

PRECLINICAL CHARACTERIZATION OF THE NON-NUCLEOSIDE POLYMERASE INHIBITOR HCV-796

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ABSTRACT

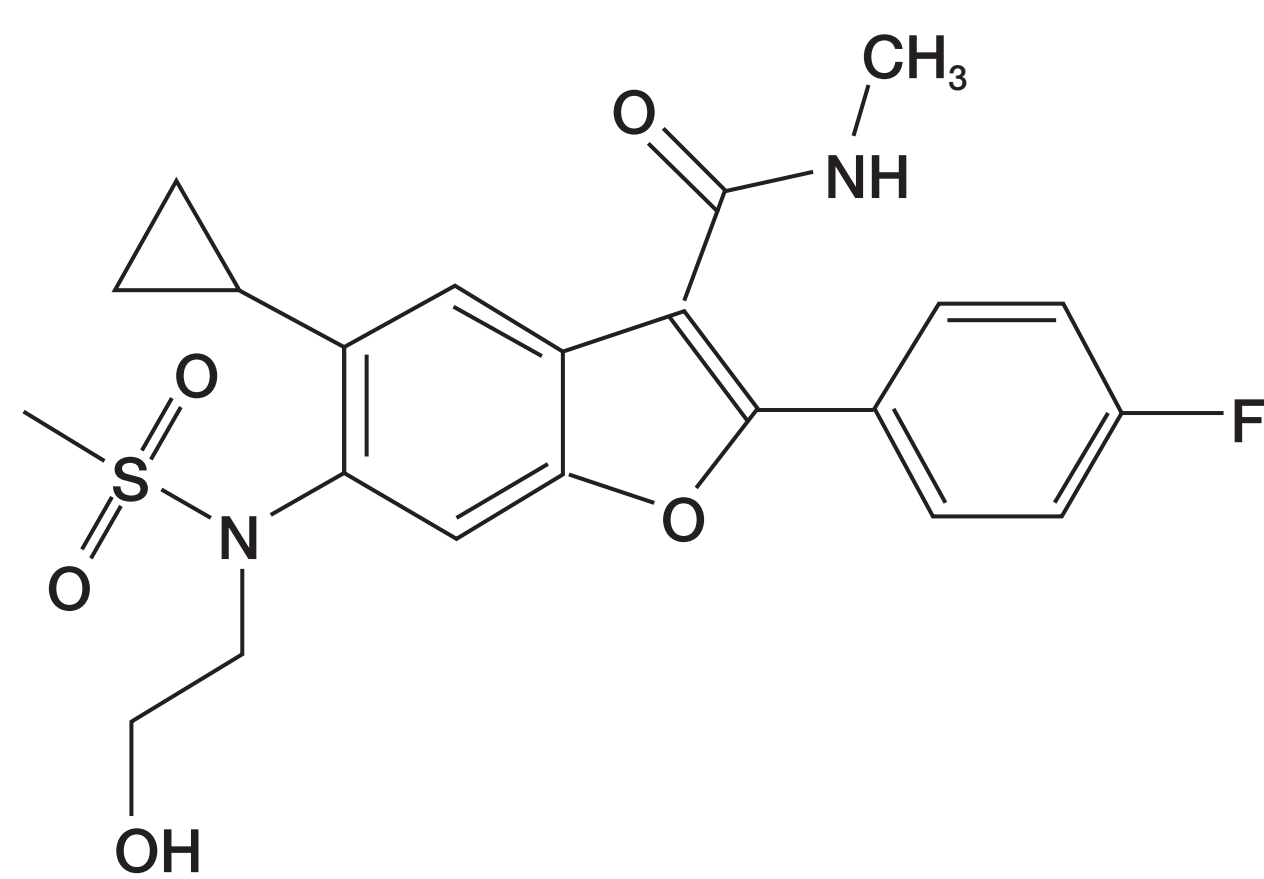
Background: Hepatitis C virus (HCV) is an enveloped, single-stranded positive sense RNA virus with an RNA dependent RNA polymerase (RdRp), NS5B, which is essential for HCV replication. A similar counterpart does not exist in mammalian cells. Consequently, an NS5B inhibitor should be an effective and selective antiviral agent for treating HCV infection.

Methods: Activity of HCV-796 against HCV RdRp and other human polymerases was evaluated in nucleotide incorporation assays using purified recombinant enzymes. Intracellular antiviral activity of HCV-796 was determined in HCV replicon cells. Cytotoxicity was assessed using a standard MTT assay. Specificity was investigated using a panel of RNA and DNA viruses.

