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Non-nucleoside inhibitors of the hepatitis C virus NS5B polymerase: discovery and preliminary SAR of benzimidazole derivatives

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Abstract—Benzimidazole 5-carboxamide derivatives from a combinatorial screening library were discovered as specific inhibitors of the NS5B polymerase of the hepatitis C virus (HCV). Initial hit-to-lead activities taking advantage of high-throughput parallel synthetic techniques, identified a 1,2-disubstituted benzimidazole 5-carboxylic acid scaffold as the minimum core for biological activity. Potent analogues in this series inhibit the polymerase at low micromolar concentrations and provide an attractive 'drug-like' lead structure for further optimization and the development of potential HCV therapeutics.

The hepatitis C virus was discovered more than a decade ago,[1] and the prevalence of the infection is now estimated at 1–3% of the world population (ca. 170 million people).[2] Chronic HCV infections can lead to fatal liver disorders such as cirrhosis and hepatocellular carcinomas that eventually require organ transplantation.[3] Therapies for the treatment of chronic HCV infection are currently limited to various forms of interferon, in combination with the broad spectrum antiviral ribavirin.[4] A significant proportion of the patient population does not respond to therapy and there is an urgent demand for novel strategies addressing this clearly unmet medical need.[5a]

HCV is a small, enveloped virus whose ~9.6 Kb single-strand (+)-sense-RNA genome encodes a polypeptide of approximately 3000 amino acids.[6] The C-terminal two-thirds of the polyprotein consists of non-structural (NS) proteins (NS2→NS5) whose enzymatic functions are essential for viral replication.[7] The NS5B segment encodes a 65 kd RNA-dependent RNA polymerase (RdRp).[8] Because of the central role this enzyme plays in the replication of HCV RNA, it has become a target of choice for the screening and design of small molecule inhibitors which, in principle, should interfere with viral replication.[5]

Our sample collection was screened in an HCV polymerase assay using an N-terminally histidine-tagged recombinant full-length NS5B protein from a **1b** genotype.[9] We identified a set of benzimidazole derivatives from a combinatorial library (as exemplified by compounds **1** and **2**) as specific[10] inhibitors of the polymerase (Fig. 1).[11]

In order to confirm that inhibition was mediated by specific interactions of the compounds with NS5B, NMR differential line broadening (DLB) and

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transferred-NOESY experiments were performed with **2** in the presence of a soluble form of the polymerase.[12]

Figure 2A shows a representative ^{1}H resonance of **2** (H_{14}) that broadens upon incremental additions of NS5B, which would be expected for rapid and reversible binding of a small ligand to a large macromolecule. Figure 2B shows other resonances of **2** that are also subject to DLB (shown in red on the trace and the structure), indicating regions of the molecule that are likely contacting the protein ('epitope' mapping). The observation of transferred-NOESY cross-peaks[12] (data not shown) provided further evidence that **2** binds to HCV polymerase. The observation of DLB for the resonances of the cyclohexyl substituent (H_{11}s), the C-2 aryl group and parts of the right-hand side of the inhibitor (H atoms shown in red) suggested that these segments contact the protein in the bound state. In contrast, the left-hand portion of **2** (H_{1-7}) lacks DLB and does not appear to interact with the protein, in agreement with the SAR (vide infra). These results, combined with the observed specificity of the inhibitors for HCV NS5B indicated that the observed enzymatic inhibition resulted from specific interactions of the ligands with the polymerase rather than through undesired mechanisms such as binding to the RNA primer/template substrate.

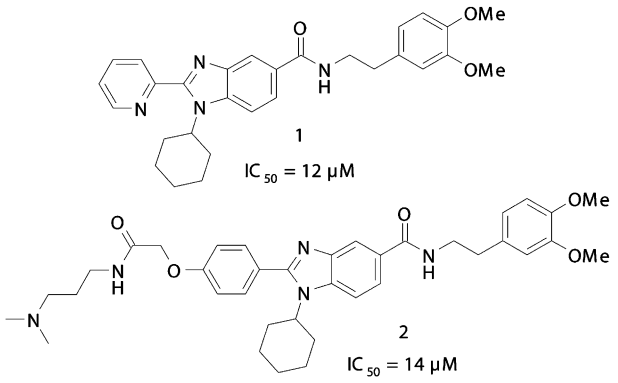


Figure 1. Original hits from a combinatorial screening library.

