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Non-nucleoside inhibitors of the hepatitis C virus NS5B polymerase: discovery of benzimidazole 5-carboxylic amide derivatives with low-nanomolar potency

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Abstract—Optimization of benzimidazole 5-carboxamide derivatives previously identified as specific inhibitors of the NS5B polymerase of the hepatitis C virus (HCV) has led to the discovery of potent analogues that inhibit the enzyme at low-nanomolar concentrations. Greater than 800-fold improvement in potency from the original lead structure was achieved through the combined effects of conformational rigidification, molecular size extension and the identification of previously unexploited interactions. Furthermore, these inhibitors retain specificity for HCV polymerase relative to other viral and mammalian RNA polymerases.

The hepatitis C virus has infected an estimated 1–3% of the world population, exceeding 170 million individuals.[1,2] In up to 70–80% of incidences the infection is chronic and may progress to conditions that require orthotopic liver transplantation as a life-saving measure.[2] Current therapies based on combinations of pegylated interferons and the broad spectrum antiviral ribavirin are ineffective in a significant proportion of cases and are associated with the occurrence of severe side effects.[3] A growing patient population is in urgent need for novel therapies that could address a clearly unmet medical need.

The HCV NS5B RNA-dependent RNA polymerase is a central enzyme in the replication of the virus and has become a target of choice for the screening and design of small molecule inhibitors which, in principle, should interfere with viral replication.[4] We recently described the discovery and initial optimization of benzimidazole 5-carboxylic acid derivatives which are specific inhibitors of HCV polymerase.[5] Compound **1**, a representative of this novel class of non-nucleoside inhibitors, had an IC_{50} in the low-micromolar range and delineates the minimum core for activity against the enzyme (Fig. 1).

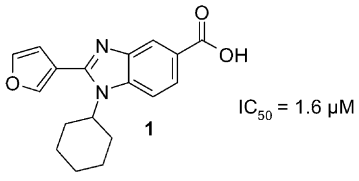

Figure 1. Benzimidazole-based inhibitor of HCV NS5B polymerase.

This class of inhibitors was shown to inhibit polymerase activity in a non-competitive fashion with respect to nucleotide incorporation. Based on evidence obtained from several biochemical experiments, the most likely mode of action is believed to result from interference with productive binding of the RNA substrate to the enzyme.[6] This highlights the potential of a novel mechanism for inhibition of the polymerase, distinct from active site-directed inhibitors such as nucleoside

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analogues. The existence of allosteric binding sites to which small molecules can bind in a productive fashion represents an attractive avenue for the discovery of specific and novel antiviral agents.[4] Unfortunately, compound **1** did not inhibit replication of HCV replicons in a cell-based assay.[7] Factors responsible for this lack of efficacy had to be addressed in order to advance further towards the development of HCV therapeutics. The modest inhibition by these compounds in our in vitro enzymatic assay was likely an important liability and our objectives focused on improving potency.

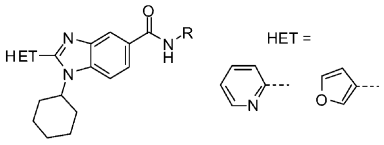
In our initial studies leading to the discovery of compound **1**,[5] substitution of the benzimidazole scaffold at N^1 and C-2 by a cyclohexyl ring and a 3-furyl moiety respectively, were optimal. SAR had revealed the former to be rather intolerant to manipulation whereas small aromatic heterocycles with a variety of substitutions were favored at the C-2 position. Since the minimum core for inhibitory activity, as exemplified by compound **1**, offered no obvious possibilities for further improvements, the amide derivative **2** (Table 1), which was also identified as an inhibitor ($IC_{50} = 7$ μM) during preliminary hit-to-lead activities,[5] was selected as the starting point for further optimization. Taking advantage, once again, of parallel synthesis techniques established in our previous work,[5] we describe modifications to the right-hand side of the molecule leading to 800-fold increases in potency.[8]