Results: HCV-796 was effective against HCV polymerases derived from genotypes 1a, 1b, 2, 3, and 4. HCV-796 exhibited no activity against a panel of human and other unrelated viral polymerases. A single application of HCV-796 in cells containing genotypes 1a and 1b replicons led to a dose-dependent reduction of HCV RNA levels (EC₅₀s < 10 nM). Multiple treatments of cells with 1 μM HCV-796 for 16 days reduced HCV RNA levels by ~4-log₁₀. An additive antiviral effect was observed in cells treated with HCV-796 plus pegylated interferon. No cytotoxicity was observed in 6 cell lines under dividing and non-dividing conditions. HCV-796 inhibited HCV RdRp non-competitively with respect to substrate NTPs and RNA. Results from single and continuous processive cycle polymerase reactions suggest that HCV-796 may interfere with the initiation event of RNA polymerization, consistent with x-ray crystallography studies.

Conclusion: HCV-796 possesses all the hallmarks of an effective and selective antiviral agent against HCV infection.

Figure 1: HCV-796 Structure



In Vitro Biochemical Activities of HCV-796

Table 1: Activity of HCV-796 Against NS5B RdRp From Various HCV Genotypes

Genotype Enzyme Isolate ^a	HCV-796 IC ₅₀ (μM) ± SD	Amino Acid Sequence Divergence From Genotype 1b BB7 NS5B	
		%	# of Amino Acids
Genotype 1b			
BB7	0.04 ± 0.02 (N = 35)	-	-
BK	0.14 ± 0.05 (N = 33)	3.7	22
4a	0.05 ± 0.04 (N = 9)	2.7	16
FC 1-20	0.05 ± 0.03 (N = 7)	4.7	28
320-10-3	0.02 ± 0.01 (N = 8)	3.6	21
J4	0.16 ± 0.05 (N = 10)	4.7	28
Genotype 1a			
H77	0.04 ± 0.01 (N = 8)	11.8	70
A5	0.03 ± 0.01 (N = 7)	11.5	68
207-247	0.013 ± 0.006 (N = 9)	11.8	70
Genotype 2			
J6	1.71 ± 1.03 (N = 4)	25	145
Genotype 3			
167-A1	0.22 ± 0.08 (N = 10)	24	142
Genotype 4			
	0.67 ± 0.30 (N = 8)	21	125

a. All NS5Bs are histidine-tagged fusion proteins with 21 amino acid carboxyl terminal deletions; divergence is based on the 591 amino acids of the full-length proteins.
 HCV = Hepatitis C virus; IC₅₀ = Concentration that inhibits 50% of activity; N = Number (refers to independent determinations in this study); NS5B = Nonstructural protein 5B; RdRp = RNA-dependent RNA polymerase, SD = Standard deviation.