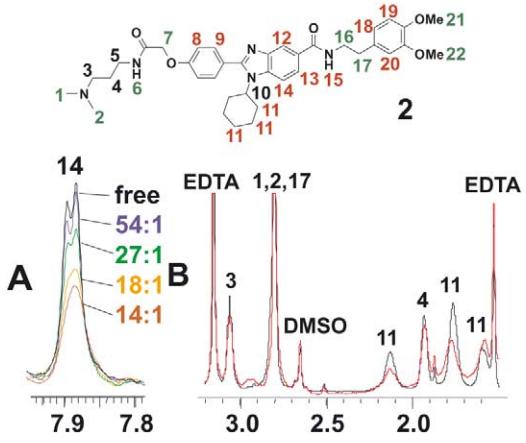


Figure 2. The structure of **2** is provided with color-coded numbering that summarizes the DLB NMR data observed upon addition of NS5B to a solution containing the inhibitor: red, black and green correspond to DLB, lack of DLB and no observation possible due to resonance overlap, respectively. A shows DLB for H_{14} upon addition of incremental amounts of the polymerase (**2**/NS5B ratio from 54:1 to 14:1). B provides a selected portion of the aliphatic region showing DLB for the cyclohexane protons (H_{11}) at an inhibitor/NS5B ratio of 27:1 (red trace).

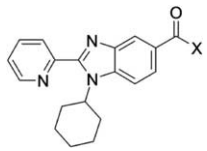
Based on the similar inhibitory potencies of **1** and **2** and on the NMR data (vide supra), it was evident that the basic left-hand side of **2** contributed very little to the observed activities. Hence, we chose derivative **1** as a starting point for optimization studies. Preliminary hit-to-lead activities consisted of systematically truncating the right-hand side amide substituent which eventually lead us to a minimal core that is required for significant biological activity.

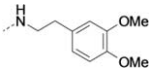
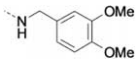
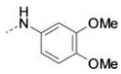
As shown in Table 1, excision of a methylene group from **1** resulted in a small increase in potency (**3**), yet further truncation to anilide **4** was not tolerated. Remarkably, amides **5** and **6** showed partial activity, which was further improved in the case of the free carboxylic acid. Compound **7** had comparable potency to benzylic amide **3** and was 3-fold more potent than the original hit molecule **1**. The feasibility of substituting the neutral amide right-hand side of compounds such as **1** or **3** with a charged carboxylic acid group as in **7** without loss in potency, suggested that inhibitors may exploit diverse interactions with the protein. Despite the substantial size reduction and modifications in chemical properties resulting from this change, inhibitor **7** maintained specificity for HCV polymerase as compared to both the poliovirus and mammalian calf thymus (CT) RNA polymerases.

Isomeric 4-, 6- and 7-carboxy-benzimidazole derivatives had little or no inhibitory activity in the assay (results not shown). Furthermore, the homologous phenylacetic acid derivative (5-CH_2COOH) corresponding to **7** was also inactive. This suggested that the carboxylic acid function of **7** is implicated in a well-defined and specific interaction with the protein. Whereas replacement of the 5-carboxylic acid group of **7** by the isosteric tetrazole decreased potency 6-fold, removal of the 5-COOH substituent or replacement by other functional groups such as CN, CF_3 resulted in a complete loss of activity (results not shown). Compound **7**, showing low μM specific activity against NS5B (IC_{50} = 4.3 μM) and improved physicochemical properties relative to **1** and **3** (solubility), became the starting point for further optimization studies.

