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Pyridine Carboxamides: Potent Palm Site Inhibitors of HCV NS5B Polymerase

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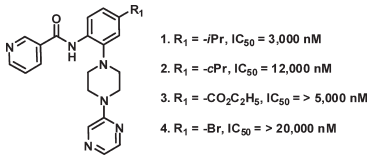
ABSTRACT Pyridine carboxamide-based inhibitors of the hepatitis C virus (HCV) NS5B polymerase were diversified and optimized to a variety of topologically related scaffolds. In particular, the 2-methyl nicotinic acid scaffold was developed into inhibitors with improved biochemical (IC_{50}-GT1*b* = 0.014 μM) and cell-based HCV replicon potency (EC_{50}-GT1*b* = 0.7 μM). Biophysical and biochemical characterization identified this novel series of compounds as palm site binders to HCV polymerase.

KEYWORDS Hepatitis C virus, HCV NS5B polymerase, palm site inhibitors, direct-acting antiviral (DAA), automated ligand identification system (ALIS), 2D ^{15}N-HSQC



Chronic infection by the hepatitis C virus (HCV) afflicts an estimated 2−3% of the world population and represents a serious health issue.[1] Currently, it is treated with a combination of pegylated interferon-α and ribavirin, which results in sustained virologic response (SVR) in only approximately 40−50% of patients infected with genotype 1 virus.[2] With major advances in our understanding of this pathogen that came from the identification of essential viral functions,[3] the development of subgenomic replicons allowing HCV RNA replication in Huh-7 cells,[4] and the ability to replicate specific viral strains in the laboratory,[5] several direct-acting antivirals (DAA) against HCV have now achieved encouraging results in phase 1 to 3 clinical trials.[6] A major target of DAA against HCV is the viral nonstructural protein 5B (NS5B), which possesses RNA-dependent RNA polymerase (RdRp) activity essential for viral genome replication. Several potent, selective, structurally distinct non-nucleoside inhibitors of NS5B, binding at palm sites 1 and 2 and thumb sites 1 and 2, have demonstrated clinical proof of concept.[7] Clinical efficacy was first demonstrated with HCV-796, which targets palm site 2,[8,9] although safety signals precluded further development of this compound. Several palm site 1 (e.g., ABT-333[10] and ANA598[11]), thumb site 1 (MK-3281[12]), and thumb site 2 binders (e.g., PF-868554/filibuvir[13] and VX-222[14]) have since entered clinical testing. However, as monotherapy with DAA is expected to engender development of viral resistance, new small-molecule inhibitors of HCV remain a focus of intense drug discovery efforts in search of candidate components of combination therapy.

A medicinal chemistry effort to optimize small molecule inhibitors of NS5B was initiated following high-throughput screening of NS5B(Δ21)-genotype 1b (Con1) enzyme using our proprietary Automated Ligand Identification System (ALIS) platform, an affinity selection−mass spectrometry platform for label-free, high-throughput screening of mixture-based combinatorial libraries.[15] The screen yielded several classes of compounds, one of which was recently reported in detail.[16] Here, we describe another distinct series that is based on a nicotinamide-based scaffold and inhibits through binding at the palm site of NS5B.

N-(4-Isopropyl-2-(4-(pyrazin-2-yl)piperazin-1-yl)phenyl)-nicotinamide **1** (Figure 1), a hit from the initial screen, was a modestly potent compound in the biochemical assay but did not appreciably inhibit HCV RNA replication in the cell-based replicon assay. The decline in potency when the *i*-Pr group of **1** was replaced with the similar *c*-Pr group (**2**) or a Br (**4**) group suggested that this position was very sensitive to changes and important for activity. Synthesis of compounds in the nicotinic carboxamide series began with oxidation of 2-bromo-4-isopropylaniline (**5**) as outlined in Scheme 1. We therefore surveyed the substitution patterns on nicotinic acid of **1** and other varieties of scaffolds (Table 1) to provide compounds **10**−**45** with a substantial improvement in potency, quickly establishing structure−activity relationship (SAR) and improving upon activity in both the biochemical and the cell-based replicon assays.

