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Studies on acyl pyrrolidine inhibitors of HCV RNA-dependent RNA polymerase to identify a molecule with replicon antiviral activity

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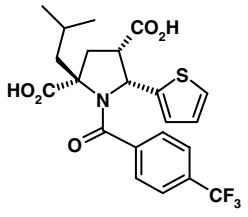
Abstract—The SAR development is described for a series of *N*-acyl pyrrolidine inhibitors of the Hepatitis C virus RNA-dependent RNA polymerase, NS5B, from tractable Δ21 enzyme inhibitors to an example with antiviral activity in a cellular assay (HCV replicon).

Hepatitis C virus (HCV), a positive strand RNA virus of the Flaviviridae family, is the major etiological agent of non-A, non-B hepatitis chronically infecting an estimated 2–3% of the world population.[1] Current treatments of HCV include interferon or pegylated interferon in combination with ribavirin which target stimulation of host defence mechanisms to result in viral clearance. Improvements in sustained response rate, which is presumed to be dependent on viral RNA load and genotype, are still needed.[2] Attempts to identify alternative options for the treatment of chronic hepatitis C virus infections are ongoing.



(±) **1**

We recently described the identification of a series of *N*-acyl pyrrolidines (e.g. **1**) as inhibitors of the NS5B RNA-dependent RNA polymerase.[3] A developable drug for the treatment of HCV must be able to penetrate infected cells to inhibit replication of the virus. In addition, favourable in vivo distribution, metabolism and pharmacokinetic properties are essential for efficacy in the patient.

A crystal structure of NS5B of genotype 1b, isolate J4, with 21 amino acid C-terminal truncation (Δ21) showed that (+) **1** binds in the palm region of the polymerase enzyme.[4] This supports the hypothesis that the polymerase enzyme inhibition of these *N*-acyl pyrrolidines is through binding in a specific pocket and not via a promiscuous or non-specific mechanism. The chemistry efforts described here were undertaken to improve the potency and replace one of the carboxylic acid functions to address the poor cell penetration and poor 'drug-like properties' of these tractable hit diacidic molecules. For efficiency, these two objectives were pursued in parallel.

Initially analogues were assayed for their inhibitory activity against the recombinant HCV NS5B enzyme of genotype 1b, isolate J4, with 21 amino acid C-terminal truncation (Δ21) using the biochemical assay described previously.[5] As the programme progressed and

Keywords: Hepatitis C virus; RNA-dependent RNA polymerase; HCV NS5B; *N*-Acyl pyrrolidines; HCV replicon; SAR.
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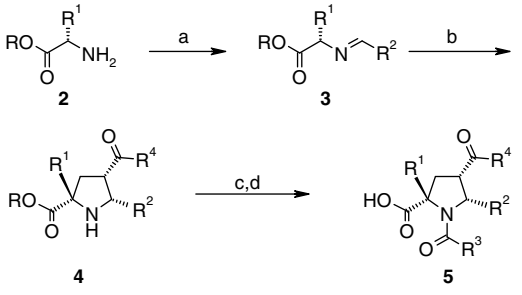
more-active molecules were identified, a similar assay was used, which employs full-length recombinant HCV NS5B enzyme (genotype 1b, isolate RB01).[6] Although less sensitive than the Δ21 enzyme assay, this second assay offered a greater dynamic range for more potent inhibitors and had proved more suitable to identify analogues for progression to the cellular replicon assay.[7]

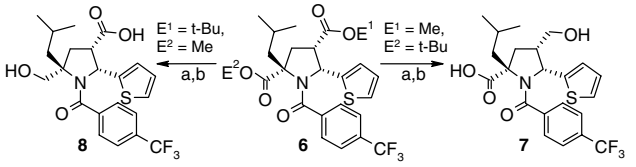
Compound synthesis was achieved using the solid phase chemistry described previously or similar solution phase chemistry (Scheme 1) using judicious choices of amino acid derivatives, aldehydes and acrylates.[3,8] It was also found advantageous to modify the thermal pyrrolidine ring formation with a Lewis acid catalysed reaction as described by Grigg et al.[9] Silver nitrate or lithium bromide in the presence of triethylamine at room temperature overnight gave excellent results in our hands. Both solid phase and solution chemistry yields, although somewhat dependent on the substitutions, were usually between 20% and 50% over the four steps. Combinatorial techniques were employed wherever possible to examine the variation of R^1, R^2 and R^3 alongside the changes to the acid groups in a similar way to that described earlier.[3] For clarity, however, the data and discussion presented below maintains R^1 derived from leucine, R^2 from thien-2-yl- or thiazol-2-yl-aldehyde and R^3 as 4-trifluoro- or 4-*tert*-butyl-benzoyl.

Both isomers in which one of the carboxylic acids had been reduced to a primary alcohol were prepared by the reduction of mixed methyl *tert*-butyl di-esters with sodium borohydride in the presence of lithium chloride; this selectively reduced the methyl ester leaving the *tert*-butyl ester unchanged. Reduction of the hindered 2-methyl ester proved difficult, ~7% yield isolated after 10 days reaction, whereas the less hindered isomer gave a 67% yield after 7 days. The *tert*-butyl ester of each was then deprotected to provide the acids **7** and **8** for testing (Scheme 2).

