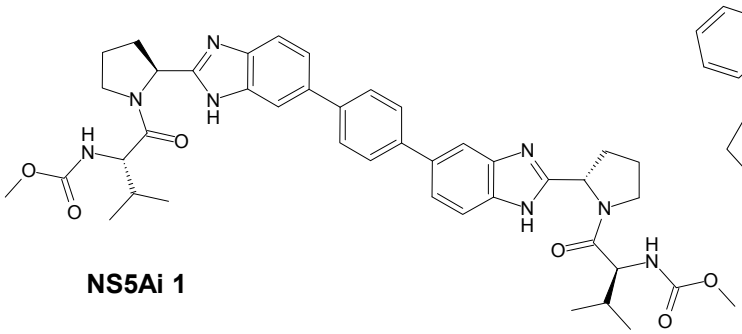
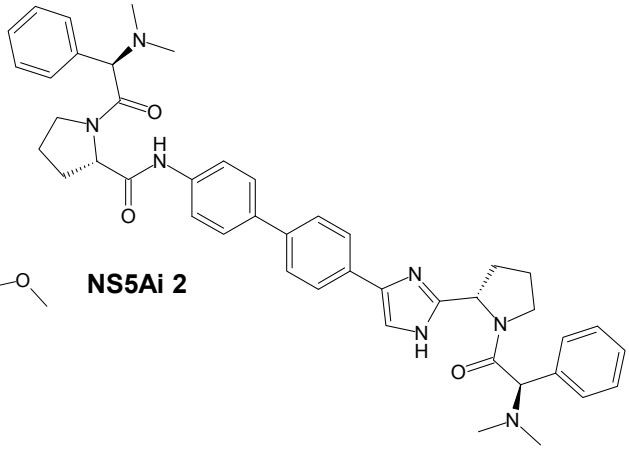
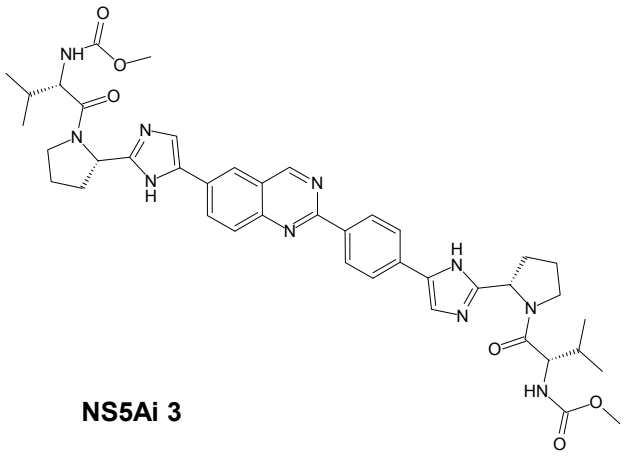
Fig. S1



