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Discovery of BI 207524, an indole diamide NS5B thumb pocket 1 inhibitor with improved potency for the potential treatment of chronic HCV infection

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ABSTRACT

The development of interferon-free regimens for the treatment of chronic HCV infection constitutes a preferred option that is expected in the future to provide patients with improved efficacy, better tolerability and reduced risk for emergence of drug-resistant virus. We have pursued non-nucleoside NS5B polymerase allosteric inhibitors as combination partners with other direct acting antivirals (DAAs) having a complementary mechanism of action. Herein, we describe the discovery of a potent follow-up compound (BI 207524, **27**) to the first thumb pocket 1 NS5B inhibitor to demonstrate antiviral activity in genotype 1 HCV infected patients, BILB 1941 (**1**). Cell-based replicon potency was significantly improved through electronic modulation of the pKa of the carboxylic acid function of the lead molecule. Subsequent ADME-PK optimization lead to **27**, a predicted low clearance compound in man. The preclinical profile of inhibitor **27** is discussed, as well as the identification of a genotoxic metabolite that led to the discontinuation of the development of this compound.

INTRODUCTION

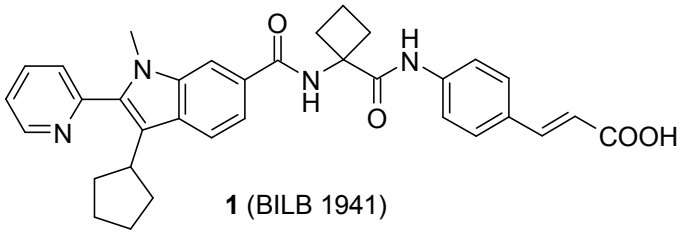
HCV is a blood-borne pathogen that causes serious and often fatal liver damage and is the leading cause of liver transplantation in industrialized nations. An estimated 130 – 170 million people are infected worldwide and conditions associated with HCV infection represent a significant economic burden on health care systems.[1] The viral functions encoded by the HCV genome have been the focus of intense drug discovery efforts since the isolation and characterization of the virus 25 years ago.[2,3] The recent approval in 2011 of the first HCV Direct Acting Antivirals (DAAs) has provided improved treatment options for patients suffering from chronic hepatitis C virus infection. First generation NS3/4A protease inhibitors (boceprevir/Victrelis™ from Merck and telaprevir/Incivek™ from Vertex Pharmaceuticals) have resulted in improved efficacy for the treatment of genotype-1 (gt1) HCV infection when complementing the previous standard of care (SoC) consisting of pegylated interferon-α (PegIFN) and ribavirin (RBV), a broad-spectrum antiviral agent.[4] However, despite improved overall outcomes, these treatments suffer from the combined side effects of the SoC and the new drugs themselves, resulting in significant discontinuations of therapy. Furthermore, the treatment remains ineffective in up to 20 to 30% of treatment naïve gt1 patients which predominates in North American, Europe and Japan. The lack of efficacy in some patient populations is caused in part by rapid emergence of virus that is resistant to the new agents.[5] Second-generation protease inhibitors with improved efficacy, lower dosing requirements and increased tolerability such as faldaprevir (Boehringer Ingelheim, Phase 3) and recently approved simeprevir/Olysio™ (Medivir/Tibotec), as well as the nucleotide NS5B inhibitor sofosbuvir/Solvadi™ (Gilead) have provided patients with improved outcomes (80 to >90% cure rates in gt1), but still require combination with SoC to reduce emergence of resistant virus in this difficult to treat population.[6]

The development of interferon-free regimens would represent a preferred option and relies on combination of potent DAAs with complementary modes of action, which through a concerted assault on virus-essential functions, are expected to inhibit HCV replication while minimizing selection of resistant variants. Such regimens would provide improved tolerability by eliminating the necessity for interferon-based immune co-therapy. Examples of DAA combinations in late stage clinical investigation include permutations of NS3/4A protease, nucleoside/nucleotide and non-nucleoside NS5B polymerase and NS5A function inhibitors.[7]

We have been actively pursuing non-nucleoside NS5B allosteric inhibitors as potential combination partners with other DAAs.[7a,b] The HCV-encoded NS5B RNA-dependent RNA polymerase is a key component of the replication machinery of the virus and has provided fertile grounds for the discovery of antiviral agents.[8,9] In addition to nucleoside and nucleotide substrate-based inhibitors, this enzyme which catalyzes the replication of viral RNA, has proven susceptible to inhibition by agents that interfere with conformational changes that are important in the early steps of viral RNA synthesis. Molecules that bind to one of 5 distinct allosteric sites (denoted thumb sites 1 and 2 and palm sites 1, 2 and 3) have been identified and the mechanisms through which they inhibit enzymatic activity have been validated both in cell-based replicon assays that reproduce replication of subgenomic HCV RNA[10] and subsequently in the clinic for all but Palm site 3.[8] We recently described the discovery of **1** (BILB 1941, Figure 1), the first thumb pocket 1 NS5B inhibitor to demonstrate antiviral activity in patients infected with gt1 HCV. Compound **1** is an indole-based cinnamic acid derivative that displays moderate antiviral activity in gt1a/1b replicon assays (EC_{50} = 153 and 84 nM respectively) and favorable pharmacokinetics in preclinical animal species.[11] In a 5-day multiple rising dose monotherapy trial in gt1-HCV infected patients, **1** produced significant antiviral activity (up to 2.5 $\log_{10}$

reduction in viremia) when dosed at 450 mg q8h.[12] Unfortunately, GI intolerance precluded testing of the compound at higher doses but this proof-of-concept trial suggested that more potent analogs may improve antiviral response. We describe herein the discovery of a follow-up candidate from this class, which displayed improved potency compared to **1** in both gt1a/1b replicons while maintaining a similarly favorable PK profile.

Figure 1. Structure of the first thumb pocket 1 NS5B inhibitor with antiviral activity in man



1 (BILB 1941)

1b NS5B IC_{50} = 60 nM
1a/1b replicon EC_{50} = 153 / 84 nM

RESULTS and DISCUSSION

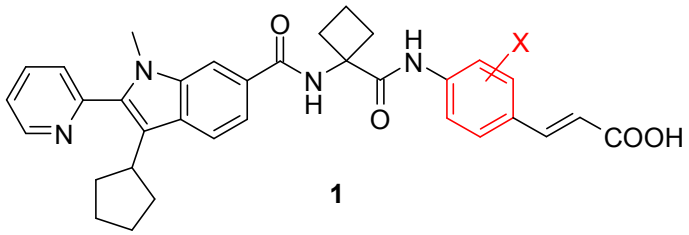
As mentioned above, a follow-up candidate to **1** requires potency improvement while maintaining a similar or better PK profile (e.g., longer human $t_{1/2}$). Since the preclinical toxicological profile of **1** in animals was not predictive of the GI intolerance side effects that were observed at doses required to provide a uniform response across HCV gt1a and gt1b infected subjects, we postulated that further structural optimization leading to increased potency could translate into improved response, increased tolerability and/or reduced dosing requirements. SAR which led to the discovery of compound **1** provided the foundation for an

optimization strategy of this lead. For example, the cyclopentyl ring at the 3-position of the indole scaffold was maintained even though replacement by a cyclohexyl ring was known to provide 2 – 3 fold potency improvements, but at the expense of metabolic stability due to CYP450-mediated oxidative metabolism of that substituent.[11] Likewise, no advantage was anticipated from expanding further from the indole nitrogen methyl substituent.[13] On the other hand, the C-2 heterocyclic substituent, the central amino acid linker and the right-hand-side cinnamic acid moiety were shown to be determinants and modulators of potency and ADME-PK profiles.[11] Since the presence of the cinnamic acid moiety was beneficial for potency and solubility (compound **1** apparent pKa = 4.5 as derived from a pH-titration profile) and did not compromise Caco-2 permeability, cellular activity or oral absorption, we investigated the effect of adding substituents on the cinnamic acid aromatic ring in an attempt to improve the profile of **1**. Both the steric and electronic impact of substituents on this part of the molecule (e.g., conformational rigidification, pKa of carboxylic acid group) were investigated and initial outcomes are reported in Table 1.[14]

