

# The Mechanism of Action of $\beta$ -D-2'-Deoxy-2'-fluoro-2'-C-methylcytidine Involves a Second Metabolic Pathway Leading to $\beta$ -D-2'-Deoxy-2'-fluoro-2'-C-methyluridine 5'-Triphosphate, a Potent Inhibitor of the HCV RNA-Dependent RNA Polymerase

Poster #

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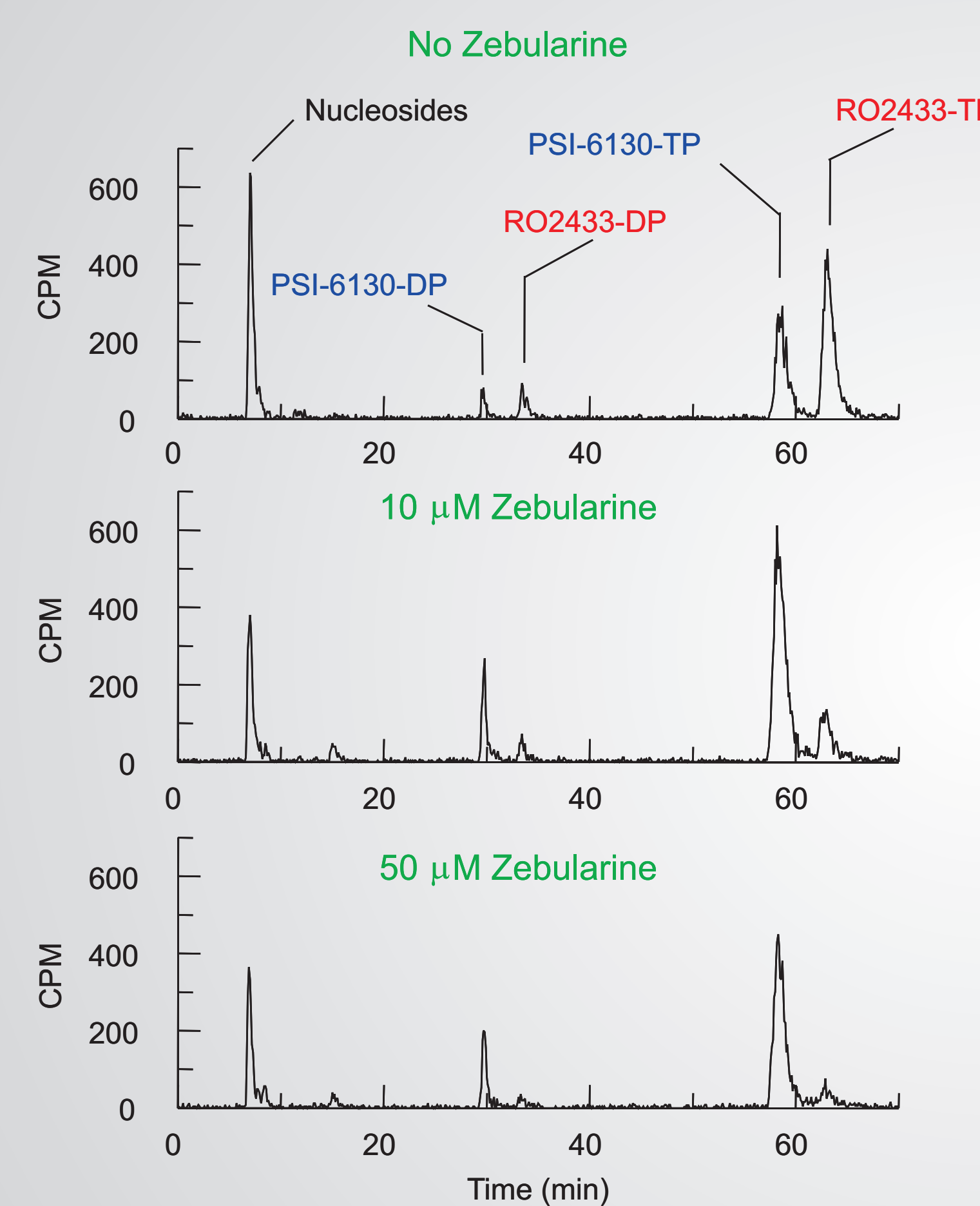


