

Enhanced Antiviral Activity Of IDX375, A Novel HCV Non-Nucleoside Inhibitor, In Combination With Other HCV Antiviral Agents In The Genotype 1b Replicon *In Vitro*

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ABSTRACT

Objectives:

- The paradigm for future hepatitis C virus (HCV) treatment will be a combination of multiple antiviral agents with distinct modes of action. This study evaluated (i) the *in vitro* effects of IDX375 (a palm-binding non-nucleoside inhibitor of HCV polymerase) in combination with IDX184 (a nucleotide prodrug), interferon- α (IFN- α), ribavirin (RBV) or an IDX protease inhibitor (PI) and (ii) the mutations within the NS5B polymerase that may confer resistance to IDX375.

Methods:

- Three-day combination assays were performed in the HCV replicon and evaluated by three mathematical analyses. Longer-term combination effects of IDX375 with IDX184 or IFN- α were further determined by RT-qPCR and colony formation assays. Variants selected in HCV genotype 1b replicons by continuous culturing of replicon-bearing cells in the presence of IDX375 and G418 were identified by sequencing.

Results:

- In short and long-term HCV replicon assays, combinations of IDX375 and IDX184, IFN- α , RBV or an IDX PI were additive. The colony formation assay further suggested combination treatment was more effective at inhibiting the emergence of resistant colonies compared to single agents. While complex and variable patterns of mutations were observed in multiple IDX375-resistant cell lines, the NS5B M414T mutation emerged as the predominant and consistent genotypic change in the genotype 1b background; phenotypic assays confirmed that the M414T mutation confers drug resistance to IDX375 *in vitro*.

Conclusions:

- These results suggest that combinations of IDX375 with other direct-acting HCV antiviral classes or standard-of-care agents could enhance antiviral activity and limit the emergence of resistance.

INTRODUCTION

- Hepatitis C virus (HCV) is a common blood-borne pathogen. Currently, an estimated 170 million people are infected globally, representing a nearly 5-fold greater prevalence than human immunodeficiency virus.¹

- The current standard-of-care therapy, a combination of pegylated interferon and ribavirin, is effective in approximately 40-50% of patients infected with HCV genotype 1 and is associated with significant side effects.² Thus, there remains a need for new, more effective and better tolerated HCV treatment options.

- The HCV polymerase is an attractive antiviral target. Nucleoside analogs, or more recently pro-nucleotides such as IDX184, are classically used to target the active site of the enzyme.^{3,4} In another approach, multiple classes of non-nucleoside polymerase inhibitors (NNIs), which target different allosteric sites in the enzyme, are under investigation.

- IDX375 is a novel NNI developmental candidate that targets the palm pocket of the NS5B polymerase. The low nanomolar *in vitro* potency of IDX375 (IC_{50} = 18 nM; EC_{50} = 2.3 nM; genotype 1b) may allow for lower therapeutic doses. Such low doses could be compatible with fixed dose combination therapy using different classes of inhibitors.

- This study evaluated the *in vitro* antiviral activity of IDX375 in combination with standard-of-care agents, the nucleotide prodrug IDX184, or an IDX protease inhibitor (PI). Furthermore, resistance selection and cross-resistance profiles of IDX375 were determined in cell-based assays.

METHODS

HCV replicon assay: Huh-7 cells stably expressing a HCV genotype 1b replicon containing the luciferase transgene were seeded onto 96-well plates, cultured for 3 days in the presence of IDX375 (alone or in the presence of IFN- α , RBV, IDX184 or an IDX PI) and subjected to a luciferase assay. EC_{50} values were calculated from one-site dose-response curves. The *in vitro* cytotoxicity of IDX375 was measured by a standard MTS cell proliferation assay.

Long-term treatment assay: A replicon cell line was treated with compound in the absence of 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, whereupon the cells were stained and multicellular colonies counted.

Colony formation assay: GS4.1 cells stably expressing a bicistronic replicon were seeded onto 6-well plates and cultured in the presence of drug (alone or in combination) and G418 for 3 weeks. Compound was replaced every 3-4 days. The cells remaining in each well were visualized by staining with crystal violet.