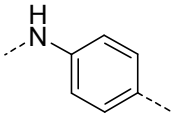
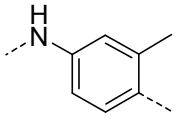
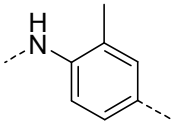
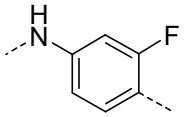
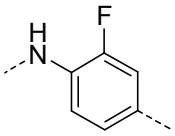
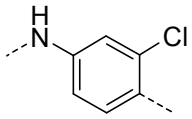
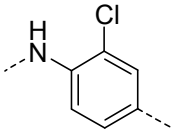
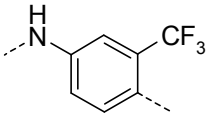
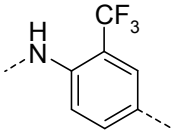
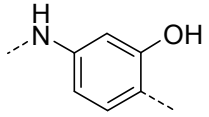
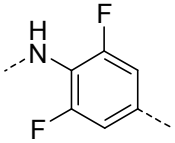
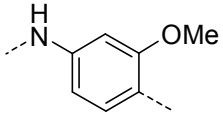
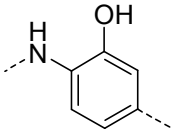
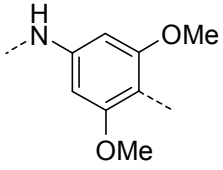
As can be seen in Table 1, substitution in the ortho-position relative to the nitrogen atom (compounds **2** – **7**) reduced potency compared to unsubstituted analog **1**, particularly in the case of bulky substituents (e.g., CF_3, **5**) suggesting that substitutions affecting co-planarity of the amide linkage relative to the phenyl ring were detrimental to interactions with NS5B.[15] On the other hand, introduction of substituents in the ortho-position relative to the acrylic acid moiety (compounds **8** – **14**) were well tolerated, even for bulky groups such as CF_3 that remained within 3-fold potency (compound **11**) of **1**. The introduction of electron donating alkoxy groups ortho relative to the acrylic acid function provided the most benefit. For example, a methoxy substituent produced a 2.5-fold improvement in replicon potency (compound **13**). Furthermore,

an additive effect was observed upon methoxylation of both ortho-positions (compound **14**) which led to an analog that exhibited close to a 10-fold improvement in potency (EC_{50} = 8 nM) relative to our previous clinical candidate **1**. The improved antiviral potency of alkoxy-cinnamic acid derivatives such as **13** and **14** cannot be rationalized on the basis of their intrinsic potency as measured in our biochemical assays or lipophilicity (calculated logP = 5.4 and 5.3 for **1** and **13** respectively). On the other hand, the electronic impact of electron-donating alkoxy substituents on the ionizability (i.e., pKa as derived from pH titration curves) of the cinnamic acid is significant (measured pKa = 4.5 and 6.5 for **1** and **15** respectively) and may account for the increased ability of alkoxy-derivatives to permeate hepatocyte membranes and inhibit replication of subgenomic RNA in cell-based replicon assays. This hypothesis is corroborated by cell permeability measurements in a Caco-2 assay where apparent apical $\rightarrow$ basolateral permeability values of 11.2 X 10^{-6} cm/sec and 19.0 X 10^{-6} cm/sec were observed for **1** and **15** respectively.

Table 1. Initial exploration of substituted right-hand-side cinnamic acid derivatives



1

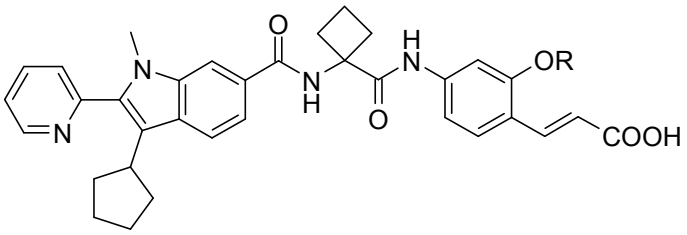
Entry	cinnamic acid	IC_{50} (μM)[a,b]	EC_{50} (μM)[a,c]	Entry	cinnamic acid	IC_{50} (μM)[a,b]	EC_{50} (μM)[a,c]
1		0.060	0.084	**8**		0.069	0.079
2		0.37	1.03	**9**		0.061	0.13
3		0.23	0.56	**10**		0.10	0.11
4		0.30		**11**		0.18	0.23
5		2.25	> 2.5	**12**		0.044	0.17
6		1.32	>2.8	**13**		0.047	0.032
7		0.44	0.25	**14**		0.34	0.008

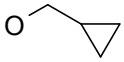
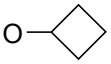
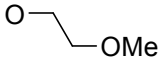
[a]values are an average of at least two determinations. [b]Full length NS5B enzymatic assay.[14a] [c]Luc reporter assay[14c,d]

Although compound **14** had reasonable stability in the presence of human and rat liver microsomes (HLM/RLM $T_{1/2}$ = 121/42 min) and good permeability as measured in a Caco-2 cell assay (apical → basolateral = 17.6 x 10^{-6} cm/sec) **14** was poorly absorbed when administered

orally to rats in a cassette screen (mixture of 4 compounds, see experimental section for details) at 4 mg/kg, with plasma inhibitor concentrations reaching 0.4 and 0.3 μM at 1 and 2 h time points respectively. Mono-substituted methoxy analog **13**, with EC_{50} = 32 nM and $C_{1h/2h}$ = 1.1/0.5 μM in a 4 mg/kg rat cassette screen, became a prototype for further investigation of alkoxycinnamic acid derivatives as shown in Table 2.

Table 2. Alkoxy cinnamic acid SAR



Compound	OR	IC_{50} (μM)[a,b]	EC_{50} (μM)[a,c]	HLM $T_{1/2}$ (min)[d]	calcd log P[e]	plasma $C_{1h/2h}$ (μM)[f]
13	OMe	0.047	0.032	82	5.3	1.1/0.5
15	OEt	0.048	0.024	64	5.7	0.9/0.4
16	OnPr	0.13	0.036	124	6.1	0.4/0.2
17	OiPr	0.11	0.036	114	6.2	1.3/0.8
18	OiBu	0.42	0.055	>300	6.4	
19		0.25	0.046	231	6.0	
20		0.28	0.065	50	6.2	
21		0.056	0.027	87	5.1	

[a]values are an average of at least two determinations. [b]Full length NS5B enzymatic assay.[14a] [c]Luc reporter assay[14c,d]. [d]Human liver microsome stability. [e] JChem 5.0.0 (http://www.chemaxon.com). [f] Following oral administration to rats as mixtures of four compounds at a dose of 4 mg/kg each.

Variations in intrinsic potency of compounds (Table 2) in the enzymatic NS5B assay apparently were not reflected in the cell-based replicon assay as all compounds had comparable potency (EC_{50} = 24 – 65 nM). Thus, increasing the size of the alkoxy substituent (e.g., **16** – **20**) did not provide advantages over smaller groups such as methoxy and ethoxy (compounds **13** and **15**). In addition to simple alkyl ethers, functionalized ethers were also examined. For example, methoxyethoxy analog **21** had reduced lipophilicity and a comparable profile to **13** and **15**. Ether side chains containing basic substituents such as ethylamino, picolinyl and ethylmorpholino were also investigated but did not provide any potency advantage and in some cases, compromised

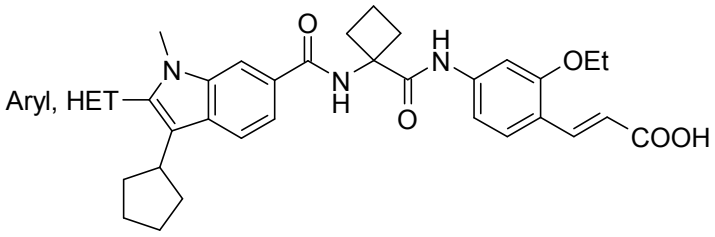
other key parameters such as Caco-2 permeability when zwitterionic species were generated (results not shown). As cell-based potency improved, the optimization strategy shifted some focus to identifying analogs that also displayed favorable ADME and PK properties, thus advancing their overall profile toward eventual selection of a follow-up compound to **1**.