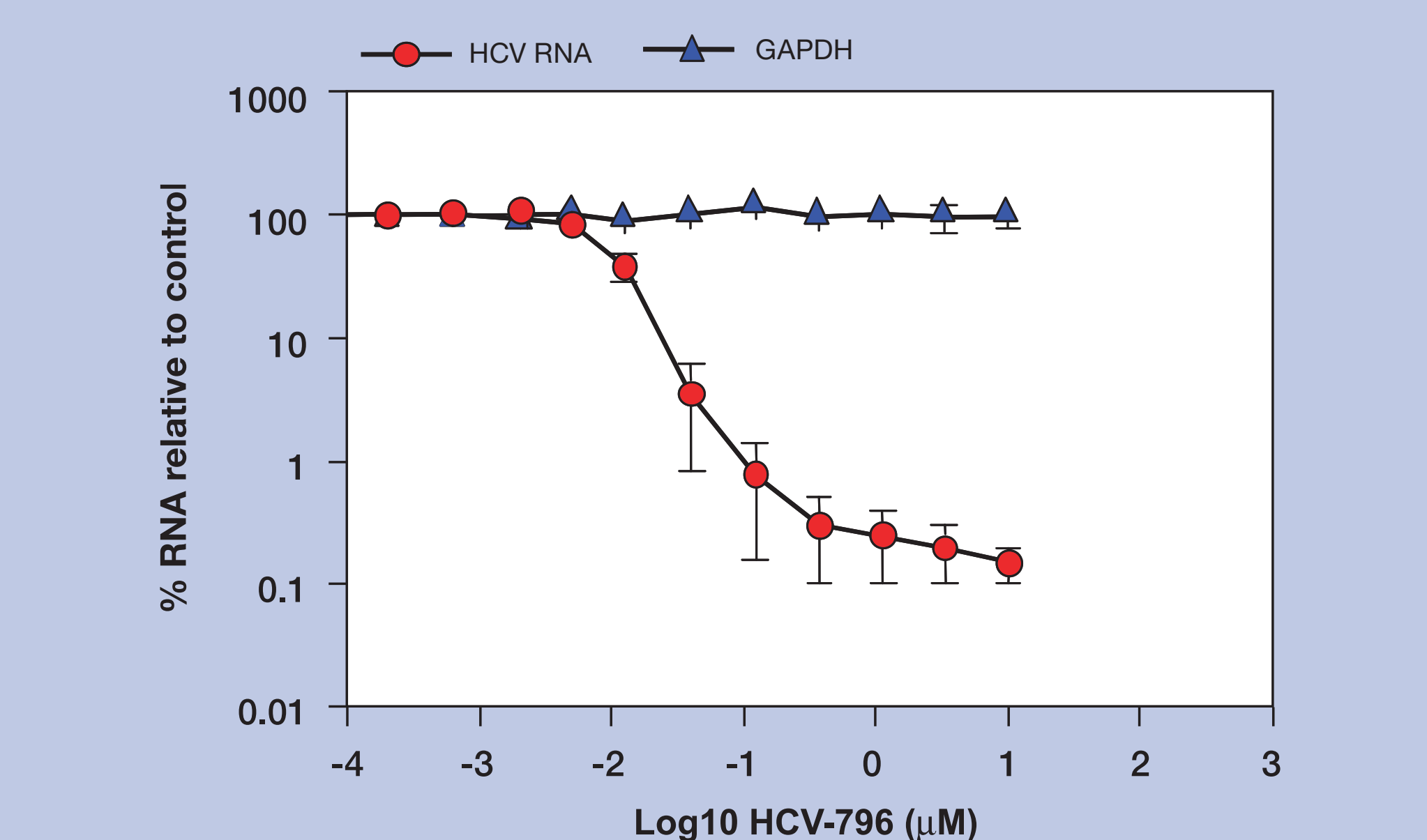
Table 2: Biochemical Selectivity of HCV-796

Enzyme	IC ₅₀ (μM)
HCV NS5B (Genotypes 1a, 1b, 2, 3, and 4)	0.01 - 1.7
Human DNA polymerase α	> 30 ^a
Human DNA polymerase β	> 56 ^a
Human DNA polymerase γ	> 56 ^a
Human RNA polymerase II	> 30 ^a
Calf thymus DNA polymerase α	> 30 ^a
HIV reverse transcriptase	> 30 ^a
RNA Intercalation	> 30 ^a
Selectivity Index	> 18 - > 5600

a. Each value represents at least 2 independent determinations. Substrate concentrations in the above enzyme assays were adjusted to the corresponding K_m levels.
 HCV = Hepatitis C virus; HIV = Human immunodeficiency virus; IC₅₀ = Concentration that inhibits 50% of activity; K_m = Michaelis-Menton constant; NS5B = Nonstructural protein 5B.

Intracellular Antiviral Activities

FIGURE 2a. Effect of Single Treatment (3 days) With HCV-796 on Intracellular HCV and GAPDH RNA Levels



Compound	HCV RNA EC ₅₀ (nM) ± SD ^a	HCV Protein EC ₅₀ (nM) ± SD	GADPH CC ₅₀ (μM) ± SD	Cell Cytotoxicity CC ₅₀ (μM) ± SD	Therapeutic Index ^a
1a replicon	4.5 ± 2.0 (N = 17)	19 ± 14 (N = 12)	≥ 10 (N = 7)	ND	> 2000
1b replicon	8.6 ± 4.0 (N = 14)	14 ± 15 (N = 25)	≥ 10 (N = 8)	≥ 50	> 1100

a. Based on HCV RNA and GAPDH.
 CC₅₀ = Concentration of compound that inhibits 50% of cell viability/functions; EC₅₀ = 50% effective concentration; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase; HCV = Hepatitis C virus; N = Number (refers to independent determinations in this study); ND = Not done; SD = Standard deviation.

