

# In Vitro Antiviral Activity of IDX320, a Novel and Potent Macrocyclic HCV Protease Inhibitor

L.B. Lалlos, J. McCarville, B. Li, J.P. Bilello, M. La Colla, J. Jakubik, M.A. Soubasakos, J. Gillum, I. Serra, L. Hubbard, A. Bonin, M. Seifer, M. Liuzzi, C. Parsy, D. Surleraux, D.N. Standing  
Idenix Pharmaceuticals, Inc., Cambridge, MA, USA and Montpellier, France

768

## INTRODUCTION

- HCV infection remains a significant health problem, infecting three to four million people worldwide annually.<sup>1</sup>
- NS3/4A protease is a validated antiviral target for the treatment of HCV infection.
- We have identified a novel macrocyclic compound, IDX320, that specifically inhibits HCV NS3/4A protease with subnanomolar to nanomolar potency. IDX320 is in early clinical development.
- This study evaluated the *in vitro* antiviral activity of IDX320 in biochemical and replicon assays. In addition, the resistance and cross-resistance profiles were evaluated.

## METHODS

**Biochemical determinations:** Cleavage of a synthetic peptide by purified, recombinant HCV NS3/4A protease from genotypes 1a, 1b, 2a, 3a and 4a, or various cellular proteases, was measured in the presence of compound. The binding kinetics of IDX320 to NS3/4A (Con1) were determined by surface plasmon resonance.

**HCV activity assays:** Luciferase-replicon cells (genotype 1a or 1b) were seeded onto 96-well plates, cultured for 3 days in the presence of compound and subjected to a luciferase assay. HPC cells were infected with JFH-1 (genotype 2a) and treated with compound for 4 days (virus inoculum was removed after 16 hours); remaining virus was measured by an anti-HCV core ELISA. Compound cytotoxicity was measured in parallel using a colorimetric proliferation assay.

**Long-term treatment assay:** Replicon cells (genotype 1b) were treated with compound, without G418, for 14 days and the level of replicon RNA was measured at multiple time points. At the end of the 14-day treatment, cells were cultured in the absence of compound  $\pm$  G418 in 10 cm dishes for 21 days. Surviving cell colonies were then stained with crystal violet and counted.

**Resistance selection:** Replicon cells (genotype 1a or 1b) were treated with various concentrations of IDX320 in the presence of G418. Treatment-emergent genotypic changes were identified by population sequencing of NS3 (both protease and helicase regions). The activity of IDX320 and other compounds was evaluated by NS5A western blot (after selection) or luciferase assay (during and after selection).

**HCV transient transfection assay:** Mutations were introduced into a genotype 1b luciferase-replicon by site-directed mutagenesis. Compound activity was measured in cells transiently transfected with *in vitro* transcribed wild-type or mutant luciferase-replicon RNA by luciferase assay after 4-day treatment. Replication capacity was determined in untreated transfected cells by comparing the luciferase activity at 4 hours and 4 days post-transfection.

## RESULTS

### IDX320 is a potent and selective inhibitor of HCV NS3/4A protease

- IDX320 inhibited NS3/4A proteases from genotypes 1a, 1b, 2a and 4a ( $IC_{50}$  values from 0.8 to 1.9 nM; **Table 1**), as well as from genotype 3a ( $IC_{50}$  value of 23 nM).
- IDX320 did not inhibit nine tested cellular proteases ( $IC_{50} > 10 \mu\text{M}$ ; data not shown), indicating high selectivity.

**Table 1. In vitro activity of IDX320 against NS3/4A proteases from multiple genotypes**

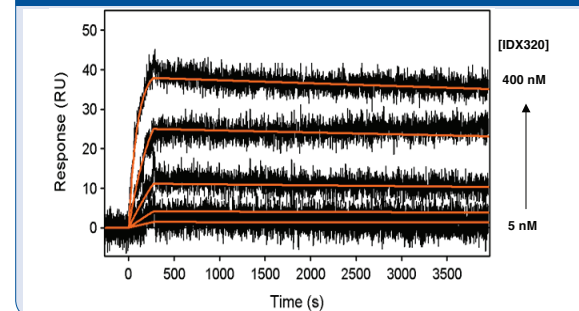
HCV protease	IDX320 $IC_{50}$ (nM)
Genotype 1a	1.1 $\pm$ 0.1
Genotype 1b	1.2 $\pm$ 0.1
Genotype 2a	1.9 $\pm$ 0.5
Genotype 3a	23 $\pm$ 1.9
Genotype 4a	0.81 $\pm$ 0.03

Mean  $\pm$  standard deviation  $IC_{50}$  values were derived from 3 independent experiments.

