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Design, synthesis and evaluation of novel anti-HCV molecules that deliver intracellularly three highly potent NS5A inhibitors

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Abstract

The design and synthesis of new non-symmetrical NS5A inhibitors with sulfur containing amino acids is reported along with their ability to block HCV replication in an HCV 1b replicon system. These compounds display EC₅₀ values in the picomolar range with a large therapeutic index (>10⁶). Moreover, cellular pharmacology studies show that our preferred compounds intracellularly deliver three potent NS5A inhibitors.

Graphical Abstract

Keywords

NS5A; Antiviral; HCV

Hepatitis C virus (HCV) is a global health problem, with 3% of the world's population currently infected. As most of the infected persons are not yet aware of their status, the virus continues to spread with up to 80% of symptomatic acute cases progressing into further hepatic diseases such as liver cirrhosis, steatosis, and hepatocarcinoma.¹ As a consequence, chronic hepatitis C infection remains one of the major causes of premature mortality,

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causing more than 200,000 deaths worldwide each year.² Initial efforts towards the discovery of antiviral agents were focused on the NS3/4A serine protease³ and the NS5B RNA dependent polymerase⁴ but the non-structural NS5A protein emerged as an attractive target with discovery of the picomolar inhibitor Daclastavir (BMS-790052). This highly potent compound paved the way for the design of fixed dose combinations with a better resistance profile.^{5,6,7,8,9,10} In fact, in October 2014, Harvoni,¹¹ a combination of the NS5A inhibitor Ledipasvir (GS-5885) and Sofosbuvir (a nucleoside NS5B inhibitor) was approved by the US FDA for treatment of chronic genotype 1 hepatitis C infection in adults. In December 2014, Viekira Pak, a four direct-acting antiviral agent (DAA) regimen that includes an NS5A inhibitor Ombitasvir (ABT-267), received approval when used with or without ribavirin.¹² Despite their remarkable potency (EC_{50} in the picomolar range), NS5A inhibitors lead to quick development of resistance mutations when given as a single agent. Thus, BMS-790052 rapidly selects mutant virus; with up to 15 thousand fold resistance *versus* the L31V and Y93H double mutation detected during clinical trials in subjects with persistent Gt1b-infections.¹³

In our continuing efforts to identify more effective DAA, we hypothesized that a single drug capable of delivering several distinct DAA intracellularly could overcome the resistance selection problem. Thus, based on the fact that thioether derivatives are prone to be intracellularly metabolized to their corresponding sulfoxide and sulfone analogs by enzymes such as cytochrome P450 enzymes,^{14,15} we decided to introduce various sulfur containing amino acids onto NS5A scaffolds. By doing so we expect to produce up to three active compounds (a thioether, a sulfoxide and sulfone derivative) that could potentially have a complementary resistance profile (Figure 2)¹⁶

Carbamates **2a-d** were prepared from the corresponding commercially available amino acids **1a-d** by reaction with methylchloroformate in a solution of sodium hydroxide and sodium carbonate (Scheme 1).^{17,18}

The biphenyl core of the targeted compounds was prepared according to general Scheme 2 as previously described.¹⁹ Diketone **3** was first converted to the α,α' -dibromodiketone **4** using bromine in dichloromethane then coupled with *N*-Boc protected proline in the presence of *N,N*-diisopropylethylamine (DIPEA). Refluxing the corresponding ester intermediate with ammonium acetate in toluene allowed for the formation of the imidazole ring and lead to Boc-protected intermediate **5** in 79% yield. After removal of the two *tert*-butyl carbamate groups using 6 N HCl, intermediate **6** was coupled with 1 equivalent of *N*-methylcarbamate protected *L*-valine, using *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxybenzotriazole (HOBt) in the presence of *N,N*-diisopropylethylamine (DIPEA). (It is noteworthy that the reaction was not completely selective and leads to the formation of some the undesired disubstituted symmetrical product). The resulting compound **7** was finally reacted with protected sulfur-containing amino acids **2a-d** under the same EDC/HOBt peptidic coupling conditions described above to provide the target compounds **8a-d**.

Compounds **8a-d** were evaluated for inhibition of HCV RNA replication in Huh7 cells that contain a subgenomic HCV 1b replicon system (Table 1). Cytotoxicity was determined

simultaneously with anti-HCV activity by extraction and amplification of both HCV RNA and ribosomal RNA (rRNA). Cytotoxicity was also determined in peripheral blood mononuclear (PBM) cells, CEM cells (human T-lymphoblastoid derived cell line), and Vero cells (kidney epithelial cells from African green monkey).