Utilizing **1** as a starting point, we began the optimization process by investigating the substitution pattern on the nicotinic acid and other scaffolds (Table 1). Attempts to substitute the pyridine ring with polar substituents (**10**−**12**) resulted in lesser potency relative to monofluorinated (**13**) nicotinic or 2-aminonicotinic (**14**) analogues. 2-Trifluoromethyl analogue (**15**) retained potency, while the 6-trifluoromethyl (**16**) and

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4-trifluoromethyl (**17**) nicotinic analogues were weak inhibitors. The addition of a bulky group to the pyridine ring of R_2 as in the 2-methyl vinyl (**18**) and phenyl (**19**) analogues resulted in a loss of potency. The isomeric pyridines **20** and **21** also were weaker inhibitors than the starting **1**.

Attempts to replace nicotinic acid with pyrimidine (**22**), pyridazine (**23**), or pyrazine (**24**) analogues or to replace with saturated heterocycles (**25**−**27**) resulted in less potent compounds as well as with H-bond donors, for example, 3-methylpyrazole (**34**) and aza-indole (**35**), relative to five-membered ring heterocyclic analogues (**28**−**33**). Surprisingly, replacement of the 5-methylisoxazole (**31**) analogue with a 3-methylisoxazole (**36**) resulted in a notable decrease in activity. A breakthrough was uncovered upon difluorination of the phenyl ring. Whereas the 2-methyl-3,6-difluoro (**37**) and 3,5-difluoro-4-methyl analogues (**38**) were uniformly less active than the 2-methyl-3,5-difluoro analogue (**39**), the 2-methyl-3,5-difluoro derivative (**39**), with an IC_{50} = 540 nM, displayed a remarkable 6-fold improvement in potency relative to **1**. At this point, we incorporated the 2-methyl substitution on the nicotinic acid (**40**) as well as some five-membered ring heterocyclic analogues (**41**) and were pleased to observe a similar effect. Compounds **40** and **41** inhibited NS5B with an IC_{50} = 520 and 580 nM, respectively. No significant potency improvements were observed by introducing methyl group in different positions (**42** and **43**) or by replacing methyl with a cyclopropyl substituent (**44**) or with a quinoline analogue (**45**).

The SAR of *N*-substitution (R_3) is presented in Table 2, and the chemistry route is outlined in Scheme 2. BOC-protected piperazine (**48**), amides (**51** and **52**), urea (**53**), and sulfonamide (**54**) substitutions led to analogues with greatly diminished activity relative to **1**, but the original activity was restored by attaching five-membered heterocycles (**55**−**58**). Replacement of a pyrazine with a 2-pyridine (**59**) or addition of a phenyl substituent (**60**) also caused a notable decrease in activity. The addition of a straight chain or small alkyl substituent had a positive effect on potency (**61** and **62**) relative to parent compound (**1**), as did pyrimidine replacements (**63** and **64**) and substituted pyrimidines (**65** and **66**). The intrinsic potency was improved moderately; however, despite measurable cell-based effects indicating compound penetration, it was not possible to differentiate anti-HCV replicon activity from overlapping cytotoxicity.

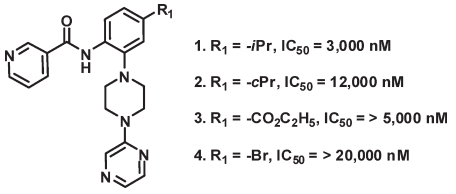
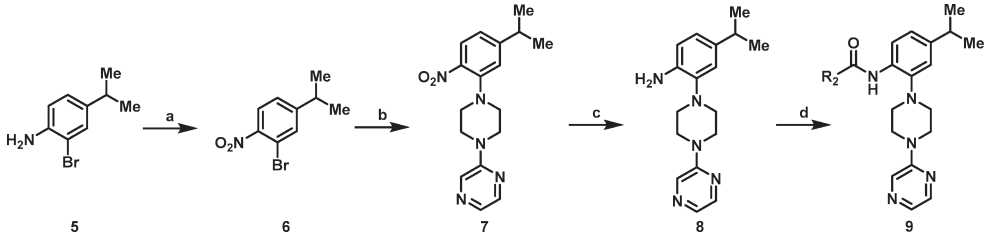


Figure 1. Initial nicotinamide-based NS5B inhibitors.