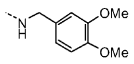
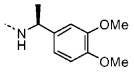
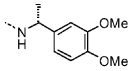
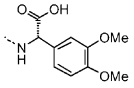
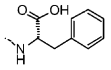
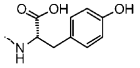
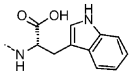
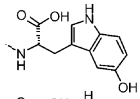
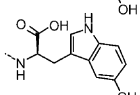
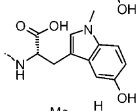
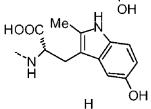
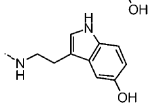
Addition of a methyl group at the benzylic position of **2** generated a pair of enantiomers (compounds **3** and **4**, Table 1). The (*R*)-enantiomer was approximately 4 times more potent than the (*S*)-isomer and about 2-fold more potent than unsubstituted benzylic amide **2**. The increased potency could be due to a productive interaction of the methyl group with the protein, but is more likely due to an orienting effect that helped rigidify the free-state of the inhibitor towards a bound-like conformation.[9] Support for the latter proposal derives from replacement of the neutral methyl group with an ionized carboxyl group of identical configuration that resulted in an analogue of similar potency (**5**), despite large differences in electronic character between the two groups. One advantage of incorporating the α-carboxyl group was that it also improved aqueous solubility of this compound class.[10]

Compound **5** being the coupling product between the 5-benzimidazole carboxylic acid derivative and a phenylglycine analogue, a set of commercially available α-amino acids with diverse side chains were coupled to *N*-cyclohexyl-2-(2-pyridyl) benzimidazole 5-carboxylic acid to further expand the SAR in this new direction. Most compounds inhibited the polymerase with IC_{50} values in the 2-35 μM range (examples included: Ala, Leu, Gly, Ile, Val, Asp, Glu, Ser, Thr, His, phenylglycine, *tert*-butylglycine; results not shown).

The most improved results in this survey were obtained with amino acid derivatives bearing aromatic side chains. For example, whereas phenylalanine amide **6** was 3-fold less potent than phenylglycine analogue **5**, addition of a hydroxyl group to the aromatic ring (tyrosine analogue **7**) provided a 4- to 5-fold increase in potency over a hydrogen atom (**6** versus **7**). Extending the aromatic surface of the amino acid from a simple phenyl ring (**6**) to an indole gave tryptophan analogue **8** which was also 3- to 4-fold more potent. The 5-hydroxytryptophan analogue **9** was then prepared with the expectation that the beneficial effect of the hydroxyl group (seen with **6** versus **7**) was reproducible in this derivative. Indeed the two effects, expanding the size of the aromatic surface and addition of a hydroxyl group were additive. Thus, the first inhibitor in this class to inhibit the polymerase activity at sub-micromolar concentrations was identified. Compound **9** had an

Table 1. Benzimidazole 5-carboxamide SAR



HNR		HCV NS5B IC_{50} (μM)[a]		
		HET = 2-pyridyl		HET = 3-furyl
	**2**	7±1.5		
	**3**	12±5		
	**4**	3±1		
	**5**	2±0.8	**10**	0.4±0.2
	**6**	7±3	**11**	4±1
	**7**	1.5±0.7	**12**	0.4±0.1
	**8**	1.9±0.5	**13**	0.4±0.1
	**9**	0.14±0.05	**14**	0.05±0.01
			**15**	0.7±0.08
			**16**	0.16±0.02
			**17**	0.46±0.18
			**18**	1.5±0.3

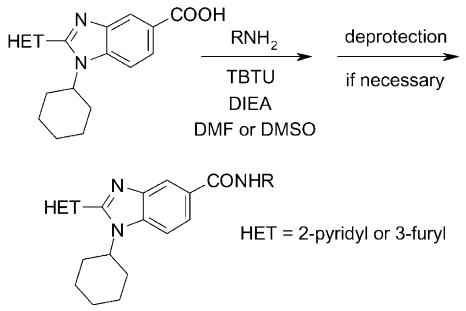
[a] Values are the mean of duplicate experiments with two separate weighings.

$IC_{50} = 0.14$ μM, and represented a 50-fold increase in potency from the starting benzylic amide **2**.