## Introduction

Nearly 2% of the U.S. population and an estimated 170 million people worldwide are HCV carriers. The current standard of care, a combination of pegylated interferon and ribavirin, has limited efficacy. We have previously reported that  $\beta$ -D-2'-deoxy-2'-fluoro-2'-C-methylcytidine (PSI-6130), is a specific, potent and non-cytotoxic inhibitor of HCV in the subgenomic replicon assay (1). To inhibit the HCV NS5B polymerase, PSI-6130 must be phosphorylated to the 5'-triphosphate. The enzymes involved in the phosphorylation of PSI-6130 and inhibition of HCV NS5B were investigated. PSI-6130 is activated to the triphosphate form by 2'-deoxycytidine kinase (dCK), UMP-CMP kinase (YMPK), and nucleoside diphosphate kinase (NDPK). PSI-6130-TP is a potent inhibitor of HCV RNA-dependent RNA polymerase (RdRp) and acts as a non-obligate chain-terminator (2). PSI-6130 was found to be a substrate for cytidine deaminase (CDA) which generated the uridine metabolite, RO2433 (PSI-6206). When tested in the replicon assay RO2433 was found to be inactive. However, in cell-based metabolism assays with [ $^3$ H]-PSI-6130 using primary human hepatocytes, the triphosphate of RO2433 (RO2433-TP) was seen (3). In subsequent metabolism studies in Clone A replicon cells RO2433-TP was also seen. None of the known pyrimidine nucleoside kinases could phosphorylate RO2433 *in vitro*, but RO2433-MP and RO2433-DP were phosphorylated by YMPK and NDPK, respectively, suggesting that PSI-6206-TP is formed via deamination of PSI-6130-MP. Enzyme studies with cloned human deoxycytidylate deaminase (DCTD) show that this enzyme was capable of deaminating PSI-6130-MP to RO2433-MP. This result was confirmed by cell based metabolism studies using zebularine, a non-toxic and specific inhibitor of DCTD. In the presence of zebularine, the amount of RO2433-TP significantly decreased whereas PSI-6130-TP increased. Finally, *in vitro* studies showed that both PSI-6130-TP and RO2433-TP are potent inhibitors of wild-type HCV RdRp and the S282T mutant enzyme was approximately 7.5- and 23.7-fold less sensitive to inhibition by PSI-6130-TP and RO2433-TP, respectively.

## Methods & Results

Figure 2: Zebularine inhibits formation of RO2433-TP in Clone A Cells



PSI-6130-MP and RO2433-MP peaks were close to the background level with the retention times of 8.57 min and 11.83 min, respectively.

Zebularine[1-( $\beta$ -D-ribofuranosyl)pyrimidin-2-one] is a specific inhibitor of CDA and DCTD. 50  $\mu$ M zebularine diminished the formation of RO2433-TP. However, the total amount of triphosphate was not affected by zebularine up to 50  $\mu$ M. Taken together with the kinase results, this strongly suggests that DCTD is the enzyme that converts PSI-6130-MP to RO2433-MP.

### Methods:

1. Clone A cells were treated with 5  $\mu$ M [ $^3$ H]-PSI-6130 in the presence or absence of zebularine for 48 hours.
2. Cells were washed 3 times with cold PBS.
3. After trypsinization, cells were counted, resuspended in 1ml of cold 60% methanol and incubated overnight at -20°C.
4. The samples were centrifuged and supernatant was collected and dried under a gentle nitrogen flow and then stored at -20°C.
5. Residues were resuspended in 100  $\mu$ l of water, and 50  $\mu$ l aliquots were injected into HPLC.