To continue the investigation of the SAR at R_3, we prepared several analogues incorporating the 2-methylpyridyl group (Scheme 2), which as exemplified by compound (**40**) enhanced both intrinsic potency and replicon activity with a ∼10-fold cytotoxicity window. We changed to the more potent 4-pyrimidine analogue combined with 2-methyl nicotinic carboxamide (**70**) as a benchmark (Table 3) and found that whereas pyrazolopyrimidine analogue (**71**) and 2-methylthiomethylpyrimidine (**72**) provided a modest improvement in enzyme potency without gaining cellular activity, thienopyrimidine (**73**) afforded a 7-fold enhancement in enzyme potency (IC_{50} = 14 nM) and yielded submicromolar replicon activity (EC_{50} = 0.7 μM) with a >20-fold cytotoxicity window, representing a potency gain of 200- and 30-fold in biochemical and cell-based HCV replicon activities, respectively, from the initial hit (**1**).

Three experimental approaches were employed to characterize the binding site of the pyridine carboxamide inhibitor series. First, we conducted an analysis of inhibitor-induced chemical shift changes in 2D ^{15}N-HSQC NMR spectra of ^{15}N-labeled HCV NS5B polymerase. Because chemical shift assignments of NS5B are not readily obtained due to its large size (64 kDa), peaks of residues within interesting subsites of NS5B were mapped using various NS5B binders whose binding sites were previously known from X-ray crystal structures. Such chemical shift perturbation maps were then used to infer the binding sites of newly discovered HCV NS5B inhibitor series. As detailed in the Supporting Information, the thienopyrimidine inhibitor (**73**) induced chemical shift perturbation patterns similar to those of a benzofuran inhibitor, HCV-796, a known palm site 2 binder,[17] suggesting that the two inhibitors share an overlapping but not identical binding site in the palm domain. By contrast, a distinctly different chemical shift perturbation pattern was induced by a dihydropyranone derivative previously shown to bind at thumb site 2,[18,19] indicating that the pyridine carboxamides are unlikely to be thumb site binders. Limited aqueous solubility of a benzothiadiazine analogue, A-848837, precluded

Scheme 1. Synthesis of 4-Isopropyl-2-(4-(pyrazin-2-yl)piperazin-1-yl)aniline and Amidation[a]



[a] Reagent and conditions: (a) *m*-CPBA, toluene, reflux. (b) 2-(Piperazin-1-yl)pyrazine, DIEA, DMF, 200 °C for 20 min in microwave. (c) Zn, $CaCl_2$, EtOH, 4 h. (d) Carboxylic acid, HATU, DIEA, DMF.

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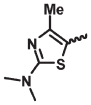
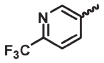
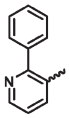
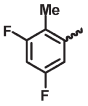
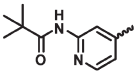
Table 1. Scaffold Replacements in Nicotinic Carboxamide Analogues of Compound **9** at R_2

Compound	R_2	NS5B IC_{50} (μM)[a,b]	Replicon EC_{50} (μM)[a,b,c]	Cell CC_{50} (μM)[a,b]	Compound	R_2	NS5B IC_{50} (μM)[a,b]	Replicon EC_{50} (μM)[a,b,c]	Cell CC_{50} (μM)[a,b]
1		3	20	54	**29**		7	6.5	28
10		> 200	ND	ND	**30**		10	6.9	>10
11		> 20	ND	ND	**31**		13	ND	ND
12		46	—	17	**32**		14	ND	ND
13		2.2	—	17	**33**		15	ND	ND
14		1.0	—	30	**34**		> 200	ND	ND
15		2.3	—	4.5	**35**		> 20	ND	ND
16		> 20	ND	ND	**36**		> 200	ND	ND
17		> 20	ND	ND	**37**		54	—	11
18		> 200	ND	ND	**38**		5.6	> 50	> 50
19		> 200	—	15	**39**		0.54	—	20
20		> 20	ND	ND	**40**		0.52	2.8	22
21		> 20	ND	ND	**41**		0.58	—	25
22		> 20	ND	ND	**42**		2.3	—	13
23		> 20	ND	ND	**43**		14	—	11
24		> 20	ND	ND	**44**		2	—	21
25		> 20	ND	ND	**45**		6.7	1.8	6
26		> 200	ND	ND					
27		20	ND	ND					
28		3	> 10	> 10					

