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Potent Hepatitis C Virus NS5A Inhibitors Containing a Benzidine Core

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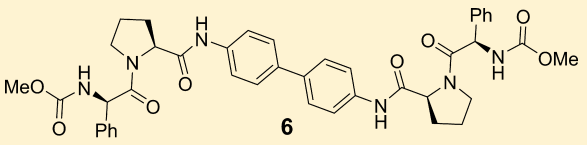
Supporting Information

ABSTRACT: Here we report the discovery of a series of potent hepatitis C virus (HCV) NS5A inhibitors based on the benzidine prolinamide backbone. Taking a simple synthetic route, we developed a novel inhibitor structure, which allows easy modification, and through optimization of the capping group, we identified compound **6** with highly potent anti-HCV activity. Compound **6** is nontoxic and is anticipated to be an effective HCV drug candidate.



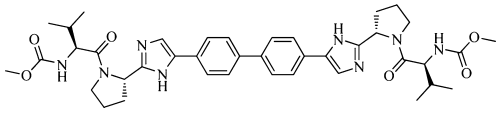
6

Type 2a: EC_{50} = 0.26 nM, G-1b: EC_{50} = 0.028 nM

KEYWORDS: *HCV, NS5A inhibitor, benzidine, antiviral agent, structure−activity relationship*

Approximately 170 million people, including almost 4 million in the United States, are estimated to be infected with hepatitis C virus (HCV).[1,2] It has been estimated that 75−85% of those infected with HCV will develop chronic hepatitis and more serious diseases such as liver cirrhosis and hepatocellular carcinoma.[3] Until recently, therapies for HCV patients have consisted of subcutaneous injections of pegylated interferon-α (PEG-IFN-α) in combination with oral doses of ribavirin (RBV).[4] This interferon based therapy has a limited sustained virologic response (SVR), especially in patients infected with genotype 1 HCV. In 2011, the US Food and Drug Administration (FDA) approved boceprevir (Merck) and telaprevir (Vertex Pharmaceuticals and Johnson & Johnson) as antiviral agents directly targeted against HCV NS3/4A protease, in combination with PEG-IFN-α and RBV. In genotype 1 patients, very promising results have been reported when either telaprevir or boceprevir are added to the standard of care.[5] Although the introduction of these direct-acting antivirals (DAAs) into the regimen improves therapeutic outcome, their possible limitations include a low genetic barrier, which may result in the appearance of drug-resistant mutants during long-term treatment. The development of effective and safe small molecule antiviral agents aimed at a variety of gene targets is therefore warranted.[6]

HCV, which belongs to the *Hepacivirus* genus in the Flaviviridae family, contains a 9.8 kb, single-stranded positive sense RNA genome encoding a polyprotein of approximately 3000 amino acids. This polyprotein encompasses structural proteins (Core, E1, and E2), virion particles, and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) participating in RNA replication.[7−9] Although the precise enzymatic role of NS5A has not been clarified, it has been shown to be essential for HCV replication, virus assembly, and the host immune response related to viral resistance to IFN-α therapy. NS5A is zinc-binding phosphoprotein (56−58 kDa), consisting of 447 amino acids,[10,11] and associated with the membrane through an *N*-terminal amphipathic α-helix region.[12] The three principle domains in NS5A are domain I, containing a zinc binding motif required for viral RNA replication;[13] domain II, which interacts with NS5B and cellular proteins such as PKR and PI3K; and domain III, which plays a role in infectious virus assembly, but not in RNA replication.[14]



A novel NS5A inhibitor, daclatasvir (**1**, shown above; BMS-790052), was recently reported to exhibit strong anti-HCV activity, particularly in the case of the HCV genotype 1. The effective concentration (EC_{50}) of daclatasvir was shown to be in the picomolar range in vitro, and in clinical trials, a single dose treatment of 100 mg reduced the HCV RNA level about 3.3 $\log_{10}$ without apparent toxicity.[15,16] Subsequently, pharmaceutical companies and numerous research groups have focused on the development of a series of NS5A inhibitors.[17−21] In addition to daclatasvir, currently known candidates in this series include GS-5885, ABT-267, PPI-461, AZD-7295, BMS-824393, ACH-2928, IDX-719, PPI-1301, and EDP-239.[22] Daclatasvir (**1**) has a biphenyl core connected to an imidazole moiety, a proline moiety, and a capping group of amino acid derivatives. In 2012, Schinazi and co-workers reported on inhibitors containing a variety of extended biphenyl linkers with small modifications at both ends of the inhibitors.[23−25] Here, we report on a new class of potent inhibitors based upon a benzidine prolinamide skeleton.