## Methods & Results

Figure 1: Structure of PSI-6130 and RO2433

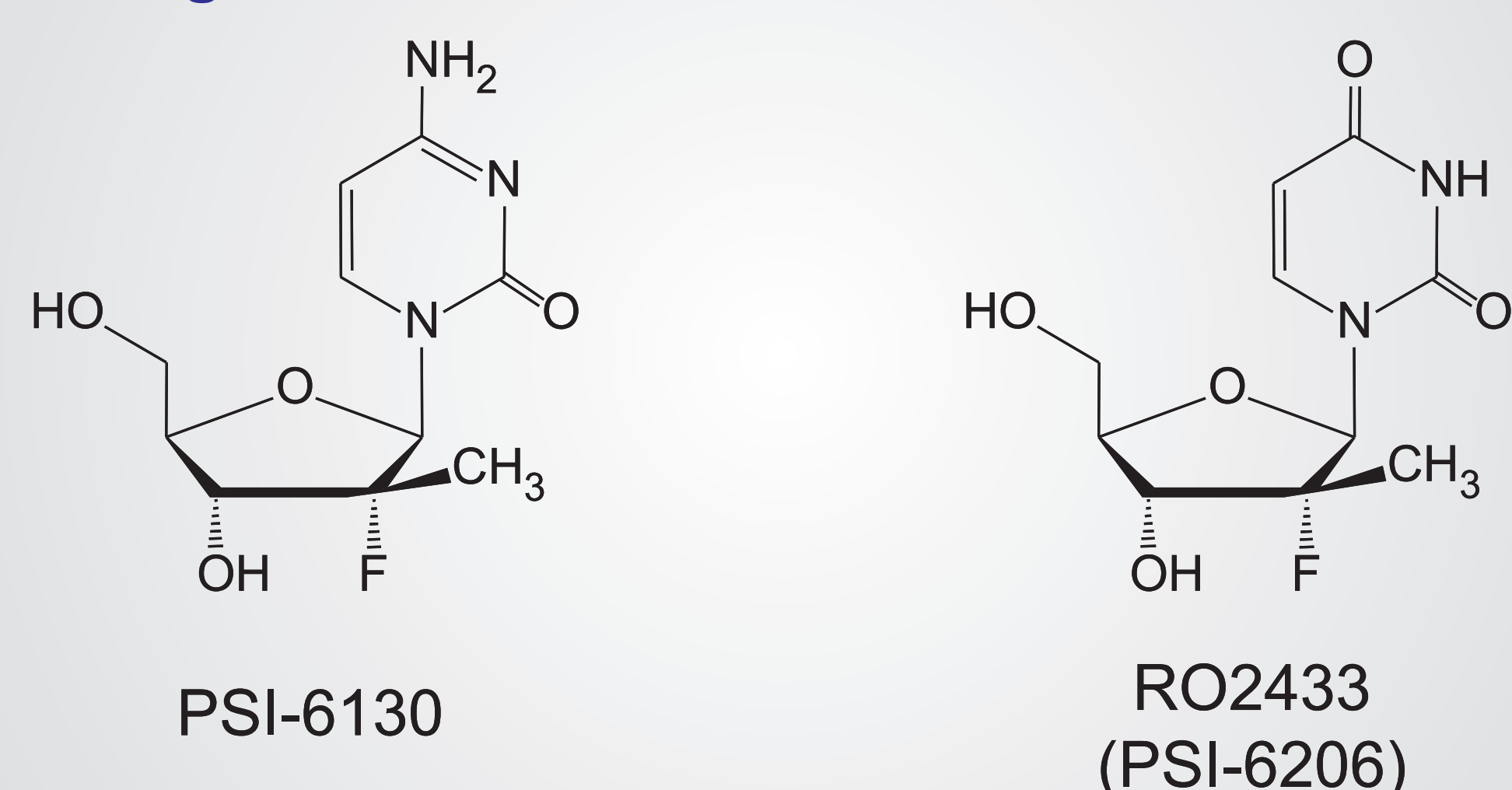


Table 4: Effect of zebularine on anti-HCV replicon activity

[Zebularine]	Wild-type, EC <sub>90</sub> ( $\mu$ M)	S282T, EC <sub>90</sub> ( $\mu$ M)	EC <sub>90</sub> <sup>S282T</sup> /EC <sub>90</sub> <sup>WT</sup>
0	7.86 $\pm$ 0.51	41.30 $\pm$ 1.19	5.25
25 $\mu$ M	6.46 $\pm$ 0.27	24.79 $\pm$ 5.30	3.84
50 $\mu$ M	5.05 $\pm$ 1.02	18.55 $\pm$ 2.55	3.67
100 $\mu$ M	3.94 $\pm$ 0.64	10.82 $\pm$ 1.22	2.75

In the presence of zebularine, which reduces the level of RO2433-TP and increases the level of PSI-6130-TP, the activity of PSI-6130-TP was increased. This suggests that PSI-6130-TP is a more potent inhibitor of the RdRp than is RO2433-TP.

Table 5: Inhibition of HCV NS5B RNA polymerase

Inhibitor	Enzyme	K <sub>i</sub> ( $\mu$ M)	K <sub>m</sub> ( $\mu$ M) <sup>*</sup>	K <sub>i</sub> /K <sub>m</sub>	Fold Resistance
PSI-6130-TP	Wild-type	0.059 $\pm$ 0.011	0.074 $\pm$ 0.014	0.80	---
	S282T	0.31 $\pm$ 0.05	0.052 $\pm$ 0.008	6.0	7.5
RO2433-TP	Wild-type	0.42 $\pm$ 0.04	0.068 $\pm$ 0.007	6.2	---
	S282T	22 $\pm$ 2	0.15 $\pm$ 0.01	147	23.7

<sup>\*</sup>K<sub>m</sub> values for CTP are shown for PSI-6130-TP and values for UTP are shown for RO2433-TP.

