to be inhibitors of HCV replication in the subgenomic replicon system (8–13). Prodrugs of two nucleoside analogs, 2′-C-methylcytidine (NM107) and 4′-azidocytidine (R1479), have successfully progressed into clinical development and shown efficacy in HCV-infected patients (14, 15). The corresponding nucleotide triphosphate analogs are substrates for HCV polymerase NS5B and inhibit RNA synthesis activity of HCV NS5B *in vitro*. The incorporation of the nucleotide analogs into nascent HCV RNA strongly reduces the efficiency of further RNA elongation by NS5B,

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed: Roche Palo Alto LLC, 3431 Hillview Ave., Palo Alto, CA 94304. Tel.: 650-852-3190; Fax: 650-354-7554; E-mail: han.ma@roche.com.

[2] The abbreviations used are: HCV, hepatitis C virus; MP, 5′-monophosphate; DP, 5′-diphosphate; TP, 5′-triphosphate; HPLC, high performance liquid chromatography.

resulting in termination of the nascent RNA product. Therefore, these nucleoside analogs are non-obligatory chain terminators despite the presence of a 3′-hydroxyl group.

Recently, β-D-2′-deoxy-2′-fluoro-2′-C-methylcytidine (PSI-6130) has been identified as a potent and selective inhibitor of HCV replication in the subgenomic replicon system with little or no cytotoxicity in various human cell lines or bone marrow precursor cells (16). The corresponding triphosphate of PSI-6130 is an inhibitor of HCV NS5B competitive with natural CTP (17). Conversion to the active 5′-triphosphate form by cellular kinases is an important part of the mechanism of action for nucleoside analogs. In this study we determined the metabolism of β-D-2′-deoxy-2′-fluoro-2′-C-methylcytidine in primary human hepatocytes isolated from several donors. We show that β-D-2′-deoxy-2′-fluoro-2′-C-methylcytidine was converted to β-D-2′-deoxy-2′-fluoro-2′-C-methylcytidine 5′-triphosphate and β-D-2′-deoxy-2′-fluoro-2′-C-methyluridine 5′-triphosphate via deamination of the phosphorylated cytidylates. Furthermore, we determined the kinetics of the formation of the two active triphosphates and the potency of the two triphosphates against the native HCV replicase and NS5B as well as the molecular mechanism of action of the two triphosphates.

EXPERIMENTAL PROCEDURES

Compounds—β-D-2′-Deoxy-2′-fluoro-2′-C-methylcytidine (PSI-6130) was provided by Pharmasset, Inc. (18). A stock solution of 10 mM PSI-6130 was prepared in Dulbecco's phosphate-buffered saline and stored at −20 °C. Tritium-labeled PSI-6130 was synthesized at Roche Palo Alto LLC. The tritiated compound was dissolved in 50% (v/v) ethanol at the concentration of 0.97 mCi/ml with a specific activity of 25.78 Ci/mmol. The stock solution was stored at −20 °C. The phosphorylated derivatives of PSI-6130, namely PSI-6130-MP, -DP, and -TP, were provided by Pharmasset, Inc. β-D-2′-Deoxy-2′-fluoro-2′-C-methyluridine (RO2433) was synthesized at Roche Palo Alto LLC. RO2433-MP, -DP, and -TP were synthesized by TriLink BioTechnologies (San Diego, CA). Compound stock solutions were prepared in nuclease-free H_2O and stored at −20 °C. 3′-dCTP was purchased from TriLink BioTechnologies.

Cell Culture of Primary Human Hepatocytes—Plated fresh human hepatocytes or hepatocyte suspensions were obtained either from CellzDirect, Inc. or from In Vitro Technologies, Inc. Fresh human hepatocytes obtained from each company were plated or cultured on 6-well collagen coated plates (BD Biosciences #356400) at 1.5 million cells per well using complete serum containing medium obtained from the respective companies. Cells were allowed to recover for at least 18 h before the addition of the compound. All incubations were carried out at 37 °C in a humidified 5% CO_2 atmosphere.

To determine the time course of uptake and phosphorylation of PSI-6130, human primary hepatocytes were incubated with ^{3}H-labeled PSI-6130 at a final concentration of 2 μM and 10 μCi/ml. The compound was added 72, 48, 24, 16, 6 and 1 h before cell harvesting. All time points and untreated cell controls were set up in duplicates.

To determine the dose response of the phosphorylation of PSI-6130, human primary hepatocytes were incubated with ^{3}H-labeled PSI-6130 at 0, 2, 10, 25, 50, 100, and 250 μM for 24 h. Final concentrations of PSI-6130 were achieved by supplementing ^{3}H-labeled PSI-6130 with non-radiolabeled PSI-6130. Duplicate cell samples were harvested after 24 h of incubation.

To determine the half-life of the triphosphates of PSI-6130 and RO2433, human primary hepatocytes were incubated for 24 h with ^{3}H-labeled PSI-6130 at 2 μM and 10 μCi/ml. The cell monolayer was washed once with the cell culture medium without PSI-6130 and then incubated with fresh medium without PSI-6130 at 0-, 0.5-, 1-, 2-, 4-, 6-, 8-, 24-, 48-, and 72-h time points after the removal of PSI-6130. Duplicate cell samples were set up for each time point. The viable cell numbers of the untreated cell controls for each experiment were determined at the end of the experiment using the trypan blue exclusion method.

Preparation of Cell Extract for High Performance Liquid Chromatography (HPLC) Analysis—At the time of cell harvest the cell culture medium was aspirated, and the cells were washed once with cold phosphate-buffered saline. The cells were scraped into 1 ml of pre-chilled 60% (v/v) methanol and extracted in methanol for 24 h at −20 °C. The extracted samples were then centrifuged at 10,000 × *g* for 15 min to remove cell debris. The supernatant was transferred to new tubes and evaporated in a speed vacuum at room temperature. The pellets were stored at −80 °C until analysis.

The dried pellets of cell extracts were dissolved in H_2O and filtered though a nanosep MF centrifugal device (Pall Life Sciences #ODM02C34). Before HPLC analysis, cell extract samples were spiked with unlabeled reference standards PSI-6130, RO2433, and their phosphorylated derivatives.

HPLC—The phosphorylated derivatives of PSI-6130 were separated by ion exchange HPLC with a Whatman Partisil 10 SAX (4.6 × 250 mm) column coupled to a radiometric detector (β-RAM, IN/US Systems, Inc.). The mobile phase gradient changed linearly from 0% buffer A (H_2O) to 100% buffer B (0.5 M KH_2PO_4 + 0.8 M KCl) between 4 and 8 min. 100% buffer B ran from 8 to 18 min and changed back to 100% A in 1 min. Buffer A ran until 25 min. The flow rate was 1 ml/min. A ratio of 5:1 Flo Scint IV or Ultima-Flo™ AP (PerkinElmer Life Sciences) to column eluent was used for the detection of radiolabeled species in the β-RAM detector (IN/US Systems, Inc.).