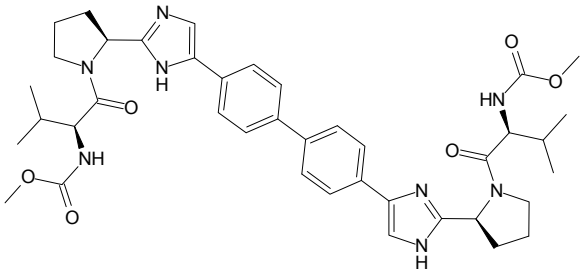
NS5Ai 1



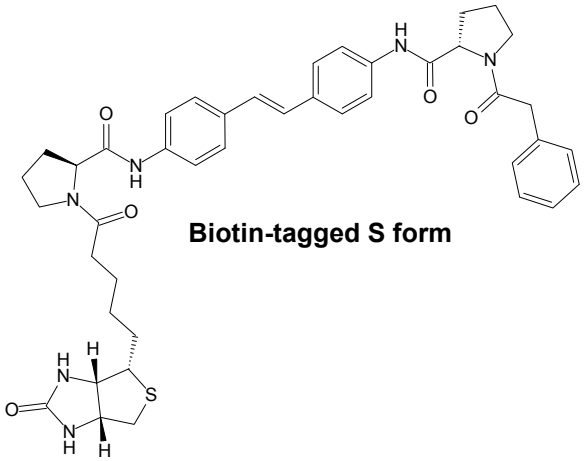
NS5Ai 2



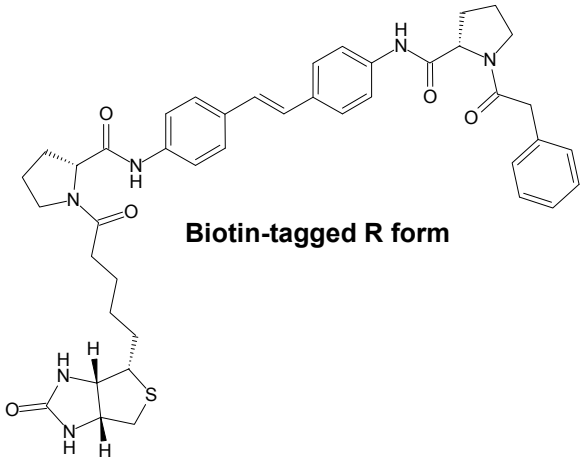
NS5Ai 3



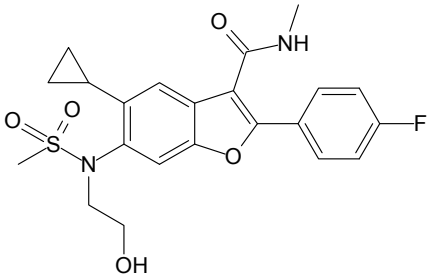
NS5Ai 4 (BMS-790052)



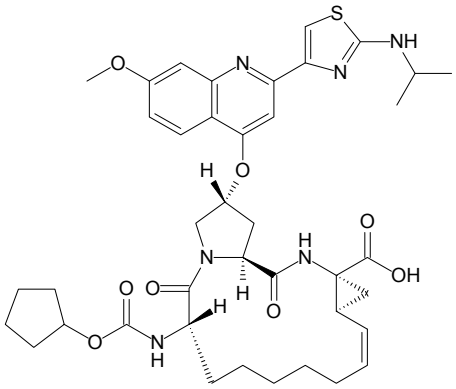
Biotin-tagged S form



Biotin-tagged R form



HCV-796



BILN 2061

Figure S1. Structures of compounds used in the present study.

Supplemental Table. S1

Sample	Changes in NS5A primary amino acid sequence																	
Con1																		
DMSO																		
NS5Ai A 8x		Q54Q/H	Y93Y/H	V196V/A														
NS5Ai A 20x	L31L/V		Y93Y/H															
NS5Ai B 8x	L31L/V		Y93Y/H	V196V/A														
NS5Ai B 20x	L31L/V		Y93Y/H															
NS5Ai 1 8x	L31L/V		Y93Y/H															
NS5Ai 1 20x	L31L/V		Y93Y/H															
NS5Ai C 8x	L31V		Y93C			P224L		A300T					T392A		Y413C			C446R
NS5Ai C 20x	L31F		Y93H															
NS5Ai D 8x																		
NS5Ai D 20x	L31L/V		Y93Y/H								T377T/A							
NS5Ai E 8x	L31L/V																	
NS5Ai E 20x	L31L/V		Y93Y/H															
NS5Ai 4 8x	L31L/V		Y93Y/H			P224P/L							T392T/A	T394T/A	Y413Y/C			C446C/R
NS5Ai 4 20x	L31L/V		Y93Y/H														S437S/R	
NS5Ai F 8x			Y93H						K358K/E	A376A/S	T377T/I	S382S/N						C446C/S
NS5Ai F 20x			Y93H				S230S/L											
HCV-796 25x					I209I/V											P426P/S	S437S/R	

Table S1. Changes in the NS5A primary sequence in response to prolonged treatment with NS5A-targeting molecules. Replicon 1b cells were incubated with NS5A-targeting molecules (at 8x or 20x their respective IC_{50} values) as described in Materials and Methods. Following RT-PCR amplification of the NS5A gene, NS5A amplicons were subjected to population sequencing and sequence deviations from WT con1 are indicated in the table. Examples such as Y93Y/H indicated a mixed sequence (Y93 and H93) were evident in the population of NS5A amplicons derived from cells serially passaged with the respective compounds. Highlighted in yellow are NS5Ai 1 and NS5Ai 4, whose structures are exemplified in Fig. S1. NS5Ai A-F are series derivatives of NS5Ai 1. Con1 (Replicon 1b cells serially passaged in unmodified medium), DMSO (Replicon 1b cells serially passaged in equivalent concentrations of DMSO), and HCV-796 (Replicon 1b cells serially passaged in 25x IC_{50} HCV-796) served as negative controls. Note this was population-based amplicon sequencing; not sequencing of individual clones. Thus, data presented for each compound represents a summary of all sequence variation from WT evident in the amplicon population.

