Discovery of Proline Sulfonamides as Potent and Selective Hepatitis C Virus NS5b Polymerase Inhibitors. Evidence for a New NS5b Polymerase Binding Site

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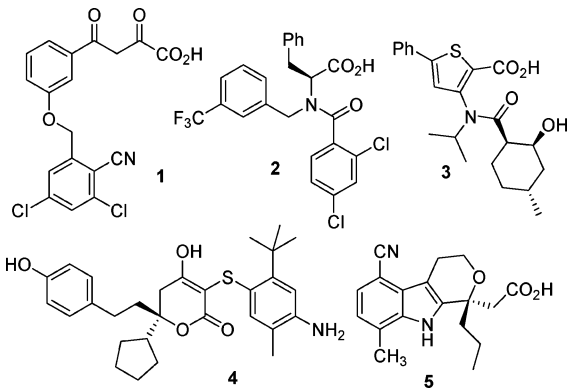
Abstract: Through high throughput screening, substituted proline sulfonamide **6** was identified as HCV NS5b RNA-dependent RNA polymerase inhibitor. Optimization of various regions of the lead molecule resulted in compounds that displayed good potency and selectivity. The crystal structure of **6** and NS5b polymerase complex confirmed the binding near the active site region. The optimization approach and SAR are discussed in detail.

Hepatitis C virus (HCV) infection is one of the causes for liver cirrhosis and hepatocellular carcinoma leading to liver failure and as such is a growing medical problem worldwide.[1] Currently approved therapies involve pegylated interferon-α as a single agent or in combination with ribavirin.[2] The current therapeutic approach is not aimed at any particular viral target. Lack of specificity of current therapies against the known HCV subtypes underscores the need for direct inhibition of viral targets in an effort to significantly improve patient outcomes.

HCV is a positive strand RNA virus, and the genome consists of 9600 base pairs that encode several structural and nonstructural proteins.[3] Recently, BILN-2061, the NS3/4A serine protease inhibitor, has provided proof-of-concept clinical data.[4] Non-nucleoside inhibitors of NS5b RNA dependent RNA polymerase such as HCV-371[5] have advanced to the clinic, and several other distinct chemical classes[6] (Chart 1) have received much attention as potential therapeutic agents for treatment of HCV infection. An important feature of allosteric inhibitors of HCV NS5b polymerase is defined by the nature of the binding pocket in the target enzyme. HCV-371 **5** and phenylalanine analogues **2** have been shown to bind at a region distant from the catalytic site. In this manuscript we disclose the discovery of certain substituted proline sulfonamides as potent inhibitors of HCV NS5b polymerase with the evidence for a new binding site close to the catalytic site.

High throughput screening of various libraries to identify NS5b polymerase inhibitors led to the proline sulfonamide **6** (Figure 1) with an IC_{50} of 3.1 μM. This compound was found to be selective for HCV NS5b polymerase over human polymerase β (IC_{50} > 100 μM), calf thymus polymerase α (IC_{50} > 100 μM), HCV helicase ((IC_{50} > 75 μM), and HIV reverse transcriptase (IC_{50} > 100 μM). Employing fluorescence spectroscopy techniques, we determined the apparent K_D of 1.6 μM from the changes in the endogenous tryptophan fluorescence

Chart 1. Examples of NS5b Polymerase Inhibitors. Diketo Acid 1,[6a] Phenylalanine Derivative **2**,[6b] Thiophene Carboxylic Acid **3**,[6c] Pyranone **4**,[6d] and Pyranoindole (HCV-371) **5**[5]



of the enzyme upon inhibitor binding, at the emission and excitation wavelengths of 340 and 295 nm, respectively. From these experiments and NMR binding studies, the stoichiometry of binding was found to be a 1:1 ratio, indicative of specific binding. This compound was then considered as a lead for further optimization.

All compounds described in this study were prepared in parallel either by solid-phase or solution-phase routes as described in Schemes 1 and 3. Solid-phase methodology was employed for varying the amino acid region of the molecule while the solution phase route was used for varying the sulfonamide region of the molecule.