The separation of PSI-6130 and RO2433 was performed by reverse phase chromatography with a Zorbax SB-C8 column (4.6 × 250 mm, 5 μm) coupled to a radiometric detector (β-RAM). The gradient changed linearly from 100% buffer A (0.01 M heptane sulfonic acid, sodium salt, 0.1% (v/v) acetic acid in water) to 10% buffer B (0.01 M heptane sulfonic acid sodium salt, 0.1% (v/v) acetic acid in 1:1 methanol water) between 0 and 3 min and then changed linearly from 10% buffer B to 95% buffer B between 3 and 18 min. 95% buffer B ran from 18 to 22 min and changed back to 100% A in 0.1 min. Buffer A ran until 25 min. The flow rate was 1 ml/min. PSI-6130 and its intercellular metabolites were identified by comparison of the retention times of the intracellular species in the radiochromatogram with the retention times of nonradioactive reference standards spiked in the cell extract samples and detected by UV absorption at 270 nm.

Acid Phosphatase Treatment of Cell Extracts—Hepatocyte cell extracts were incubated with acid phosphatase (Sigma #P-0157) at a final concentration of 0.05 mg/ml (23.9 units/ml) at 37 °C for 2.5 h to dephosphorylate any phosphorylated metabolites of PSI-6130. After digestion the samples were analyzed by reversed phase HPLC.

HCV Replicon Assay—The 2209-23 cell line containing a bicistronic HCV subgenomic replicon (genotype 1b, Con1 strain), which expresses a Renilla luciferase reporter gene as an index of HCV RNA replication, has been described before (9). The analysis of inhibition of HCV replication by nucleoside analogs and IC_{50} determinations were performed as described (12).

HCV Replicase Assay—The membrane-associated native HCV replication complexes were isolated from 2209-23 replicon cell lines as described (6). The inhibition of the RNA synthesis activity of the HCV replicases by PSI-6130-TP was determined as described (6) except that 5 μl of cytoplasmic replicase complex (2.5×10^6 replicon cell equivalent) was added to a 20-μl reaction for 60 min. The inhibition of the RNA synthesis activity of the HCV replicases by RO2433-TP was determined in reactions containing 6.25 μl of cytoplasmic replicase complex (3.1×10^6 replicon cell equivalent), 50 mM HEPES, pH 7.5, 10 mM KCl, 10 mM dithiothreitol, 5 mM $MgCl_2$, 20 μg/ml actinomycin D, 1 mM ATP, GTP, and CTP, 24 μCi of (0.4 μM) [α-^{33}P]UTP (PerkinElmer #NEG607H), 1 units/μl SUPERase. In (Ambion), 10 mM creatine phosphate, 200 μg/ml creatine phosphokinase with or without the nucleotide triphosphate inhibitor in a final volume of 20 μl for 90 min.

HCV Polymerase Assay—The inhibition potency of PSI-6130-TP on the RNA-dependent RNA polymerase activity of recombinant NS5B570-Con1 (genotype 1b, GenBank™ accession number AJ242654) was measured as the incorporation of radiolabeled nucleotide monophosphate into acid-insoluble RNA products as described (6) with the following modifications; IC_{50} determinations were carried out using 200 nM *in vitro* transcribed complementary internal ribosome entry site RNA template, 1 μCi of tritiated UTP (42 Ci/mmol), 500 μM ATP, 500 μM GTP, 1 μM CTP, 1× TMDN buffer (40 mM Tris-HCl, pH 8.0, 4 mM $MgCl_2$, 4 mM dithiothreitol, 40 mM NaCl) and 200 nM NS5B570-Con1. The inhibition potency of RO2433-TP was determined as described above with the following modification of NTP concentrations: 1 μCi of tritiated CTP (39 Ci/mmol), 500 μM ATP, 500 μM GTP, 1 μM UTP. The compound concentration at which the enzyme-catalyzed rate is reduced by 50% (IC_{50}) was calculated using equation,

$$Y = \%\ \text{Min} + \frac{(\%\ \text{Max} - \%\ \text{Min})}{\left(1 + \frac{X}{(IC_{50})}\right)} \qquad \text{(Eq. 1)}$$

where Y corresponds to the relative enzyme activity, % Min is the residual relative activity at saturating compound concentration, % Max is the relative maximum enzymatic activity, and X corresponds to the compound concentration.

The apparent Michaelis constants ($K_{m(app)}$) for UTP or CTP were measured using assay conditions above with the following modifications; $K_{m(app)}$ for CTP was measured using 2 μCi of tritiated UTP (0.93 μM), 4.07 μM unlabeled UTP, 50 μM ATP, 50 μM GTP, and 5 nM to 50 μM CTP; $K_{m(app)}$ for UTP was measured using 2 μCi of tritiated CTP (1.67 μM), 3.33 μM unlabeled CTP, 50 μM ATP, 50 μM GTP, and 5 nM to 50 μM UTP. Apparent $K_{m(app)}$ values were calculated by nonlinear fitting using Equation 2,

$$Y = \frac{(V_{max(app)})X}{K_{m(app)} + X} \qquad \text{(Eq. 2)}$$

where Y corresponds to the rate of RNA synthesis by NS5B, $V_{max(app)}$ is the maximum rate or RNA synthesis at saturating substrate concentration, and X corresponds to CTP or UTP concentration.

K_i values were derived from the Cheng-Prusoff Equation (Equation 3) for competitive inhibition,

$$K_i = \frac{IC_{50}}{\left(1 + \frac{[NTP]}{K_{m(app)}}\right)} \qquad \text{(Eq. 3)}$$

where [NTP] is the concentration of CTP or UTP, and $K_{m(app)}$ is the apparent Michaelis constant for CTP or UTP. Mean K_i values were averaged from independent measurements at 0.2, 1, 5, and 25 μM CTP or UTP concentrations in triplicate experiments.

Gel-based Nucleotide Incorporation Assay—The RNA template-directed nucleotide incorporation and extension of nucleotide triphosphates and nucleotide triphosphate analogs by HCV polymerase was performed with a 19-nucleotide RNA oligo (5′-AUGUAUAAUUAUUGUAGCC-3′) under assay conditions as described (9). The incorporation and extension of CTP and CTP analogs were determined with 5′-end-radiolabeled GG primer and nucleotide triphosphates at the indicated concentrations. The incorporation and extension of UTP and UTP analogs were performed similarly with the same RNA oligo template, 5′-end-radiolabeled GGC primer and nucleotide triphosphates at the indicated concentrations. The radiolabeled RNA products were separated on a TBE-urea acrylamide gel and analyzed using phosphorimaging (GE Healthcare).

RESULTS

Metabolic Profile of PSI-6130—Cellular extracts from primary human hepatocytes incubated with tritium-labeled β-D-2′-deoxy-2′-fluoro-2′-C-methylcytidine (PSI-6130) were resolved by ion exchange HPLC. PSI-6130 and metabolites derived from PSI-6130 were identified by comparing the retention times of radiolabeled species with the retention times of unlabeled reference compounds (Fig. 1*A*). As shown in Fig. 1*B*, PSI-6130 (3.0 min) and the 5′-phosphorylated derivatives PSI-6130-DP (13.2 min) and PSI-6130-TP (16.8 min) were identified in human hepatocytes incubated with PSI-6130. In addition, metabolites with retention times corresponding to those of the deaminated product of PSI-6130, β-D-2′-deoxy-2′-fluoro-2′-C-methyluridine (RO2433, 3.8 min) and its corresponding 5′-phosphates RO2433-DP (12.5 min) and RO2433-TP (14.8 min), were detected (Fig. 1*B*). The monophosphates of the cytidine and uridine analogs PSI-6130-MP and RO2433-MP were not separated sufficiently under the