FIGURE 2b. Effect of Multiple Treatments (22 days) With HCV-796 on Intracellular HCV RNA Levels in Genotype 1b Replicon

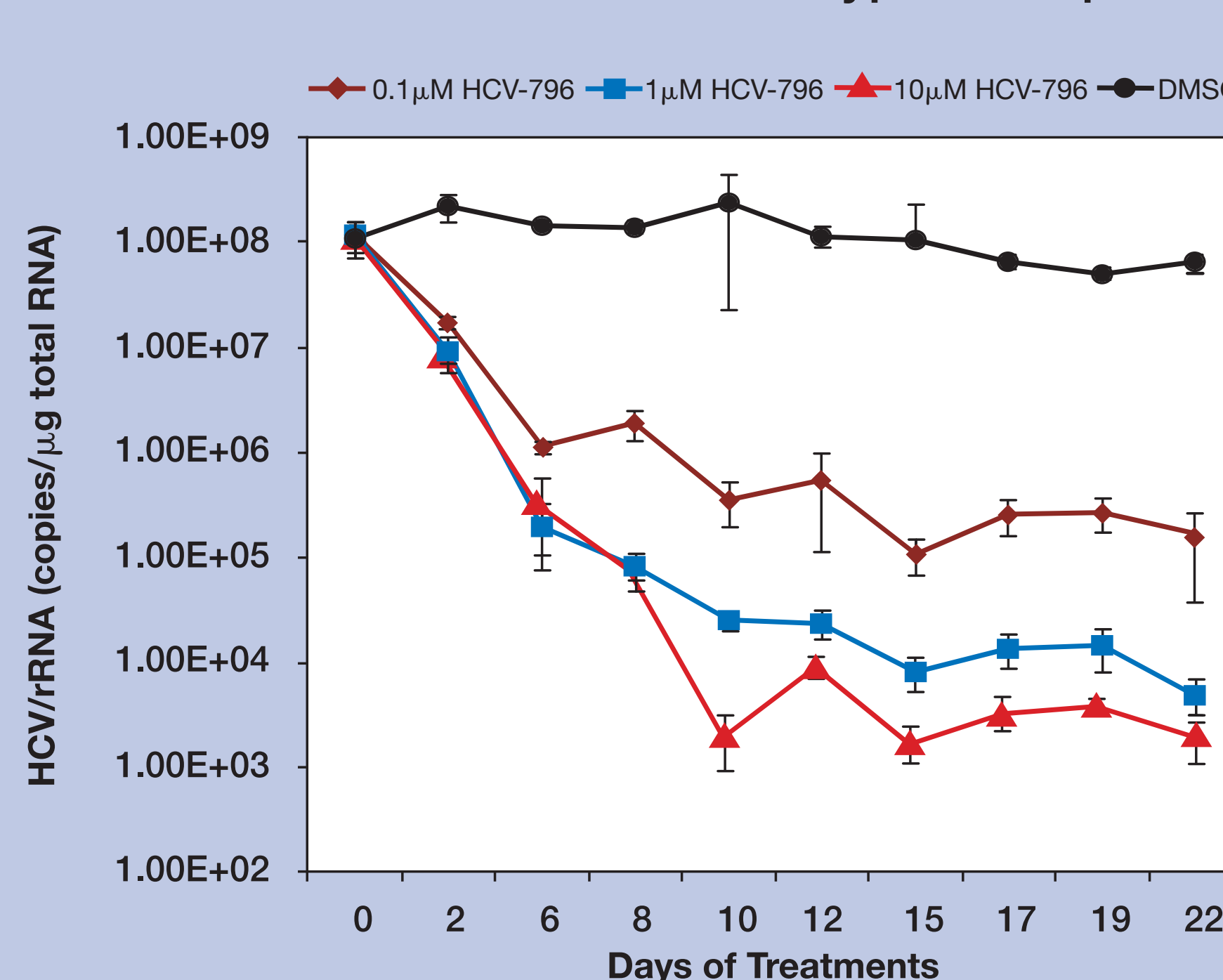
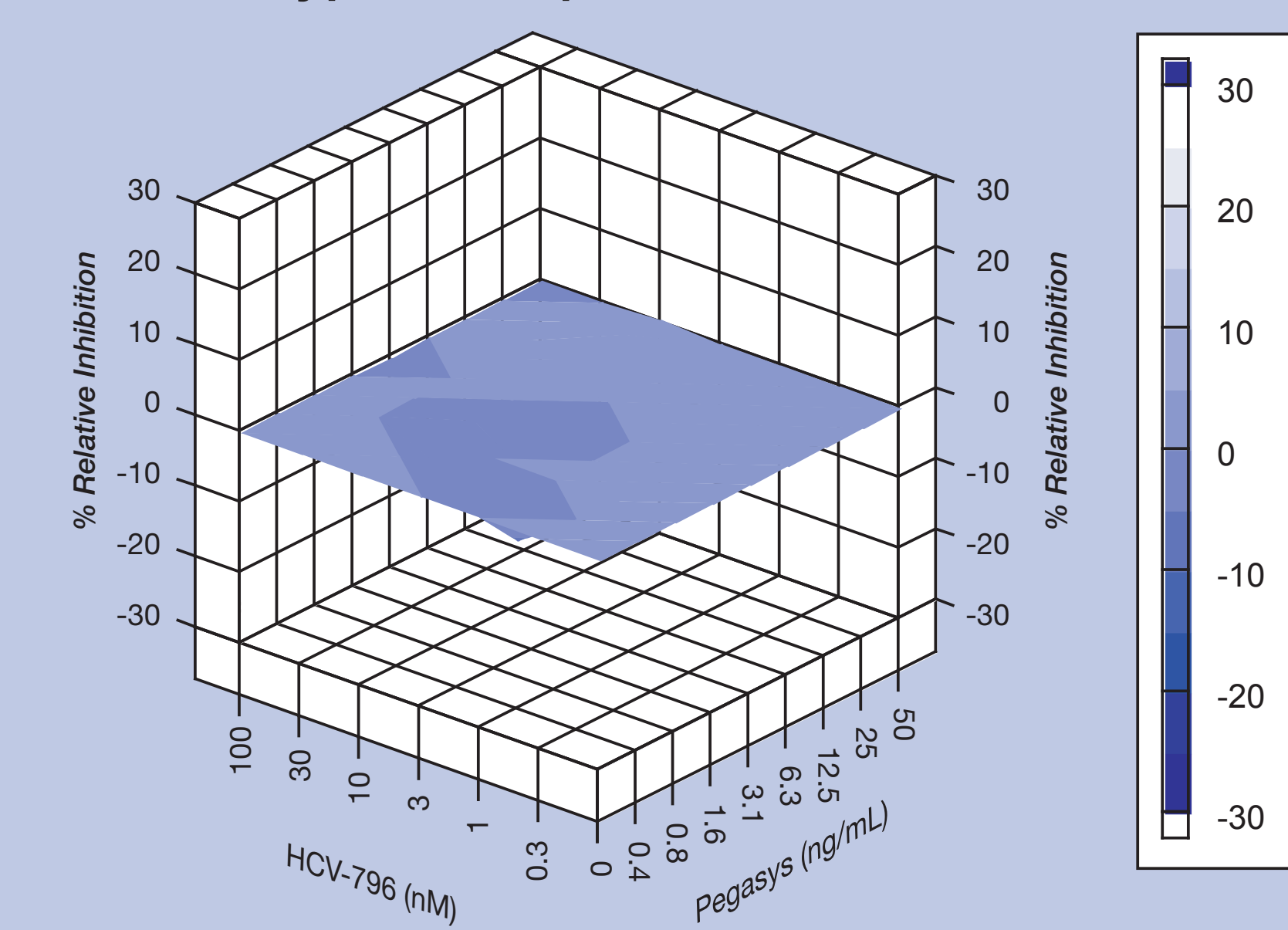