From initial studies varying the ester function, it rapidly became apparent that the 2-carboxylic acid is very sterically hindered and therefore difficult to modify. Construction of the pyrrolidine ring with the required 2-carboxyl substitution already in place on the amino acid avoided this difficulty. This strategy of introducing



Scheme 1. Reagents and conditions: (R = resin for solid-phase or *t*-Bu for solution synthesis): (a) R^2CHO, toluene, 80 °C, ~12 h; (b) acrylate ester, $AgNO_3$ or LiBr, NEt_3, MeCN or THF, ~12 h; (c) R^3COCl, base, DCM, 2–3 h; (d) TFA, DCM, 0.25 h.

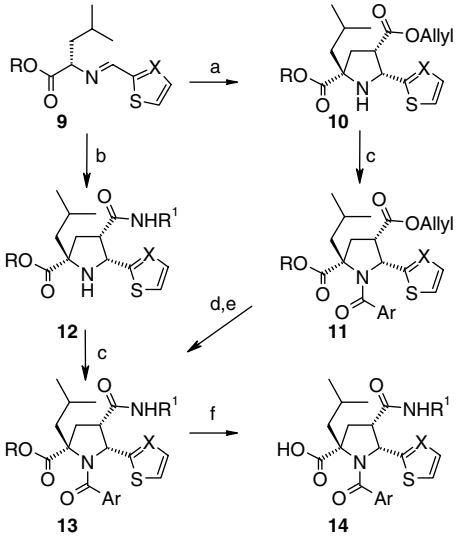


Scheme 2. Reagents and condition: (a) $NaBH_4$, LiCl, THF, ~10 days; (b) TFA.

the group to be varied at the start was also found to be a useful alternative for some position 4 substitutions.

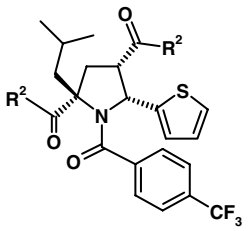
Thus amides of the less hindered 4-carboxylic acid were accessed either by use of an appropriately substituted acrylamide, readily available from acryloyl chloride and the corresponding amine, or by using differential ester protection of the two acids to readily provide the 4-carboxylic acid for amide formation (Scheme 3).

Conversion of either, or both, of the carboxylic acid groups of (±) **1** to an ester resulted in the loss of inhibition of NS5B, see compounds **15**–**18** (Table 1). Thus it appeared necessary to retain hydrogen bond donators in the carboxylic acid replacements which suggested examining amides or primary alcohols. Although the primary amide at position 4 (**19**) or the corresponding 2-amide (**20**) showed reduced NS5B inhibition, they did retain some activity. However a series of secondary amides at position 2 all showed less enzyme inhibition than the primary amide, compounds **21**–**25**. The primary alcohol analogue, **7**, retained some weak activity (51% at 10 μM) but **8** was inactive (0% at 10 μM). These data suggest that any change to the 2-carboxyl group essentially suppresses inhibitory activity.



Scheme 3. Reagents and condition: (R = resin for solid-phase or *t*-Bu for solution synthesis): (a) allyl acrylate, $AgNO_3$ or LiBr, NEt_3, MeCN or THF; (b) acrylamide, $AgNO_3$ or LiBr, NEt_3, MeCN or THF; (c) R^3COCl, base; (d) $Pd^{[0]}$, PPh_3; (e) amine (R^1NH_2), HOAt, DIC, DIEA; (f) TFA.

Table 1. NS5B inhibitory activity of *N*-acyl pyrrolidine esters

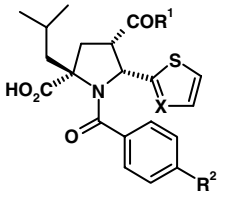


Compound	R^1	R^2	NS5B J4 Δ21 IC_{50} (μM)
1	OH	OH	0.3
15	OMe	OH	>20
16	OCH_2Ph	OH	>20
17	OH	OMe	>20
18	OMe	OMe	>20
19	OH	NH_2	4.4
20	NH_2	OH	8.1
21	NHMe	OH	>20
22	NHEt	OH	>20
23	$NHCH_2Ph$	OH	>20
24	NH*i*-Pr	OH	>20
25	NHPh	OH	>20

Meanwhile, studies to expand the SAR of the series had continued with examination of alternate 1-substitution. Benzyl and sulphonamide analogues of compound **1** were inactive while its urea derivative showed reduced potency (IC_{50} = 2.1 μM).

More rewarding was the continued investigation of additional *N*-acyl groups and 5-heterocycles performed in a combinatorial manner with small arrays. This uncovered a synergistic effect from the combination of the 1-*tert*-butylbenzoyl and 5-thiazoyl groups **28** compared with **1**, **26** and **27** (Table 2). Indeed, when applied to the amide series this effect was also observed (**32** cf. **30** and **31**) and resulted in a significant advance for the study, identifying the mono-acid **32** with similar potency to its di-acid analogue **28** capable of showing measurable inhibition of full-length enzyme (NS5B RB01 FL).