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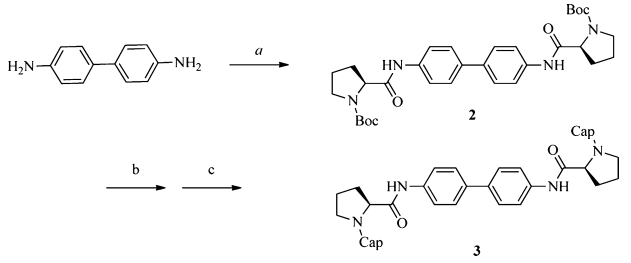
The symmetrical structure of the benzidine scaffold greatly simplified our synthetic route. Coupling of Boc-L-proline with benzidine resulted in excellent yield of an almost pure product, obviating the need for column chromatographic purification. After removal of the Boc group using trifluoroacetic acid (TFA) in CH_2Cl_2, a series of capping groups was added to the free amine using either peptide coupling (EDCI and Hünig's base in CH_2Cl_2) or reductive amination, to furnish the target compounds, **4**–**11** (Scheme 1).[26,27]

Scheme 1. Synthesis of Benzidine Prolinamide Skeleton[a]

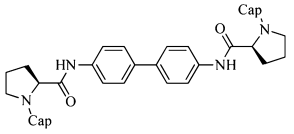


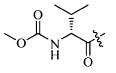
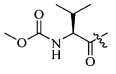
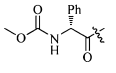
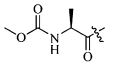
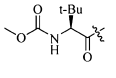
[a]Reagents and conditions: (a) *N*-Boc-L-proline, EDC, DCM, 94%; (b) TFA, DCM; (c) capping group, EDC, DIPEA, DCM 22–49% (2 steps).

To determine the inhibitory activities (EC_{50}'s) of the compounds, dose–response experiments were performed using an infectious HCV cell culture system (HCVcc) (see also Supporting Information). JFH 5a-Rluc-ad34 is a derivative of JFH1 containing a Renilla luciferase reporter and cell culture adaptive mutations.[28] Huh 7.5.1 cells were inoculated with the HCVcc for 3 h and then cultivated for 3 d in the presence of the indicated compounds at serially diluted concentrations. After cell harvesting, EC_{50}'s were calculated by sigma plot analysis of the luciferase activities in the cells.

The assay data indicate that the inhibitory activity of these compounds is greatly dependent upon the structure of the flanking end groups (Table 1).[29,30] When both enantiomers of *N*-methyloxycarbonyl-protected valine were attached, a clear difference in inhibitory activity between the two epimers was observed, indicating the importance of the stereochemistry of the capping group. The inhibitory activity of the D-isomer (compound **4**) was 8-fold higher than that of the L-epimer (compound **5**, Table 1, entries 1 and 2, respectively). Next, we identified the optimal capping group through a series of structure–activity relationship (SAR) studies. When phenylglycine derivatives were incorporated on both ends, extremely high inhibitory activities were observed, regardless of the *N*-protecting groups. The highest inhibition was observed for the compound containing an *N*-methyloxycarbonylphenylglycine group (Table 1, entry 3), while *N*,*N*-dimethyl- and *N*,*N*-diethyl phenylglycine derivatives also had inhibitory EC_{50}'s in the low nanomolar range. Compounds **6**–**8** were potent inhibitors of HCV proliferation, with EC_{50}'s of 260 pM, 2.3 nM, and 2.2 nM in infectious HCVcc, respectively, and none had detectable cytotoxic effects at 25 μM of concentration (Table 1, entries 3–5, respectively). In contrast, compound **9** which contains a *N*-methyloxycarbonyl-L-alanine moiety, had no detectable inhibition at 1 μM. Inhibitors capped with *N*-methyloxycarbonyl-L-*tert*-leucine and *N*-2(*R*)-tetrahydrofurylcarbonyl groups inhibited HCV proliferation with EC_{50}'s of 24 and 400 nM inhibition, respectively (entries 7 and 8, Table 1).

Table 1. Structure–Activity Relationships of Inhibitors Containing a Benzidine Prolinamide Skeleton against HCV Type 2a



entry	Compound	Cap	EC_{50} (nM)	Cytotoxi-city	SI (Cytotoxicity/EC_{50}× 1000)
1	**4**		15		
2	**5**		120		
3	**6**		0.26	>25μM	>96
4	**7**		2.3	>25μM	>11
5	**8**		2.2	>25μM	>11
6	**9**		>1 μM		
7	**10**		24		
8	**11**		400		

To assess the inhibitory activity of compounds on HCV replication, we measured EC_{50}'s using an HCV replicon containing a HCV nonstructural protein, NS3-NS5B, and the Renilla luciferase reporter gene.[31,32] Compound **6** showed the most potent inhibition in the replicon assay (EC_{50} = 28 pM), while compounds **7** (EC_{50} = 0.43 nM) and **8** (EC_{50} = 0.27 nM) also exhibited subnanomolar inhibition (Table 2, entries 1–3).

