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Discovery of thiophene-2-carboxylic acids as potent inhibitors of HCV NS5B polymerase and HCV subgenomic RNA replication. Part 2: Tertiary amides

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Abstract—Further SAR studies on the thiophene-2-carboxylic acids are reported. These studies led to the identification of a series of tertiary amides that show inhibition of both HCV NS5B polymerase in vitro and HCV subgenomic RNA replication in Huh-7 cells. Structural insights about the bioactive conformation of this class of molecules were deduced from a combination of modeling and transferred NOE (trNOE) studies.

The severe morbidity associated with infection by the hepatitis C virus (HCV) together with the lack of an efficacious and well-tolerated agent has generated intense research efforts in the discovery of novel anti-HCV chemical entities.[1] In the preceding paper,[2] the discovery of a novel class of low molecular weight HCV NS5B RNA dependent RNA polymerase inhibitors was reported and several analogues such as **1** were also inhibitory to the replication of HCV RNA subgenomic replicons in Huh-7 cells (replicon assay). Structure–activity relationship studies on the 5-phenyl and sulfonamide moieties have provided insights on the requirement for activity in both in vitro HCV polymerase and in surrogate HCV replication assays. Only compounds with substituents in the precise 2,3,5 arrangement depicted in **1** showed consistent activity and it was demonstrated that sulfonamides were optimum whereas secondary amides, ureas and carbamates were less active. Electron withdrawing substituents at the *para* position of 5-phenyl were also beneficial as they also served the purpose of reducing cytotoxicity and thereby increasing selectivity indices. It also became apparent that in vitro inhibition of NS5B polymerase did not necessarily correlate with inhibition of HCV RNA replication in the replicon cell line. The exact cause of this discrepancy is not known but the artificial conditions utilized for the in vitro assay (e.g., a homopolymeric template) and the association of enzyme with viral and cellular proteins in the replication assay are probably key factors. Recent reports have provided evidence that the activity of HCV NS5B polymerase is modulated by NS3, NS4B and NS5A non structural proteins.[3] These proteins are not present in the enzymatic assay; it is thus possible that in the biochemical assay, the conformation and/or accessibility of the binding site is different from that of the replication model. As a result, the affinity of certain analogues for the NS5B binding site may be different in the more complex cellular environment. The goal of this study was therefore 2-fold; the first objective was to conduct further SAR studies to optimize the profile of the compounds such as potency enhancement in both biochemical and replication assays. Since analogues that were active in both the enzymatic and cell-based assays could be identified in the previous study, the second objective was therefore to identify structural features that show consistent and predictable correlation between these two assays.

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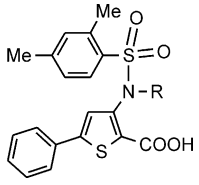
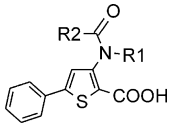
N-Methylation of peptidic amides is an established tool that has traditionally been employed in medicinal chemistry to both, improve pharmacological properties, such as in vivo stability and to alter the overall conformation and biological profile of peptides.[4] Unfortunately, when a similar transformation was applied to sulfonamide **1**, *N*-methyl sulfonamide **2** showed a 5- to 7-fold loss in HCV polymerase inhibition as well as in the cell-based assay indicating that there is no beneficial effect in alkylating the sulfonamide nitrogen (Table 1). However, it was gratifying to observe that when the amide nitrogen of *p*-toluamide **3** was methylated, tertiary amide **4** showed a 6-fold enhancement in polymerase inhibition (Table 2). Furthermore, IC_{50} of 4 in replicon cells was 22 μM and no toxicity was observed at 200 μM. It is also worthwhile to note that the secondary amide is inactive in the replicon cell line ($IC_{50} > 100$ μM). Having thus identified a new direction for the discovery for potential anti-HCV agents, the optimization effort was therefore concentrated towards identifying optimum amide as well as *N*-alkyl moieties. The compounds in this study were prepared according to the methods described in Scheme 1.