Optimization, so far, was carried out on a benzimidazole scaffold carrying a 2-pyridyl substituent at C-2. In our previous work,[5] we found that a 3-furyl group at C-2 provided a 2- to 3-fold improvement in potency. A similar effect was anticipated in the current benzimidazole amide series. The outcome of this modification is also shown in Table 1. In all cases, replacement of the 2-pyridyl group by a 3-furyl group increased potency an additional 2- to 5-fold (compare **5**–**9** and **10**–**14**). The most potent analogue in this series (**14**) had an $IC_{50} = 50$ nM. The parallel SAR displayed by the two C-2 heterocycles is an indication that despite large structural differences between the original benzimidazole 5-carboxylic acid series[5] and the current amide series, the two classes of inhibitors have similar modes of action and bind similarly to the polymerase. Interestingly, potent inhibitors appear to be more sensitive to configurational changes at the asymmetric carbon center. Whereas a 4-fold difference in potency was previously observed between enantiomers **3** and **4**, the gap increased to 14-fold in the case of the more potent 5-hydroxytryptophan derivatives **14** and **15**. This result suggests that complementarities between the ligands and the protein binding site were improved, and in turn, increased compound sensitivity to conformational effects. Methylation of the indole nitrogen or the C-2 indolic carbon of the tryptophan residue resulted in a 3- and 9-fold decrease in potency, respectively. In the latter case, methylation affects the free-state conformation. The importance of conformational contributions to compound potency was evident from the 30-fold loss in inhibitory activity (compound **18**) when the α-carboxyl group of **14** was removed. Although, at this stage, we could not exclude minor binding contributions from the carboxyl group.

Following the discovery of benzimidazole–tryptophan conjugates with inhibitory potency in the 50 nM range, further improvements were examined through SAR at the easily accessible 5-position of the tryptophan indole nucleus of compounds such as **13**. As discussed above, the introduction of a phenolic hydroxyl group at the 5-position of the tryptophan indole nucleus increased potency 8-fold (compound **14**). Groups not capable of providing hydrogen bonds such as NO_2, Me, F and OMe were all significantly less potent (**19**–**22**). The beneficial effect of an OH group added to the indole is not likely due to changes in electronic properties of the ring system, as the OMe derivative (**22**) was equipotent to **13**. All compounds with functional groups capable of donating hydrogen bonds, on the other hand, had IC_{50}s < 150 nM, independent of their electronic properties or ionization state: basic groups (aniline **23**), neutral sulfonamides (**24** and **25**), anilides (**26**), carboxamides (**29**) and acidic functions (**27**, **28**, and **30**–**32**) all behaved similarly. Oxalic amide (**27**) was the most potent analogue identified in this series with an IC_{50} < 10 nM.

It is tempting to invoke the identification of a new pharmacophore on the right-hand side of the molecules that may be responsible for the observed increases in potency resulting from addition of hydrogen-bond donors to the 5-position of the tryptophan ring. However, such conclusions await further confirmation from structural studies (e.g., X-ray crystallography).

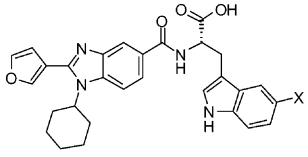


Scheme 1. General synthesis of inhibitors.

Potent analogues in Table 2 (**14** and **23**–**32**) were tested for specificity against another RNA-dependent RNA polymerase from polio virus and a DNA-dependent RNA polymerase isolated from calf-thymus.[5,8] Specificity windows of >1500-fold were seen for all compounds.