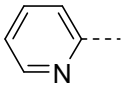
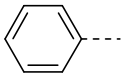
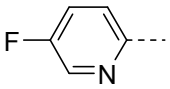
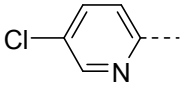
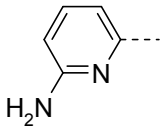
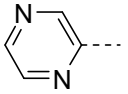
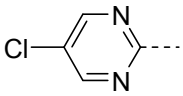
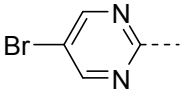
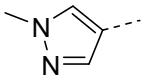
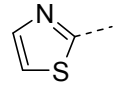
The metabolic stability of the analogs presented in Table 2 ranged from satisfactory to excellent when incubated with human liver microsomes ($t_{1/2}$ = 50 – >300 min) and inhibitors **15** – **17** had excellent Caco-2 permeability (Caco-$2_{A\rightarrow B}$ = 19 – 23 x 10^{-6} cm/sec). A cassette of 4 compounds (**13**, **15** – **17**) was administered orally to rats at a dose of 4 mg/kg each and compound plasma exposures were measured at 1 h and 2 h time points. Consistent with the Caco-2 permeability data, alkoxycinnamic acid derivatives displayed promising oral absorption in rats (plasma C_{1h} = 0.9 – 1.3 μM), except for *n*Pr analog **17** which showed reduced exposure and was eliminated from further consideration. While both methoxy and ethoxy cinnamic acid derivatives were subsequently pursued in parallel for some time, analogs bearing an ethoxy substituent often displayed improved properties over corresponding methoxy analogs and efforts were eventually focused on further optimization of **15**.

Studies that lead to the discovery of **1** had revealed the ability of indole C-2 substituents to modulate the physicochemical properties and ADME-PK profile of this class of inhibitors.[11] This position thus became the next focus of exploration in our attempts to identify potent analogs that maintain an overall PK profile minimally comparable **1**. Approximately 30 variations were examined, much of the selection building on SAR previously established in the unsubstituted cinnamic acid series.[11] These included alkyl, aryl, 5- and 6-membered heterocycles and a selection of those analogs is presented in Table 3. Although a variety of C-2 heterocycles provided compounds with good antiviral potency in the gt1b luciferase replicon assay (EC_{50} < 30

nM), only a limited set of analogs were identified that combined good cell-based potency with a satisfactory *in vitro* ADME profile and maintained lipophilicity in a satisfactory range. In particular, halo-pyridine and halo-pyrimidine derivatives (e.g., **24**, **27**, **28**) displayed good to excellent metabolic stability upon incubation with human or rat liver microsomes (HLM $t_{1/2}$ = 273 – >300 min; RLM $t_{1/2}$ = 104 – >300 min) and excellent Caco-2 permeability (A $\rightarrow$ B > $13x10^{-6}$ cm/sec). The *in vitro* ADME profiles translated into good oral plasma exposure at the 1 h time point when compounds were screened in rats as a mixture of 4 compounds, each dosed at 4 mg/kg. In particular, halo-pyrimidine analogs **27** and **28**, as well as fluoropyridine **23** displayed high exposures (C_{1h} = 3.4 and 1.8 μM for pyrimidines **27** and **28** and 1.3 μM for fluoropyridine **23**).

Table 3. C-2 optimization of 2-ethoxycinnamic acid analogs



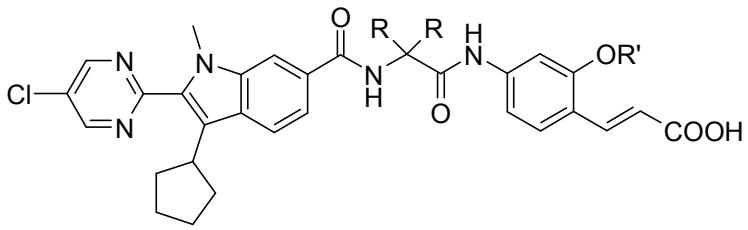
Compound	C-2	IC_{50} (μM)[a,b]	EC_{50} (μM)[a,c]	HLM $T_{1/2}$ (min)[d]	calcd log P[e]	Caco-2 (cm/sec)[f]	plasma $C_{1h/2h}$ (μM)[g]
15		0.048	0.024	64	5.7	19 x 10^{-6}	0.9/0.4
22		0.41	0.021		6.9		
23		0.11	0.016	72	5.9	17 x 10^{-6}	1.3/0.8
24		0.33	0.026	81	6.3	18 x 10^{-6}	0.5/0.3
25		0.33	0.010	78	5.6	9.4 x 10^{-6}	0.1/0.1
26		0.048	0.021	46	5.0		
27		0.11	0.013	102	5.3	13 x 10^{-6}	3.4/2.3
28		0.46	0.019	154	5.5	13 x 10^{-6}	1.8/2.1
29		0.19	0.012	44	5.4		0.4/0.1
30		0.15	0.012	36	5.1		

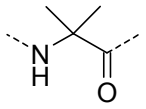
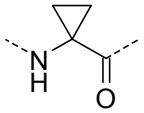
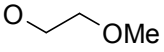
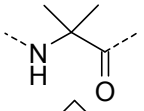
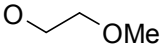
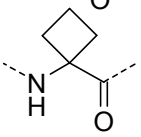
[a]values are an average of at least two determinations. [b]Full length NS5B enzymatic assay.[14a] [c]Luc reporter assay[14c,d]. [d]Human liver microsome stability. [e] JChem 5.0.0 (http://www.chemaxon.com). [f]Apical to basolateral direction.[g]Following oral administration to rats as mixtures of four compounds at a dose of 4 mg/kg each.

Previous studies performed in the unsubstituted cinnamic acid series had shown that a cyclobutane 2-aminocarboxylic acid linker in the central portion of the molecule provided

superior PK profiles (e.g., compound **1**).[11] Once again in this series, while *gem*-dimethyl linkers had excellent cell-based potency (e.g., **31** and **33**), these analogs displayed significantly reduced oral exposure in rat cassettes when compared to the corresponding cyclobutane derivatives (**27** and **34**). Similarly, cyclopropane derivatives (e.g. **32**) exhibited reduced potency as a result of slightly altered dihedral angles (as determined by NMR studies) in the central portion of the unbound molecule compared to the NS5B-bound conformation of inhibitors.[15] As shown in Table 4, inhibitor **34** had an overall well-balanced profile encompassing potency, *in vitro* ADME properties and *in vivo* rat exposure.

Table 4. Central amino acid linker modifications

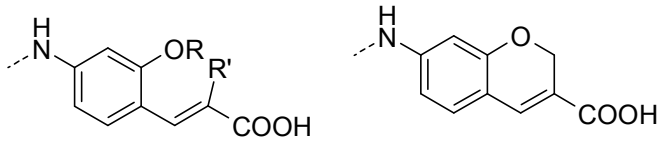


Compound	OR'		IC_{50} (μM)[a,b]	EC_{50} (μM)[a,c]	HLM $T_{1/2}$ (min)[d]	calcd log P[e]	Caco-2 (cm/sec)[f]	plasma $C_{1h/2h}$ (μM)[g]
31	OEt		0.027	0.008		5.3	24 x 10^{-6}	0.5/0.1
32	OEt		0.38	0.034	195	4.9		
33			0.20	0.010	129	4.7	12 x 10^{-6}	0.2/0.0
34			0.20	0.013	>300	4.7	17 x 10^{-6}	1.7/1.1

[a]values are an average of at least two determinations. [b]Full length NS5B enzymatic assay.[14a] [c]Luc reporter assay[14c,d].[d]Human liver microsome stability. [e] JChem 5.0.0 (http://www.chemaxon.com). [f]Apical to basolateral direction.[g]Following oral administration to rats as mixtures of four compounds at a dose of 4 mg/kg each.

As depicted in Figure 2, additional structural modifications to the right-hand-side alkoxycinnamic acid moiety were explored, including substitution of the cinnamic acid double bond with methyl, ethyl or fluorine groups (**35**) in an attempt to explore steric shielding of the acrylic acid moiety and electronic effects, as well as conformational rigidification (**36**). While these modifications were relatively well tolerated, none provided an advantage over the original alkoxycinnamic acid moiety (results not shown).

Figure 2. Cinnamic acid double bond substitution and rigidification



35 **36**

R = Me, Et
R' = Me, Et, F

Three analogs which had improved replicon potency compared to **1** and also showed promising preliminary ADME-PK characteristics were selected for advanced profiling. Individual rat PK profiles for compounds **23**, **27** and **34** are shown in Table 5 and compared to that of clinical candidate **1**. Compound **27** displayed comparable exposure in rat to compound **1** albeit with lower bioavailability, but clearance was reduced and half-life was increased from 1.5 to 2.4 h in this rodent species. Characteristic of carboxylic acid containing molecules, the volume of distribution remained low but comparable to **1**. In rats, compound **27** showed favorable distribution from plasma to the liver target organ with liver/plasma ratio ~5 at 6 h post dosing.

Table 5. Single compound Rat PK parameters.