[a] Values are means of duplicate experiments on two separate weightings. NS5B enzyme, replicon, and cytotoxicity assay protocols are described in the Supporting Information. [b] ND, not determined. [c] Dashed lines, anti-HCV replicon activity masked by overlapping cellular CC_{50}.

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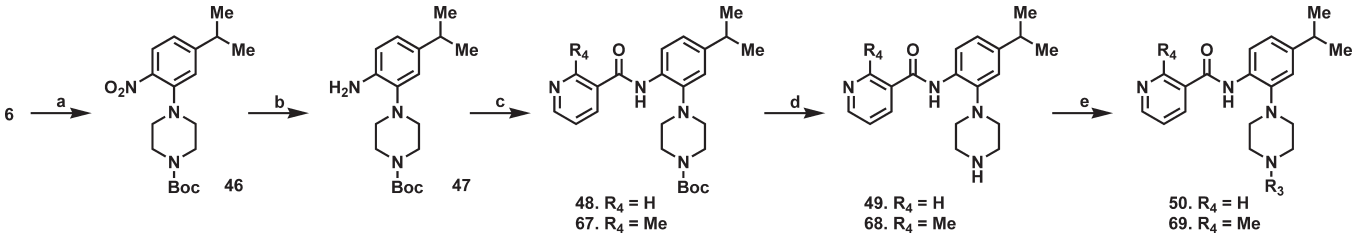
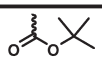
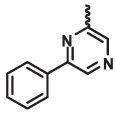
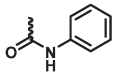
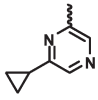
Scheme 2[a]



[a] Reagents and conditions: (a) *tert*-Butyl piperazine-1-carboxylate, DIEA, DMF, 200 °C for 20 min in the microwave. (b) H_2, 10% Pd/C, MeOH. (c) Nicotinic acid or 2-methyl niconic acid, HATU, DIEA, DMF. (d) TFA. (e) R_3X, DIEA, DMF, 200 °C for 20 min in the microwave.

Table 2. SAR Development of Compound **50** at R_3

Compound	R_3	NS5B IC_{50} (μM)[a,b]	Replicon EC_{50} (μM)[a,b,c]	Cell CC_{50} (μM)[a,b]	Compound	R_3	NS5B IC_{50} (μM)[a,b]	Replicon EC_{50} (μM)[a,b,c]	Cell CC_{50} (μM)[a,b]
48		> 200	ND	ND	**60**		17	ND	ND
51		> 200	ND	ND					
52		> 200	ND	ND	**61**		0.5	—	23
53		> 100	ND	ND					
54		> 200	ND	ND	**62**		1	—	1
55		2.5	> 50	> 50	**63**		0.56	—	27
56		15	ND	ND	**64**		2.3	—	37
57		5	> 10	> 10	**65**		0.16	—	25
58		3.9	6	27	**66**		1.5	—	14
59		7.3	> 50	> 50					

[a] Values are means of duplicate experiments on two separate weightings. NS5B enzyme, replicon, and cytotoxicity assay protocols are described in the Supporting Information. [b] ND, not determined. [c] Dashed lines, anti-HCV replicon activity masked by overlapping cellular CC_{50}.

evaluation of this known palm site 1 binder[20] using the bio-NMR approach.