As shown in Scheme 1, Fmoc-protected proline was attached to the Wang resin using standard peptide coupling conditions. Fmoc was then removed, and the free amine was reacted with suitably protected sulfonyl chlorides to form the resin-bound

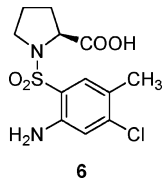
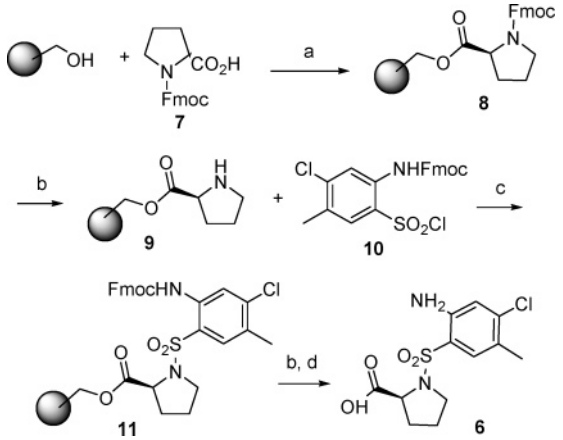


Figure 1. High throughput hit for NS5b polymerase.

Scheme 1[a]



[a] Method A. Reagents: (a) EDCI, HOBT, DIEA, DMF, RT, 6 h; (b) 20% piperidine in DMF, RT, 20 min; (c) pyridine, RT, 12 h; (d) 1:1 TFA−DCM, RT, 2 h.

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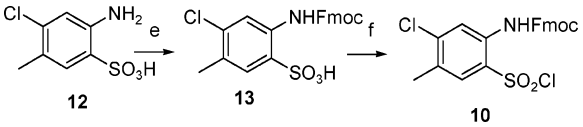
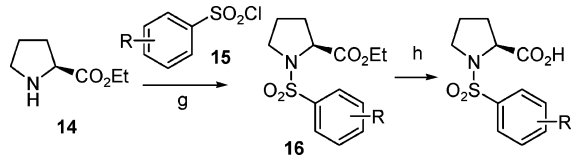
Scheme 2[a]



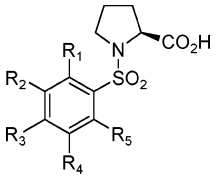
[a] Method B. Reagents: (e) $NaHCO_3$, H_2O, Fmoc-Cl, THF, 12 h; (f) $SOCl_2$, DMF, RT, 4 h.

Scheme 3[a]



[a] Reagents: (g) Pyridine, THF, RT, 8 h; (h) aq NaOH, EtOH, RT, 8 h.

Table 1. NS5b Polymerase Activity of Proline Sulfonamides: Optimization of Sulfonamide Region



compd	R_1	R_2	R_3	R_4	R_5	IC_{50}(μM)
6	NH_2	H	Cl	Me	H	3.1
17	NH_2	H	H	H	H	>20
18	NH_2	H	Me	Cl	H	5.95
19	NHCOMe	H	Me	Cl	H	>20
20	NHCOPh	H	Me	Cl	H	>20
21	OH	Cl	H	Cl	H	0.77
22	H	Cl	H	Cl	H	>20
23	OH	Cl	H	F	H	2.1
24	OH	Br	H	Cl	H	0.26
25	OH	Cl	Cl	Cl	H	0.08

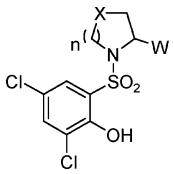
sulfonamides. The desired compound was cleaved from the resin using TFA cleavage conditions. The Fmoc-protected sulfonyl chlorides employed were synthesized as shown in Scheme 2.

Solution-phase parallel synthesis employed proline ester **14** as the starting material. A number of sulfonyl chlorides were introduced, and the resulting esters **16** were hydrolyzed to provide analogues with variation on the sulfonamide region of the molecule.