	Oral parameters (10 mg/kg)[a]			IV parameters (2 mg/kg)[b]			
	C_{max} (μM)	AUC (μM*h)	MRT (h)	V_{ss} (L/kg)	CL (mL/kg/min)	$T_{1/2}$ (h)	F%
1	7.5	23	4.1	0.48	8.3	1.5	59
23[c]	2.2	6.1	3.0	0.42	6.2	1.5	17
27[c]	6.3	19	3.6	0.38	2.6	2.4	19
34[c]	4.1	10.6	4.1	0.29	3.7	2.1	16

[a] Compound dosed as an oral suspension in 0.5% Methylcellulose and 0.3% Tween-80. [b] Bolus injection prepared in 70% PEG-400:30% water. [c] 1% N-Methylpyrrolidone (NMP) was added to the oral dosing solution.

Compound **27** was profiled in dog and monkey to provide cross-species data for human PK predictions and the results are compared to **1** in Table 6. Compound **27**'s PK parameters in dogs

and monkeys were comparable to compound **1**. As in rats, a low volume of distribution was observed but clearance was slightly reduced almost two-fold in both species relative to **1**. While plasma exposures following oral administration were superior to compound **1** (likely as a result of reduced clearance) bioavailability for **27** was lower in dogs and comparable in monkeys to those previously reported for compound **1**.[11] Based on its cross-species PK profile and following allometry principles,[16] compound **27** was predicted to be a low clearance compound in human (estimated IV Cl = 0.7 mL/min/kg, estimated $t_{1/2}$ ~8.5 h, suitable for BID or TID dosing regimens).

Table 6. Dog and rhesus monkey PK profiles for 1 and 27.

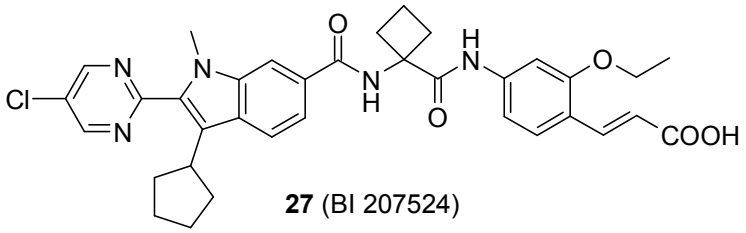
	Oral parameters (10 mg/kg)[a]			IV parameters (2 mg/kg)[b]			
	C_{max} (μM)	AUC (μM*h)	MRT (h)	V_{ss} (L/kg)	CL (mL/kg/min)	$T_{1/2}$ (h)	F%
1 (dog)	15.3	99	5.4	0.54	1.8	4.3	64
27 (dog)[c]	8.1	76	8.0	0.38	1.0	4.5	29
1 (monkey)	6.4	20.5	2.9	0.70	9.5	1.7	70
27 (monkey)[c]	9.6	53	6.1	0.60	3.6	3.0	72

[a] Compound dosed as an oral suspension in 0.5% Methylcellulose and 0.3% Tween-80. [b] Bolus injection prepared in 70% PEG-400:30% water. [c] 1% N-Methylpyrrolidone (NMP) was added to the oral dosing solution.

Additional profiling data for compound **27** (BI 207524) is summarized in Table 7. In a biochemical assay using a NS5BΔ21 polymerase construct,[14a] compound **27** had an IC_{50} = 84 nM (comparable to **1**) and was selective against poliovirus RNA-dependent RNA polymerase and a mammalian DNA-dependent RNA polymerase isolated from calf thymus (IC_{50} = 124 and 70 μM respectively).[14a] In a gt1b replicon assay where subgenomic RNA was quantified by Taqman assay,[14b] **27** was approximately 7-fold more potent than **1** with an EC_{50} = 11 nM. As expected for

thumb pocket 1 NS5B inhibitors, a ~3-fold shift was observed in a gt1a replicon assay (EC_{50} = 29 nM) relative to gt1b. In Huh-7 cells, the compound was not significantly cytotoxic (CC_{50} = 38 μM in a MTT assay). This class of compound is typically highly protein bound (>99% for **27** and **1** in human plasma) but only slight shifts in EC_{50} values were observed in replicon assays performed in the presence of up to 40% human serum (3.8-fold for **27**). Compound **27** exhibited moderate lipophilicity as reflected by a measured logD (pH 7.4) = 4.1 and was only weakly acidic (pKa = 6.5) which is in line with the observed improvements in cell-based potency and the high Caco-2 permeability value (13 x 10^{-6} cm/sec). Metabolic stability in human liver microsomes was moderate ($t_{1/2}$ = 102 min) and comparable to that of compound **1** while the CYP450 inhibition profile was improved with the lowest IC_{50} values measured against the 2C9 isozyme (IC_{50} = 13.5 μM) .

Table 7. Structure and profile of compound 27 (BI 207524).



27 (BI 207524)

	Compound **1**	Compound **27**
Gt1b NS5BΔ21 IC_{50} (nM)[14a]	67	84
Gt 1a/1b replicon EC_{50} (nM)[14b]	153 / 84	29 / 11
MTT TC_{50}	93 μM	38 μM
Serum shift in 40% human serum	3 fold	3.8 fold
Human plasma protein binding	99.2%	99.6%
logD (pH 7.4) / pKa	3.3 / 4.5	4.1 / 6.5
HLM $t_{1/2}$ (min)	90	102
CYP450 inhibition: IC_{50} (μM) 1A2/2C9/2C19/2D6/3A4	13/3.5/2.9 >30/>30	>30/13.5/>30 >30/>30
Caco-2 permeability (cm/sec)[a]	11 x 10^{-6}	13 x 10^{-6}

[a] apical pH 6.0/basolateral pH 7.4

Compound **27** was non-mutagenic in the Ames test in the presence or absence of metabolic activation at dose levels up to maximum solubility. The compound showed no inhibition of the hERG channel at 0.8 μM (solubility limit) and no effect in the action potential duration assay in isolated guinea pig papillary muscle at 90% repolarization (APD90). In conscious telemeterized dogs, no effects were observed on cardiovascular function and electrocardiographic parameters up to the highest dose of 200 mg/kg (plasma C_{max} = 18 μM). Results from *in vivo* general pharmacology studies performed in rats and a receptor binding assay screen did not lead to findings that would preclude advancement into more advanced toxicology studies and further development.

We recently reported a model to predict the pharmacokinetic-pharmacodynamic relationship for direct acting anti-HCV agents in humans, based on the liver partition coefficient-corrected inhibitory quotient (LCIQ) derived from *in vitro* hepatocyte partition coefficients and animal *in vivo* liver/plasma exposures.[17] Available clinical data for HCV DAAs, suggests that a C_{min} LCIQ ≥ 500 is generally predictive of a strong antiviral response. According to this model and based on its gt1a antiviral potency and predicted human liver partitioning coefficient, regimens of 400 mg of compound **27** administered twice a day or 230 mg administered three-times a day should maintain C_{min} liver concentrations 500-fold gt1a EC_{50} ($LCIQ_{500}$) predictive of a strong antiviral response in humans.

However, during *in vitro* and *in vivo* cross-species metabolite studies performed in support of the development of **27**, small amounts of a genotoxic 4-amino-2-ethoxycinnamic acid metabolite were observed. This metabolite resulted from cleavage of the anilide bond and was also detected in animal plasma samples from PK studies and upon incubation of **27** with simulated gastric

