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Identification of small molecule inhibitors of the hepatitis C virus RNA-dependent RNA polymerase from a pyrrolidine combinatorial mixture

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Abstract—HTS of the compound collection for inhibition of the HCV RNA dependent RNA polymerase identified two 168 member *N*-acyl pyrrolidine combinatorial mixture hits. Deconvolution and expansion of these mixtures by solid phase synthesis to establish initial SAR and identify a potent inhibitor is reported.

1. Introduction

Hepatitis C virus (HCV), a positive strand RNA virus of the Flaviviridae family, is the major etiological agent of post-transfusion and sporadic non-A, non-B hepatitis.[1] An estimated 2–3% of the world population are chronically infected with HCV which causes significant liver disease and can eventually lead to the development of hepatocellular carcinoma. Currently, the gold-standard for treatment of HCV, interferon with ribavirin, targets stimulation of host defence mechanisms to result in viral clearance. Recently, pegylated interferon has displaced the use of recombinant IFN, by virtue of its enhanced efficacy and tolerability profile. Nonetheless, improvements in sustained response rate, which is presumed to be dependent on viral RNA load and genotype, are still needed.[2] Hence, attempts to identify alternative treatment options for the treatment of chronic hepatitis C virus infections are ongoing.

In infected cells, translation of the viral RNA yields a 3011 residue polyprotein chain[3] which is subsequently cleaved to generate envelope and core proteins, required for assembly of new virus particles and nonstructural enzymes essential for viral replication.[4] The NS protein products include a serine protease activity (NS3 along with the NS4A co-factor), a helicase activity (NS3) as well as an RNA-dependent RNA polymerase (RdRp) activity (NS5B).[5] These enzymatic proteins represent attractive targets for the development of novel, direct inhibitors of HCV.[6] We report herein the discovery of a series of pyrrolidine derivatives identified from a combinatorial mixture library that are reversible NS5B inhibitors and have the potential to be effective anti-viral agents. Furthermore, we illustrate the application of a library based approach to rapid optimisation of the substituents on the pyrrolidine core to deliver potent and selective polymerase inhibitors.

2. HTS and chemistry

High-throughput screening (HTS) of the GlaxoSmithKline compound collection using an RdRp assay with oligo-rG primed poly-rC substrate and C-terminal 21 amino acid deleted NS5B (J4; genotype 1b; HCV Δ21)[7] identified two racemic *N*-benzoyl pyrrolidine

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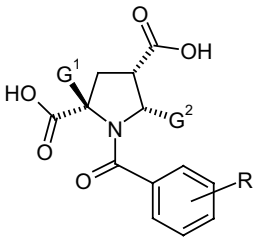
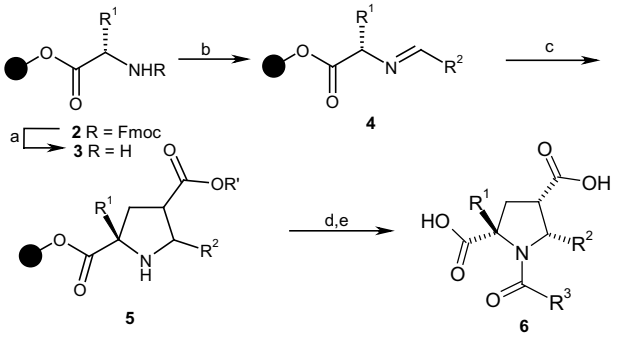


Figure 1.

libraries that differed in the substituent on the benzoyl phenyl ring (Fig. 1). Both libraries were present as a mixture of 168 components ($7 \times G^1$, $12 \times G^2$, racemic) and originated from a combinatorial [3+2] cycloaddition reaction of acrylate ester, a series of aromatic aldehydes and resin bound amino acid esters[8] followed by N-acylation (Scheme 1).

Although the mixtures **1** had only relatively modest inhibitory activity, it was considered worthy of deconvolution to the individual components on the basis that if the observed activity was due to a single component, this compound was likely to be of much higher potency. Critical to a successful deconvolution was the availability, at the outset, of a robust solid phase synthetic methodology amenable to automation. Using this validated chemistry allowed for rapid synthesis of the individual components of the mixture hits for biological assay. In addition, simply by judicious choice of reagents, the SAR around this template could be established and expanded in parallel to the library deconvolution further demonstrating the power of the library approach.