Table 2. NS5B inhibitory activity of *N*-acyl pyrrolidines



Compound	X	R^1	R^2	NS5B J4 Δ21 IC_{50} (μM)	NS5B RB01 FL IC_{50} (μM)
1	C	OH	CF_3	0.33	20
26	N	OH	CF_3	0.45	
27	C	OH	*t*-Bu	0.75	
28	N	OH	*t*-Bu	0.07	3.8
29	C	NH_2	CF_3	4.40	
30	C	NH_2	*t*-Bu	0.33	
31	N	NH_2	CF_3	2.05	
32	N	NH_2	*t*-Bu	0.08	4.4

Separation of the racemic mixtures of thiazole di-acid **28** and acid amides **29** and **32** by chiral HPLC readily provided the pairs of enantiomers which exhibited data similar to that described for compound **1** where the inhibition resided in the (+) isomer (Table 3) and in agreement with the enantiomer bound in the enzyme crystal structure.[10] Although this was a significant advance in enzyme potency, the amides demonstrated only marginal, if any, detectable cellular activity in line with low permeability similar to that seen for the di-acids (as measured in an artificial membrane permeability assay, <0.3 nm/s).[11]

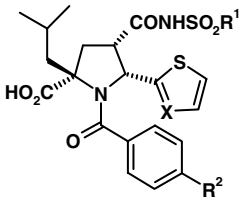
Following the progress described above, further investigation of substituted amides was considered. Initially amides which retained an acid proton such as acyl sulphonamides were examined. These acid isosteres retained a good level of enzyme inhibition, particularly when combined with the 4-*tert*-butylbenzoyl acyl group (Table 4). However, as these do not address the permeability issue of di-acids (artificial membrane permeability assay for compound **36**, <0.3 nm/s) so attention turned to examining non-acidic amide analogues.

A series of 4-N-substituted amide analogues of **32** was examined whilst keeping the other groups constant with the aim of improving cell permeability and retaining potency (Table 5). The benzyl **39** and methyl **40** amides showed a significant drop in potency; however the phenyl amide **41** retained significant inhibition of the Δ21 and

Table 3. NS5B inhibitory activity of separated enantiomers

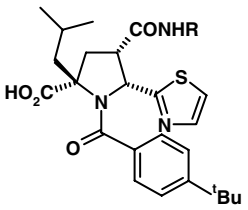
Compound	NS5B J4 Δ21 IC_{50} (μM)	NS5B RB01 FL IC_{50} (μM)
(+) **1**	0.30	11.9, 20
(−) **1**	18	
(+) **28**	0.03	2.1 (*n* = 6)
(−) **28**	2.36	
(+) **29**	2.23	
(−) **29**	>30	
(+) **32**	0.07	3.8 (*n* = 6)
(−) **32**	2.50	

Table 4. NS5B inhibitory activity of *N*-acyl sulphonamides



Compound	X	R^1	R^2	NS5B J4 Δ21 IC_{50} (μM)	NS5B RB01 FL IC_{50} (μM)
33	C	Me	CF_3	0.23	
34	C	CF_3	CF_3	0.20	
35	N	Me	CF_3	0.41	
36	N	Me	*t*-Bu	0.04	
37	N	Ph	*t*-Bu	0.50	3.15
38	N	CH_2Ph	*t*-Bu	0.55	4.23

Table 5. NS5B inhibitory activity of substituted amides



Compound	R	NS5B J4 Δ21 IC_{50} (μM)	NS5B RB01 FL IC_{50} (μM)	HCV replicon IC_{50} (μM)
39	CH_2Ph	8.29	68	
40	Me		22	
41	Ph	0.16	3.1 ($n = 4$)	45[a], 77[a]
(+) **41**	Ph		0.5 ($n = 4$)	47[a], 30[a]
(−) **41**	Ph		>100	>200[a]

[a] $CC_{50} > 200$ μM, some cell toxicity seen at the highest dose.

full-length enzymes. Encouraged by detectable permeability in the artificial membrane permeability assay (2.2 nm/s), compound **41** was tested in the cell based HCV replicon assay. Indeed a weak antiviral effect could be detected which was judged sufficient to encourage further study.[12] Once again, after separation by chiral HPLC, the (+) enantiomer showed superior activity in both the enzyme and replicon assays (Table 5).

Medicinal chemistry studies towards an antiviral agent for the treatment of HCV infection are described. Hit molecule **1** had been identified from the follow-up of a high-throughput screening combinatorial mixture hit.[3] The identification of a *N*-acyl pyrrolidine **41** which demonstrated an antiviral effect in the replicon cell based assay provided a lead for further medicinal chemistry optimisation to identify a candidate molecule for the treatment of HCV infections.[12]

Acknowledgments

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