Initial SAR studies were focused on determining the importance of the substituents on the aryl group (Table 1). Removal of the lipophilic substituents as in compound **17** rendered the molecule inactive. However, switching the position of the chloro and methyl group did not alter the potency substantially as shown by compound **18**. Acylating the amino group with a small acetyl group or a bulky benzoyl group as in compounds **19** and **20** displayed greatly reduced activity. However, a significant boost in activity was obtained by replacing the amino group with a hydroxyl group in conjunction with the dichloro substituents as in compound **21**.

Compound **21** with ortho substitution demonstrated the importance of the hydroxyl group since the activity was rather weak in the absence of the hydroxyl group, even in the presence of the dichloro substituents. Replacing one of the chloro groups with a more electron-withdrawing smaller fluoro group (compound **23**) reduced the activity while a bromo group was well tolerated (compound **24**). Another large increase in potency was observed when an additional chloro substituent was added to the aromatic group as in compound **25** which displayed an IC_{50} value of 80 nM.

Table 2. NS5b Polymerase Activity of Proline Sulfonamides: Optimization of Proline Region



compd	W	stereochem	X	*n*	IC_{50}(μM)
21	COOH	S	CH_2	0	0.77
26	COOH	R	CH_2	0	7.9
27	COOMe	S	CH_2	0	>20
28	CN	S	CH_2	0	1.4
29	tetrazole	S	CH_2	0	0.56
30	H	-	CH_2	0	4.8
31	COOH	S	CH_2	1	2.0
32	COOH	R	CH_2	1	7.1
33	COOH	S	S	0	0.24
34	COOH	R	S	0	1.7

Since the original hit was derived from L-proline, it was of interest to see if the D-proline analogue had any impact on the potency of the molecule. Toward this end, compound **21** was compared with its antipode **26**. This resulted in a 10-fold loss of potency. Capping the carboxylic acid as an ester **27** was not favorable. However, a carboxylic acid isostere such as tetrazole **29**, was equipotent, while the cyano intermediate **28** was less potent. Complete removal of the acid reduced the activity significantly (compound **30**). Expanding the proline ring to a six-membered piperidine ring **31** was not preferred, but substituting the proline ring with a thioproline was well tolerated. We have consistently found 3−10-fold greater activity for the L-amino acids stereoisomers (Table 2). Despite the excellent potency against NS5b polymerase of compounds **21**, **24**, and **25**, they displayed moderate activity in cellular replicon. Compounds exhibited very low permeability in the PAMPA assay (PAMPA @ pH 7.4 for compound **25** $Pe = 0.02 \times 10^{-6}$ cm/s), and the compounds showed lower permeability than atenolol in the Caco-2 measurement (A→B for compound **25** $Pe = 0.4 \times 10^{-6}$ cm/s). However, the compounds were not cytotoxic in the MTS assay of growing Vero cells or MTS assay of growing clone A cells (compound **25** IC_{50} value >100 μM in both assays).[5b]

Following the determination of the 1:1 binding stoichiometry from 1D NMR experiments for the protein and compound **6** complex, the crystal structure of **6** and NS5b polymerase complex was determined by soaking of preformed NS5b-delta 21 crystals (PDB ID: 2GC8). With resolution of 2.2 Å, the compound was seen to bind approximately 10 Å from the Motif D catalytic aspartic acids in the active site (Figure 2), making interactions with residues from both the palm and thumb domain of NS5b. The most significant interactions are made from the loop containing Try448. This displays hydrogen bonds to the sulfonamide and carboxylic acid regions of compound **6**. The sulfonamide group is oriented such that one sulfonamide oxygen forms a hydrogen bond to the backbone amide of Tyr448 (Figure 3) and the other to a bound water molecule (not shown).