Supplemental Table. S2

Compound	IC_{50} (pM)		
	Con1 BB7 (n=4)	BB7 with WT 1b NS5A gene (n=2)	BB7 with WT 1a NS5A gene (n=2)
HCV-796	6223 ± 627	16559 ± 1383	4953 ± 463
NS5Ai 3	3.87 ± 0.27	10.4 ± 0.95	20.0 ± 1.42
NS5Ai 4	3.76 ± 0.45	11.3 ± 0.70	21.4 ± 1.42

Table S2. The S2197P tissue culture-adaptive mutation in NS5A does not affect NS5A inhibitor efficacy. The Con1 BB7-based replicon used in transient replication assays contained tissue culture-adaptive mutations in NS3 (HCV polyprotein residues E1202G and T1280I) and NS5A (HCV polyprotein residue S2197P) open reading frames. To ensure compound efficacy was equivalent in transient replication assays when assessed against HCV replicons that did not contain the adaptive mutation in the NS5A primary amino acid sequence, the BB7 replicon sequence was engineered to convert the S2197P tissue culture adaptive mutation back to WT sequence. To compensate for any resulting decrease in the replicative capacity of the subsequent replicon, a tissue culture adaptive mutation in the NS4B gene was inserted into the BB7 sequence (HCV polyprotein residue K1846T). For genotype 1a, the WT H77 NS5A sequence was cloned into the modified BB7 replicon background to create a chimeric replicon. IC_{50} values are presented with calculate standard deviations.

Fig. S2

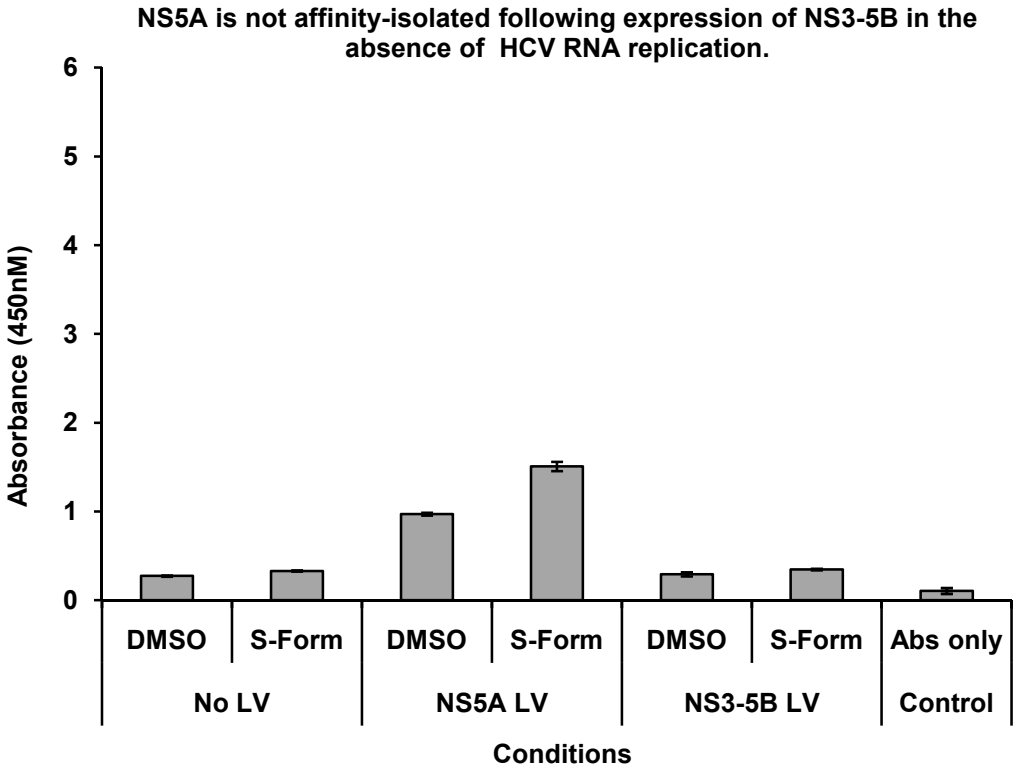


Figure S2. NS5A is not affinity-captured from Huh-7.5 cells expressing a NS3-5B polyprotein. Huh-7.5 cells were infected with recombinant lentiviruses expressing either NS5A alone or a NS3-5B polyprotein. Cells were then treated with the S-form biotin-tagged NS5A-targeting compound. Bound NS5A was detected following capture of protein-compound complexes, within cell lysates, on streptavidin-coated ELISA plates as described in Materials and Methods (Abs, NS5A antibodies; LV, lentivirus).