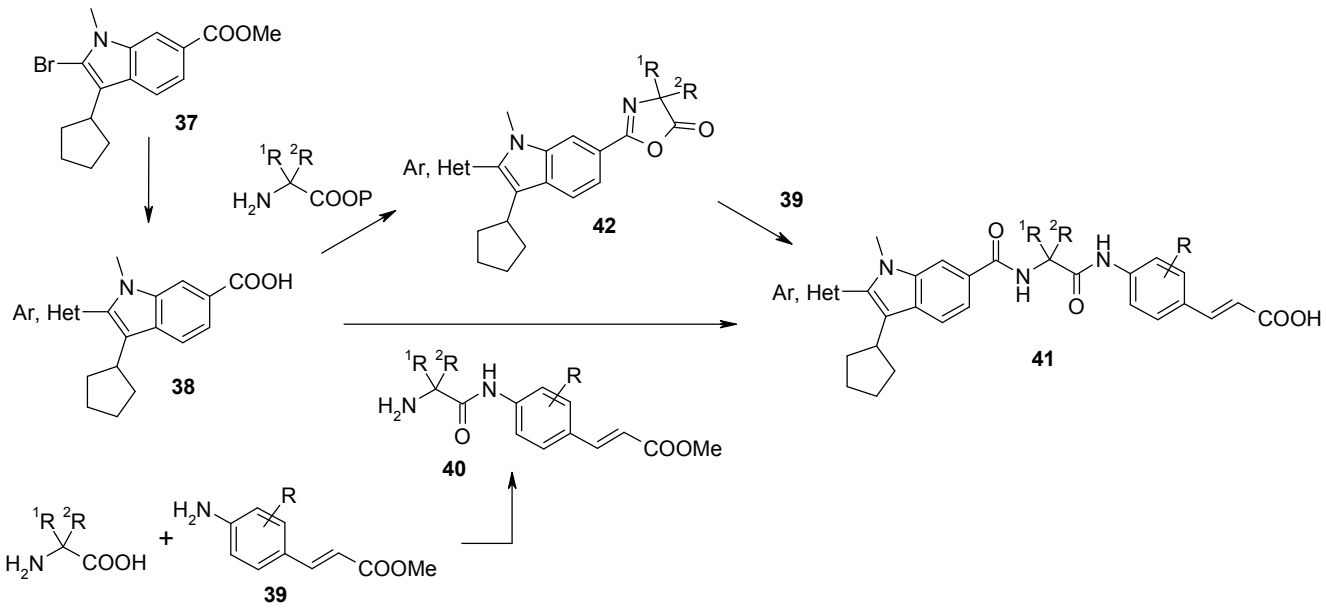
fluids (SGF). In human liver microsomes, the metabolite was formed at a rate of ~1 ppm/min in a NADPH-independent manner. In light of the challenges associated with the progression of such a compound,[18] development of **27** was discontinued and efforts focused on compounds without liabilities associated with the formation of genotoxic metabolites.

SYNTHESIS OF INHIBITORS

Inhibitors were synthesized in a convergent fashion through amide bond coupling between indole-6-carboxylic acid derivatives and amine fragments as shown in scheme 1 and as previously described for compound **1**.[11]

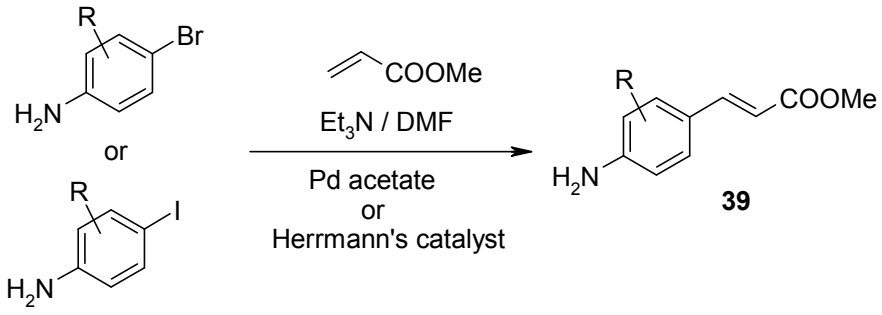
6-Indolecarboxylic acid derivatives **38** were prepared from protected 2-bromoindole **37** and commercially available aryl or heteroaryl halides using standard cross-coupling protocols as previously described.[11] Amide bond coupling with amines **40**, themselves derived from condensation of α,α-disubstituted amino acids with 4-aminocinnamate esters **39** provided directly inhibitors **41** after deprotection of the carboxylic acid function by saponification. Alternatively, Indoles **38** could be coupled in a stepwise fashion to protected α,α-disubstituted amino acid esters to provide azalactones **42** and then condensed with 4-amino cinnamate esters to provide **41** after deprotection.

Scheme 1. General synthetic scheme



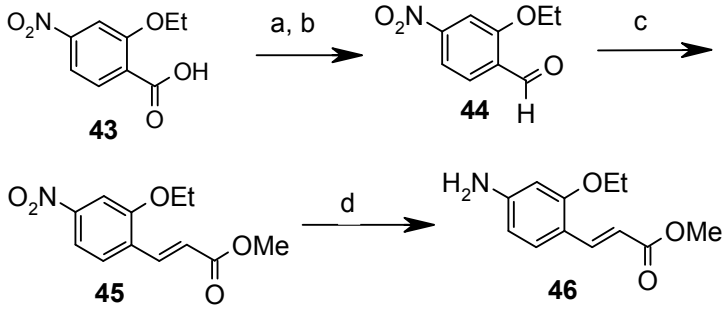
Cinnamate esters **39** for inhibitors **2** – **11** were prepared using a palladium-catalyzed Heck cross-coupling reaction using commercially available substituted 4-bromo or 4-iodoanilines and methyl acrylate as shown in Equation 1.

Equation 1. Preparation of substituted 4-aminocinnamate ester building blocks for the synthesis of inhibitors 2 – 11



4-Amino-ethoxycinnamic acid methyl ester **46** was prepared from 2-ethoxy-4-nitrobenzoic acid **43** using a sequence involving reduction-oxidation to the corresponding aldehyde **44** followed by Horner-Wittig to give nitrocinnamate **45** and reduction of the nitro group to the corresponding aniline using iron as described in scheme 2. Cinnamate ester containing α-alkyl substituents (e.g., **35**) were prepared in the same manner using substituted phosphonoacetates.

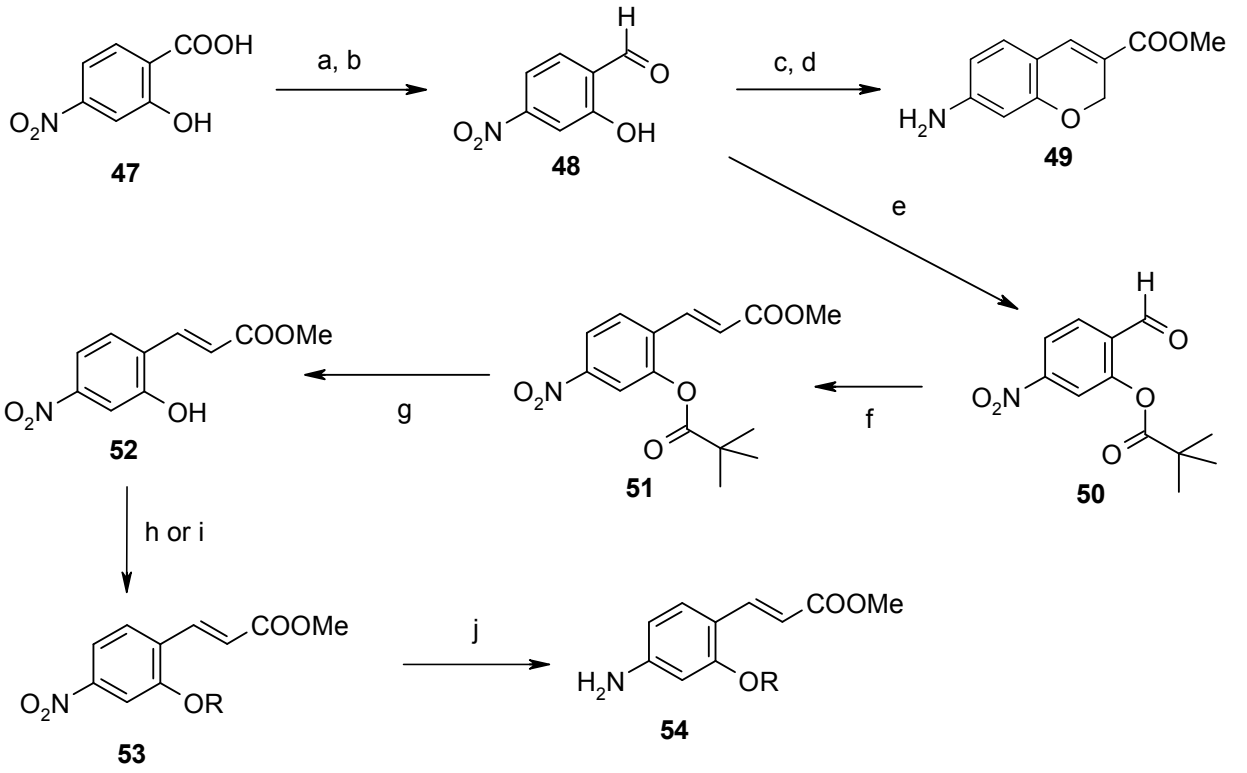
Scheme 2. Preparation of 4-amino-2-ethoxycinnamic acid methyl ester 46



[a] (a) $BH_3.Me_2S$, THF 0 °C to RT; (b) MnO_2, $CHCl_3$, reflux; (c) trimethylphosphonoacetate, NaH, THF, RT; (d) Fe powder, NH_4Cl, EtOH, reflux.