As shown in Scheme 1, the synthesis of the *N*-acylpyrrolidines was accomplished on solid support and started with deprotection of Fmoc amino acids on Wang resin (**2**) to furnish the free amine (**3**). Imine (**4**) formation with aryl and heterocyclic aldehydes in toluene followed by [3+2] cycloaddition with *tert*-butyl acrylate in 5% acetic acid toluene were each achieved at 80 °C to form the resin bound pyrrolidines (**5**). In a final diversity adding step, the pyrrolidine nitrogen was acylated with a range of acid chlorides. Cleavage of the products (**6**) from the resin with concurrent removal of the *tert*-butyl ester using strong acid provided the *N*-acylpyrrolidines which were purified by automated reverse phase HPLC.[9] As expected,[10] and confirmed by NMR studies, the major products isolated corresponded to the all *cis* relative stereochemistry as shown in (**6**).[11]



Scheme 1. Solid-phase synthesis of *N*-acylpyrrolidines. Reagents and conditions: (a) 20% piperidine, DMF; (b) R^2CHO, toluene, 80 °C; (c) *tert*-butyl acrylate, 5% acetic acid/toluene, 80 °C; (d) R^3COCl, pyridine; (e) TFA.

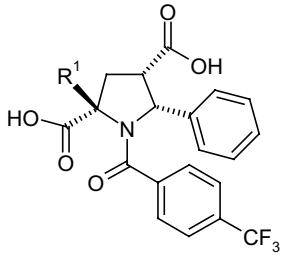
3. Results and discussion

The *N*-acylpyrrolidines (**6**) were assayed for their inhibitory activity against recombinant HCV NS5B Δ21 using the biochemical assay outlined above and described previously.[7] At R^1, either isobutyl (**7**) or benzyl (**8**) substituents, derived from the amino acids leucine and phenylalanine, were favoured over smaller (**9**, alanine) or non-branched (**10**, norleucine) alkylgroups. More polar functionality, as derived from the amino acids lysine (**11**), glutamic acid (**12**) or tyrosine (**13**) was not tolerated (Table 1).

The pyrrolidine 5-position (R^2) appeared to be the most promiscuous and a wide range of substitution was tolerated including small heterocyclic (**14**,**18**,**19**), aryl (**7**,**15**,**16**,**17**,**20**) or alkyl (**21**) (Table 2). This suggested that this position was not involved in making extensive contacts with the polymerase and could be hypothesised to be, at least partly solvent exposed.

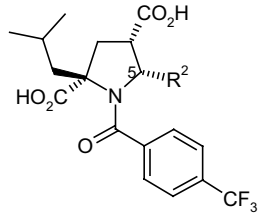
A range of substituted aryl groups proved acceptable for the N-acyl substitution (Table 3). Among those, lipophilic, electron withdrawing groups such as trifluoromethyl were preferred with substituents at the *meta* (**25**) and *para* (**14**) positions being particularly favoured. In contrast, the *ortho* isomer (**26**) was much less active suggesting an important role in the torsion angle be-

Table 1. NS5B inhibitory activity of acylpyrrolidines with various R^1 groups



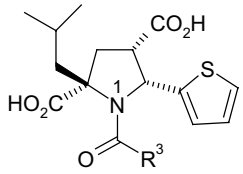
Compound	R^1	NS5B Δ21 IC_{50}, μM
7	isobutyl	0.7
8	$PhCH_2$	1.9
9	Me	>20
10	*n*-Butyl	3.3
11	$(CH_2)_3NH_2$	>20
12	$(CH_2)_2CO_2H$	>20
13	4-$HOPhCH_2$	>20