We then performed kinetic competition with inhibitors of known binding sites and analyzed by the method first described by Yonetani-Theorell.[21] Compounds that share identical or overlapping binding sites will yield a mutually exclusive inhibition pattern, indicated by a series of parallel lines in a plot of the reciprocal of enzyme velocity (V_0/V_i) versus the concentration of a titrated inhibitor. Compounds that bind independently at distinct sites of the target enzyme will exhibit nonparallel, convergent lines in such a Yonetani—Theorell plot, representing additive or synergistic inhibition. As shown in Figure 2A, a mutually exclusive pattern of inhibition was clearly evident when a pyridine carboxamide compound (**73**) was added in the presence of the palm site 2 binder HCV-796, similar to that seen with the palm site 1 binder A-848837 (data not shown). In contrast, an additive pattern of inhibition was observed when **73** was titrated with TS2, a known thumb site 2 binder (Figure 2B), or with an indole derivative previously shown to bind at thumb site 1 (data not shown).[22] Results from the inhibitor competition

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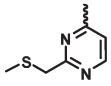
Table 3. Further SAR Development of Compound **69** at R_3

Compound	R_3	NS5B IC_{50} (μM)[a]	Replicon EC_{50} (μM)[a]	Cell CC_{50} (μM)[a]
70		0.1	1.1	20
71		0.032	3.0	>10
72		0.027	2.2	19
73		0.014	0.7	15

[a] Values are means of duplicate experiments on two separate weightings. NS5B enzyme, replicon, and cytotoxicity assay protocols are described in the Supporting Information.

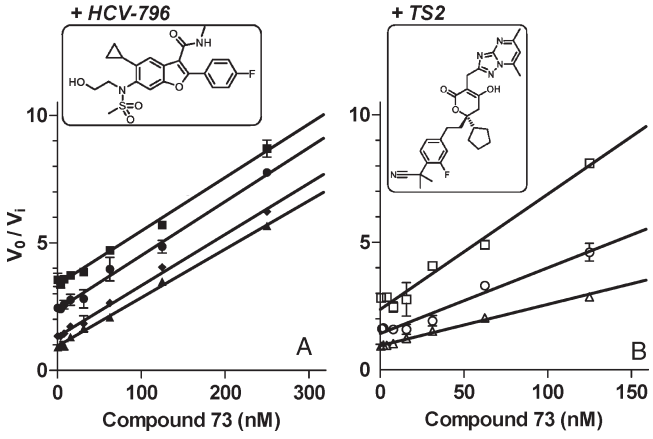


Figure 2. Yonetani−Theorell plots of inhibition of HCV NS5B polymerase by a combination of inhibitors. The NS5B polymerase activity was measured as described[23] (see the Supporting Information) in the presence of various concentrations of **73** and (A) 10, 20, 80, or 160 nM HCV-796; (B) 12.5, 50, or 100 nM TS2.

analysis thus strengthen the conclusion from bio-NMR data that the pyridine carboxamides share an overlapping binding site with HCV-796 in the palm domain of the polymerase. Furthermore, these biochemical studies suggest that compounds in this novel series may be useful in combination with inhibitors targeting other polymerase binding sites and result in at least additive inhibition of HCV RNA replication.