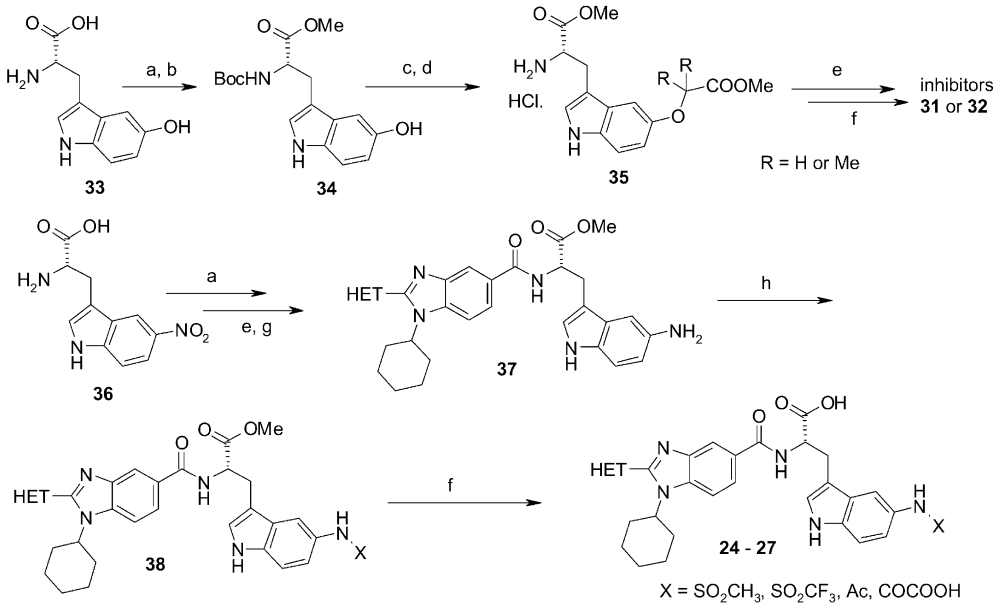
Several inhibitors generated during this optimization study were also tested in an HCV cell-based replicon assay of RNA replication.[7] Compound **31**, despite good intrinsic potency, had only marginal efficacy (i.e., 63% inhibition at 50 μM) which may reflect its inability to permeate cells effectively and reach the intracellular enzymatic target. The presence of a highly ionized carboxyl group at physiological pH is presumably a major cause for this lack of efficacy. Further support for this hypothesis is provided by the measured log D values for compounds **14** and **31**, which were found to be 0.45 and

Table 2. SAR at the 5-position of the tryptophan indole nucleus



Compds	X	HCV NS5B IC_{50} (μM)[a]	Poliovirus and CT RNA polymerases IC_{50} (μM)
13	H	0.4±0.1	200, 316
14	OH	0.05±0.01	79, >200
19	NO_2	2.0±1	
20	Me (dl)	2.2±0.3	
21	F (dl)	2.9±1.9	
22	OMe (dl)	0.4±0.1	
23	NH_2	0.09±0.04	>500, >250
24	$NHSO_2CH_3$	0.12±0.04	>500, –
25	$NHSO_2CF_3$	0.15±0.05	>250, >250
26	NHAc	0.06±0.01	>125, >250
27	NHCOCOOH	0.008±0.002	>250, >250
28	COOH	0.02±0.008	>200, >250
29	$CONH_2$	0.037±0.01	>200, >250
30	C_5-tetrazole	0.015±0.005	>200, >250
31	OCH_2COOH	0.019±0.005	100, >200
32	$OC(Me)_2COOH$	0.10±0.02	>200, —

[a] Values are the mean from at least duplicate experiments on two separate weighings.



Scheme 2. Synthesis of functionalized tryptophan derivatives: (a) $MeOH/SOCl_2$.. (b) Boc_2O. (c) $BrCH_2COOMe$ or $BrC(CH_3)_2COOMe/Cs_2CO_3$ (2 equiv)/acetone/50 °C. (d) HCl-dioxane. (e) 5-benzimidazole carboxylic acid derivative (e.g., **1**)/TBTU/*i*Pr_2EtN/DMSO or DMF. (f) NaOH then AcOH or TFA. (g) $SnCl_2$ (5 equiv)/3:1 DMF–water/60 °C. (h) Acid chloride, sulfonyl chloride or anhydride/pyridine/DMSO or DMF.

−1.62, respectively.[11] Future efforts in this series, focused on addressing the issue of cell permeability and activity in the replicon, will be reported in due course.

Inhibitors were prepared in solution-phase by condensation of 1,2-disusbtituted benzimidazole 5-carboxylic acids[5,12] with amine derivatives, using standard amide bond forming reagents (Scheme 1).[13] Inhibitors were purified by reversed-phase HPLC to >90% homogeneity (isolated as TFA salts in most cases). Compounds gave spectral data (electrospray MS and ^{1}H NMR) consistent with their assigned structures.