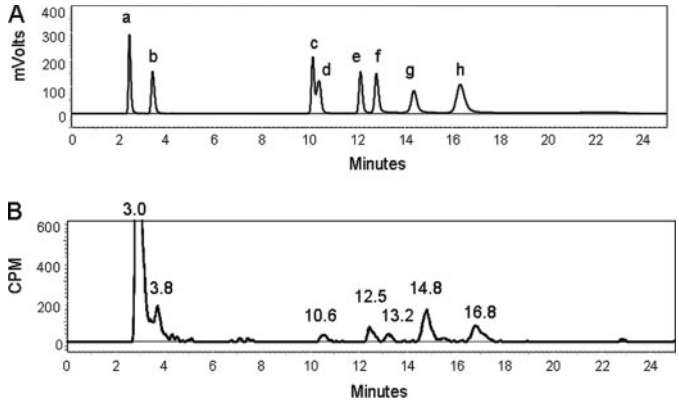

FIGURE 1. **Ion exchange HPLC profile of an extract of primary human hepatocytes incubated with PSI-6130.** *A*, HPLC separation and retention times of reference compounds PSI-6130 (*a*, 2.5 min), RO2433 (*b*, 3.4 min), RO2433-MP (*c*, 10.2 min), PSI-6130-MP (*d*, 10.4 min), RO2433-DP (*e*, 12.2 min), PSI-6130-DP (*f*, 12.8 min), RO2433-TP (*g*, 14.4 min), and PSI-6130-TP (*h*, 16.3 min). *B*, HPLC profile of an extract from hepatocytes incubated with ^{3}H-labeled PSI-6130 at 2 μM for 24 h. The retention times in min of the intracellular species are indicated *above* the *radioactive peaks*. Intracellular PSI-6130 and its metabolites were identified by comparing the retention times of the radioactive peaks with those of the UV absorption peaks of reference compounds. There is a calibrated 0.3–0.4-min delay in the retentions times of radioactive trace compared with those of UV trace due to sample traveling from UV detector to radiometric detector.

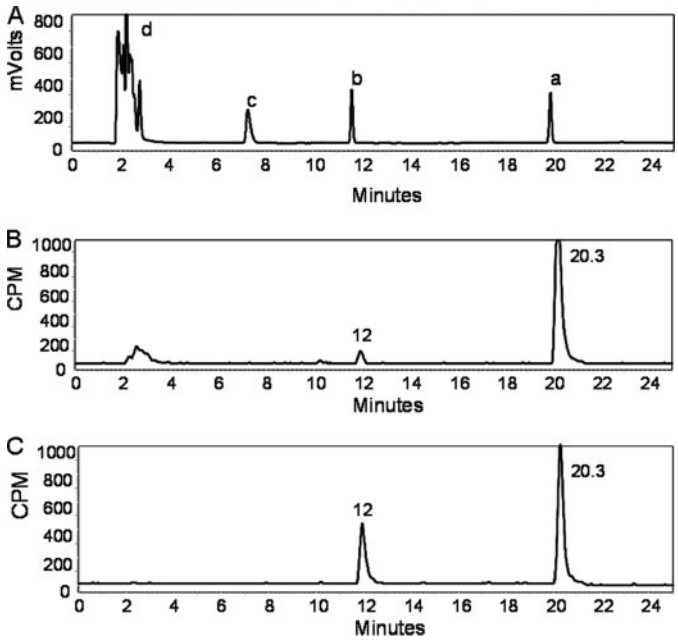

FIGURE 2. **Identification of PSI-6130 and its metabolites by reversed phase HPLC and acid phosphatase treatment.** *A*, reversed phase HPLC separation of standard compounds: PSI-6130 (*a*, 20 min), RO2433 (*b*, 11.7 min), PSI-6130-MP (*c*, 7.5 min), (*d*, PSI-6130-DP, PSI-6130-TP, RO2433-MP, RO2433-DP, and RO2433-TP). *B*, reversed phase HPLC profile of an extract from hepatocytes incubated with 25 μM extracellular PSI-6130 for 24 h. *C*, reversed phase HPLC profile of the same extract after acid phosphatase treatment.

chromatography conditions and, therefore, co-eluted as a single radioactive peak at 10.6 min. It has been reported that 2′-O-methylcytidine was extensively metabolized to CTP and UTP due to deamination coupled with demethylation of the 2′-substituent or base swapping after glycosidic bond cleavage (19). None of the intracellular metabolites of PSI-6130 was eluted with retention time corresponding to those of 2′-C-methyl-CTP, CTP, and UTP (data not shown). Therefore, there was no evidence for metabolism of PSI-6130 at the 2′-position or evidence for hydrolysis at the glycosidic bond. These data suggest that the primary routes of PSI-6130 metabolism in human hepatocytes were phosphorylation at the 5′-position and deamination at the base.

The hepatocyte extracts were also analyzed by reversed phase HPLC to identify unphosphorylated metabolites of PSI-6130. Two unphosphorylated species with retention times corresponding to PSI-6130 (20.3 min) and its uridine metabolite RO2433 (11.9 min) were observed, with PSI-6130 being the predominant intracellular species (Fig. 2*B*). There was no evidence of formation of uracil, uridine, cytosine, cytidine, or 2′-deoxycytidine (data not shown). These data agree well with the ion exchange HPLC analysis result and suggest the absence of metabolism at the 2′ position and at the glycosidic bond. The phosphorylated metabolites, with the exception of PSI-6130-MP, were not resolved by reversed phase HPLC and co-eluted early as a single broad peak (Fig. 2, *A*, and B). Acid phosphatase treatment completely converted all the intracellular metabolites to PSI-6130 and RO2433. Therefore, all detected intracellular metabolites represent phosphorylated derivatives of PSI-6130 and RO2433. These results established that PSI-6130 could be phosphorylated to its pharmacologically active 5′-triphosphate analog and that PSI-6130 and/or its phosphates could be deaminated to the corresponding uridine analogs in primary human hepatocytes.

In Vitro Potency of RO2433 and RO2433-TP—As described above, incubation of human hepatocytes with PSI-6130 resulted in the formation of substantial concentrations of the triphosphate of its uridine analog, RO2433-TP. Next we determined whether the PSI-6130-derived uridine analog RO2433 could inhibit HCV replication targeting NS5B polymerase. Huh7 cells containing a subgenomic genotype 1b Con1 strain HCV replicon were incubated with RO2433 or PSI-6130 for 72 h, and dose-dependent inhibition of luciferase reporter activity was determined. RO2433 did not inhibit the HCV replication in the HCV subgenomic replicon system at concentrations up to 100 μM, whereas PSI-6130 inhibited HCV replication with a mean IC_{50} of 0.6 μM under the same assay conditions (Table 1). The lack of potency in the replicon could be related to inefficient compound phosphorylation. To address whether the triphosphate of RO2433 directly inhibits the HCV RNA polymerase, the RNA synthesis activity of the native membrane-associated HCV replication complexes isolated from the same replicon cells was tested in the presence of RO2433-TP. RO2433-TP inhibited the RNA synthesis activity of HCV replicase with a mean IC_{50} of 1.19 μM, whereas PSI-6130-TP inhibited HCV replicase with a mean IC_{50} of 0.34 μM (Table 1). RO2433-TP also inhibited the RNA synthesis activity of the recombinant HCV Con1 NS5B on a heteropolymeric RNA template derived from the 3′-end of the negative strand of the HCV genome with an IC_{50} of 0.52 μM and K_i of 0.141 μM, as compared with an IC_{50} of 0.13 μM and K_i of 0.023 μM for PSI-6130-TP under the same assay conditions (Table 1). These results established that both RO2433-TP and PSI-6130-TP are intrinsically potent inhibitors of RNA synthesis by HCV polymerase.