Initial benzimidazole hits from our screening campaign that showed specificity for HCV polymerase[10] all carried a cyclohexyl substituent at the N^1-position and hence, the importance of this position was explored (Table 2). Only a few select examples of the extensive SAR carried out at this position are presented in Table 2. Removal of the N^1-substituent (compound **8**) obliterated inhibitory activity. Increasing ring size to a seven-membered ring (**9**) was detrimental to activity, but replacement with a cyclopentyl ring resulted only in a 3-fold decrease in potency (**10**). Further ring-size reduction or cleavage of the ring to produce acyclic variants was unfavorable (**11**–**13**).

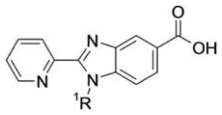
Table 1. Identification of the minimum core required for biological activity in the NS5B polymerase enzymatic assay



Compd	X	HCV NS5B IC_{50} (μM)[a]	Poliovirus IC_{50} (μM)
1		12±3	>500
3		7.1±1	>500
4		>7[b]	
5	$NHCH_3$	28±6	
6	NH_2	27±8	
7	OH	4.3±1	>500

[a] Values are means of duplicate experiments on two separate weighings. Actual compound concentrations determined by HPLC using standards.
[b] 13–30% inhibition at maximum soluble concentration (7 μM).

Table 2. SAR at the 1-position of the benzimidazole scaffold



Compd	^1R	HCV NS5B IC_{50} (μM)[a]	Poliovirus IC_{50} (μM)
7		4.3±1	>500
8	H	>500	
9		49±19	
10		12±4	>500
11		76±15	
12		139±46	
13		408±12	
14		50±13	
15		13±4	>500
16		360±14	

[a] Values are means of duplicate experiments on two separate weighings. Actual compound concentrations determined by HPLC using standards.

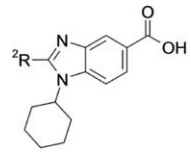
Substitution of the cyclohexane ring with methyl groups (**14** and **15**) reflected rather tight steric requirements for the 4-position (incorporation of an adamantane ring at this position resulted in a >20-fold decrease in potency; result not shown); however, the 2-position of the cyclohexane ring appeared to be more permissive. Nevertheless, no net beneficial effect resulted from these susbtitutions. Aromatization of the ring (**16**) also resulted in a significant loss in potency. These findings and NMR experiments discussed above confirm that the cyclohexyl substituent is optimally interacting with a very well defined lipophilic pocket on the polymerase enzyme. Similar results were observed when this exercise was performed with the neutral benzylic amide series using compound **3** as a reference point, suggesting a similar mode of binding at this position by both series (results not shown).

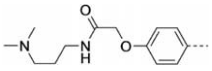
The C-2 position on the benzimidazole scaffold, in contrast to the N^1-position, was very permissive. SAR patterns were difficult to establish and the main feature that was extracted from the examples presented in Tables 3 and 4 is that small heterocycles were preferred. More than 100 alternatives were evaluated at the 2-position and only select compounds are presented in the tables.

The necessity for a substituent at C-2 was obvious, as unsubstituted derivative **17** was approximately 40-fold less potent. Incorporation of the basic entity found in one of the initial hit compounds (**2**) to give **18** resulted in a 3-fold loss in potency relative to the 2-pyridyl substitution (**7**). This was consistent with the DLB findings for **2** discussed above, that suggested minimal interaction with the protein for segments of inhibitors that extend beyond the first aromatic ring. Replacement of the 2-pyridyl group in **7** with a neutral phenyl ring (**19**) resulted in a 2-fold decrease in potency. Substitution of this ring with a variety of single or multiple functional groups (OH, OR, halo, alkyl, CN, CONHR, COOH, NO_2, NHCOR, NR_2) did not produce any inhibitors with IC_{50}s below 10 μM (results not shown).