Selection of IDX375-resistant replicons: IDX375-resistant (375R) HCV replicon-bearing cell lines were generated by treating HCV replicon cells with varying concentrations of IDX375 in the presence of G418 to ensure maintenance of the replicon. Treatment-emergent genotypic changes were identified by population sequencing.

HCV transient transfection assay: Polymerase inhibitor-associated resistance mutations were introduced into a luciferase-replicon by site-directed mutagenesis. The activity of compound was measured in cells transiently transfected with *in vitro* transcribed wild-type or mutant luciferase-replicon RNA by luciferase assay after 4-day treatment.

RESULTS

Additive activity is achieved when IDX375 is combined with other agents

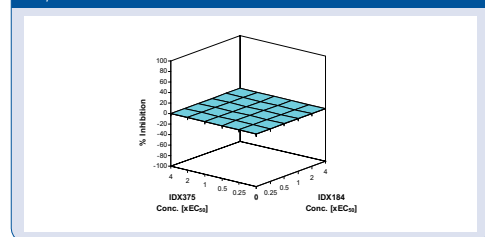
- As seen in Table 1 and Figure 1, additive activity was observed when IDX375 was combined with IDX184, an IDX PI, IFN- α , or RBV in short-term (3-day) replicon studies.

Table 1: Combination effects of IDX375 with other agents

	Bliss Independence	Loewe Additivity
IDX375 + IDX184	Additive	Additive
IDX375 + IDX PI	Additive	Additive
IDX375 + IFN- α	Additive	Additive
IDX375 + RBV	Additive	Additive

No cytotoxicity was observed

Figure 1: Effect of IDX375 in combination with IDX184 as seen by the Bliss Independence model⁵



⁵ A representative graph is shown. Similar results were obtained for IDX375 in combination with an IDX PI, IFN- α or RBV.

Additive activity of IDX375 and IDX184 after long-term treatment

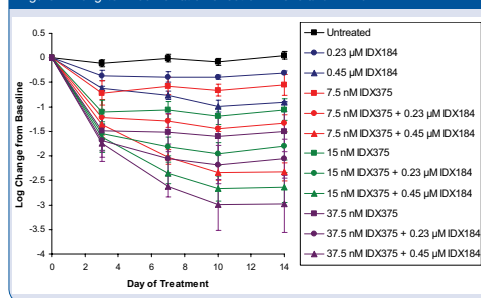
- During 14-day treatments of replicon cells with IDX375 in combination with IDX184, the decline in replicon RNA and in the number of replicon-bearing colonies was greater than treatment with either drug alone, consistent with an enhanced (additive) antiviral effect (Table 2 and Figure 2).

Table 2: Effect of long-term treatment with IDX375 and IDX184 on replicon RNA (Day 14)

IDX375 concentration (nM)	IDX184 concentration (μ M)	Log ₁₀ reduction ^a	Number of colonies ^b
0	0	-0.1	>400
	0.23	0.3	>400
	0.45	0.9	>400
7.5	0	0.6	>396
	0.23	1.3	>255
	0.45	2.3	34
15	0	1.1	>220
	0.23	1.8	130
	0.45	2.6	32
37.5	0	1.5	248
	0.23	2.1	66
	0.45	3.0	6

^a Values represent the mean log₁₀ reduction values across three independent experiments. The log₁₀ reduction was calculated by subtracting the average log₁₀ HCV replicon copies/ng RNA of the sample at Day 14 from the average log₁₀ copies/ng RNA of the untreated control at Day 0.
^b Values represent the mean number of colonies counted across three independent experiments.

Figure 2: Long-term combination effect of IDX375 and IDX184



Additive activity of IDX375 and IFN- α after long-term treatment

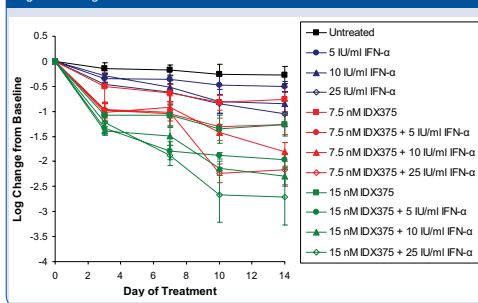
- Similarly, 14-day treatments of replicon cells with IDX375 in combination with IFN- α resulted in a greater decline in replicon RNA and in the number of replicon-bearing colonies than treatment with either drug alone, consistent with an enhanced (additive) antiviral effect (Table 3 and Figure 3).