TABLE 1

Potency of PSI-6130 and RO2433 and their 5′-triphosphates

Values presented are the mean ± S.D. from greater than three independent experiments. ND, not determined.

Compound	IC_{50}			K_i NS5B GT1b	K_m NS5B GT1b
	Replicon GT1b	NS5B GT1b	Replicase GT1b		
		μM		μM	μM
PSI-6130	0.6 ± 0.04	ND	ND	ND	ND
RO2433	>100	ND	ND	ND	ND
PSI-6130-TP	ND	0.13 ± 0.01	0.34 ± 0.1	0.023 ± 0.002	0.22 (CTP)
RO2433-TP	ND	0.52 ± 0.11	1.19 ± 0.09	0.141 ± 0.03	0.37 (UTP)

To investigate the molecular mechanism of HCV polymerase inhibition by PSI-6130-TP and RO2433-TP, we measured their incorporation and chain -termination properties. The incorporation of nucleotide and nucleotide analogs by HCV polymerase NS5B was determined in a gel-based assay using a short RNA template (Fig. 3*A*). Incorporation of CMP, PSI-6130-MP, and 3′-dCMP was initiated from a ^{33}P-labeled GG primer (Fig. 3*A*). Incorporated CMP (Fig. 3*B*, *lane 6*) could be further extended in the presence of the next nucleotide UTP (Fig. 3*B*, *lanes 7–10*). PSI-6130-TP and 3′-dCTP could also serve as substrates for HCV NS5B and were incorporated into the nascent RNA product (Fig. 3*B*, *lane 11* and *16*). After incorporation of PSI-6130-MP or 3′-dCMP, further extension in the presence of the next nucleotide UTP was completed blocked even when UTP was present at concentrations up to 1 mM (Fig. 3*B*, *lane 12–15* and *lanes 16–20*, respectively). Incorporation of UMP, RO2433-MP, and 3′-dUMP was initiated from a ^{33}P-labeled GGC primer using the same RNA template (Fig. 3*A*). Incorporated UMP (Fig. 3*C*, *lane 6*) could be further extended in the presence of the next nucleotide ATP (Fig. 3*C*, *lanes 7–10*). Full-length RNA product was observed in the presence of UTP and ATP but absence of CTP (Fig. 3*C*, *lanes 7–10*), possibly due to misincorporation by NS5B through G-U wobble base-pairing. HCV NS5B was also able to incorporate RO2433-MP (Fig. 3*C*, *lane 11*) and 3′-dUMP (Fig. 3*C*, *lane 16*) but was unable to further extend the incorporated RO2433-MP and 3′-dUMP in the presence of the next nucleotide ATP (Fig. 3*C*, *lanes 12–15* and *lanes 17–20*, respectively). The control samples with GG primer and UTP-only as well as GGC primer and ATP-only did not lead to further extension of the primers, suggesting the incorporation of PSI-6130-MP and RO2433-MP was base-specific. Taken together, these results demonstrate that PSI-6130-TP and RO2433-TP serve as alternative substrates for HCV NS5B and act as functional chain terminators once incorporated into nascent RNA. While this manuscript was under preparation it was reported that the incorporation of PSI-6130-MP into the nascent RNA by HCV polymerase led to chain termination, in agreement with our observations (17).

Kinetics of Phosphorylation of PSI-6130 in Primary Human Hepatocytes—To determine the steady state level of the two triphosphates in hepatocytes after exposure to PSI-6130, primary human hepatocytes from 4 different donors were incubated with 2 μM PSI-6130 for up to 72 h. The uptake of PSI-6130 by human hepatocytes was fast, and total intracellular activity reached steady state levels at 1 h of PSI-6130 incubation, the earliest time point in this study (Table 2). PSI-6130-TP was detectable in hepatocytes from all 4 donors at 6 h after PSI-6130 incubation and increased with time to reach steady state levels

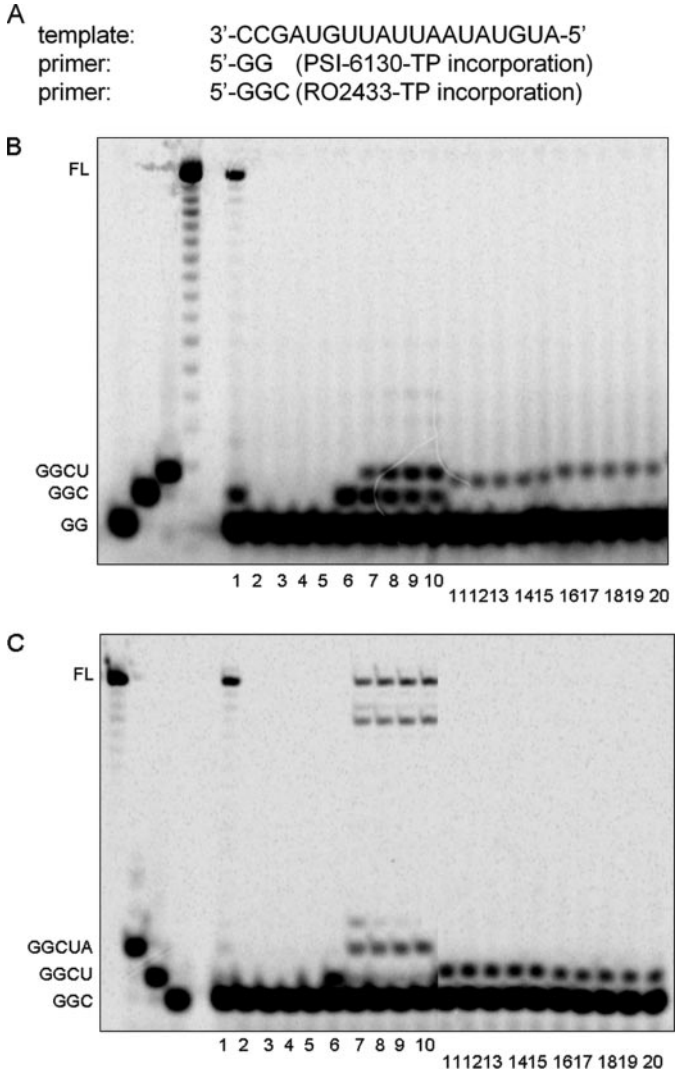

FIGURE 3. **PSI-6130-TP and RO2433-TP are substrates of HCV polymerase and chain-terminators.** *A*, sequence of the RNA template and primers. *B*, the incorporation of CTP and CTP analogs was initiated with GG primer according as described under "Experimental Procedures." The nucleotide triphosphates included in the reactions were as follows: 10 μM CTP, ATP, and UTP (*lane 1*); 2, 10, 100, and 1000 μM UTP (*lanes 2–5*); 10 μM CTP without UTP (*lane 6*) or with 2, 10, 100, and 1000 μM UTP (*lanes 7–10*); 10 μM PSI-6130-TP without UTP (*lane 11*) or with 2, 10, 100, and 1000 μM UTP (*lanes 12–15*); 10 μM 3′-dCTP without UTP (*lane 16*) or with 2, 10, 100, and 1000 μM UTP (*lane 17–20*). *FL*, full-length. *C*, incorporation of UTP and UTP analogs initiated with GGC primer. The nucleotide triphosphates included in the reactions were as follows: 100 μM CTP, ATP, and UTP (*lane 1*); 20, 50, and 200 μM ATP (*lanes 2–5*); 100 μM UTP without ATP (*lane 6*) or with 2, 20, 50, and 200 μM ATP (*lanes 7–10*); 100 μM RO2433-TP without ATP (*lane 11*) or with 20, 50, and 200 μM ATP (*lanes 12–15*); 100 μM 3′-dUTP without ATP (*lane 16*) or with 20, 50, and 200 μM ATP (*lanes 17–20*).