ortho-Substituted aromatics were particularly detrimental to potency as shown for the example with trimethylated phenyl derivative **20**. Presumably, *ortho*-substituents can sterically influence positioning of the critical cyclohexane ring at the N^1-position within its well-defined lipophilic pocket in the enzyme. Larger bicyclic substituents such as benzofuran **21** (as well as quinolines and indoles) were significantly less potent relative to **7**. Likewise, aliphatic substituents such as cyclohexyl analogue **22** (and others: *tert*-butyl, cyclopropyl, isobutyl) resulted in a minimum 5-fold decrease in potency. Vinylic substituents (e.g., **23**) were slightly

Table 3. SAR at the 2-position of the benzimidazole scaffold



Compd	^{2}R	HCV NS5B IC$_{50}$ (μM)[a]	Poliovirus IC$_{50}$ (μM)	CT IC$_{50}$ (μM)
7		4.3±1	>500	
17	H	186±76		
18		13±3		
19		8.6±2	250	>500
20		120±29		
21		58±24		
22		20±9		
23		19±5	>500	
24		62±35		
25		44±9		

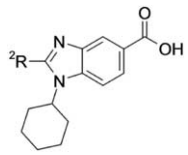
[a] Values are means of duplicate experiments on two separate weighings. Actual compound concentrations determined by HPLC using standards.

better than their saturated counterparts, yet, potency was decreased at least 5-fold compared to **7**. Other sp^2-hybridized groups such as ester **24** and ketone **25** were also examined but conferred no benefit.

Particularly interesting compounds discovered to date in this series are those depicted in Table 4, which incorporated small heterocycles at the 2-position of the benzimidazole scaffold.

Replacement of the 2-pyridyl ring in **7** with small five-membered heterocycles (e.g., furans, pyrroles, thiophenes, imidazoles, thiazoles) either increased potency slightly (ca. 3-fold: **29** and **32**) or had no effect. In general, substitution at various positions of these small heterocycles with alkyl, halogen, COOH or other groups led to significant decreases in potency (some examples are shown in Fig. 3). Though no clear SAR trends were apparent for the C-2 position, we believe that steric factors are an important determinant for inhibitor activity.

Table 4. SAR at the 2-position of the benzimidazole scaffold: most potent analogues



Compd	^{2}R	HCV NS5B IC$_{50}$ (μM)[a]	Poliovirus IC$_{50}$ (μM)	CT IC$_{50}$ (μM)
7		4.3±1	>500	>500
26		28±14	>250	>500
27		17±6	>500	>500
28		3.7±1.3	>500	>500
29		1.6±0.6	>250	>500
30		5.9±2	>500	>500
31		10±4.5	>250	>500
32		1.8±0.4	>500	>500
33		6.3±4	>250	>500
34		3.4±1.5	>250	>500
35		4.6±0.2	>500	>500

[a] Values are means of duplicate experiments on two separate weighings. Actual compound concentrations determined by HPLC using standards.

Bulky groups such as phenyl rings may likely influence the conformation or positioning of the critical cyclohexyl substituent on the neighboring N^1-position, due to the presence of two *ortho*-hydrogens. Smaller five-membered aromatic heterocycles or the placement of a heteroatom *ortho* to the point of attachment on six-membered rings (as exemplified by compound **7** relative to **26** and **27**) may allow for partial relief of detrimental steric interactions with the cyclohexyl ring and generally led to more active compounds.

Electronic factors do not appear to play a major role as neutral (**28**–**30** and **32**–**34**) and more basic groups (e.g.,