With EC₅₀ values between 30 and 60 pM, methylcysteine, *tert*-butylcysteine and methionine derivatives **8a**, **8b** and **8d** were 7 to 15 times less potent than BMS-790052 and displayed low micromolar toxicities in PBM, CEM and Vero cells. On the other hand, compound **8c** with an EC₅₀ of 20 pM is only 5 times less active than BMS-790052, but appeared 8.5 times less toxic in CEM cells. In addition compound **8c**, unlike BMS-790052, does not show signs of toxicity up to 100 µM in PBM and Vero cells (BMS-790052 CC₅₀'s = 19 and 21 µM in PBM and Vero cells, respectively).

Activities of compounds **8b-d** were also assessed against a panel of genetically diverse HCV replicons including genotypes 1a and 2a (Table 2). While types 1a and 1b are the most common, accounting for about 60% of global infections, genotype 2 represents about 10% of the HCV infected population, but can be the prominent genotype in countries like Korea or Japan.²⁰ Compounds **8b-d** appeared to be as potent *versus* GT 2a as they were against GT 1b while still displaying picomolar activities against GT 1a.

Since it is known that thioether derivatives can be intracellularly metabolized into their corresponding sulfoxides and sulfones, cellular pharmacology studies were undertaken with derivatives **8b-d**. Thus, compounds **8b**, **8c**, and **8d** were incubated in Huh7 cells at 50 µM for 4 h at 37 °C. The cells were washed with phosphate-buffered saline and the intracellular metabolites were extracted with 70% ice-cold methanol in water and identified by LC-MS. After 4 h, we detected the formation of small amounts of the corresponding sulfoxide and sulfone. For instance, **8c**, our best compound, was metabolized to the sulfoxide derivative **9** (3.4%) and to the sulfone analog **10** (0.02%).

Considering that conversions could potentially be much higher in liver tissue *in vivo*, we decided to further study these metabolites and prepared sulfoxide **9** and sulfone **10** to evaluate their potency *versus* HCV replication. Sulfoxide **9**, as a diastereomeric mixture, was readily obtained by treating **8c** with sodium periodate and sulfone **10** was prepared by oxidation of **8c** with hydrogen peroxide in presence of sodium tungstate **10** (Scheme 3).²¹ Interestingly, both of these compounds maintained picomolar anti-HCV activity with little to no toxicity in PBM, CEM or Vero cells (Table 3).

It is well established that NS5A inhibitors rapidly select for L31V and Y93H mutations in genotype 1b NS5A which markedly reduce their clinical efficacy.²² To further characterize the compounds, thioether derivatives, compounds **8b**, **8c** and **8d** were tested in HCV 1b L31F and Y93H transient replicon transfection assays in Huh7 cells (Table 4). We were encouraged to find that compounds **8b** and **8d** maintained their low picomolar potency versus the L31V mutation, but **8c** was > 3 times less potent against that specific mutation. On the other hand, compounds **8b**, **8c** and **8d** activities against Y93H mutation significantly decreased, following BMS-790052's trend.

We then evaluated the SAR utilizing a new method for asymmetric modeling of the NS5A protein receptor *in silico*.²³ Force field minimized analog **8c**, with CH₂SBn substitution, formed favorable packing interactions with R30, L31, and Y161 (blue arrow) of Site-2 consistent with low picomolar WT activity (Figure 3a). However, the CH₂SOBn substitution of the minimized sulfoxide **9** (*R*) isomer formed direct electronic interactions with R30 and destabilized the benzyl packing with the aromatic Y161 group (orange arrow) - consistent with the reduced potency of **9** relative to **8c** (Figure 3b, Table 3). Minimized analog **10**, with CH₂SO₂Bn substitution, restored interactions with Y161 of the WT receptor and has similar potency to **8c** (Figure 3c, Table 3).

In general, our models predict these asymmetric thioether substitutions can fit tightly within the Site 2 hinge interface between L31 and Y93 of the NS5A dimer, while the unsubstituted portion binds the Site 1 Y93/Y93 interface at the dimer core similarly to the BMS compound (Figure 4). Unfortunately, this class of asymmetric inhibitor is not predicted to interact as well with either the L31V or Y93H mutation, consistent with the observed loss of activities relative to the BMS parent scaffold (Table 4).

Analog **10** is predicted to interact with WT receptor via two asymmetric sites similar to the symmetric BMS dimer described previously.²² The asymmetric CH₂SO₂Bn substitution fits between L31 and Y161 of Site 2, but not the Y93-N-term Site 1 at the dimer core. (Figure 4 - orange arrow) Mutation L31V in hinge Site 2 changes steric packing against membrane-bound W11 and causes distal repositioning of chain A (gold) N-term. The mutation Y93H at core Site 1 repositions the N-term of both chains simultaneously. The proposed binding mechanism is consistent with resistance patterns observed for all analogs tested (Table 4).