Table 3: Effect of long-term treatment with IDX375 and IFN- α on replicon RNA (Day 14)

IDX375 concentration (nM)	IFN- α concentration (IU/ml)	Log ₁₀ reduction ^a	Number of colonies ^b
0	0	0.3	>400
	5	0.5	>400
	10	0.8	>400
	25	1.0	>400
7.5	0	0.8	>400
	5	1.3	>400
	10	1.8	>400
	25	2.2	>400
15	0	1.3	>400
	5	2.0	173
	10	2.3	285
	25	2.7	81

^a Values represent the mean log₁₀ reduction values across three independent experiments. The log₁₀ reduction was calculated by subtracting the average log₁₀ HCV replicon copies/ng RNA of the sample at Day 14 from the average log₁₀ copies/ng RNA of the untreated control at Day 0.
^b Values represent the mean number of colonies counted across three independent experiments.

Figure 3: Long-term combination effect of IDX375 and IFN- α



Additive activity of IDX375 and IDX184 or IFN- α after 3-week treatment in colony formation assay

- Upon 3 week combination treatment with IDX375 and IDX184 (Figure 4) or IFN- α (Figure 5), the suppression of replicon-bearing cells was greater than either treatment alone. This observation agrees with results obtained upon 3-day combination treatment of luciferase replicon-bearing cells showing that combination treatment of IDX375 and IDX184 or IFN- α was additive (Table 1 and Figure 1). This effect is not due to cytotoxicity of IDX375; the CC_{50} of IDX375 is >100 μ M, which is well above the top concentration of IDX375 used in this study.
- Furthermore, the combination of IDX375 and IDX184 or IFN- α more effectively suppresses the emergence of breakthrough colonies than treatment with each drug alone.

Figure 4: Colony formation assay following combination treatment of replicon-bearing cells with IDX375 and IDX184

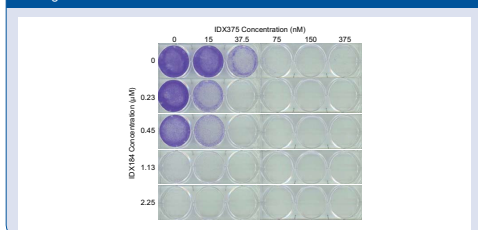
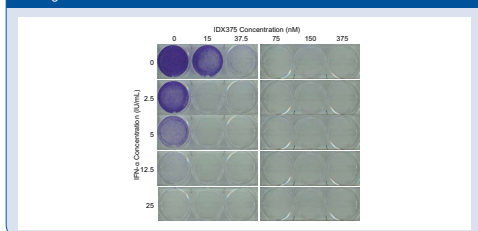


Figure 5: Colony formation assay following combination treatment of replicon-bearing cells with IDX375 and IFN- α



Crystal violet staining of HCV replicon cells after 3 weeks of treatment with the indicated concentrations of each compound in the presence of G418 (n=3 or 4; a representative experiment is shown).

Identification of NS5B mutations following selection of replicon-bearing cells with IDX375

- Prolonged exposure of genotype 1b replicon-bearing cells to varying regimens of IDX375 selected several different mutations in NS5B.
- M414T was the predominant mutation selected, emerging in all 7 IDX375-selected cell lines (375Rs) either as a dominant or minor variant (Table 4).
- The NS5B A442T and I585T mutations were also selected by IDX375; however, *in vitro* phenotypic assays demonstrate they are fully susceptible (<3-fold resistance) to IDX375 when expressed individually in an otherwise wild-type replicon background (Table 5).
- Minor variants in NS5B, such as C445F and C316Y, which confer resistance (>3-fold resistance) to IDX375 in *in vitro* phenotypic assays, were each selected in one or two IDX375-resistant cell lines.
- Other minor variants were also observed in NS5B following IDX375-selection. Their roles are unclear and they are currently being phenotyped.
- Various combinations of dominant and minor mutations within NS5B in the 375R cell lines conferred >100-fold resistance to IDX375.