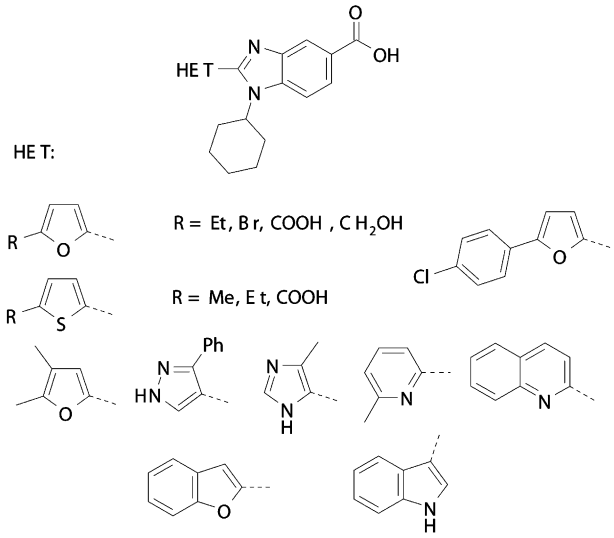
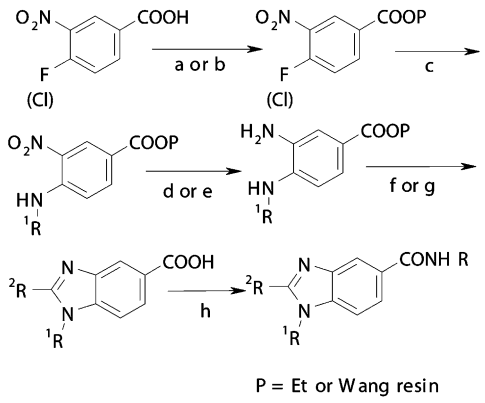


Figure 3. Less potent C-2 heterocyclic substituents ($IC_{50} > 30$ μM).

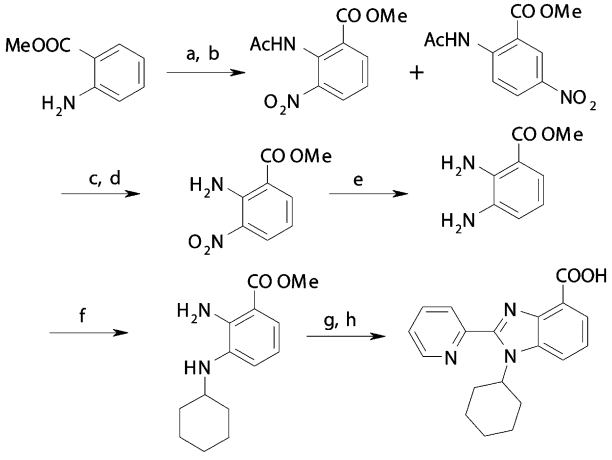
7, **26**, **27**, **31**, and **35**) are equally tolerated at this position. As can be seen from the data presented in Table 4, the compounds still retained their specificity for HCV polymerase relative to the polio virus RdRp and, more importantly, the mammalian DdRp II isolated from calf thymus (CT).

Recently, cell lines that support high levels of self-replicating sub-genomic HCV RNAs (HCV replicons) in Huh-7 cells have become available.[13] This viral replication surrogate cell-based assay is useful for evaluating the potential for compounds to inhibit their target (in this case NS5B polymerase) within a cellular environment. None of the selected compounds from the present study (**7**, **29**, **33**, and **34**) showed inhibition of HCV RNA replication at non-toxic concentrations ($EC_{50} > 100$ μM). Some of the factors that may explain this lack of efficacy include: the compounds are not reaching the target enzyme in the cell (lack of cellular permeation); the mechanism by which they inhibit the polymerase is not relevant in vivo; the compounds are not potent enough. Further optimization of these specific HCV NS5B inhibitors will be required in order to promote this class of compounds as potential HCV therapeutics. Further optimization of benzimidazole-based HCV polymerase inhibitors will be reported in due course.

A general procedure for the synthesis of the benzimidazole inhibitors is shown in Scheme 1. Since screening hits originated from a combinatorial library, well-established methodologies for solid- or solution-phase, parallel synthesis of 5-carboxy-benzimidazole derivatives were used.[14] For compounds prepared in solution phase (P = Et), a newly developed protocol using oxone® as an oxidant was used,[14c] and found to be particularly convenient when larger quantities of material were required. For *rapid* optimization of substituents at N^1 and C-2, solid-phase synthesis (P = Wang resin) became the method of choice. The isomeric 4-COOH benzimidazole derivative was prepared as shown in Scheme 2.