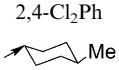
Table 1. Activity of *N*-methylsulfonamide **2**



	R	HCV NS5B polymerase IC_{50} (μM)	Replicon IC_{50} (μM)	Huh-7 CC_{50} (μM)
1	H	1	5	57
2	Me	5	35	115

Table 2. Activity against HCV polymerase and replicon cells[10]

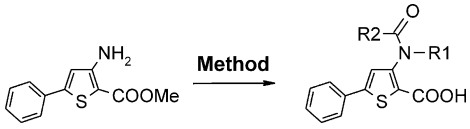


	R_1	R_2	IC_{50} (μM) HCV NS5B polymerase	IC_{50} (μM) Replicon	CC_{50} (μM) Huh-7
3	H	4-MePh	12	>100	118
4	Me	4-MePh	2	22	>200
5	Me	3-MePh	>50	>100	>200
6	Me	2-MePh	21	ND	ND
7	Me	4-ClPh	1.2	19	88
8	Me	4-EtPh	>50	>100	ND
9	Me	4-CF_3Ph	34	>50	ND
10	Me	Ph	>32	ND	ND
11	Me	3,4-Me_2Ph	>50	ND	ND
12	Me	2,4-Me_2Ph	1.4	16	160
13	Et	4-MePh	2	12	100
14	*i*Pr	4-MePh	3	5	>200
15	*i*Pr	2,4-Cl_2Ph	1.2	8.9±2.4	62±10
16	*i*Pr		1.5	0.6 0.3[a]	123
17	*i*Pr		24	>50	ND
18	*i*Pr		9	15	69

[a] Real time PCR.

Although substitution of the amide nitrogen with methyl resulted in a dramatic increase in potency of *para*-toluamide **4**, no such effect was observed with the *ortho* and *meta* toluamides **5** and **6** indicating that the beneficial effect of methylating the amide nitrogen was limited to the presence of a *para* susbtituent. This is in contrast to the sulfonamide class where a single *ortho* methyl substitution was generally sufficient to provide active entities in both enzymatic and cellular assays. In addition, among all the *para* substitutions evaluated only chloro **7** and methyl **4** retained activity, substitution with ethyl **8** or even trifluoromethyl **9** significantly reduced activity against HCV polymerase. Unsubstituted benzamide **10** was also inactive underscoring the necessity of having a substituent at the *para* position. The chloro analogue was also equipotent to the methyl analogue in the replication assay albeit with higher toxicity. A *meta* methyl substituent in addition to the *para* methyl group (analogue **11**) was detrimental to activity but the corresponding 2,4 disubstituted derivative **12** had a similar profile to a single *para* methyl substitution. Increasing the bulk of the alkyl substituent on the amide nitrogen was in general beneficial for activity. *N*-Ethyl derivative **13** was approximately equipotent to the corresponding methyl substituent, whereas introduction of an isopropyl unit (**14**) resulted in a 4-fold increase in activity in the replicon assay. It is also interesting to note that no corresponding increase in the inhibition of polymerase activity in the enzymatic assay was observed for these compounds. Substitution with a 2,4-dichlorobenzamide moiety (**15**) which was found to be important for activity in the phenylalanine series,[5] also yielded an active compound.

The precise requirement of the substitution pattern on the benzamide moiety indicates that this group is likely to be accommodated in a structurally well-defined pocket on the enzyme. Since all the amides described so far in this study are aromatic and thus planar, the effect of replacing the benzamides with a non-planar and bulkier cyclohexylcarboxamide moiety was examined as this can provide structural insight about the binding site. As substitution at the *para* position was shown to