TABLE 2

Time course of uptake and phosphorylation of PSI-6130

Levels of total intracellular species, PSI-6130-TP, and RO2433-TP were determined in hepatocytes incubated with 2 μM PSI-6130 for the indicated lengths of time. Data shown are mean ± S.D. derived from four independent experiments using hepatocytes from four donors. BQL, below the quantification limit.

Time	Total intracellular species	PSI-6130-TP	RO2433-TP
h	*pmol/million cells*	*pmol/million cells*	*pmol/million cells*
1	30.4 ± 18.8	BQL	BQL
6	32.9 ± 16.8	0.6 ± 0.5	0.3 ± 0.01[a]
16	34.4 ± 20.8	0.7 ± 0.6	0.6 ± 0.3
24	36.4 ± 21.3	1.1 ± 0.7	1.3 ± 1.1
48	31.9 ± 17.1	1.3 ± 0.6	2.0 ± 1.1
72	34.6 ± 15.4	1.0 ± 0.4	2.0 ± 1.3

[a] Mean ± S.D. derived from 2 donors as RO2433-TP was only detectable in 2 out of 4 donors at the 6-h time point.

at 24–48 h. The formation of the triphosphate of the uridine metabolite, RO2433-TP, demonstrated a delayed time course relative to that of PSI-6130-TP. RO2433-TP was detectable in hepatocytes from only 2 of 4 donors at 6 h and in hepatocytes of all 4 donors at 16 h. RO2433-TP formation reached steady state levels at 48–72 h. RO2433-TP concentrations were lower than those of PSI-6130-TP at time points earlier than 16 h but surpassed those of PSI-6130-TP at 24 h and beyond. The mean steady state level concentrations of PSI-6130-TP and RO2433-TP after incubation with 2 μM PSI-6130 were 1.3 ± 0.6 and 2.0 ± 1.1 pmol/10^6 cells at 48 h, respectively. Unchanged PSI-6130 was the major intracellular species at all time points tested after incubation of human hepatocytes with radiolabeled PSI-6130 (Fig. 4).

To determine whether increasing the exposure of hepatocytes to PSI-6130 will lead to increased uptake of PSI-6130 and formation of PSI-6130-TP and RO2433-TP, primary human hepatocytes from 3 different donors were incubated with PSI-6130 at different concentrations up to a maximal concentration of 250 μM for 24 h. Total intracellular species, determined by total intracellular radioactivity in the cell extracts, increased linearly with the extracellular PSI-6130 up to 250 μM (Table 3). The mean total intracellular species at 250 μM extracellular PSI-6130 reached 3591 pmol/10^6 cells, with the unphosphorylated PSI-6130 being the predominant species (data not shown). The formation of both PSI-6130-TP and RO2433-TP increased linearly with the extracellular PSI-6130 concentrations up to 100 μM (Fig. 5). The concentrations of PSI-6130-TP and RO2433-TP at 100 μM extracellular PSI-6130 were 34.3 and 71.2 pmol/10^6 cells, respectively. At 250 μM extracellular PSI-6130, PSI-6130-TP and RO2433-TP levels were slightly increased over the levels obtained at 100 μM extracellular PSI-6130, but the relationship was no longer linear. PSI-6130-TP and RO2433-TP were the major intracellular phosphorylated species at all extracellular PSI-6130 concentrations (Table 3), suggesting a high capacity of the hepatocytes to convert the parent compound to the triphosphate forms and no accumulation of either mono- or diphosphates. At all concentrations of extracellular PSI-6130, RO2433-TP levels were higher than those of PSI-6130-TP.

Intracellular Stability of PSI-6130-TP and RO2433-TP—Primary human hepatocytes from 4 different donors were incubated for 24 h with 2 μM PSI-6130, at which point extracellular PSI-6130 was removed, and the concentrations of intracellular

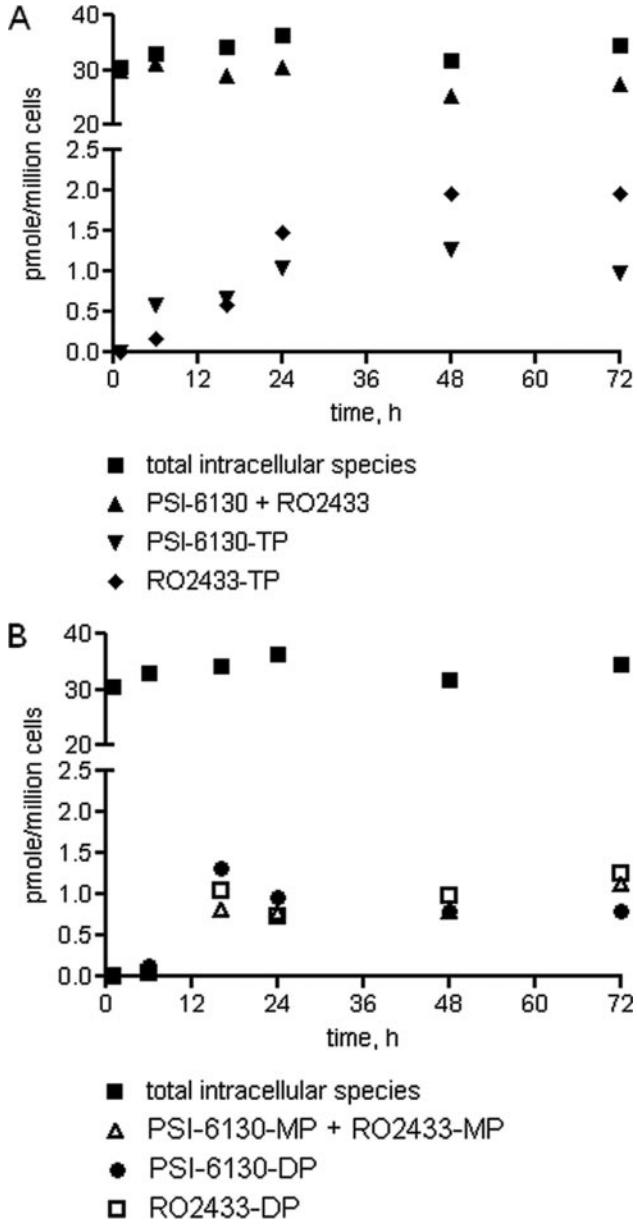

FIGURE 4. **Time course of uptake and metabolism of PSI-6130.** *A*, time course of uptake of PSI-6130 and formation of PSI-6130-TP and RO2433-TP. *B*, time course of formation of PSI-6130-MP + RO2433-TP, PSI-6130-DP, and RO2433-TP. The data show mean values from four experiments performed with primary hepatocytes from four donors.

PSI-6130-TP and RO2433-TP were quantified at different time points up to 72 h (see "Experimental Procedures").