Scheme 1. Synthesis of 5-carboxybenzimidazole inhibitors. Reagents and conditions: (a) EtOH, $SOCl_2$, reflux; (b) oxalyl chloride, cat. DMF, CH_2Cl_2 then Wang resin, DIEA; (c) $^1RNH_2$, DIEA, DMSO, 60–80 °C; (d) H_2 (1 atm), 20% $Pd(OH)_2/C$, MeOH; (e) $SnCl_2$ dihydrate, water, DMF; (f) ^2RCHO, chloranil or oxone®, DMF, water then NaOH, water/MeOH then AcOH; (g) ^2RCOOH, HATU, DIEA, DMF or DMSO then TFA; (h) RNH_2, TBTU or HATU, DIEA, DMF or DMSO.



Scheme 2. Synthesis of 4-carboxybenzimidazole inhibitor. Reagents and conditions: (a) Ac_2O, heat; (b) 90% HNO_3, Ac_2O, cat. concd H_2SO_4, 0–5 °C then rt then separate by flash chromatography; (c) 2 N KOH, heat then 4 N HCl; (d) CH_2N_2, ether; (e) H_2 (1 atm), 20% $Pd(OH)_2/C$, MeOH; (f) cyclohexanone, BH_3·pyridine complex, MeOH, rt; (g) 2-pyridinecarboxaldehyde, oxone®, DMF–water, rt; (h) KOH, MeOH, 60 °C then HCl.

The 6-COOH and 7-COOH analogues were prepared from commercially available 3-fluoro-4-nitrobenzoic and 2-chloro-3-nitrobenzoic acids, respectively, using a similar sequence to that described in Scheme 1.

All inhibitors were purified by reversed-phase HPLC to >90% homogeneity and isolated as TFA salts in most cases. Compounds gave spectral data (electrospray MS and ^1H NMR) consistent with their assigned structures.

We have discovered a series of small 5-carboxybenzimidazole derivatives that specifically inhibit the NS5B RNA-dependent RNA-polymerase of the hepatitis C virus with IC_{50}s in the low micromolar range. Preliminary SAR studies revealed strict requirements for a small C_5–C_6 carbocyclic substituent at the N^1-position. At the 2-position of the benzimidazole scaffold, small aromatic heterocycles were preferred.

Although the moderate potency of these compounds and their physicochemical properties may be limiting their efficacy in a cell-based HCV RNA replication assay, they do nevertheless, represent an attractive starting point in our search for more potent molecules and HCV therapeutics.

Acknowledgements

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9. Full length HCV NS5B was purified as a N-terminal hexa-histidine fusion protein from baculovirus infected insect cells. Compounds were tested for inhibitory activity using a 12 nucleotide RNA oligo-uridylate primer modified with biotin at the free 5′C position and a complementary poly-adenylate template of heterogeneous length (1000–10,000 nucleotides). Polymerase activity was measured as incorporation of UMP-[5,6 ^{3}H] into the chain elongated from the oligo-U primer using SPA-bead capture and quantification (TopCount).[11a] A more detailed description of this assay will be published else where (McKercher, G. et al.).
10. Compounds were tested against polio virus RNA-dependent RNA polymerase and a mammalian DNA dependent RNA polymerase II isolated from calf thymus in an assay format similar to that described for HCV polymerase.[9,11a]
11. (a) Austel, V., Beaulieu, P. L., Fazal, G., Gillard, J., Kukolj, G. US patents 6,448,281 B1 and 6,479,508 B1, 2002, to Boehringer Ingelheim (Canada) Ltd. (b) A recent patent application from Japan Tobacco Inc. also discloses derivatives of benzimidazole 5-carboxylic acids as HCV polymerase inhibitors: Hashimoto, H., Mizutani, K., Yishida, A. EP 1 162 196 A1, 2001.
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