Amines were obtained from commercial sources or prepared using standard protocols as described in the literature. Amino acid derivatives were coupled as their methyl esters (prepared using $MeOH/SOCl_2$) and required subsequent hydrolysis of ester functionalities using hydroxide.[14] The synthesis of some ring-functionalized tryptophan derivatives is illustrated in Scheme 2. Commercially available (*S*)-5-hydroxytryptophan **33** was esterified to the methyl ester and *N*-protected to give carbamate **34**. Alkylation of the phenolic hydroxyl group with bromoacetate derivatives and cleavage of the Boc protecting group under acidic conditions gave ethers **35** (R=H or Me), precursors to inhibitors **31** and **32**. For the synthesis of inhibitors **24**–**27**, (*S*)-5-nitrotryptophan **36**[15] was esterified to the methyl ester hydrochloride and coupled in the usual manner to benzimidazole **1**. Reduction of the nitro group then gave 5-aminotryptophan derivative **37**. Compound **37** was reacted with acid chlorides, sulfonyl chlorides or anhydrides to provide protected derivatives **38**, which upon saponification of the methyl esters provided the desired inhibitors. 5-Carboxy and 5-carboxyamide analogues **28** and **29** were prepared from 5-cyanotryptophan[16] by hydrolysis under acid conditions (concd HCl/80 °C), which yielded a mixture of the free 5-carboxyl group and the corresponding primary amide in a 2:1 ratio respectively. Following esterification of carboxyl groups ($MeOH/SOCl_2$), the mixture of tryptophan derivatives was coupled to the left-hand side scaffold and following deprotection, **28** and **29** were separated by preparative HPLC. 5-Tetrazolyl analogue **30** was prepared by condensation of 5-cyano-Trp-OMe[16] with the left-hand side benzimidazole scaffold and the 5-cyano group converted to a tetrazole ring using tri-*n*-butyltin azide in DMSO at 80 °C.[17] Saponification of the protected inhibitor in the usual manner gave **30**.

This study describes the optimization of a previously discovered series of substituted 5-carboxybenzimidazole inhibitors of HCV NS5B polymerase. An 800-fold increase in intrinsic potency relative to the initial hit compound was achieved, leading to the discovery of inhibitors with low nanomolar potency in our in vitro enzymatic assay. This result was achieved through the combined effects of conformational rigidification and the discovery of a potential new pharmacophore on the right-hand side of the molecule, favoring hydrogen-bond donors. Potent compounds in this series retain specificity for inhibition of HCV polymerase. Additional studies are required to address the issue of cellular permeation with this class of inhibitors and improve their efficacy in a cell-based HCV-replicon assay.

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8. The in vitro enzymatic assay used to evaluate the potential of compounds as inhibitors of HCV polymerase is described in reference 6 and US patents 6,448,281 B1 and 6,479,508 B1 (**2002**) to Boehringer Ingelheim (Canada) Ltd.
9. NMR studies on the bioactive conformation of this class of inhibitors when bound to NS5B will be reported separately: LaPlante, S. et al. (manuscript in preparation).
10. The solubility of compounds **4** and **10** was measured in aqueous pH 7. 2 phosphate buffer and were found to be 5 μg/mL and >991 μg/mL, respectively. The low solubility of neutral molecules such as **2**–**4** and others (not shown) was considered a liability at this stage, in terms of establishing solid SAR patterns. For this reason, optimization studies were pursued in the more soluble carboxylic acid class.
11. The methyl ester of compound **14** was also prepared and tested in the replicon assay. Despite its good inhibitory potency against the enzyme ($IC_{50}=0.11$ μM) and a substantial increase in lipophilicity resulting from the removal of ionizable functions (log $D=3.07$, solubility $=0.6$ μg/mL in pH 7.2 phosphate buffer), only 30% inhibition was measured at the highest non-cytotoxic concentration (3.4 μM). No hydrolysis of the methyl ester was detected under assay conditions.
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