Alkoxycinnamic acid building blocks with variations of the alkoxy moiety for the synthesis of inhibitors **16** – **21** were prepared from 2-hydroxy-4-nitrobenzoic acid **47** through the sequence depicted in scheme 3 using a similar reduction-oxidation sequence to generate benzaldehyde **48** which served as a key intermediate providing access to chromene analog **49** or protected 2-hydroxycinnamate **52** following protection of **48** as the pivaloate ester and Horner-Wittig condensation. Phenol **52** was etherified to alkoxy derivatives **53** by direct alkylation using alkyl halides and Cs_2CO_3 or via a Mitsunobu protocol. Subsequent reduction with sodium hydrosulfite or iron powder provided the corresponding aniline building blocks **54**.

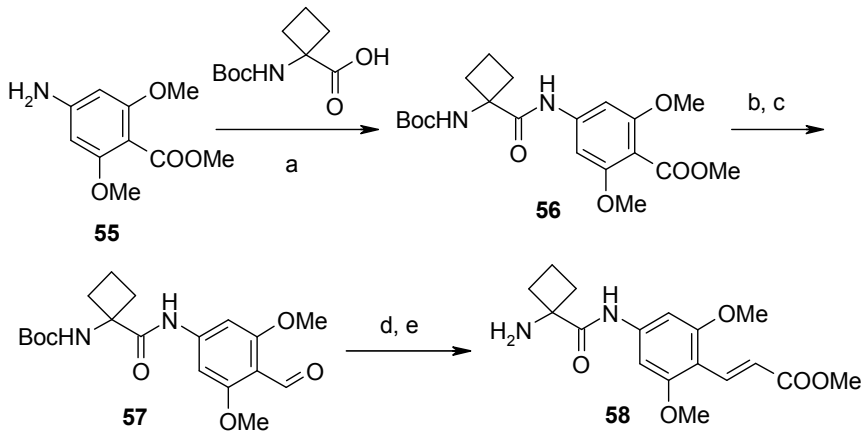
Scheme 3. Preparation of chromene 49 and 2-alkoxycinnamate building blocks (inhibitors 16 – 21) by alkylation and Mitsunobu etherification protocols[a]



[a] (a) $BH_3.Me_2S$, THF 0 °C to RT; (b) MnO_2, $CHCl_3$, reflux; (c)methylacrylate, DABCO, 50°C; (d) Fe powder, NH4Cl, EtOH, reflux; (e) Piv-Cl, Et_3N, CH_2Cl_2, RT; (f) trimethylphosphonoacetate, NaH, THF, RT; (g) conc. H_2SO_4; (h) ROH, DEAD, Ph_3P, THF, RT; (i) RI or RBr, Cs_2CO_3, DMF, RT or 60 °C; (j) Fe, NH_4Cl, EtOH-watre, 80 °C or K_2CO_3, $NaHSO_3$, EtOH-water, RT.

2,6-Dimethoxycinnamate analog **14** was prepared from amine **58** prepared as shown in scheme 4 from methyl 4-amino-2,6-dimethoxybenzoate **55**,[19] by condensation with N-Boc-1-aminocyclobutane carboxylic acid to provide protected amino acid derivative **56** followed by the usual reduction-oxidation-Horner/Wittig and deprotection to access **58** as the hydrochloride salt via intermediate benzaldehyde **57**.

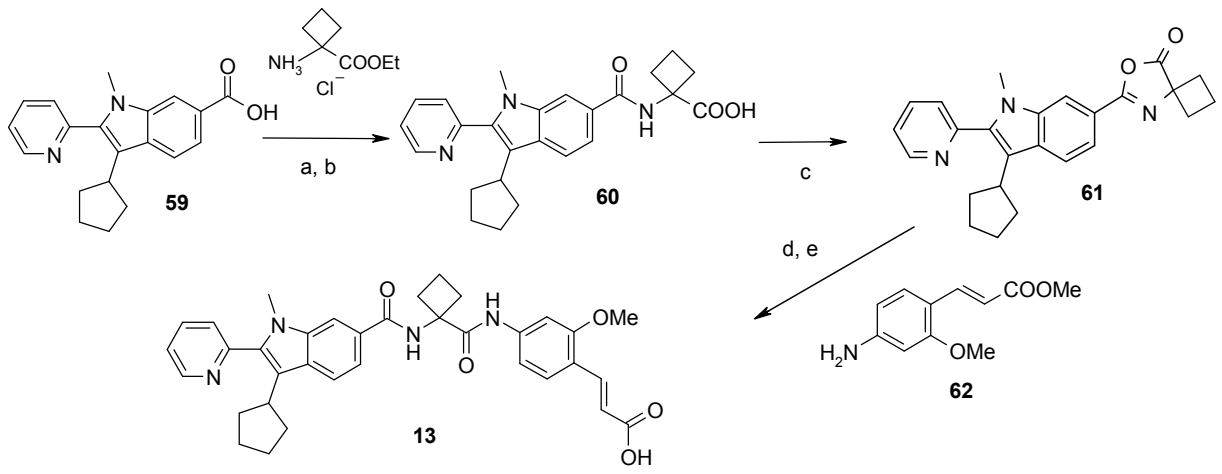
Scheme 4. Preparation of 2,6-dimethoxycinnamate building block for the synthesis of inhibitor 14[a]



[a] (a) TBTU, Et_3N, DMSO, RT; (b) $LiAlH_4$, THF, 0 °C to RT; (c) MnO_2, THF, reflux; (d) trimethylphosphonoacetate, NaH, THF, RT; (e) 4N HCl, dioxane, RT

Inhibitors **16** – **21** were prepared using an alternative assembly sequence illustrated in Scheme 5. 6-Indole carboxylic acid **59**[11] was condensed with the ethyl ester of 1-aminocyclobutane carboxylic acid and the resulting amide product was saponified to provide carboxylic acid **60**. Treatment with acetic anhydride provided azalactone **61** as a stable material that was used in condensation reactions with a variety of 4-aminocinnamic ester building blocks **39** (e.g. **62**) to provide final inhibitors (e.g. **21**) following deprotection of the cinnamate ester.

Scheme 5. Alternative assembly of inhibitors[a]



[a] (a) TBTU, Et_3N, DMF, RT; (b) 1N NaOH, MeOH-THF-water, RT; (c) Ac_2O, 100 °C; (d) Aniline **62**, toluene, AcOH, 85 °C; (e)1N NaOH, DMSO.

CONCLUSION

We have described the discovery of a potent HCV NS5B thumb pocket 1 follow-up compound to our initial clinical candidate, **1**. Cell-based potency in the subgenomic replicon was improved 7 to 8-fold by decreasing the acidity of **1** by ~100x (pKa = 4.5 → 6.5) through electronic effects mediated by the introduction of alkoxy substituents in conjugation to the acrylic acid moiety. Subsequent ADME-PK optimization led to the discovery of **27**, an analog that was predicted to be a low clearance compound in man based on its cross-species PK profile. Based on its improved potency, cross-species PK profile and predicted human liver partitioning, doses of 400 mg BID or 230 mg TID of **27** (corresponding to LCIQ = 500) are predicted to provide a stronger antiviral response in man than achieved with **1** at 450 mg TID (LCIQ ~ 100).[17] However, the identification of a genotoxic aniline metabolite released through the action of liver peptidases

and/or chemical hydrolysis of the anilide bond in biological fluids led to discontinuation of the development of **27**.