In conclusion, extremely potent new NS5A inhibitors with a wide therapeutic index (> 10⁶) have been prepared and profiled. The synthesized compounds displayed extremely potent activity in the picomolar range against HCV GT 1a, 1b and 2a. Among these compounds, **8c** showed an improved therapeutic window compared to BMS-790052 in the cell systems tested, and was found to deliver three different potent molecules intracellularly. However, this compound lost significant activity *versus* in replicons containing the L31F and Y93H mutations relative to BMS-790052; which may preclude further preclinical development. Finally, improved, non symmetrical and sulfur containing NS5A inhibitors are currently being evaluated and will be subject of future publications.

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Figure 1.
Structures of BMS-790052, ABT-267 and GS-5885.

Figure 2.
Delivery of three active molecules intracellularly.

Scheme 1.

Reagents and conditions: (a) ClCOOMe, NaOH 1 M, Na₂CO₃, 0 °C – rt, 18 h, 41-68%.

Scheme 2.

Reagents and conditions: (a) Br₂, CH₂Cl₂, rt, overnight, 85%; (b) (i) Boc-*L*-Proline, DIPEA, rt, 5 h; (ii) NH₄OAc, toluene, 130 °C, 4 h, 79%; (c) 6 N HCl, CH₃OH, 50 °C, 5 h, 89%; (d) *N*-methoxycarbonyl-*L*-Valine, EDC, HOBt, DIPEA, CH₃CN, 0 °C – rt, 3 h, 40%; (e) **2a-d**, EDC, HOBt, DIPEA, CH₃CN, 0 °C – rt, 3 h, 35-51%.

Scheme 3.

Reagents and conditions: (a) NaIO_4 , $\text{MeOH}/\text{H}_2\text{O}$, rt, 3 h, 71%; (b) H_2O_2 , $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$, $\text{BnN}(\text{Et})_3\text{Cl}$, 3 h, 49%;

Figure 3.

Predicted binding of compounds **8c** (a), **9** (b) and **10** (c) to Site-2 of NS5A associated with GT1b activity. NS5A dimer: chain A- gold ribbon and atoms; chain B- blue ribbon and atoms. Residues within 4.5Å of the inhibitor are displayed.

a) Force field minimized analog **8c**, with CH₂SBn substitution, forms favorable packing interactions with R30, L31, and Y161 (blue arrow) of Site-2 consistent with low picomolar activity. b) CH₂SOBn substitution of the minimized analog **9** (*R*) isomer is thought to reduced potency by destabilizing Bn packing with aromatic Y161 (orange arrow) c)

Minimized analog **10**, with CH₂SO₂Bn substitution, has restored predicted interactions with Y161 of WT receptor and similar potency to **8c**.

Figure 4. Energy minimized binding of sulfone **10** in a two site model provides a structural rational for potent anti-HCV activity

Table 1Chemical Structure, Anti-HCV Activity and Cytotoxicity of Compounds **8a-d**.

cmpd	X	Anti-HCV activity (pM)		Cytotoxicity, CC ₅₀ (μM)			
		EC ₅₀	EC ₉₀	PBM	CEM	Vero	Huh7
8a	-CH ₂ SMe	61	100	31	11	18	ND
8b	-CH ₂ S <i>t</i> -Bu	30	100	18	10	39	> 330
8c	-CH ₂ SBn	20	32	> 100	82	>100	> 330
8d	-(CH ₂) ₂ SMe	30	100	41	11	40	> 330
BMS-790052	- <i>i</i> -Pr	4	20	19	10	21	> 15

ND: not determined.

Table 2Activity of Compounds **8b-d** in GT1a and GT2a HCV Replicons.

cmpd	HCV Replicon in Huh-7 cells (pM)	
	GT 1a	GT 2a
	EC ₅₀	EC ₅₀
8b	140	55
8c	100	39
8d	59	27
BMS-790052	48	24

Table 3Structure, Anti-HCV Activity and Cytotoxicity of **8c** and its Metabolites **9** and **10**.

compd	X	Anti-HCV activity (pM)		Cytotoxicity, CC ₅₀ (μM)		
		EC ₅₀	EC ₉₀	PBM	CEM	Vero
8c	-CH ₂ SBn	20	32	> 100	82	> 100
9	-CH ₂ S(O)Bn	100	300	42	11	4.0
10	-CH ₂ S(O) ₂ Bn	20	100	21	> 100	> 100
BMS-790052	-iPr	4.2	20	19	9.6	21

Table 4Activity of compounds **8b-d** versus the L31F and Y93H HCV mutations.

cmpd	Anti-HCV activity (EC ₅₀ , pM)		
	WT	L31V replicon	Y93H replicon
8b	140	72	330
8c	100	390	> 330
8d	59	50	> 110
BMS-790052	48	35	180