The PSI-6130-TP level remained constant up to 1 h after the removal of extracellular PSI-6130. Thereafter, PSI-6130-TP decreased after a single-phase exponential decay kinetics pattern with a mean half-life of 4.7 ± 0.6 h (Fig. 6, Table 4). The PSI-6130-TP level was below quantification limit at 48 h after the removal of extracellular PSI-6130. The RO2433-TP level increased slightly to reach steady state level at 1–2 h after extracellular PSI-6130 removal and remained at steady state level for up to 6 h before decreasing toward base-line levels. RO2433-TP decreased with a mean half-life of 38.1 ± 16.1 h (Fig. 6, Table 4). The levels of unphosphorylated species PSI-6130 and RO2433 decreased rapidly after removal of extracellular RO1656 (data

TABLE 3

Dose response of uptake and phosphorylation of PSI-6130

Levels of total intracellular species, PSI-6130-TP, and RO2433-TP were determined in hepatocytes incubated with 2, 10, 25, 50, 100, or 250 μM PSI-6130 for 24 h. Data shown are the mean ± S.D. of values of three experiments using hepatocytes from three donors. BQL, below the quantification limit.

Extracellular PSI-6130	Total intracellular species	PSI-6130-MP + RO2433-MP	PSI-6130-DP	RO2433-DP	PSI-6130-TP	RO2433-TP
μM	*pmol/million cells*	*pmol/million cells*	*pmol/million cells*	*pmol/million cells*	*pmol/million cells*	*pmol/million cells*
2	34.5 ± 9.8	0.3 + 0.3	0.3 + 0.4	0.9 ± 0.7	1.4 ± 1.3	2.4 ± 1.5
10	165.5 ± 38.2	1.1 + 1.0	2.4 + 2.7	4.8 ± 2.7	5.6 ± 5.8	9.6 ± 6.4
25	439.1 ± 150.4	5.0 + 5.9	3.4 + 4.3	9.5 ± 4.9	11.3 ± 13.0	21.7 ± 13.4
50	847.9 ± 327.7	8.2 + 8.2	8.2 + 7.3	20.3 ± 12.2	18.7 ± 17.7	34.9 ± 17.2
100	1529.9 ± 413.1	3.4 + 5.9	13.4 + 15.4	34.7 ± 25.0	34.3 ± 36.3	71.2 ± 14.7
250	3591.3 ± 1102.5	BQL	19.6 + 19.6	40.9 ± 13.2	40.4 ± 35.4	84.0 ± 28.7

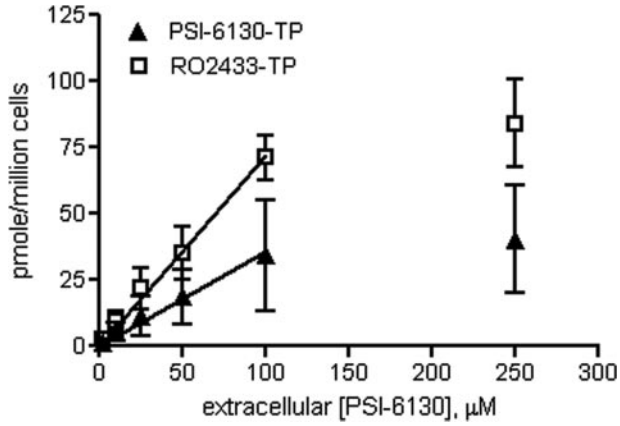

FIGURE 5. **Dose response of the formation of PSI-6130-TP and RO2433-TP.** Primary human hepatocytes were incubated with PSI-6130 for 24 h at 2, 10, 25, 50, 100, 250 μM concentrations. The data plotted represent the mean values and S.D. of experiments with hepatocytes from three donors.

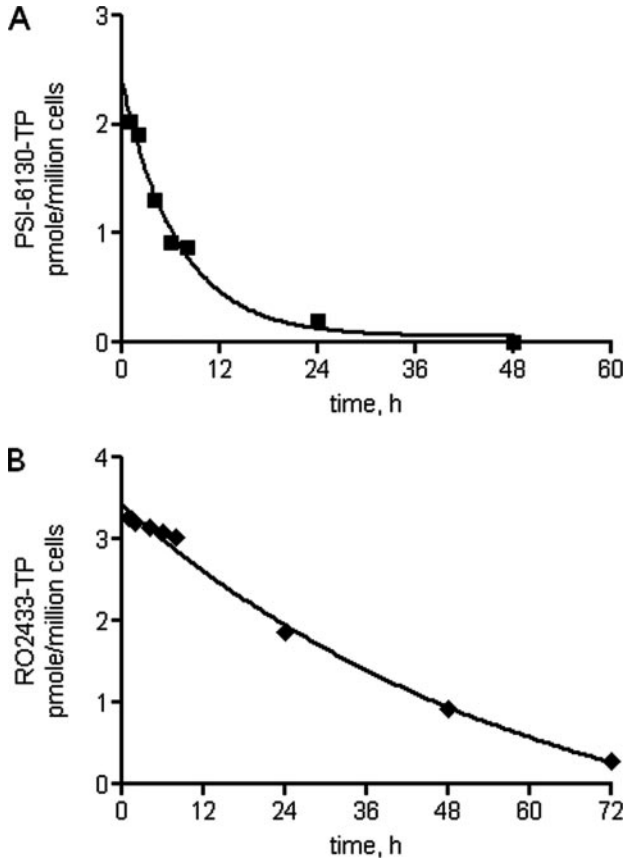

FIGURE 6. **Intracellular stability of PSI-6130-TP and RO2433-TP.** Primary human hepatocytes were incubated with PSI-6130 at 2 μM for 24 h, at which point extracellular PSI-6130 was removed, and PSI-6130-TP and RO2433-TP levels were determined at 1, 2, 4, 6, 8, 24, 48, and 72 h after extracellular PSI-6130 removal. Representative kinetic profiles of PSI-6130-TP (*A*) and RO2433-TP (*B*) elimination in hepatocytes from one donor are shown.

not shown). Unphosphorylated PSI-6130 and RO2433 were reduced by more than half within 0.5 h after extracellular PSI-6130 removal, suggesting rapid equilibration of uncharged nucleoside analogs across the hepatocyte cell membrane.

DISCUSSION

The antiviral activity of a nucleoside inhibitor is the combined outcome of uptake of the nucleoside into the host cells, conversion of the nucleoside to the active triphosphate, intracellular stability of the triphosphate, and the ability of the triphosphate form to interfere with the RNA synthesis activity of the viral polymerase. In this study we performed experiments to address each of the steps involved in the mechanism of action of β-D-2′-deoxy-2′-fluoro-2′-C-methylcytidine.

The analysis of radiolabeled PSI-6130 incubated with primary human hepatocytes demonstrated the conversion of PSI-6130 into its 5′-phosphorylated derivatives (mono-, di-, and triphosphate) and to the uridine analog R2433 and its corresponding 5′-phosphorylated derivatives. Unmodified PSI-6130 remained the major intracellular species at all time points studied. These results suggest that PSI-6130 is only subjected to intracellular 5′-phosphorylation and base deamination, with no evidence for metabolism at other positions. In contrast, another cytidine analog, 2′-O-methylcytidine, demonstrated intracellular conversion with significant efficiency to CTP and UTP instead of 2′-O-methyl-CTP due to deamination combined with extensive demethylation at the 2′ position or base swapping after deglycosylation (8, 19). The intracellular instability of 2′-O-methylcytidine most likely accounts for the poor potency of this cytidine analog in the HCV replicon system (8, 19). Importantly, both triphosphates of PSI-6130 and RO2433 were potent inhibitors of the RNA synthesis activity of the native HCV replicase and recombinant NS5B (Table 1), suggesting

TABLE 4

Intracellular half-life of PSI-6130-TP and RO2433-TP

The half-life ($t_{1/2}$) values were calculated by nonlinear fitting of intracellular triphosphate concentrations to a single phase exponential decay equation. $t_{1/2}$ was defined as the time needed for the triphosphates to be reduced to 50% that of the highest level of the triphosphates after extracellular parent compound removal. Data shown are the mean ± S.D. of values of four independent experiments using hepatocytes from four donors.