Fig. S3

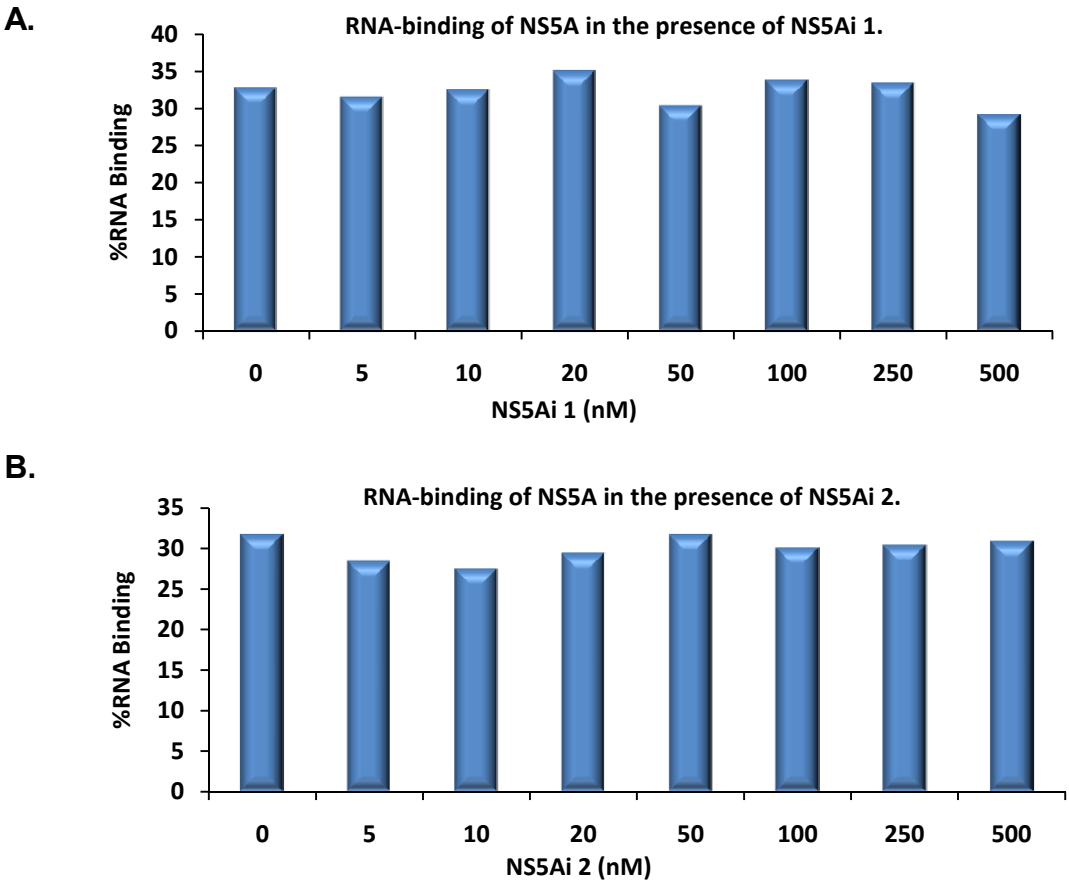


Figure S3. NS5A inhibitors do not affect the RNA-binding ability of NS5A. The ability of NS5Ai 1 (A) and NS5Ai 2 (B) were examined for their capacity to inhibit the HCV 3' UTR RNA-binding function of purified full-length NS5A as described in Foster et al, 2010. No compound-mediated decrease on the RNA-binding capacity of NS5A at inhibitor concentrations of up to 500nM were noted, which is approximately 15000- and 1300-fold greater than the IC_{50} values for NS5Ai 1 and 2 in Replicon 1b cells respectively.