

INTRODUCTION

- Hepatitis C virus (HCV) is a common blood-borne pathogen infecting three to four million people worldwide annually. Currently, an estimated 170 million people are infected worldwide, 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 curative in approximately 40-50% of patients infected with HCV genotype 1 and is frequently associated with significant side effects.² Thus, additional therapies, including potent and safe direct-acting antiviral agents (DAAs), need to be developed for new, more effective and tolerable HCV treatment options.
- IDX184 is a liver-targeted nucleotide inhibitor (NI), which demonstrated anti-HCV activity and safety in a 3-day proof-of-concept study and is currently in Phase II clinical trials.
- IDX375 is a non-nucleoside inhibitor (NNI) that targets the palm pocket of the HCV NS5B polymerase and is currently in Phase I clinical trials.
- IDX320 is a macrocyclic protease inhibitor (PI) of the HCV NS3/4A protease. IDX320 exhibited multi-genotypic coverage *in vitro* and is currently in Phase I clinical trials.
- This *in vitro* study examined the potential for combining 3 DAAs by evaluating the antiviral activity of triple combinations of IDX184 with IDX320, IDX375 or a prototype NS5A inhibitor (IDX-NS5A).

METHODS

HCV replicon assay: Luciferase replicon-bearing cells (genotype 1b) were seeded onto 96-well plates, cultured for 3 days in the presence of compound(s) and subjected to luciferase assay. IDX184, IDX320, IDX375 and IDX-NS5A individually inhibited HCV replicon replication *in vitro* in a dose-dependent manner with EC₅₀ values of about 120, 0.7, 6 and 0.01 nM, respectively. The effects of drug combination were evaluated using MacSynergy software (Bliss Independence Model), and CalcuSyn software (Combination Index). Compound cytotoxicity was measured in parallel using a standard colorimetric proliferation assay.

14-day treatment assay: Replicon cells (genotype 1b) were treated with 1x EC₅₀ of compound(s), without G418, for 14 days and the level of replicon RNA was measured at multiple time points. IDX184, IDX320, IDX375 and IDX-NS5A individually reduced HCV RNA with EC₅₀ values of about 450, 1, 15 and 0.01 nM, respectively. At the end of the 14-day treatment, cells were cultured in the absence of compound(s) ± G418 in 10 cm dishes for 21 days, whereupon the surviving cell colonies were stained with crystal violet and counted.

RESULTS

Triple combinations of IDX320 and IDX375 with IDX184 show synergistic activity after 3-day treatment

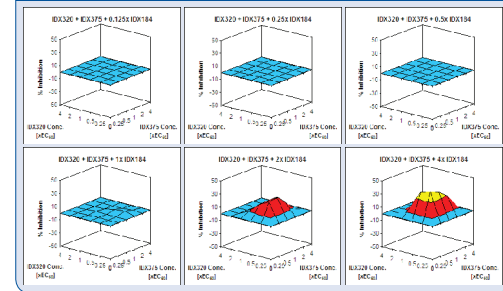
- IDX320 and IDX375 were serially diluted 1:2 from 4x to 0.25x their EC₅₀ values for five point titrations and combined in a constant ratio; IDX184 was titrated at 0.125x, 0.25x, 0.5x, 1x, 2x, 4x its EC₅₀ value and added to the IDX320/IDX375 combination.
- As shown in Table 1, additive antiviral activity was observed when IDX320 was combined with IDX375.
- A triple combination of IDX320 and IDX375 with IDX184 showed an additive to synergistic effect in the Bliss Independence model and Combination Index (CI) analysis (Table 1 and Figure 1).
- The strength of synergy was dose dependent with respect to IDX184.
- At IDX184 concentrations ranging from 0.25x to 1x EC₅₀, the triple combination showed evidence of synergy, according to the CI analysis.
- With IDX184 at 2x or 4x EC₅₀, the triple combination showed strong or even very strong synergy.