### IDX320 demonstrates tight binding to HCV NS3/4A protease

- IDX320 bound protease tightly as determined by surface plasmon resonance (**Figure 1**).
- Association was fast ( $2.7 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ ) and dissociation was very slow ( $2.1 \times 10^{-6} \text{ s}^{-1}$ ), with an equilibrium constant of 0.8 nM and a dissociation half-life of over 9 hours.

**Figure 1. Binding of IDX320 to NS3/4A by surface plasmon resonance**



### IDX320 is a potent and selective inhibitor of HCV in cell culture

- IDX320 potently inhibited HCV replicons from genotype 1a and 1b, as well as a genotype 2a virus, with low cytotoxicity leading to high selectivity indices (**Table 2**).

**Table 2. Activity of IDX320 in cell culture**

	$EC_{50}$ (nM)	$CC_{50}$ ( $\mu\text{M}$ )	Selectivity Index
1b replicon	0.5 $\pm$ 0.1	25.2 $\pm$ 5.3	50,400
1a replicon	3.4 $\pm$ 1.1	> 77	> 22,647
2a virus	4.4 $\pm$ 0.6	11.3 $\pm$ 0.1	2,568

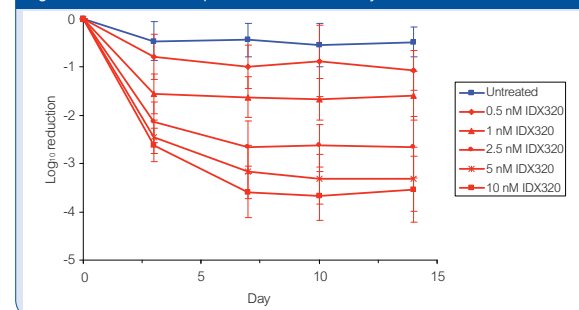
Mean  $\pm$  standard deviation values were derived from 3-6 independent experiments.

### Treatment of replicon cells over 14 days with IDX320 results in a multi-log reduction in replicon RNA

The antiviral activity of IDX320 was examined in a 14-day treatment assay in the absence of G418.

- A dose-dependent decline in replicon RNA was observed (**Figure 2**); a mean maximal RNA reduction of 3.7  $\log_{10}$  was obtained with 10 nM of IDX320.
- No RNA rebound or cytotoxicity was observed.

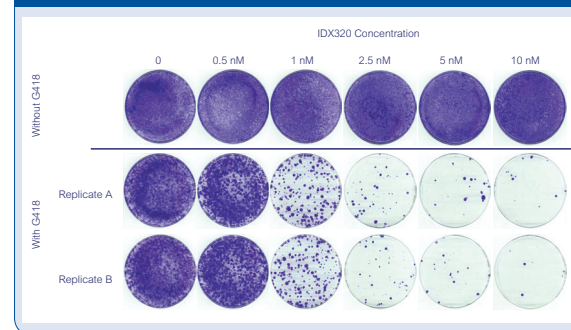
**Figure 2. Reduction in replicon RNA after 14-day treatment with IDX320**



- The treated cells shown in **Figure 2** were further cultured without compound  $\pm$  G418 to quantitate the remaining replicon-bearing cells; only these cells survive G418 selection.
- As expected, a lawn of cells (confluency) was observed in the absence of G418 selection pressure, indicating that cells remained viable after treatment (**Figure 3**, top row).

- When cells were cultured in the presence of G418, a dose-dependent effect of IDX320 on the number of replicon-bearing colonies was observed for concentrations of IDX320  $\geq 1 \text{ nM}$  (**Figure 3**).
- Only 6 colonies remained on average in response to 14-day treatment with 10 nM of IDX320. These data indicate that most of the replicon RNA present in the initial culture had been eliminated, consistent with the loss of replicon RNA demonstrated in **Figure 2**.