Table 4: Summary of NS5B mutations selected by IDX375 in GS4.1 cells

Cell Line	Dominant mutations ^a	IDX375 selection concentration (nM)
375R-A	M414T	25 - 50
375R-B	M414T	250 - 500
375R-C	-	20 - 1600
375R-D	A442T ^b	20 - 1600
375R-E	A442T ^b	100 - 1600
375R-F	M414T	20
375R-G	M414T	400

^a Dominant mutations that consistently emerged in more than a single 375R line are listed.
^b Replicons bearing the A442T mutation were fully susceptible to IDX375 in phenotypic assays (see Table 5).

In vitro phenotypic and cross-resistance analyses of IDX375

- Resistance of replicons bearing individual NS5B mutations and their replication capacity compared to wild-type replicon was determined following transient transfection (Table 5).
- Three mutants replicated at or above wild-type capacity: M414T, C445F and I585T. Four mutants replicated at an intermediate level: C316Y, M423T, A442T and I482L. Six mutants replicated at a low level relative to wild-type: S282T, S365T, M423V, C445Y, Y448H and P495L.
- Replicons bearing the NS5B S282T, M423T/V, A442T, I482L, P495L and I585T mutations were fully susceptible (<3-fold resistance) to IDX375.
- However, HCV replicons bearing the C316Y, S365T, M414T, C445F/Y and Y448H mutations in the genotype 1b HCV polymerase were resistant (>3-fold resistant) to IDX375 to varying degrees.

Table 5: Activity of IDX375 against replicons bearing resistance mutations to polymerase inhibitors

Replicon	Confers resistance to ^a	EC ₅₀ Fold-change	Replication Capacity ^b (% of WT)
S282T	2'-C-Methyl-nucleosides ^c	2.68	5
C316Y	Benzofurans/benzothiadiazines ^c	738	29
S365T	Benzofurans ^c	12.6	2
M414T	Benzothiadiazines ^c	26.8	122
M423T	Thiophenes ^c	1.04	40
M423V	Thiophenes/Pyranindoles ^c	1.12	11
A442T	Pyranindoles ^c	0.83	60
C445F	Benzofurans ^c	5.59	196
C445Y	N/A ^d	64.8	14
Y448H	Benzothiadiazines ^c	89.4	10
I482L	Thiophenes ^c	1.32	73
P495L	Benzimidazole-based ^c	1.69	16
I585T	N/A ^d	2.01	239

The average EC₅₀ of the wild-type replicon against IDX375 in the transient transfection assay was 1.53 nM.
^a Resistance profiles for the polymerase inhibitors examined were based on published reports and were independently verified when applicable (data not shown).
^b Replication capacity was evaluated at least 3 times for each mutant replicon.
^c WT = wild-type
^d N/A (not applicable); published evidence has not directly demonstrated that this mutation confers resistance to HCV antivirals

CONCLUSIONS

- Short-term (3-day) combination treatment of replicon-bearing cells with IDX375 and either IDX184, an IDX PI, IFN- α or RBV was additive.
- Similarly, long-term (14- to 21-day) combination treatment of replicon-bearing cells with IDX375 and either IDX184 or IFN- α was additive.
- Combination treatment of IDX375 and IDX184 or IFN- α was more effective at inhibiting the emergence of drug resistant colonies compared to each drug alone.
- Continuous treatment of genotype 1b replicon-bearing cells with IDX375 selected for several mutations within NS5B, with M414T being the predominant mutation found to confer resistance to IDX375.
- HCV replicons bearing the M414T mutation have a higher replication capacity and are resistant to IDX375, whereas HCV replicons bearing the S282T mutation have a significantly reduced replication capacity and are fully susceptible to IDX375.
- Minor variants (C316Y or C445F) were detected by population sequencing following IDX375 selection and were shown to confer resistance to IDX375 by *in vitro* phenotypic testing. The contributions of other minor variants selected to IDX375-resistance are currently under investigation.
- In vitro* phenotypic data demonstrated that replicons bearing A442T or I585T were fully susceptible to IDX375; the roles of these mutants in IDX375-resistance remain unclear.
- Therefore, IDX375, like other NNIs, appears to have a relatively low barrier to resistance.
- The potency of IDX375, coupled with its lack of cross resistance with agents such as nucleotides and PIs (data not shown), suggest that it could find a useful place in HCV therapy when combined with standard of care or newer agents that may help to suppress resistance.

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Disclosures

All authors are current employees of Idenix Pharmaceuticals, Inc.