Table 1: Combination effects of IDX320 and IDX375 with IDX184

IDX320 + IDX375 + xEC ₅₀ IDX184	Bliss Independence	Combination Index
0	Additive	Additive
0.125x	Additive	Additive
0.25x	Additive	Moderate Synergy
0.5x	Additive	Synergy
1x	Additive	Synergy
2x	Strong Synergy	Strong Synergy
4x	Strong Synergy	Very Strong Synergy

3-day luciferase assay
No cytotoxicity was observed

Figure 1: Combination effects of IDX320 and IDX375 with IDX184 using the Bliss Independence model



A horizontal plane at 0% indicates an additive interaction. Any peaks above this plane indicate synergy or greater than additive interactions. Peak volumes were calculated and used to quantitate synergy; 99.9% confidence level values are presented here. These results are based upon 5 independent experiments.

Triple combinations of IDX320 and IDX-NS5A with IDX184 show synergistic activity after 3-day treatment

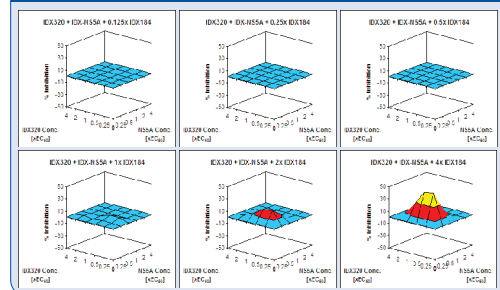
- IDX320 and a prototype NS5A inhibitor (IDX-NS5A) were serially diluted 1:2 from 4x to 0.25x their EC₅₀ values for five point titrations and combined in a constant ratio; increasing concentrations of IDX184 (0, 0.125x, 0.25x, 0.5x, 1x, 2x, 4x its EC₅₀ value) were added to the IDX320/IDX-NS5A combination.
- As shown in Table 2, additive to synergistic activity was observed when IDX320 was combined with IDX-NS5A.
- Triple combinations of IDX320 and IDX-NS5A with IDX184 showed additive to synergistic effects in the Bliss Independence model and synergistic effects in the CI analysis (Table 2 and Figure 2).
- The strength of synergy was dose dependent with respect to IDX184.
- By Bliss Independence analysis at concentrations of 2x and 4x EC₅₀ of IDX184, the triple combination showed moderate to strong synergy.
- By CI analysis, strong to very strong synergy was observed for triple combinations containing IDX184 at 1x to 4x EC₅₀.

Table 2: Combination effects of IDX320 and IDX-NS5A with IDX184

IDX320 + IDX-NS5A + xEC ₅₀ IDX184	Bliss Independence	Combination Index
0	Additive	Synergy
0.125x	Additive	Synergy
0.25x	Additive	Synergy
0.5x	Additive	Synergy
1x	Additive	Strong Synergy
2x	Moderate Synergy	Strong Synergy
4x	Strong Synergy	Very Strong Synergy

3-day luciferase assay
No cytotoxicity was observed

Figure 2: Combination effects of IDX320 and IDX-NS5A with IDX184 using the Bliss Independence model

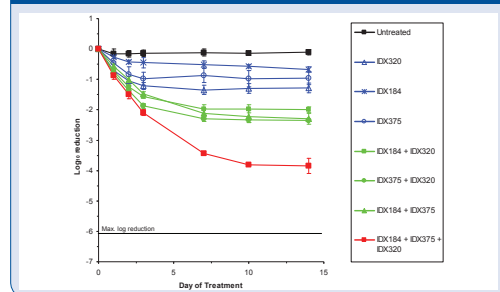


A horizontal plane at 0% indicates an additive interaction. Any peaks above this plane indicate synergy or greater than additive interactions. Peak volumes were calculated and used to quantitate synergy; 99.9% confidence level values are presented here. These results are based upon 5 independent experiments.

IDX184, IDX320 and IDX375 triple combination gives greater than additive activity after 14-day treatment

- During 14-day treatment of replicon cells, a greater decline in replicon RNA was observed for the double combinations (green lines) when IDX184 was combined with IDX320 or IDX375 and when IDX320 was combined with IDX375 (Figure 3 and Table 3), as compared with single agent treatment (blue lines), consistent with an additive antiviral effect.
- When IDX184 was combined with IDX320 and IDX375 (Figure 3 and Table 3), the decline in replicon RNA was 0.2 to 0.8 log₁₀ greater for the triple combination (red line) than the predicted additive antiviral effect.
- No HCV RNA rebound or cytotoxicity was observed in these studies.