$t_{1/2}$, h (mean ± S.D. (n = 4))	
PSI-6130-TP	RO2433-TP
4.7 ± 0.6	38 ± 16

that the antiviral potency of PSI-6130 is not compromised by intracellular deamination.

The formation of the active triphosphates PSI-6130-TP and RO2433-TP increased with time and reached steady levels at 48 h after exposure to parent PSI-6130. The mean steady state intracellular concentrations of PSI-6130-TP and RO2433-TP at 2 μM extracellular PSI-6130 were 1.3 and 2.0 pmol/10^6 cells, respectively (Table 2). These values are equivalent to about 0.43 μM for PSI-6130-TP and 0.67 μM for RO2433-TP, based on a 3-μl volume of normal human liver parenchymal cells as determined by the stereological method (20). These steady state concentrations are above the K_i values of PSI-6130-TP (0.023 μM) and RO2433-TP (0.141 μM) determined for HCV NS5B polymerase and are in good agreement with the observed submicromolar IC_{50} of PSI-6130 in the HCV replicon system (Table 1).

Among the phosphorylated species of PSI-6130 and RO2433, the rank of the phosphate levels from highest to lowest intracellular concentration was: triphosphates > diphosphates > monophosphates at all time points of compound incubation and all extracellular concentrations (Fig. 4, Table 3). This suggests that the first cellular activation step to monophosphate is the rate-limiting step among the three phosphorylation steps. Recently, it has been reported that the human cellular kinases responsible for the sequential three-step phosphorylation of PSI-6130 to triphosphate are 2′-deoxycytidine kinase, uridine/cytidine monophosphate kinase, and uridine/cytidine diphosphate kinase (17). In that study the efficiency of PSI-6130 as a substrate for 2′-deoxycytidine kinase was reported to be almost 2 orders of magnitude lower than that of PSI-6130-MP and PSI-6130-DP for their respective kinases. Therefore, the results of the reported substrate efficiencies of the three kinases involved in PSI-6130-TP formation agree well with relative formation of PSI-6130 phosphates in human hepatocytes. Importantly, the intracellular concentrations of PSI-6130-TP and RO2433-TP demonstrated an excellent linear relationship with the extracellular concentration of PSI-6130 up to 100 μM, demonstrating a high capacity of human hepatocytes to form the biologically active triphosphates from PSI-6130.

Despite the intrinsic potency of RO2433-TP against HCV polymerase, RO2433 was not active in the HCV replicon system at concentrations up to 100 μM. RO2433 was either not phosphorylated in the replicon cells or could not penetrate the cell membrane. However, RO2433, when formed intracellularly from radiolabeled PSI-6130, dissociated rapidly across the cell membrane with a half-life faster than 30 min. Therefore, RO2433 is most likely not efficiently phosphorylated to form RO2433-MP. Similarly, the uridine analog of the HCV replication inhibitor R1479 (4′-azidocytidine) was inactive in the replicon system. However, when delivered as a monophosphate prodrug, 4′-azidouridine could be converted into a potent inhibitor of HCV replication, demonstrating that a block of monophosphate formation resulted in lack of antiviral activity of 4′-azidouridine (21). Assuming a likely block of RO2433 phosphorylation to its monophosphate, RO2433-MP in human hepatocytes was most likely formed through the deamination of PSI-6130-MP by the cellular dCMP deaminase and subsequently further phosphorylated to RO2433-DP and -TP by uridine/cytidine monophosphate kinase and possibly nucleoside diphosphate kinase (22–24). The proposed metabolic pathway for PSI-6130 is illustrated in Fig. 7.

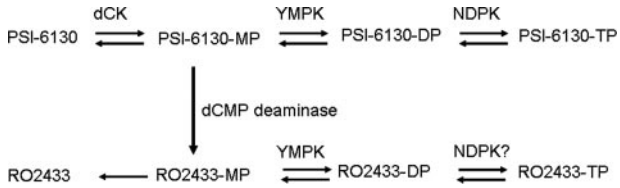

FIGURE 7. **Proposed metabolic pathway of PSI-6130.** Enzymes involved in the phosphorylation and deamination catalysis: deoxycytidine kinase (*dCK*), uridine/cytidine monophosphate kinase (*YMPK*), nucleoside diphosphate kinase (*NDPK*), deoxycytidylate deaminase (*dCMP deaminase*).

Using a primer-directed nucleotide incorporation assay mediated by HCV NS5B, we demonstrated that the incorporation of both PSI-6130-MP and RO2433-MP resulted in the complete blockage of the next nucleotide incorporation similar to that of the obligatory chain terminator 3′-dCMP and 3′-dUMP (Fig. 3, *B* and *C*). Therefore, the 2′-C-methyl-2′-Fluoro motif resulted in functional chain terminators on the respective uridine and cytidine analogs. It has been proposed that the chain termination activity of 2′-C-methyl nucleotide analogs is related to a steric clash of the 2′-methyl group with the ribose of the next incoming nucleotide substrate based on modeling of the NS5B initiation complex from bacteriophage Φ6 RNA-dependent RNA polymerase and NS5B crystal structures (10, 25). Similar steric hindrance could occur with PSI-6130-TP and RO2433-TP after incorporation due to the presence of the 2′-C-methyl group.

PSI-6130 is a potent and highly selective nucleoside inhibitor of HCV replication targeting NS5B polymerase in the HCV replicon system. Here we demonstrated that PSI-6130 was converted to two pharmacologically active triphosphate species, PSI-6130-TP and its uridine analog RO2433-TP, in primary human hepatocytes. It is worth noting that RO2433-TP demonstrated greater intracellular stability ($t_{1/2}$ = 38 h) as compared with PSI-6130-TP ($t_{1/2}$ = 4.7 h). PSI-6130 has recently entered clinical development for the treatment of HCV infected patients. The longer intracellular half-life of RO2433-TP may have pharmacologic relevance for maintaining more constant concentrations of the antiviral triphosphate over the dosing period in clinical studies.

Acknowledgments—We thank Dr. Jim Barnett and group members for providing recombinant NS5B protein and Nixy Zutshi and group members for providing HCV replicon cells. We thank Dr. Phillip A. Furman at Pharmasset, Inc. for providing PS1–6130 and the phosphorylated derivatives of PSI-6130.