A third approach to characterize the inhibition mode of this novel compound class involves evaluation of its activity against several polymerase mutant variants and genotypes and comparison with the profile of known palm site binders HCV-796 and A-848837. As shown in Table 4, the inhibitory potency of pyridine carboxamides was reduced by resistance variants selected by palm sites 1 and 2, but not thumb sites 1 and 2, binders. Resistance mutations P495L (at thumb site 1) and M423T (at thumb site 2) did not affect the activity of compounds **71**, **73**, HCV-796, or A-848837, consistent with the expected profile of compounds that do not bind at these sites. On the other hand, the inhibitor activity was affected by polymorphism at the palm site 2 residue 316, a major resistance locus for HCV-796.[8] A similar potency loss was observed for both pyridine carboxamides and HCV-796, with a moderate shift against a C316N variant and a much greater loss against C316Y. A significant shift was also observed for both compound classes against a 1b-BK variant, which contains N316. The shift for HCV-796 was in accord with a previous study of its binding kinetics that reported a ~10-fold higher equilibrium constant for the BK than for the Con1 NS5B enzyme form.[17] Unlike HCV-796, however, which is not affected by the G554D mutation and only minimally by the M414T mutation at palm site 1, the pyridine carboxamides appeared much more susceptible to these mutant variants. These results suggest that the binding pocket of the pyridine carboxamides, while overlapping with that of HCV-796, likely extends further into palm site 1 than the benzofuran derivative, consistent with the overlapping yet distinct 2D bio-NMR chemical shift perturbation patterns observed between compound **73** and HCV-796. The resistance profile of the pyridine carboxamides is, however, distinct from that of the palm site 1 binder benzothiadiazine analogue A-848837, which exhibited little or no potency shift against the C316N/Y, 1b-BK, or M414T variants but far more impacted by G554D than **71** or **73**. It should be noted that other benzothiadiazine analogues have been reported to lose significant potency against M414T, a major palm site 1 resistance locus.[20] Also in contrast to HCV-796 and A-848837, which were equipotent against the 1a enzyme, the pyridine carboxamide inhibitors lost >100-fold activity against a genotype 1a enzyme (Table 4) and were inactive up to 10 μM against genotypes 2, 3, and 4 (data not shown). It thus appears that the mechanism of action of pyridine carboxamide inhibitors is more sensitive to perturbations in their binding site than that of HCV-796 or A-848837. The activity data on enzyme mutant variants and genotypes further support findings from bio-NMR and inhibitor mutual exclusivity analyses; together, they reveal a distinct binding mode of this series of pyridine

Table 4. Activity against HCV NS5B Enzyme Variants

		fold shift over 1b-Con1							
compound	1b-Con1 IC_{50} (μM)	Con1 C316N (PS2)	Con1 C316Y (PS2)	Con1M414T (PS1)	Con1 G554D (PS1)	Con1M423T (TS2)	Con1 P495L (TS1)	1b-BK	1a-H77
71	0.032	4×	>300×	27×	8×	1×	1×	27×	270×
73	0.014	3×	>300×	21×	4×	1×	1×	14×	140×
HCV-796	0.04	3×	50×	1.5×	1×	1×	1×	6×	1×
A-848837	0.003	1×	3×	1×	100×	1×	1×	1×	1×

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carboxamide NS5B inhibitors that overlaps yet differs from that of HCV-796 in the palm domain of the HCV polymerase.

We also sought to determine if the mechanism of action of this novel series was through inhibition of NS5B-catalyzed RNA synthesis at the initiation or elongation step. As shown in the Supporting Information, while compound **73** was active against primer-initiated RNA synthesis involving multiple rounds of initiation and elongation, in a single-cycle elongation assay[16,23] it did not inhibit the elongation of preformed NS5B enzyme−primer−template complexes, similar to HCV-796 and A-848837 but unlike a chain terminator 3′-dCTP. The pyridine carboxamides thus appeared to act as inhibitors of initiation of replication, as observed with other palm site binders to NS5B.

In summary, we described a novel series of HCV NS5B polymerase inhibitors based on the nicotinic carboxamide scaffold. Beginning with relatively weak hits from the ALIS platform, we were able to provide significant potency improvements in enzymatic activity and in a cell-based replicon system of HCV RNA replication, yielding HCV polymerase inhibitors with low nanomolar potency against genotype 1b NS5B enzyme that are submicromolar inhibitors of the GT-1b HCV replicon. Mechanistic insights gained by 2D bio-NMR, inhibitor exclusivity analysis, and enzyme mutant variant characterizations identified a distinct binding mode for this new class of NS5B inhibitors in the palm domain of the HCV polymerase. Significant future lead optimization efforts will be required to generate inhibitors with broadened genotype spectrum and suitable pharmacokinetics and safety characteristics as clinical development candidates against HCV.

SUPPORTING INFORMATION AVAILABLE

Experimental synthetic procedures, spectroscopic characterization, assay protocols, inhibitor mechanism of action, and 2D bio-NMR data and discussion. This material is available free of charge via the Internet at http://pubs.acs.org.

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