Scheme 1. Preparation of compounds **3**–**18**. Method: Compounds **3**–**6**, **8**, **9**, **11**, and **12**: (i) NaH, iodomethane DMF, 0 °C→rt, 62%; (ii) LiOH, THF/MeOH/H_2O (3:2:1), 80 °C, 73%; (iii) benzoyl chloride, NaOH, dioxane, H_2O, 0 °C→rt. Compound **13** (i) 4-methylbenzoyl chloride, $(CH_2Cl)_2$, reflux, 88%; (ii) NaH, iodoethane, DMF, 0 °C→rt, 61%; (iii) LiOH, dioxane, H_2O, rt, 30%. Compounds **7**, **10**, **14**, **15**, and **18**; (i) NaH, iodoalkane, DMF, 0 °C→rt; (ii) benzoyl chloride, $(CH_2Cl)_2$, reflux; (iii) LiOH, THF/MeOH/H_2O (3:2:1), rt. Compounds **16**–**17**: (i) NaH, 2-iodopropane, DMF, 0 °C→rt, 32%; (ii) 4-methyl-1-cyclohexanecarboxylic acid, NCS, PPh_3, $(CH_2Cl)_2$, reflux; (iii) preparative TLC; (iv) LiOH, THF/MeOH/H_2O (3:2:1), rt.

be crucial for activity in both the cellular and enzymatic assays, cyclohexyl analogues with a methyl substitution at the 4-position were therefore selected. This modification resulted in the identification of the most active analogue of this study; *trans* isomer 16 had an IC_{50} of 0.6 μM in the inhibition of HCV subgenomic RNA replication. This molecule had a selectivity index (CC_{50}/IC_{50}) greater than 200 and did not show any inhibition to human polymerases α, β and γ (IC_{50} >100 μM). In addition, stable cell lines expressing only the luciferase gene or luciferase driven by either EMCV (Internal Ribosome Entry Site, IRES) or HCV IRES were used to ascertain that 16 is indeed specific to a HCV replication process in the replicon assay.[6] No activity (IC_{50} >100 μM) was detected in any of the three stable cell lines indicating that this compound is not targeting the IRES-mediated translation or a luciferase gene product. Real-time PCR analysis was also used to quantify the amount of HCV subgenomic RNA produced and by this method, IC_{50} was determined to be 0.3 μM. As for the best benzamide analogue (**14** and **15**), the enzymatic inhibition of the cyclohexylcarboxamide (IC_{50} 1.5 μM) did not correlate with the large gain of potency in the cell-based assay. In contrast, both *cis* methyl analogue **17** and unsubstituted cyclohexyl analogue **18** were significantly less potent than the *trans* isomer. This again demonstrates that substitution at the 4-position is not only necessary for activity but that group must maintain an orientation that approximates the planar orientation of a *para* substituted aromatic system.

In an attempt to provide a structural basis for the remarkable effect of the *N*-alkyl substituent, a systematic conformational searching exercise was used to generate molecular conformations[7] of the secondary *para*-toluamide analogue **3**, *N*-Me amide **4** and the bulkier *N*-isopropyl analogue **14**. The first 200 conformers in each search were then visually inspected for any shape preferences among the analogues. Two overall shapes were identified and Figure 1 depicts representative examples, which are referred to as 'closed' or 'open' amide forms. The population of each of these two shapes was also determined among the conformations generated and the results are depicted in Table 3. In the case of secondary amide **3**, all the local minima generated were from the open or the fully extended form. Introduction of an alkyl group on the amide nitrogen resulted in the appearance of a number of closed forms. Furthermore, as the bulk of the alkyl group became more significant, the population became predominantly of the closed mode; 77% of the local minima for the *N*-isopropyl analogue **14** were of the closed form compared to 37% for *N*-methyl analogue **4**.

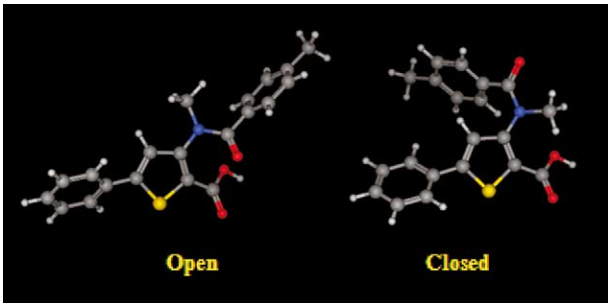
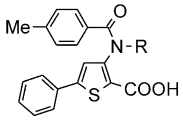


Figure 1. Open and closed conformations of **4**.