Figure 3: Effect of 14-day treatment with IDX184, IDX320 and IDX375 on replicon RNA



Replicon RNA was quantitated by RT-qPCR of 5'-UTR and normalized to GAPDH. All compounds were tested at 1x EC₅₀.

Table 3: Effect of 14-day treatment with IDX184, IDX320 and IDX375 on replicon RNA

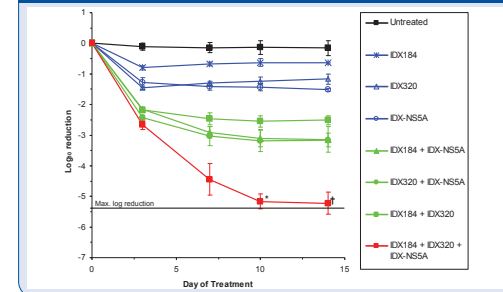
IDX184 conc. (1x EC ₅₀)	IDX375 conc. (1x EC ₅₀)	IDX320 conc. (1x EC ₅₀)	Log ₁₀ reduction in replicon RNA ^a	Number of colonies following G418 selection ^b
-	-	-	0.1	> 400
+	-	-	0.7	> 400
-	+	-	1.0	> 330
-	-	+	1.3	> 273
+	-	+	2	155
-	+	+	2.4	28
+	+	-	2.3	38
+	+	+	3.8	3

^a Values represent the mean log₁₀ reduction values across three independent experiments. The log₁₀ reduction value 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.

IDX184, IDX320 and IDX-NS5A triple combination gives greater than additive activity after 14-day treatment

- During 14-day treatment of replicon cells, a greater decline in replicon RNA was observed for the three tested double combinations (Figure 4 (green lines) and Table 4), as compared with single agent treatment (blue lines), consistent with at least an additive antiviral effect.
- When IDX184 was combined with IDX320 and IDX-NS5A (Figure 4 and Table 4), the decline in replicon RNA was greater than single agent treatment (blue lines) or double combination treatment (green lines), giving at least a 2.3 log₁₀ reduction in replicon RNA beyond the predicted additive antiviral effect.
- Since HCV RNA could not be amplified after 14 days of treatment with IDX184, IDX320 and IDX-NS5A, the 2.3 log₁₀ additional reduction is a minimum estimate of the increased activity.
- No RNA rebound or cytotoxicity was observed in these studies.

Figure 4: Effect of 14-day treatment with IDX184, IDX320 and IDX-NS5A on replicon RNA



Replicon RNA was quantitated by RT-qPCR of 5'-UTR and normalized to GAPDH. All compounds were tested at 1x EC₅₀.
* no amplification of HCV RNA (2/6 replicates) and one extrapolated value
† no amplification of HCV RNA (5/6 replicates)

Table 4: Effect of 14-day treatment with IDX184, IDX320 and IDX-NS5A on replicon RNA

IDX184 conc. (1x EC ₅₀)	IDX320 conc. (1x EC ₅₀)	IDX-NS5A conc. (1x EC ₅₀)	Log ₁₀ reduction in replicon RNA ^a	Number of colonies following G418 selection ^b
-	-	-	0.1	> 200
+	-	-	0.6	> 200
-	+	-	1.2	> 172
-	-	+	1.5	> 111
+	-	+	3.1	9
+	+	-	2.5	53
-	+	+	3.2	7
+	+	+	No amp (5/6 replic.)	0

^a Values represent the mean log₁₀ reduction values across three independent experiments. The log₁₀ reduction value 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.

Combination treatment amplifies suppression of replicon bearing cells

- The cells treated with IDX184, IDX320 and IDX375 or IDX-NS5A were further cultured without compound(s) ± G418 to quantitate the remaining replicon-bearing cells.
- As expected, in the absence of G418 selection

pressure, confluent cells were observed after treatment with all combinations of IDX184, IDX320 and IDX375 or IDX-NS5A, indicating that 14-day combination treatment did not affect cell viability (data not shown).

- In the presence of G418, the suppression of replicon-bearing cells was greater after 14-day triple combination treatment than with single or double combinations (Figures 5 and 6; Tables 3 and 4).
- This observation is consistent with results obtained from the 3-day combination treatment of luciferase replicon-bearing cells demonstrating that combination treatment of IDX184 with IDX320 and IDX375 or IDX-NS5A was synergistic (Figure 1 and 2; Table 1 and 2).

