

# IN VITRO ACTIVITY AND IN VIVO PHARMACOKINETICS OF HIGHLY POTENT PHOSPHORAMIDATE NUCLEOSIDE ANALOGUE INHIBITORS OF HEPATITIS C NS5B

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## Introduction

Phosphoramidate nucleoside analogues, or pronucleotides (ProTides), possess several pharmacological advantages over their parent nucleoside that include a significant increase in antiviral activity, higher concentrations of the triphosphate in liver, and potentially less toxicity due to reduced systemic exposure. The aims of this study were to characterize the *in vitro* and *in vivo* properties of our lead novel aminoacyl ProTides of 2'-C-methyl guanosine (2'-C-MeG).

## Methods

The antiviral activity of the ProTides was determined using the genotype 1b subgenomic replicon assay. Cell cytotoxicity was measured using an XTT based ELISA and bone marrow colonies by counting on semi-solid agar. The levels of intracellular 2'-C-MeGTP produced in primary human hepatocytes was measured using LC-MS/MS. Intrinsic clearance of ProTide (10 μM) in human microsomes in the presence of NADPH was measured by HPLC. Plasma concentrations of the ProTide and parent nucleoside in the peripheral circulation (portal vein of cannulated cynomolgus monkeys) as well as intracellular 2'-C-MeGTP from liver tissue were measured using LC-MS/MS following a single (and multiple – rodents) oral dose in rodents and primates.

## Results

ProTide candidates exhibited potent anti-HCV activity with EC<sub>50</sub>s ranging from 13-86 nM (a 13-87 fold enhancement over parent) and combined with ribavirin or interferon-2α, the anti-HCV effects were demonstrated to be synergistic (data not shown). Cytotoxicity were measured in a panel of cell lines. ProTides were evaluated for mitochondrial toxicity and genotoxicity. Candidates did not reduce the mitochondrial copy number after continuous treatment for 3 days at 100 μM or for 13 days at 5 μM and were negative in the Ames II assay (data not shown). Conversion of ProTide in hepatocytes to the triphosphate demonstrated C<sub>max</sub> levels of 83 pmol/10<sup>6</sup> cells, representing approximately a 40-fold increase over the EC<sub>50</sub>. Human microsome metabolism experiments showed intrinsic clearance rates greater in liver than intestine by 3-5 fold. PK studies in rodents and primates suggested efficient extraction by the liver as demonstrated by low systemic levels of the ProTides. Analysis of liver biopsies indicated that levels of the triphosphate exceeding the EC<sub>90</sub> were achieved following oral dosing. Multiple doses in rodents appear linear without significant accumulation with normal weight gain and blood chemistries (data not shown).

**Table 1: Potency in Genotype 1b Replicon (μM±SD)**

Compound	EC <sub>50</sub>	EC <sub>90</sub>	CC <sub>50</sub>	TI
2'-C-MeG	1.134 ± 0.309	3.408 ± 0.788	>100	88
INX-108	0.086 ± 0.040	0.306 ± 0.160	>100	1163
INX-189	0.013 ± 0.037	0.054 ± 0.143	7	538

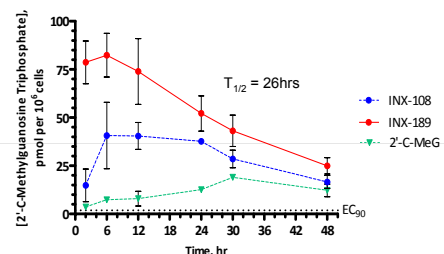
**Table 2: In Vitro Cell Cytotoxicity**  
 In Vitro Toxicity (CC<sub>50</sub> μM)

Compound	Cytotoxicity				Bone Marrow		
	LIVER Huh7b HepG2	LYMPHOID (T cell) MT-4	Kidney CEM	Kidney 293	BFU-E	CFU-GM	CFU-GEMM
GCV	>100	>100	>100	>100	12	20	10
2'-C-MeG	>100	>100	>100	>100	90	85	40
INX-108	>100	>100	>100	>100	26	40	40
INX-189	7	8.2	25	2.4	1.3	0.9	1