FIGURE 3. Drug Combinations of HCV-796 and Pegylated Interferon in HCV Genotype 1b Replicon



The antiviral activities of combinations of HCV-796 and pegylated interferon (PEGASYS[®]) were studied using the Bliss Independence null model of additivity (MacSynergy[™] II). A 3-dimensional graphical representation of these data, at the 95% confidence interval, is shown. Additive interactions appear as a horizontal plane at 0%. Any peaks above the plane are indicative of synergy, or greater than expected effects. Conversely, peaks below the plane are indicative of antagonism, or less than expected effects. The degree of synergy or antagonism in the 3-dimensional graph can be quantified using the shading scale on the right.

Therapeutic Window

Table 3: Effect of HCV-796 on Mammalian Cell Proliferation

Cell Line	Species	Tissue	Morphology	EC ₅₀ or CC ₅₀ (nM) ^a
HCV replicon Genotype 1a (H77)	Flaviviridae	Positive single strand RNA		5 ± 2 (N = 17) ^b
Genotype 1b (BB7)				9 ± 4 (N = 14) ^b
Huh-7-Clone A	Human	Liver	Epithelial	> 50,000 ^c
HeLa	Human	Cervix	Epithelial	> 50,000 ^c
HEp-2	Human	Larynx	Epithelial	> 50,000 ^c
HOS	Human	Bone	Mixed	> 50,000 ^c
CHO-K1	Hamster	Ovary	Epithelial	> 50,000 ^c
C6	Rat	Brain	Fibroblast	> 50,000 ^c
Therapeutic Index				> 5000

a. Each value represents at least 2 independent determinations, except where the number (N) of assays is specified.
 b. EC₅₀ = 50% effective concentration derived from HCV RNA in genotype 1a and 1b replicons.
 c. CC₅₀ = Concentration of compound that inhibits 50% of cell viability/functions.
 HCV = Hepatitis C virus; N = Number (refers to independent determinations in this study). Therapeutic index = CC₅₀ / EC₅₀.