Figure 5: Colony formation assay following combination treatment of replicon-bearing cells with IDX184, IDX320 and IDX375

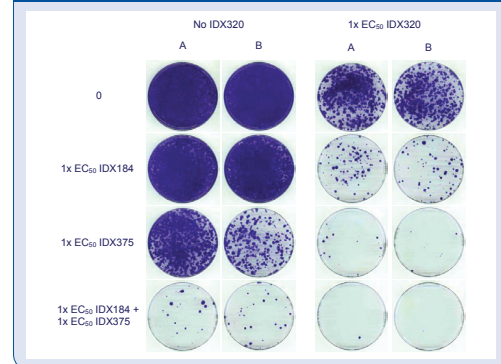
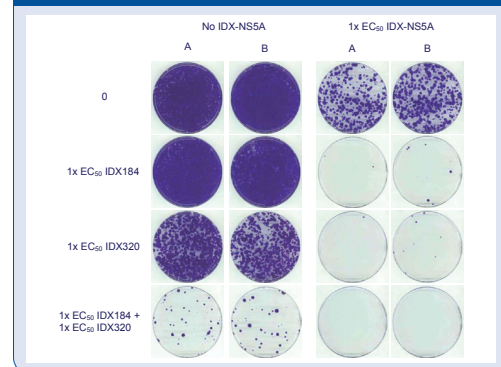


Figure 6: Colony formation assay following combination treatment of replicon-bearing cells with IDX184, IDX320 and IDX-NS5A

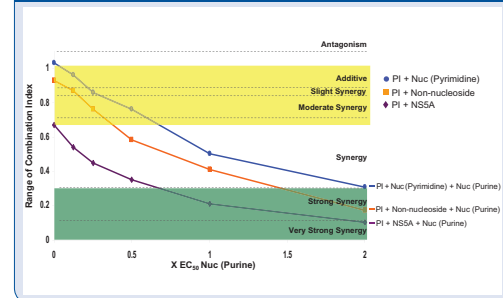


Triple combinations of DAAs with different targets and mechanisms of action (MOAs) demonstrate the most profound synergy *in vitro*

This experiment tested 3 different triple combinations in the 3-day assay (CI analysis)

- PI + 2 NIs (2 targets, 2 MOAs)
- PI + NNI + NI (2 targets, 3 MOAs)
- PI + NS5A inhibitor + NI (3 targets, 3 MOAs)
- The strength of synergy was dose dependent with respect to IDX184.
- The different double combinations demonstrated additive activity by CI analysis, with only the PI + NS5A combination demonstrating moderate synergy.
- The most profound *in vitro* synergy was achieved with triple combination of HCV DAAs with 3 different targets and 3 mechanisms of action (Figure 7).
 - PI + NS5A inhibitor + NI > PI + NNI + NI > PI + NI + NI.

Figure 7: Effect of DAAs triple combination after 3-day treatment *in vitro*



Two-drug and three-drug combinations tested at equivalent concentrations (EC₅₀) in the HCV replicon model.
■ = double combination effect
■ = triple combination effect

CONCLUSIONS

- Combination of IDX320 with IDX375 resulted in an additive effect after 3 days of treatment *in vitro*.
- Combination of IDX320 with IDX-NS5A resulted in additive to synergistic effect after 3 days of treatment *in vitro*.
- In contrast, triple combinations of IDX184 with IDX320 and IDX375 or IDX-NS5A resulted in synergistic effects after 3 days of treatment *in vitro*; the strength of synergy was dose dependent with respect to IDX184.
- Triple combinations of IDX184 with IDX320 and IDX375 or IDX-NS5A for 14 days indicated that these enhanced antiviral effects were maintained with no evidence of HCV RNA rebound or cellular cytotoxicity.
- The profound antiviral effect and the marked *in vitro* synergy of the triple DAA regimens with different mechanisms of action support the clinical evaluation of such combination therapies in HCV-infected patients.

ACKNOWLEDGMENTS

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REFERENCES

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DISCLOSURES

All authors are current employees of Idenix Pharmaceuticals, Inc.