Polymerase reaction was performed using (-)IRES as an RNA template in 50 mM Hepes Buffer pH 7.5 containing 5 mM MgCl<sub>2</sub>.

Table 1: Anti-HCV replicon activity of PSI-6130 and RO2433

Compound	Wild-type	S282T
	EC <sub>90</sub> ( $\mu$ M)	EC <sub>90</sub> ( $\mu$ M)
PSI-6130	5.3 $\pm$ 2.3	30.7 $\pm$ 11.7
RO2433	> 100	> 100

HCV replicon containing cells were seeded into 96-well plates and test compounds added immediately. After 4 days incubation, total cellular RNA was extracted and HCV replicon RNA levels quantitated by Q-RT-PCR.

Table 2: Deamination of PSI-6130 and PSI-6130-MP

Enzyme	Substrate	k <sub>cat</sub> (s <sup>-1</sup> )	K <sub>m</sub> ( $\mu$ M)	k <sub>cat</sub> /K <sub>m</sub> ( $\mu$ M <sup>-1</sup> s <sup>-1</sup> )
CDA	Cytidine	5.4	40 $\pm$ 5	0.135
	2'-Deoxycytidine	4.8	41 $\pm$ 4	0.117
	2'-F-2'-deoxycytidine	3.8	27 $\pm$ 8	0.142
	2'-C-Methylcytidine	0.18	3,100 $\pm$ 600	0.000058
DCTD	PSI-6130	0.26	4,000 $\pm$ 500	0.000065
	dCMP	246 $\pm$ 16	87 $\pm$ 22	2.83
	PSI-6130-MP	0.82 $\pm$ 0.07	2,000 $\pm$ 300	0.00041

The Kinetic values for CDA were determined using a spectrophotometric assay. An HPLC assay was employed for the DCTD reactions with PSI-6130-MP.

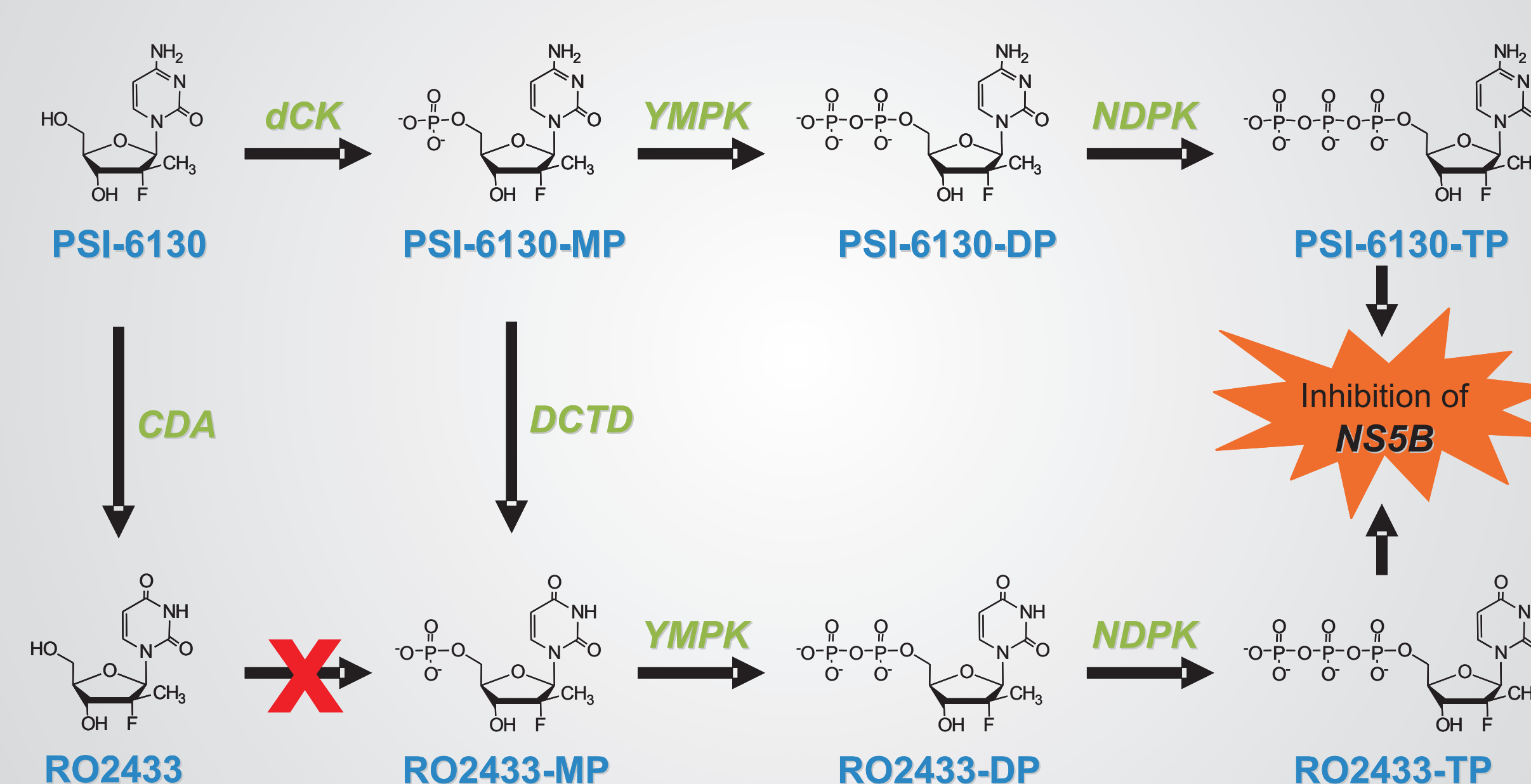
Table 3: Phosphorylation of RO2433-MP and RO2433-DP