EXPERIMENTAL SECTION

General experimental. All commercially obtained solvents and reagents were used as received without further purification. All reactions were carried out under an atmosphere of argon. Temperatures are given in degrees Celsius. Solution percentages express a weight to volume relationship, and solution ratios express a volume to volume relationship, unless stated otherwise. NMR spectra were recorded on a Bruker AVANCEII (400 MHz for ^{1}H NMR) spectrometer and were referenced to either DMSO-d_6 (2.50 ppm) or $CDCl_3$ (7.27 ppm). Data is reported as follows: chemical shift (ppm), multiplicity (s = singlet, d = doublet, t = triplet, br = broad, m = multiplet), coupling constant (J - reported to the nearest 0.5 Hz), and integration. Low resolution mass spectra were obtained on a Micromass Platform LCZ model ZMD 4000 in electrospray mode. High-resolution mass spectra (HRMS) were obtained on a Bruker micrOTOF-Q II in electrospray positive (ES+) ionization mode. Purification of crude material was performed either by flash column chromatography or by using a CombiFlash Companion using RediSep Silica or SilicaSep columns according to pre-programmed gradient and flow rate separation conditions in Hexane / EtOAc or DCM / MeOH. The final compounds were purified by preparative HPLC on a Waters 2767 Sample Manager with Pumps 2525, Column Fluidics Organizer (CFO), PDA Detector 2996 and MassLynx 4.1 using either a Whatman Partisil 10-ODS-3 column , 2.2 X 50 cm or a YMC Combi-Prep ODS-AQ column, 50 x 20 mm ID, S – 5 μm, 120 Å, and a linear gradient program from 2 to 100% AcCN /water (0.06% TFA). Fractions

were analyzed by analytical HPLC, and the pure fractions were combined**,** concentrated, frozen and lyophilized to yield the desired compound as a neutral entity or the trifluoroacetate salt for basic analogues. Inhibitor HPLC purity was measured by using a Waters Alliance 2695 Separation Module with a Waters TUV 2487 UV detector. The column was a Combiscreen ODS-AQ, 5 μm, 4.6 x 50 mm, linear gradient from 5% to 100% ACN/H2O + 0.06% TFA in 10.5 minutes, detection at 220 nm. All final inhibitors had HPLC homogeneity ≥ 95% unless noted otherwise (see supporting information).

2-Iodo-5-chloropyrimidine. 2-Hydroxypyrimidine hydrochloride (100 g, 0.754 mole) was dissolved in water (180 mL) and conc. H_2SO_4 (42 mL, 0.788 mole) was added dropwise with vigorous stirring. After stirring for an additional 30 min, water was removed under reduced pressure at 70°C and the orange residue dried under high vacuum (146 g). The residue was transferred into a 4 L flask and water (500 mL) was added. A suspension of $Ba(OH)_2$ (129 g, 0.752 mole) in water (1200 mL) was added and the cloudy suspension stirred for 30 min. The mixture was filtered through Celite™ and the water removed under reduced pressure to provide 2-hydroxypyrimidine free base as a bright yellow solid (66.4 g).

2-Hydroxypyrimidine free base (42 g, 0.44 mole) was added to AcOH (500 mL) and the mixture heated to 120°C. *N*-Chlorosuccinimide (67 g, 0.5 mole, 1.15 equivalent) was added cautiously (15 min) in small amounts to the hot solution. Stirring was continued for an additional 5 min and the reaction mixture cooled to RT. The material was concentrated under reduced pressure and the residue was stirred overnight with CH_2Cl_2 (200 mL). The suspended solid was removed by

filtration and the filtrate evaporated under reduced pressure to give 2-hydroxy-5-chloropyrimidine as a beige solid (17.3 g).

The 5-chloro-2-hydroxypyrimidine from above (8.0 g, 0.06 mole) was placed in a dry 500 mL flask under an Ar atmosphere, and $POCl_3$ (79.4 mL) was added followed by *N*,*N*-dimethylaniline (2.6 g). The mixture was heated to 120°C and stirred for 1 h. The dark brown mixture was concentrated under reduced pressure at 50°C. The residue was quenched carefully with ice water and the precipitated material was extracted with pentane (3 X 200 mL). The extract was washed with water and aqueous $NaHCO_3$ solution, and dried (Na_2SO_4). Volatiles were removed under reduced pressure with no external heating to prevent sublimation of the volatile product, to provide 2,5-dichloropyrimidine as a white solid (6 g).

A flask was charged with 57% HI (48 mL) and cooled to 0°C in an ice-salt mixture. The crude 2,5-dichloropyrimidine (6 g) was added and the mixture stirred for 4 h. The yellow suspension was treated carefully with K_2CO_3 (32 g) in water (60 mL) and the pale yellow solid was collected by filtration. The solid was washed with water and dried to give the title compound (8 g): mp 71-73 ^{o}C; ^{1}H NMR ($CDCl_3$) δ 8.47 (s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 157.1, 132.2, 125.1.

2-(5-Chloro-pyrimidin-2-yl)-3-cyclopentyl-1-methyl-1H-indole-6-carboxylic acid 38 (Het = 5-chloro-2-pyrimidinyl). 2-Bromoindole **37** was converted to the corresponding tributylstannane derivative as previously described.[11] The stannane (37.50 g, 69 mmol, 1 equiv) was charged in an oven-dried 2 L three-necked flask equipped with a mechanical stirrer and dissolved in dry DMF (300 mL). 5-Chloro-2-iodopyrimidine (91% content by ^{1}H NMR, 20.0 g,

76 mmol, 1.1 equiv) was added and the solution purged by bubbling argon gas for 1 h while stirring at RT. Solid cesium fluoride (22.50 g, 148 mmol, 2.2 equiv), solid $Pd(Ph_3)_4$ (3.41 g, 2.95 mmol, 0.036 equiv) and CuI (1.37 g, 7.2 mmol, 0.10 equiv) were added in this order and the light brown reaction mixture was stirred for 3h at RT under an argon atmosphere at which point a thick precipitate had formed and conversion was judged to be complete by HPLC analysis. The reaction mixture was then diluted with EtOAc (1 L) and the precipitated removed by filtration using additional EtOAc for washings. Water (300 mL) was added to the filtrate and the mixture was vigorously stirred to produce additional precipitate that was removed by filtration. The filtrate was again washed with water (3 x 200 mL), brine (300 mL) and dried over $MgSO_4$. After filtration, the solution was concentrated to about 300 mL under reduced pressure to give a white suspension that was cooled overnight at 5 °C. The solid was collected by filtration and dried under vacuum to give crude methyl ester as a white solid (10.76 g). The mother liquors were concentrated and partially purified by flash chromatography on silica gel using $CHCl_3$ as eluent (9.5 g). Both crops were combined and purified again by flash chromatography as described above to provide a total of 16.25 g (64% yield) of the desired indole methyl ester as a white solid that was used directly in the next step: ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.15 (s, 2H), 8.17 (s, 1H), 7.82 (d, *J*=8.6 Hz, 1H), 7.68 (dd, *J*=1.2, 8.6 Hz, 1H), 3.90 (s, 3H), 3.86 (s, 3H), 3.59-3.76 (m, 1H), 1.98-1.83 (m, 6H), 1.73-1.57 (br. m, 2H).

The indole methyl ester from above (16.10 g, 43.5 mmol, 1.0 equiv) was suspended in DMSO (400 mL) and the suspension heated to 75 °C with stirring until all solids dissolved. The solution was then cooled back to 33 °C (below this temperature, the material begins to crystallize again) and 2.5 N NaOH (70 mL, 175 mmol, 4equiv) was added dropwise over a 10 min period. The light yellow solution instantly became darker and a white suspension slowly formed which

redissolved when the addition was complete. After stirring for 30 min at RT, the reaction was judged complete by HPLC analysis and water (400 mL) was added to the reaction mixture. The solution was filtered through a 45 μm membrane to remove small amounts of undissolved material and added slowly with stirring to 0.5 N HCl (2 L) to provide an acidic suspension. After stirring for 10 min, the white-yellowish precipitate was collected by filtration, rinsed with water until neutral and dried to provide the desired indole carboxylic acid derivative as a yellow solid (15.19 g, 98% yield): mp 190-195 ^{o}C dec.; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.72 (br s, 1H), 9.12 (s, 2H), 8.15 (s, 1H), 7.78 (d, *J*=8.6 Hz, 1H), 7.67 (dd, *J*=1.4, 8.4 Hz, 1H), 3.85 (s, 3H), 3.63-3.75 (m, 1H), 1.81-1.99 (m, 6H), 1.58-1.68 (m, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.1, 157.7, 155.9, 137.3, 135.7, 128.8, 128.1, 125.0, 121.2, 120.4, 119.8, 112.6, 67.0, 36.3, 32.8, 31.6, 26.0, 25.1; HRMS calcd for $C_{19}H_{19}ClN_3O_2$ (M+H): 356.1160; found: 356.1177.

4-amino-2-ethoxycinnamic acid methyl ester 46. 2-Ethoxy-4-nitrobenzoic acid **43** (25.0 g, 118.3 mmol, 1.0 equiv) was dissolved in anhydrous THF (300 mL) and the solution cooled to 0 °C in an ice bath under an argon atmosphere. Borane-dimethylsulfide complex (10M, 26 mL, 260 mmol, 2.2 equiv) was added dropwise over 15 min (caution: gas evolution) and the solution stirred for an additional 20 min at 0 °C. The ice bath was removed and the mixture stirred for 4 h at RT. The solution was cooled again to 0 °C and water (40 mL) was added dropwise over 20 min (caution: vigorous gas evolution). After completion, the reaction mixture was stirred 30 min at RT and volatiles were removed under reduced pressure. The residue was diluted with water, extracted with EtOAc and the extract washed with 1N HCl followed by brine. After drying over $MgSO_4$, filtration and removal of solvents yielded the crude benzylic alcohol as a yellow solid (22.4 g, 96% yield) that was sufficiently pure to use in the next step.