**Table 3: Intrinsic Clearance in Human Microsomes**

Compound / Tissue	Microsome Fraction	AUC (ng·h/mL)	T <sub>1/2</sub> (min)	CL <sub>int</sub> (mL/min/g)	Ratio (CL <sub>int</sub> of liver vs. intestine)
INX-189	Liver	37.8	2.4	7.4	5.4
INX-189	Intestine	133.4	13.3	1.4	
INX-108	Liver	115.7	8.1	2.2	3.7
INX-108	Intestine	189.9	29.8	0.6	

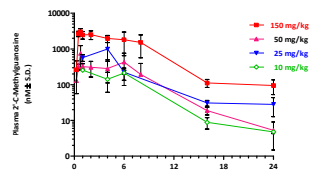
**Figure 1: 2'-C-MeGTP in 1<sup>o</sup> Human Hepatocytes**



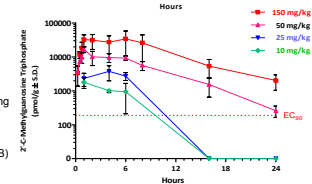
1<sup>o</sup> human hepatocytes were incubated with 2μM ProTide or 2'-C-MeG. Intracellular 2'-C-MeGTP was measured by LC-MS/MS.

**Figure 2: Single Ascending Dose PK/PD of INX-189 in Mice**

### A – Plasma Levels of 2'-C-MeG

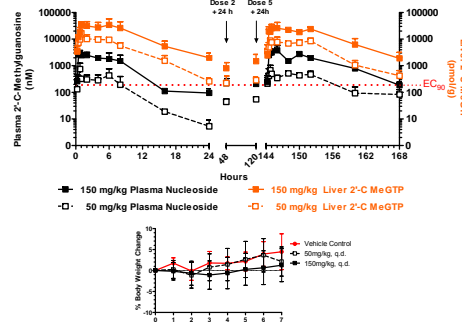


### B – Liver Tissue Levels of 2'-C-MeGTP



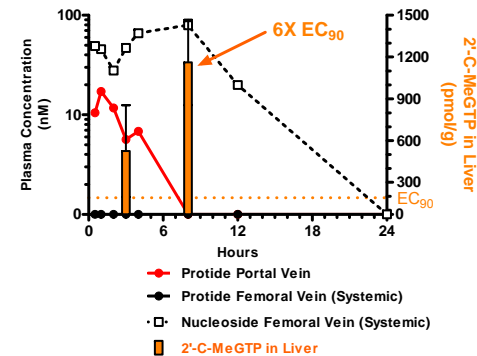
Mice were administered ascending doses PO. Liver tissue and plasma were harvested at the indicated timepoints. Concentrations of 2'-C-MeGTP (B) and nucleoside (2'-C-MeG) (A) were measured by LC-MS/MS.

**Figure 3: Multiple (7-Day) Dosing of INX-189 in Mice**



Mice were administered ascending doses PO daily for 7 days. Liver tissue and plasma were harvested at the indicated timepoints. Concentrations of 2'-C-MeGTP and nucleoside (2'-C-MeG) were measured by LC-MS/MS. Body weights were measured daily.

**Figure 4: Oral Pharmacokinetics of INX-189 in Primates**



Portal vein cannulated cynomolgus monkeys were administered ProTide orally at 25mg/kg. Portal vein and systemic plasma were sampled at the indicated time points. Liver biopsies were collected from a separate cohort for measurement of intracellular 2'-C-MeGTP. All analyses were performed using LC-MS/MS.

## Conclusions

ProTide candidates of 2'-C-MeG exhibit excellent therapeutic indices and conversion to 2'-C-MeGTP in primary human hepatocytes exceed the EC<sub>90</sub> in the genotype 1b replicon. Rodent and primate PK studies support delivery of the ProTide candidates to the liver and subsequent conversion to the triphosphate after oral administration. These data suggest that ProTides of 2'-C-MeG represent a promising class of potent compounds with high liver extraction ratios for the treatment of HCV infections.