Table 2. NS5B inhibitory activity of acylpyrrolidines with various R^2 groups



Compound	R^2	NS5B Δ21 IC_{50}, μM
14	2-Thienyl	0.3
7	Ph	0.7
15	4-ClPh	0.9
16	2-MeOPh	5.1
17	4-MePh	1.0
18	2-Thiazoyl	0.4
19	2-Furanyl	0.8
20	2-Pyridyl	0.7
21	*iso*-Propyl	0.5

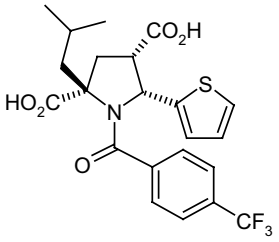
Table 3. NS5B inhibitory activity of acylpyrrolidines with various N1 acyl groups



Compound	R^3	NS5B Δ21 IC_{50}, μM
22	Ph	11.4
23	4-ClPh	1.7
24	4-NO_2Ph	3.2
14	4-CF_3Ph	0.3
25	3-CF_3Ph	0.6
26	2-CF_3Ph	>20
27	3,4-Cl_2Ph	0.6
28	4-Pyridyl	>20
29	1-Naphthyl	0.7
30	*n*-Hexyl	>20

tween the aryl and pyrrolidine rings. Polycyclic aromatics such as 1-naphthyl (**29**) were also tolerated but basic heterocycles such as 4-pyridyl (**28**) were inactive. Simple alkyl groups (**30**, *n*-hexyl) also proved to be inactive.

These initial studies identified the isobutyl and 2-thienyl groups as the preferred substituents at positions 2 and 5 of the pyrrolidine nucleus respectively. However, it is important to note that the 2-thienyl showed only marginal advantage over some other groups (Table 2). Amongst the most active N^1 substituents was the 4-(trifluoromethyl)benzoyl group and this combination (±)**14** was selected for further study. Resolution of (±)**14** by preparative chiral HPLC provided the corresponding pure enantiomers and consistent with a high degree of enantiospecificity for the enzyme–inhibitor interaction, the inhibitory activity was shown to reside exclusively with the (+) enantiomer (Fig. 2).



14
(±) IC_{50} = 300 nM
(+) IC_{50} = 190 nM[12]
(-) IC_{50} = 18000 nM

Figure 2. NS5B inhibitory activities of the separated enantiomers of (±**14**). (±) IC_{50} = 300 nM, (+) IC_{50} = 190 nM,[12] (−) IC_{50} = 18000 nM.

The mechanism of NS5B inhibition by (±)**14** was briefly investigated and, under steady state conditions, (±)**14** exhibited a kinetic behaviour consistent with a reversible, non-competitive mechanism of inhibition with respect to the nucleotide substrate, GTP (data not shown). This suggests that, like the benzothiadiazine inhibitors recently reported,[7] the acyl pyrrolidine class of inhibitor also does not bind to the nucleotide binding domain of the polymerase. Importantly, (±)**14** did not interfere with RNA synthesis via the titration or binding of RNA itself as judged by standard RNA binding displacement assays.[13]

4. Summary

HTS of the GSK compound collection to discover inhibitors of the HCV NS5B activity successfully identified a combinatorial library mixture of *N*-acylpyrrolidines with modest activity. Enumeration of the library using automated solid phase synthesis and purification techniques rapidly allowed definition of the preferred substituents on the pyrrolidine core of the inhibitor and led to the synthesis of (±)**14**, a sub-micromolar NS5B inhibitor. In addition, only one enantiomer of (±)**14** was found to possess significant biological activity. Further optimisation of (+)**14** to deliver analogues with inhibitory activity in cell based anti-HCV assays will be reported in due course.

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9. All compounds gave spectroscopic and analytical data consistent with their assigned structure.
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11. In many examples, the stereoisomer at position-5 was also obtained. Minor amounts of the **4** and **4**, **5** (**2**) stereoisomers were seen in exceptional cases. No isomer showed significant inhibition when compared to its 'all *cis*' parent.
12. The absolute stereochemistry of (+)**14** was determined by small molecule X-ray crystallography to be 2*S*,4*S*,5*R*, unpublished results.
13. The C_{50} for binding to double-stranded RNA was determined to be approximately 50 μM in a picogreen-displacement fluorescence assay described in Ref. 7.