Table 2. Antiviral Activity in an HCV Genotype 1b Replicon Assay

entry	compound	HCV replicon (type **1b**)	cytotoxicity	SI (cytotoxicity/EC_{50} × 1000)
1	**6**	0.028 nM	>25 μM	>893
2	**7**	0.43 nM	>25 μM	>58
3	**8**	0.27 nM	>25 μM	>93

Since compound **6** exhibited the highest inhibitory activity both in HCVcc infection and in the replicon system (Table 2, entry 1), we next focused our attention on further evaluation of this compound.[33] First, to evaluate the potential cardiac toxicity of compound **6** through inhibition of the inward rectifying voltage gated potassium channel encoded by the hERG gene,

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we carried out a hERG assay.[34,35] Compared to the astemizole control (EC_{50} = 1.9 nM), compound **6** (EC_{50} = 9.8 μM) was shown to bind poorly to the hERG membrane preparations, suggesting that compound **6** would have minimal cardiac toxicity (Table 3).

Table 3. hERG Ligand Binding Assay

compound	EC_{50} (μM)
6	9.8
control (astemizole)	0.0019

Next, a rat plasma stability test[36,37] of compound **6** (Table 4) showed that more than 99% of the compound was intact after 4 h, indicating high in vivo stability (Table 4).

Table 4. Stability of Compound 6 in Rat Plasma

compound **6** (2 μM)	0.5 h (%)	1 h (%)	4 h (%)
6	>99	>99	>99

Next, to evaluate potential drug−drug interactions of compound **6**, we carried out CYP450 screening (Table 5).[38]

Table 5. CYP450 Screening Assay

compound	1A2	2C9	2D6	3A4
6[a]	18.12	0	0	23.87
inhibitor[b]	98.60	75.79	95.07	95.34

[a]CYP inhibition (%) of compound **6** at 10 μM. [b]1A2: α-naphthoflavone, 2C9: sulfaphenazole, 2D6: quinidine, 3A4: ketoconazole.

A compound exhibiting greater than 50% inhibition of CYP450 activity at a concentration of 10 μM is classified as a CYP450 enzyme inhibitor. CYP1A2 (18.12% inhibition) and CYP3A4 (23.87% inhibition) were weakly inhibited in the presence of 10 μM compound **6**, while CYP2C9 and CYP2D6 retained maximal activity under the same conditions.

To determine whether compound **6** would have a synergistic or additive anti-HCV effect in combination with other HCV inhibition therapy, we treated replicon with compound **6** in the presence of a 125 nM of sofosbuvir[39,40] and monitored the effect on HCV proliferation (Figure 1). Our data show that compound **6** and sofosbuvir have a distinct additive effect on the proliferation of HCV, presumably due to the independent modes of action of compound **6** and sofosbuvir.

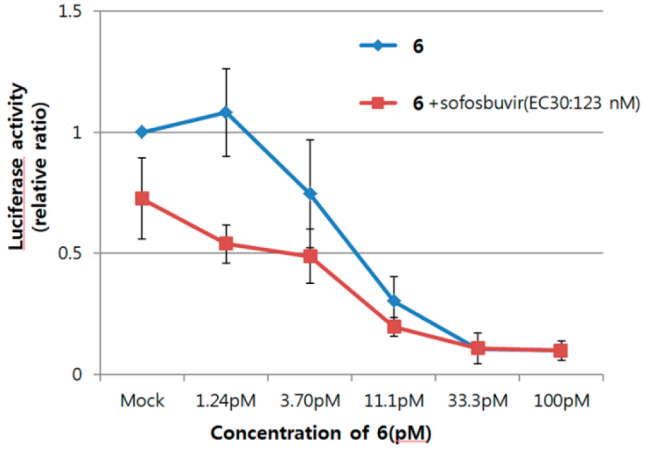

Figure 1. Effect of cotreatment of compound *6* and sofosbuvir on the HCV replicon system. All measurements were made in triplicate.

In summary, we have developed a series of inhibitors based on a new benzidine prolinamide core structure, several of which have extremely high anti-HCV activity. SAR studies using a variety of terminal capping groups showed particularly high inhibitory activities for inhibitors containing phenyl glycine capping groups, of which compound **6** was the most potent. Moreover, subsequent studies demonstrated that compound **6** has a desirable cardiac toxicity, rat plasma stability, and low inhibitory activity against representative Cyp_{450} enzymes. Our data indicate that compound **6** is a potent, safe lead compound that warrants further study for its potential in anti-HCV therapy.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, characterizations, spectra and HPLC traces of compounds **2**−**11**, and experimental protocol used to evaluate HCV inhibitors in cell and replicon assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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