Mechanism of Action

FIGURE 4. Kinetics of Inhibition of HCV NS5B Polymerase by HCV-796

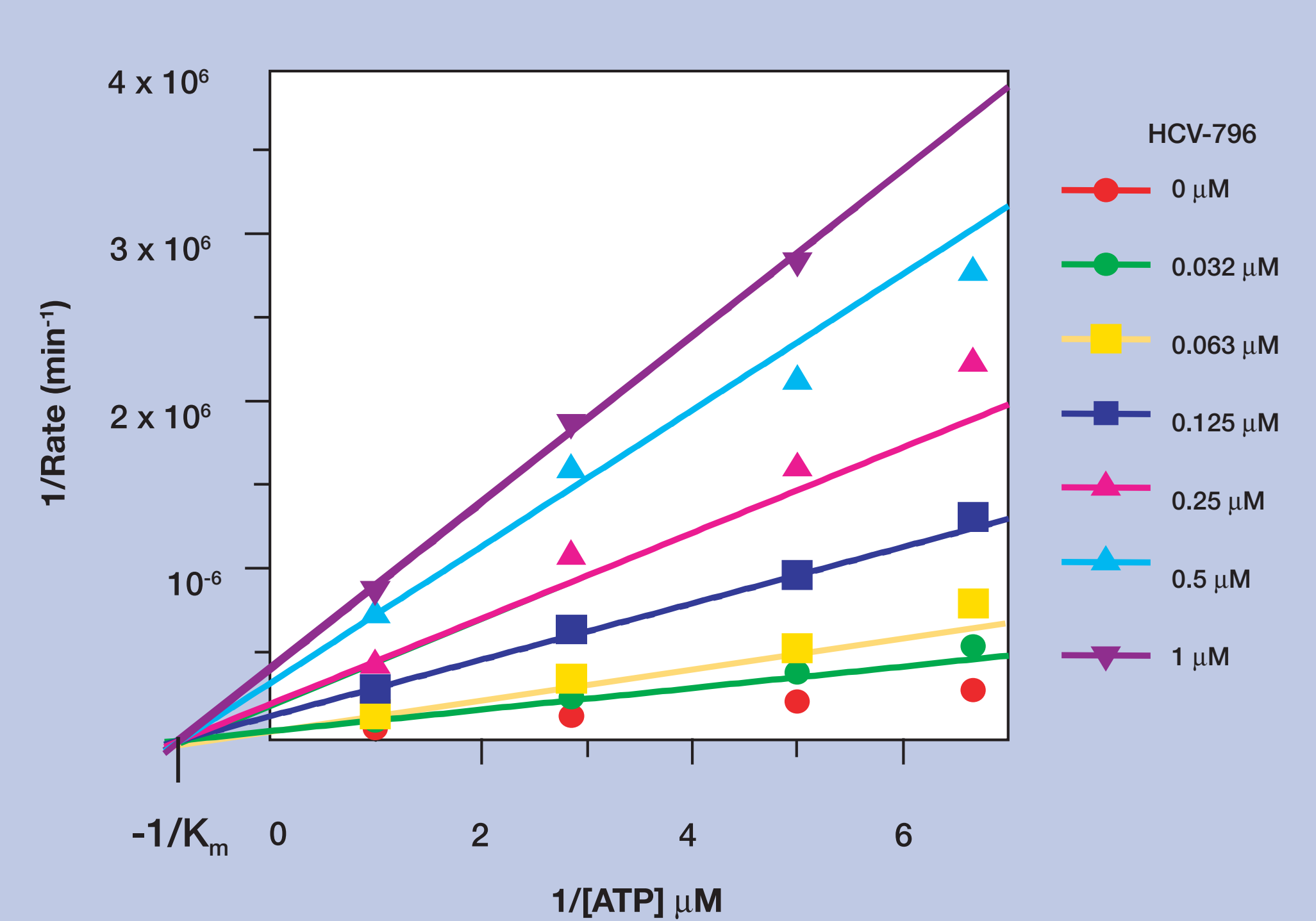


FIGURE 5a. The Activity of HCV-796 under Continuous and Single Processive Cycle Polymerization Conditions