Table 3. Relationship between shape population and activity in the replicon assay



	R	% Closed Form	HCV Replication IC_{50} (μM)
3	H	0	>100
4	Me	37	22
14	*i*Pr	77	5

An enhancement in the inhibition of HCV subgenomic RNA replication was observed although no corresponding increase in polymerase inhibition was observed in the biochemical assay. The *N*-isopropyl analogue was thus 4-fold more potent than *N*-methyl analogue in the replicon assay. It is thus possible that the bioactive character of this class of compounds resembles the closed form. In order to provide some experimental basis for this hypothesis, a transferred NOE (trNOE)[8] experiment was performed with **4** in the presence of HCV NS5B polymerase/poly rA/oligo dT complex. TrNOE's were observed between the 5-phenyl aromatic protons and the 3-benzamide protons as well as between the 4-thiophene proton and the 3-benzamide protons.[9]

Since no trNOEs were detected in the absence of enzyme, the bioactive character of this ligand most likely adopts a 'closed' or concave shape in order to allow positioning of the benzamide moiety within 5 Å to both thiophene and 5-phenyl moieties. The higher potency of the bulkier *N*-isopropyl tertiary amide likely arises from the larger population of the closed form in solution. In addition, it appears that HCV NS5B polymerase in the replication complex opts for the closed shaped ligand to a greater extent than the enzyme in the biochemical assay. Further studies are under way to exploit these findings.

In summary, optimization studies led to the discovery that *N*-alkylation of the amide nitrogen with a bulky group and the use a cyclohexylcarboxamide moiety was key in obtaining potent inhibitors of HCV sub-genomic RNA replication. These findings provide useful leads for further optimization in the search for a low-molecular weight HCV therapy. Correlation between inhibition of in vitro HCV NS5B polymerase activity and the cell-based assay is not directly proportional but nevertheless, a series that shows activity in both assays was identified. The bioactive conformation is believed to be of the 'closed' or concave form and is induced by introduction of an alkyl substituent on the amide nitrogen.

Acknowledgements

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7. All three analogues were studied by modeling each individual structure at their ionization state that could be reflected in aqueous conditions at ~pH 7. MOE (Chemical Computing Group Inc., Montreal, Canada) was used to build and energy minimize each structure with the MMFF94s force field using a gradient of 0.01 kcal/mol Å. The resulting minimized structures were used as starting points for conformational searching conducted by using the MOE implemented systematic conformational search tool. To visualize the conformational preference of each analogue, the first 200 lowest energy structures were examined for each of the representative 'closed' or 'open' shapes. The average energy difference between the 1st and 200th structure was approximately 3 kcal/mol Å.
8. (a) Clore, G. M.; Gronenborn, A. M. *J. Magn. Reson.* **1982**, *48*, 402. (b) Ni, F.; Scheraga, H. A. *Acc. Chem. Res.* **1994**, *27*, 257.
9. Details of this experiment will be reported in a forthcoming publication.
10. Polymerase IC_{50}'s were determined from dose–response curves using 11 concentrations for each compound in uniplicate. Curves were fitted to data points using nonlinear regression analysis and IC_{50}'s were interpolated from the resulting curves using GraphPad Prism software, version 2.0 (GraphPad Software, Inc. San Diego, CA, USA). A positive control was included as an internal standard in each set of experiments and results were considered accurate only when the IC_{50} value of the positive control was within 0.45 ± 0.16 μM. Similarly, IC_{50}'s in replicons were determined using 12 concentrations in duplicate and **15** was included as positive control in each experiment and data were considered accurate when IC_{50} of **15** was within 8.9 ± 2.4 μM. CC_{50}'s in Huh-7 Cells were determined from five concentrations in quadruplicate and data were taken when CC_{50} of **15** was within 62 ± 10 μM.