**Figure 3. Colony formation assay after 14-day treatment with IDX320**



A representative experiment is shown.

### Characterization of genotype 1b IDX320-resistant cell lines

Eight IDX320-resistant (320R) replicon cell lines were selected by long-term culture of genotype 1b replicon cells with 10 or 25 nM of IDX320. NS3 (amino acid 1-631) was sequenced at each passage.

- NS3 D168V was the signature mutation observed in eight 320R cell lines (**Table 3**), as observed with other macrocyclic protease inhibitors. The emergence of D168V was associated with resistance to IDX320 after  $\sim 20$  days of selection (data not shown).
- Other changes in NS3 were observed as minor variants (**Table 3**). These mutations are currently being evaluated.
- All 320R cell lines exhibited resistance to IDX320 with fold-change values  $\geq 250$  (**Table 4**).
- These resistant cell lines remained susceptible to interferon (IFN) as well as other classes of direct-acting antiviral agents.
- In addition, these 320R cell lines remained susceptible to Telaprevir (TVR).

**Table 3. NS3 genotypic changes after IDX320 selection in genotype 1b replicons**

Cell Line	IDX320 selecting conc. (nM)	Dominant mutation	Minor mutations
GS4.1 <sup>a</sup>	0	-	-
320R-A	10	D168V	D168E, E503D
320R-B	10	D168V	D168A/E, V256A, G282A, A156V
320R-C	10	D168V	Q41R, Q80R, A156V, D168H/A/E/Y/I
320R-D	25	D168V	D168A, E503D
320R-E	25	D168V	A156P, D168A/Y/E, V256A, G282A
320R-F	25	D168V	D168Y/H, G282A
Zluc <sup>a</sup>	0	-	-
320R-ZlucA	10	D168V	D168A
320R-ZlucB	25	D168V	D168A

<sup>a</sup>The GS4.1 and Zluc cell lines contain genotype 1b replicon without or with luciferase, respectively.

**Table 4. Extent of resistance after IDX320 selection in genotype 1b replicons**

Cell Line	IDX320 selecting conc. (nM)	Fold-Change <sup>a</sup>				
		IDX320 (PI)	TVR (PI)	IFN	IDX375 (NNI)	IDX184 (NI)
Zluc	0	1	1	1	1	1
320R-ZlucA	10	250 $\pm$ 66	1.0 $\pm$ 0.3	0.6 $\pm$ 0.4	0.9 $\pm$ 0.2	1.0 $\pm$ 0.2
320R-ZlucB	25	1037 $\pm$ 144	0.6 $\pm$ 0.04	0.7 $\pm$ 0.06	1.3 $\pm$ 0.4	1.1 $\pm$ 0.2

<sup>a</sup>Fold-change values were calculated by dividing the mean  $EC_{50}$  value of the resistant replicons by the mean  $EC_{50}$  value of the wild-type replicons for each experiment. Presented here are the mean  $\pm$  standard deviation fold-change values from 3 experiments. Resistance was designated as a fold-change value of  $>3$ .

- In a genotype 1a background, the dominant genotypic variant selected was D168A, instead of D168V (data not shown).
- The extent of resistance and cross-resistance of the genotype 1a 320R cell lines was similar to that of genotype 1b (data not shown).
- Interestingly, R155K, selected by several linear and macrocyclic PIs in the clinic<sup>2,3,4,5</sup>, was not selected by IDX320 in either the genotype 1a or 1b replicons.

### Resistance and cross-resistance profiles of IDX320

The activity of IDX320 was evaluated against replicons bearing single protease inhibitor-resistance mutations and compared to its activity against the wild-type replicon (**Table 5**). Each mutation confers resistance to a known PI, as reported in the literature. The resistance properties of all mutants were independently confirmed in the current work (data not shown).