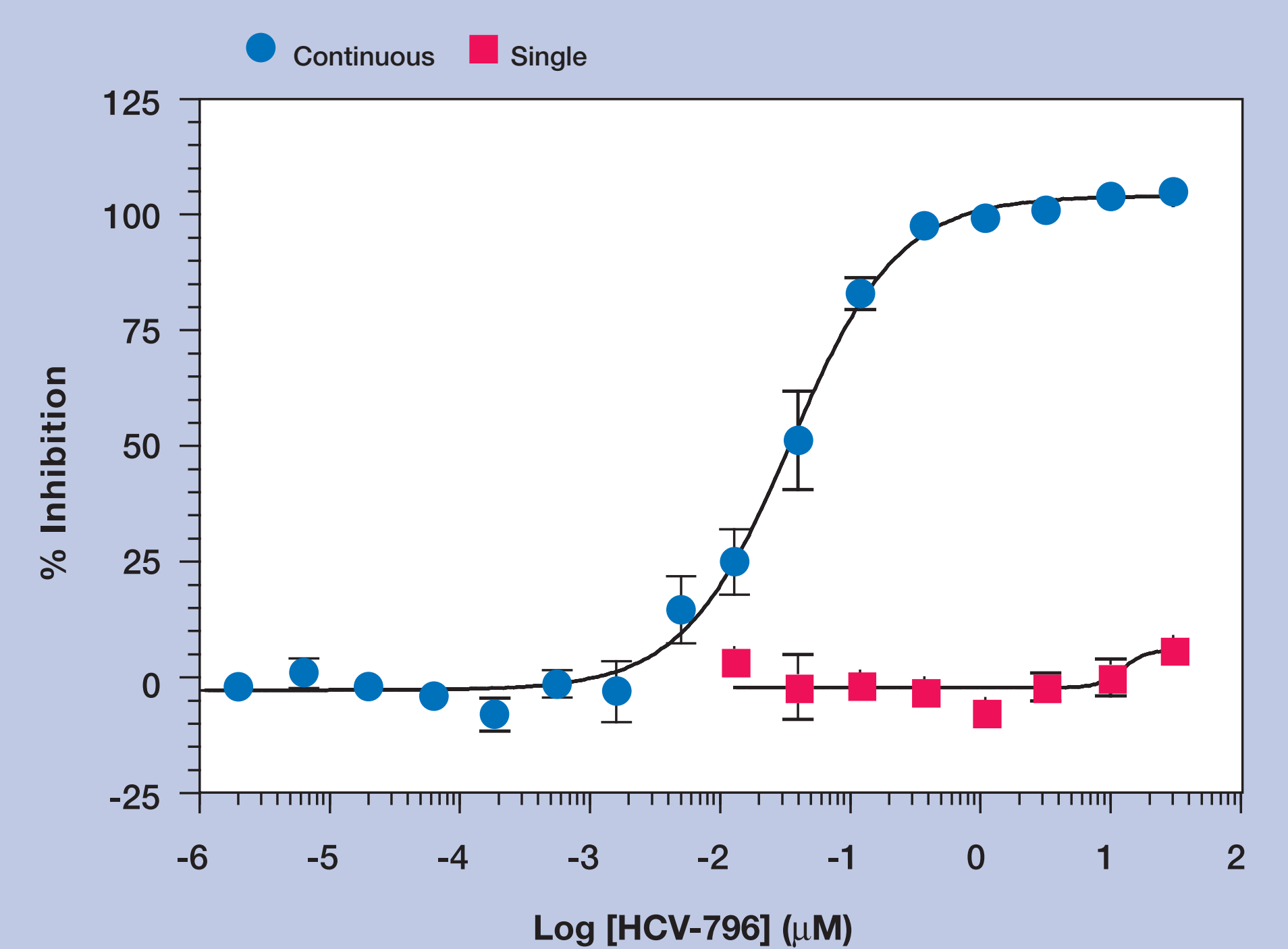
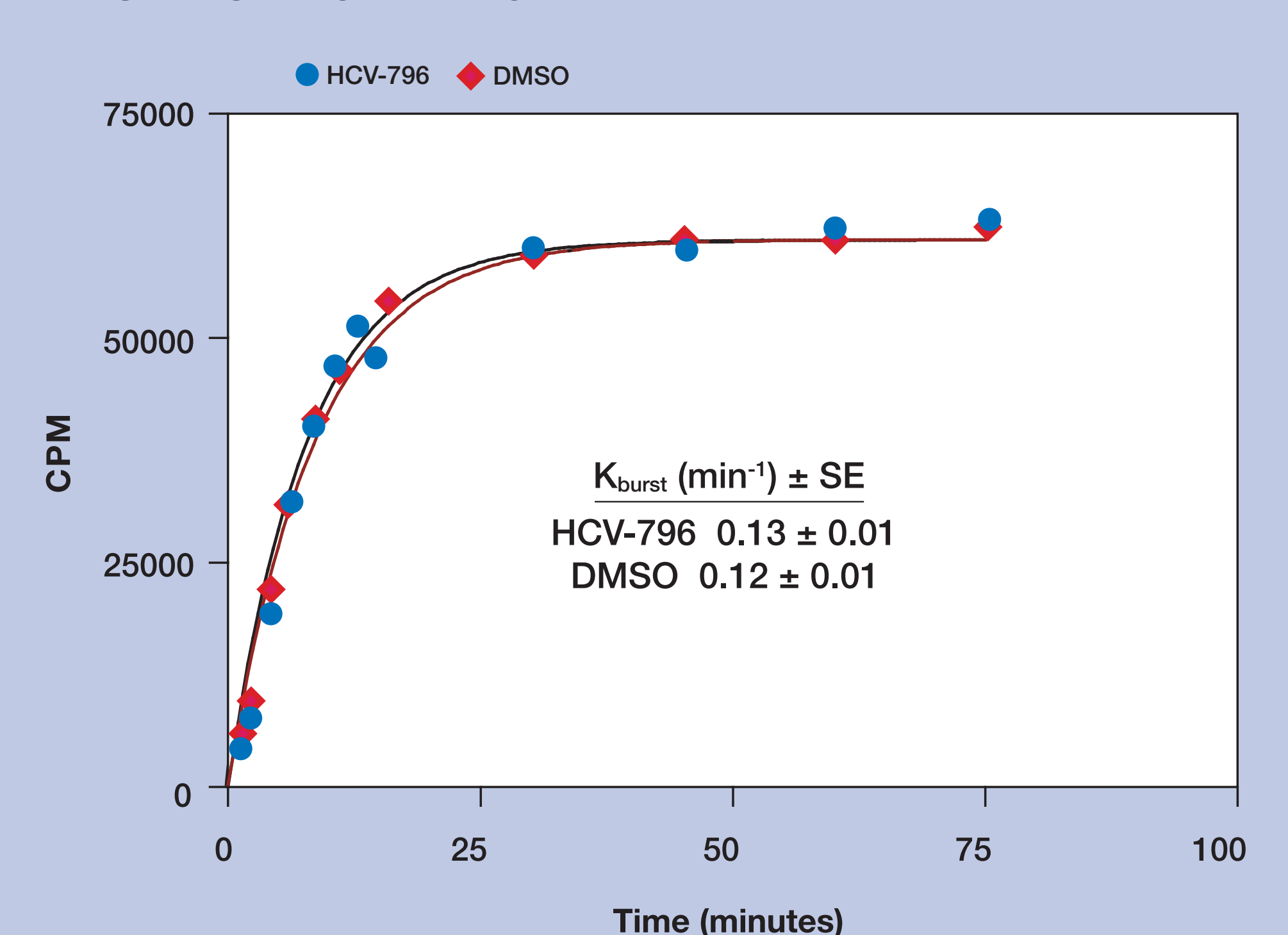


FIGURE 5b. Time Course of HCV NS5B Activity in the Presence and Absence of 30 μM HCV-796 Using Single Cycle Polymerization Conditions



CONCLUSIONS

In Vitro Biochemical Activities

- HCV-796 inhibits HCV RdRp with broad genotype activities.
- The inhibition against NS5B is specific. No activity was observed in a panel of human and other related viral polymerases.

Intracellular Antiviral Activities

- HCV-796 displays potent intracellular antiviral activities in genotype 1a and 1b replicons. Multiple treatments of cells with 1 μM HCV-796 for 16 days reduced HCV RNA levels by ~4-log₁₀.
- The combinations of HCV-796 and interferon resulted in additive antiviral activities in HCV replicon.

- No cytotoxicity was observed in a panel of cell lines. Therapeutic Index > 5000.

Mechanism of Action

- HCV-796 is a noncompetitive inhibitor of HCV NS5B with respect to substrate NTPs and RNA template.
- Results from single and continuous cycle, and burst kinetics analyses showed that HCV-796 inhibited the NS5B at the initiation step of RNA synthesis.

Wyeth