UMP-CMP Kinase (YMPK)			
Substrate	k <sub>cat</sub> (s <sup>-1</sup> )	K <sub>m</sub> ( $\mu$ M)	k <sub>cat</sub> /K <sub>m</sub> ( $\mu$ M <sup>-1</sup> s <sup>-1</sup> )
UMP	81 $\pm$ 5	151 $\pm$ 31	0.54
RO2433-MP	7.9 $\pm$ 0.5	870 $\pm$ 100	0.0091
NDP Kinase (NDPK)			
Substrate	k <sub>cat</sub> (s <sup>-1</sup> )	K <sub>m</sub> ( $\mu$ M)	k <sub>cat</sub> /K <sub>m</sub> ( $\mu$ M <sup>-1</sup> s <sup>-1</sup> )
UDP	145 $\pm$ 7	156 $\pm$ 25	0.93
RO2433-DP	27 $\pm$ 2	585 $\pm$ 104	0.046

All reactions were performed spectrophotometrically using a coupled system with pyruvate kinase and lactate dehydrogenase and following NADH oxidation at 340 nm.

RO2433 nucleoside was not phosphorylated by dCK, UCK-1, TK-1, TK-2, AK, and dGK under our experimental conditions.

Figure 3: Proposed metabolic pathway for PSI-6130



## Conclusions

1. RO2433 was not active in the HCV replicon assay.
2. CDA and DCTD deaminated PSI-6130 and PSI-6130-MP, respectively.
3. None of nucleoside kinases tested phosphorylated RO2433 to RO2433-MP.
4. RO2433-MP was subsequently phosphorylated to triphosphate by YMPK and NDPK.
5. Zebularine, an inhibitor of DCTD, inhibited formation of RO2433-TP in Clone A cells, confirming that PSI-6130-MP was deaminated by DCTD.
6. Zebularine enhanced anti-HCV replicon activity of PSI-6130.
7. RO2433-TP was a potent inhibitor of NS5B RNA polymerase and showed 23.7-fold reduction in efficacy against S282T mutant enzyme.
8. The K<sub>i</sub>/K<sub>m</sub> value for PSI-6130-TP was 8- and 25-fold lower than for RO2433-TP with wild-type and S282T RdRp, respectively.
9. Taken together our zebularine study and *in vitro* RdRp study indicate that PSI-6130-TP is a better inhibitor of the HCV RdRp than is RO2433-TP.

## References

1. Stuyver, L.J., Mc Brayer, T.R., et al. (2006) Antiviral Chem and Chemother 17: 79-87
2. Murakami, E., Bao, H., et al. (2007) Antimicrob Agents and Chemother 51: 503-509
3. Ma, H., W. R. Jiang, et al. (2007) Antiviral Research 74: A36

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