Carboxylic group oxygen forms two hydrogen bonds to the backbone amide of Gly449 and to the side chain of Gln446. The aryl group of the sulfonamide region in compound **6** packs between the hydrophobic side-chains of Tyr448 and Tyr415 and above the side chain of Met414. The R_3 chloro substituent is within 3.6 Å of the guanidinium group of Arg200. The R_4 methyl group is within 3.4 Å of the side-chain of Leu384. The importance of both these interactions is negligible given the

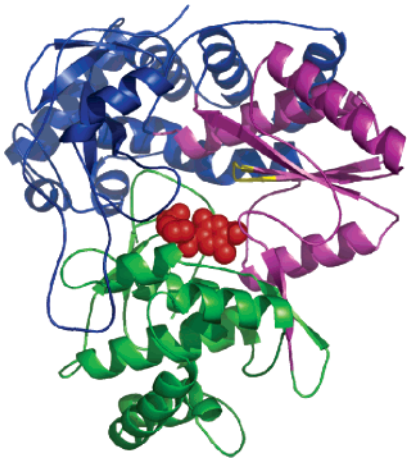


Figure 2. Ribbon diagram of NS5b complex crystal structure indicating compound **6** (red space-filling atoms) between the thumb (colored green) and palm (colored purple) domains. The fingers domain is colored blue, and active site Motif D is colored yellow.

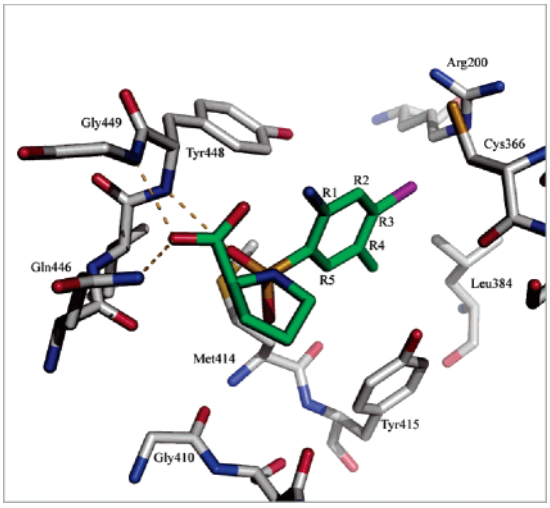


Figure 3. Atomic interactions of compound **6** with NS5b. Polar interactions closer than 3.2 Å are shown as dashed lines. Compound **6** carbons are colored green.

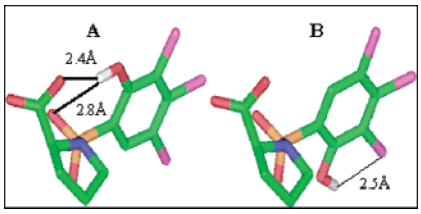


Figure 4. A. Predicted bioactive conformation of **25**; B. Alternate conformation with hydroxyl−chlorine interaction.

results of compound **18**. The amino group at R_1 is solvent-exposed; however, the proximity of the R_1 group to the compound's solvent-exposed carboxylic group oxygen (2.9 Å) may suggest a role in lowering the free energy of binding. This is consistent with an OH group (compound **21**) being preferred to larger substituents at R_1 (i.e. compounds **19** and **20**). This is further supported by the predicted bioactive conformation **A** for the most potent hydroxyl analogue **25** (Figure 4), where the hydrogen bond interaction between the hydroxyl and oxygens of sulfonamide and carboxylate results in a lower internal energy (ΔE: 8.3 kcal/mol[7]) as compared to the alternate conformation **B** involving hydroxyl−chlorine interaction.

In summary, we have identified a novel series of proline sulfonamide derivatives as potent and selective inhibitors of HCV NS5b-RNA dependent RNA polymerase. Importantly, these compounds bind in a new binding site close to the catalytic site of NS5b polymerase. Specific amino acid polymerase interactions are reported for compound **6**.

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Supporting Information Available: Experimental details for the biological assay and the synthetic procedures. This material is available free of charge via the Internet at http://pubs.acs.org

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