- Mutations at the NS3 D168 locus resulted in moderately to highly reduced susceptibility to IDX320, confirming this locus as the predominant site of resistance (**Table 5**). These D168 mutations reduced the replication capacity of the replicon (D168V, 19% of wild-type).
- The R155K mutation resulted in a mild loss of susceptibility, which may explain the lack of selection in the genotype 1a or 1b replicon cell line.
- The A156T mutation resulted in moderately reduced susceptibility to IDX320.
- IDX320 remained fully active against the T54A, R155Q and A156S mutations.

**Table 5. Resistance profile of IDX320**

NS3 Mutation	IDX320 fold-change <sup>a</sup>	Replication capacity <sup>b</sup>
T54A	1.4 $\pm$ 0.2	62 $\pm$ 23
Q80R	5.7 $\pm$ 0.9	108 $\pm$ 30
R155K	9.0 $\pm$ 1.7	60 $\pm$ 18
R155Q	0.6 $\pm$ 0.1	25 $\pm$ 21
A156S	0.5 $\pm$ 0.1	54 $\pm$ 19
A156T	27 $\pm$ 9.5	6 $\pm$ 2
D168A	575 $\pm$ 210	32 $\pm$ 10
D168E	41 $\pm$ 18	40 $\pm$ 12
D168V	4587 $\pm$ 1003	19 $\pm$ 5
D168Y	1107 $\pm$ 513	10 $\pm$ 4

<sup>a</sup>Fold-change values were calculated by dividing the mean  $EC_{50}$  value of the mutant replicon by the mean  $EC_{50}$  value of the wild-type replicon for each experiment. The mean  $\pm$  standard deviation from 3-4 experiments is presented. Resistance was designated as a fold-change value  $>3$ .

<sup>b</sup>Replication capacity was calculated by dividing the day 4 counts per second (CPS) by the 4 hour CPS for each mutant replicon and determining the percentage CPS relative to wild-type replicon values for each experiment. The mean  $\pm$  standard deviation from 8-15 experiments is presented.

The activity of IDX320 was also evaluated against replicons bearing a single polymerase inhibitor-resistance mutation. Each mutation confers resistance to a known polymerase inhibitor, as reported in the literature. The resistance properties of all mutants were independently confirmed in the current work (data not shown).

- As expected, IDX320 inhibited replicons bearing single mutations responsible for resistance to polymerase inhibitors as shown by fold-change values 0.9 to 1.7 (data not shown).

## CONCLUSIONS

- IDX320 is a potent and selective inhibitor of HCV NS3/4A protease and HCV replication in cell culture with multi-genotypic coverage.
- IDX320 bound tightly to the HCV protease enzyme with a long dissociation half-life.
- Treatment of replicon cells for 14 days resulted in a dose-dependent and sustained suppression of replicon RNA (mean maximal RNA reduction of 3.7  $\log_{10}$ ), as well as elimination of almost all replicon-bearing colonies.
- The NS3 D168V mutation was predominant in all genotype 1b 320R cell lines. This mutation resulted in a reduced replication capacity (19% of wild-type).
- In genotype 1a, D168A was selected instead of D168V.
- The commonly observed R155K mutation was not selected *in vitro* by IDX320 in either genotype 1a or 1b.
- 320R cell lines were fully susceptible to other classes of HCV agents, such as IFN, IDX375 (NNI), and IDX184 (NI), and were also susceptible to TVR (PI).
- These data, together with those presented by Good, et al. (Poster #750) and La Colla, et al. (Poster #769), support the ongoing clinical evaluation of IDX320.

## ACKNOWLEDGMENTS

The authors would like to thank Dr. Christoph Seeger for the GS4.1 HCV replicon cell line and the HCV replicon cDNA construct ZS11, and Teresa Dahlman for assistance with poster preparation.

## REFERENCES

- Lavanchy (2009) Liver International. 29(s1):74-81.
- Adiwijaya et al (2008) EASL Conference: Hepatitis B and Hepatitis C Virus Resistance to Antiviral Therapies poster #1.
- Kukolj et al (2009) EASL poster #954.
- Manns et al (2009) EASL poster #2701.
- Sarrazin et al (2009) EASL poster #964.

## DISCLOSURES

All other authors are current or former employees of Idenix Pharmaceuticals, Inc.