REFERENCES

1. Alter, M. J., Kruszon-Moran, D., Nainan, O. V., McQuillan, G. M., Gao, F., Moyer, L. A., Kaslow, R. A., and Margolis, H. S. (1999) *N. Engl. J. Med.* **341,** 556–562
2. Manns, M. P., McHutchison, J. G., Gordon, S. C., Rustgi, V. K., Shiffman, M., Reindollar, R., Goodman, Z. D., Koury, K., Ling, M., and Albrecht, J. K. (2001) *Lancet* **358,** 958–965
3. Fried, M. W., Shiffman, M. L., Reddy, K. R., Smith, C., Marinos, G., Goncales, F. L., Jr., Haussinger, D., Diago, M., Carosi, G., Dhumeaux, D., Craxi, A., Lin, A., Hoffman, J., and Yu, J. (2002) *N. Engl. J. Med.* **347,** 975–982
4. Hadziyannis, S. J., Sette, H., Jr., Morgan, T. R., Balan, V., Diago, M., Mar-

cellin, P., Ramadori, G., Bodenheimer, H., Jr., Bernstein, D., Rizzetto, M., Zeuzem, S., Pockros, P. J., Lin, A., and Ackrill, A. M. (2004) *Ann. Intern. Med.* **140,** 346–355
5. Hardy, R. W., Marcotrigiano, J., Blight, K. J., Majors, J. E., and Rice, C. M. (2003) *J. Virol.* **77,** 2029–2037
6. Ma, H., Leveque, V., De Witte, A., Li, W., Hendricks, T., Clausen, S. M., Cammack, N., and Klumpp, K. (2005) *Virology* **332,** 8–15
7. Vivet-Boudou, V., Didierjean, J., Isel, C., and Marquet, R. (2006) *Cell. Mol. Life Sci.* **63,** 163–186
8. Carroll, S. S., Tomassini, J. E., Bosserman, M., Getty, K., Stahlhut, M. W., Eldrup, A. B., Bhat, B., Hall, D., Simcoe, A. L., LaFemina, R., Rutkowski, C. A., Wolanski, B., Yang, Z., Migliaccio, G., De Francesco, R., Kuo, L. C., MacCoss, M., and Olsen, D. B. (2003) *J. Biol. Chem.* **278,** 11979–11984
9. Klumpp, K., Leveque, V., Le Pogam, S., Ma, H., Jiang, W. R., Kang, H., Granycome, C., Singer, M., Laxton, C., Hang, J. Q., Sarma, K., Smith, D. B., Heindl, D., Hobbs, C. J., Merrett, J. H., Symons, J., Cammack, N., Martin, J. A., Devos, R., and Najera, I. (2006) *J. Biol. Chem.* **281,** 3793–3799
10. Migliaccio, G., Tomassini, J. E., Carroll, S. S., Tomei, L., Altamura, S., Bhat, B., Bartholomew, L., Bosserman, M. R., Ceccacci, A., Colwell, L. F., Cortese, R., De Francesco, R., Eldrup, A. B., Getty, K. L., Hou, X. S., LaFemina, R. L., Ludmerer, S. W., MacCoss, M., McMasters, D. R., Stahlhut, M. W., Olsen, D. B., Hazuda, D. J., and Flores, O. A. (2003) *J. Biol. Chem.* **278,** 49164–49170
11. Eldrup, A. B., Prhavc, M., Brooks, J., Bhat, B., Prakash, T. P., Song, Q., Bera, S., Bhat, N., Dande, P., Cook, P. D., Bennett, C. F., Carroll, S. S., Ball, R. G., Bosserman, M., Burlein, C., Colwell, L. F., Fay, J. F., Flores, O. A., Getty, K., LaFemina, R. L., Leone, J., MacCoss, M., McMasters, D. R., Tomassini, J. E., Von Langen, D., Wolanski, B., and Olsen, D. B. (2004) *J. Med. Chem.* **47,** 5284–5297
12. Le Pogam, S., Jiang, W. R., Leveque, V., Rajyaguru, S., Ma, H., Kang, H., Jiang, S., Singer, M., Ali, S., Klumpp, K., Smith, D., Symons, J., Cammack, N., and Najera, I. (2006) *Virology* **351,** 349–359
13. Smith, D. B., Martin, J. A., Klumpp, K., Baker, S. J., Blomgren, P. A., Devos, R., Granycome, C., Hang, J., Hobbs, C. J., Jiang, W. R., Laxton, C., Pogam, S. L., Leveque, V., Ma, H., Maile, G., Merrett, J. H., Pichota, A., Sarma, K., Smith, M., Swallow, S., Symons, J., Vesey, D., Najera, I., and Cammack, N. (2007) *Bioorg. Med. Chem. Lett.* **17,** 2570–2576
14. Afdhal, N., Rodriguez-Torres, M., Lawitz, E., Godofsky, E., Chao, G., Fielman, B., Knox, S., and N., B. (2005) in *40th Annual Meeting of the European Association for the Study of the Liver*, April 13–17, 2005, European Association for the Study of the Liver (EASL), Paris, abstract 93
15. Roberts, s., Cooksley, G., Dore, G., Robson, R., Shaw, D., Berns, H., Brandl, M., Fettner, S., Hill, G., Ipe, D., Klumpp, K., Mannino, M., O'Mara, E., Tu, Y., and Washington, C. (2006) *Hepatology* **44,** (Suppl. 1), 692 (abstr.)
16. Stuyver, L. J., McBrayer, T. R., Tharnish, P. M., Clark, J., Hollecker, L., Lostia, S., Nachman, T., Grier, J., Bennett, M. A., Xie, M. Y., Schinazi, R. F., Morrey, J. D., Julander, J. L., Furman, P. A., and Otto, M. J. (2006) *Antiviral Chem. Chemother.* **17,** 79–87
17. Murakami, E., Bao, H., Ramesh, M., McBrayer, T. R., Whitaker, T., Micolochick Steuer, H. M., Schinazi, R. F., Stuyver, L. J., Obikhod, A., Otto, M. J., and Furman, P. A. (2007) *Antimicrob. Agents Chemother.* **51,** 503–509
18. Clark, J. L., Hollecker, L., Mason, J. C., Stuyver, L. J., Tharnish, P. M., Lostia, S., McBrayer, T. R., Schinazi, R. F., Watanabe, K. A., Otto, M. J., Furman, P. A., Stec, W. J., Patterson, S. E., and Pankiewicz, K. W. (2005) *J. Med. Chem.* **48,** 5504–5508
19. Tomassini, J. E., Getty, K., Stahlhut, M. W., Shim, S., Bhat, B., Eldrup, A. B., Prakash, T. P., Carroll, S. S., Flores, O., MacCoss, M., McMasters, D. R., Migliaccio, G., and Olsen, D. B. (2005) *Antimicrob. Agents Chemother.* **49,** 2050–2058
20. Duarte, M. I., Andrade, H. F., Jr., Mariano, O. N., Corbett, C. E., and Sesso, A. (1989) *J. Submicrosc. Cytol. Pathol.* **21,** 275–279
21. Perrone, P., Luoni, G., Kelleher, M. R., Daverio, F., Angell, A., Mulready, S., Congiatu, C., Rajyaguru, S., Martin, J. A., Leveque, V., Le Pogam, S., Najera, I., Klumpp, K., Smith, D. B., and McGuigan, C. (2007) *J. Med. Chem.* **50,** 1840–1849
22. Mancini, W. R., and Cheng, Y. C. (1983) *Mol. Pharmacol.* **23,** 159–164
23. Heinemann, V., and Plunkett, W. (1989) *Biochem. Pharmacol.* **38,** 4115–4121
24. Van Rompay, A. R., Johansson, M., and Karlsson, A. (2000) *Pharmacol. Ther.* **87,** 189–198
25. Carroll, S. S., and Olsen, D. B. (2006) *Infect. Disord. Drug Targets* **6,** 17–29