The benzylic alcohol from above (22.0 g, 111.5 mmol, 1 equiv) was dissolved in $CHCl_3$ (1 L) and solid MnO_2 (60 g, 690 mmol, 6.2 equiv) was added in one portion. The suspension was stirred at reflux for 2.5 h, after which the reaction was judged complete by TLC. The suspension was filtered over celite and the solids washed with CH_2Cl_2. Removal of volatiles under reduced pressure yielded the desired benzaldehyde intermediate **44** as a yellow solid (20.1 g, 92% yield) that was used directly in the next step: ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.41 (s, 1H), 7.94 (d, *J*=1.7 Hz, 1H), 7.90-7.92 (d, *J*=8.4 Hz, 1H), 7.87 (dd, *J*=1.7, 8.4 Hz, 1H), 4.34 (q, *J*=7.0 Hz, 2H), 1.43 (t, *J*=7.0 Hz, 3H).

Note: the corresponding 4-nitro-2-methoxybenzaldehyde for the synthesis of the building block required for the preparation of inhibitor **13** was obtained from commercial sources.

NaH (95%, 2.17 g, 86 mmol, 1.3 equiv) was added in small portions to a solution of trimethylphosphonoacetate (12.95 mL, 182 mmol, 1.2 equiv) in THF (550 mL) at 0 °C. After stirring for 30 min at 0 °C, a solution of aldehyde **44** (13.00 g, 66.6 mmol, 1.0 equiv) in THF (50 mL) was added dropwise to the white slurry which progressively dissolved. The cooling bath was then removed and after stirring for 4 h at RT, the reaction was judged complete by TLC. Volatiles were evaporated under reduced pressure and the residue taken up in EtOAc. The solution was washed with 1N HCl, aqueous $NaHCO_3$ (3X) and brine. After drying ($MgSO_4$) removal of solvents provided nitrocinnamate ester **45** as a yellow solid (16.6 g, 100% yield) that could be purified further by trituration with ether to remove small amounts (~3%) of the cis-isomer: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.01 (m, 1H), 7.86 (d, *J*=16.2 Hz, 1H), 7.79-7.83 (m, 2H), 6.85 (d, *J*=16.2 Hz, 1H), 4.28 (q, *J*=7.0 Hz, 2H), 3.75 (s, 1H), 1.43 (t, *J*=7.0 Hz, 3H).

Iron powder (7.30 g, 131 mmol, 3.1 equiv) and solid ammonium chloride (1.20 g, 22.4 mmol, 0.5 equiv) were added to a suspension of nitrocinnamate **45** (10.60 g, 42.2 mmol, 1.0 equiv) in a mixture of EtOH (150 mL) and water (40 mL). The resulting slurry was stirred at 85 °C for 1 h, at which point the reaction was complete by TLC. The mixture was filtered over celite and the solids rinsed with several portions of EtOH. The combined filtrate and washings were evaporated under reduced pressure, the residue taken up in CH_2Cl_2 and the solution washed with water and brine. After drying ($MgSO_4$), removal of volatiles under reduced pressure provided aniline **46** as an amber-colored gum that solidified upon standing (8.94 g, 96% yield): mp 95-98 ^oC; ^1H NMR (400 MHz, DMSO-d_6) δ 7.73 (d, *J*=16.0 Hz, 1H), 7.32 (d, *J*=8.2 Hz, 1H), 6.25 (d, *J*=16.0 Hz, 1H), 6.22-6.14 (m, 2H), 5.82 (br. s., 2H), 4.00 (q, *J*=7.0 Hz, 2H), 3.65 (s, 3H), 1.37 (t, *J*=7.0 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.9, 159.2, 153.3, 140.7, 130.7, 110.2, 110.1, 106.6, 96.6, 63.1, 50.8, 14.6; HRMS calcd. for $C_{12}H_{16}NO_3$ (M+H): 222.1125; found: 222.1134.

7-Amino-2H-chromene-3-carboxylic acid methyl ester 49. following an analogous procedure described for the preparation of ethoxybenzaldehyde **44**, 2-hydroxy-4-nitrobenzoic acid **47** was reduced-oxidized to aldehyde **48** in 70% yield for the two steps. The hydroxynitroaldehyde **48** (2.50 g, 14.96 mmol) was suspended in methyl acrylate (13.5 mL, 150 mmol, 10 equivalents) and the mixture heated to 50 °C to produce a yellow solution. DABCO (0.42 g, 3.74 mmol, 0.25 equiv) was added and the mixture heated to 90 °C for 2.5 days. The reaction mixture was cooled to room temperature and diluted with ether (150 mL). The solution was washed with 1N NaOH (2 x 100mL), 1N HCl (100 mL) and brine (100 mL). After drying (Na_2SO_4), removal of volatiles gave an orange solid (0.5 g). The combined aqueous phases were re-extracted with EtOAc (2 x 300 mL) to give 2 g of solid that was purified by flash

chromatography on silica gel using 15% EtOAc in hexane as eluent. The desired nitrochromene intermediate was obtained as an orange solid (0.47 g, 15% yield). Reduction of the nitro group to provide aniline **49** was carried out with Fe/NH_4Cl as described for derivative **46**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.37 (s, 1H), 6.98 (d, *J*=8.2 Hz, 1H), 6.16 (dd, *J*=1.9, 8.2 Hz, 1H), 6.0 (d, *J*=1.6 Hz, 1H), 5.83 (s, 2H), 4.79 (s, 2H), 3.69 (s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.7, 156.4, 153.4, 134.6, 130.7, 114.1, 109.2, 107.8, 99.5, 63.9, 51.3; HRMS calcd. for $C_{11}H_{12}NO_3$ (M+H): 206.0812; found: 206.0817.

Conversion of aldehyde 48 to hydroxycinnamate 52. aldehyde **48** (52.0 g, 0.31 mole) was dissolved in CH_2Cl_2 (1 L) under an argon atmosphere. Triethylamine (95 mL, 0.68 mole, 2.2 equiv) was added to the mixture producing a dark red coloration. Trimethylacetyl chloride (40.0 mL, 0.32 mole, 1.05 equiv) was then added dropwise over 25 min and the reaction mixture stirred for 3 h at room temperature until complete. The mixture was filtered to remove solids (CH_2Cl_2 rinses) and the filtrate concentrated under reduced pressure. The residue was diluted with EtOAc and the solution washed with 0.5 M citric acid (500 mL), water (500 mL), $NaHCO_3$ (2 x 500 mL) and brine (500 mL). After drying (Na_2SO_4), volatiles were removed under reduced pressure to yield the desired pivalate **50** as an off-white solid (104.2 g): ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.13 (s, 1H), 8.30 (dd, *J*=2.0, 8.5 Hz, 1H), 8.23 (d, *J*=2.0 Hz, 1H), 8.17 (d, *J*=8.5 Hz, 1H), 1.38 (s, 9H).

A 5 L 3-necked flask equipped with a mechanical stirrer and addition funnel was charged with NaH (60% oil dispersion, 19.1 g, 0.478 mole, 1.5 equiv) under an argon atmosphere. Dry THF (1 L) was added and the mixture cooled in an ice-salt bath to -2°C. Trimethylphosphonoacetate

(69.5 g, 0.382 mole, 1.2 equiv) was added dropwise over 35 min. The thick slurry was stirred for an additional 20 min and aldehyde **50** from above (80.0 g, 0.318 mole) in THF (600 mL) was added over 30 min followed by a THF rinse (400 mL). The orange slurry was then stirred overnight RT. The reaction mixture was quenched with water (50 mL) and THF was removed under reduced pressure. The residue was partitioned between EtOAc (800 mL) and water (800 mL) and the organic phase separated. The aqueous phase was extracted again with EtOAc (1 L) and the organic extracts combined, washed $NaHCO_3$ (2 x 1 L) and brine (1 L). After drying (Na_2SO_4), solvents were removed under reduced pressure until crystallization of the product. After cooling, crystallized material was collected by filtration and washed with cold EtOAc and hexane. After drying, a first crop of product **51** (40 g) was obtained as a light yellow solid. Filtrates and washes were combined and concentrated to give an additional 10 g of product (total yield: 50 g): ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.13-8.25 (m, 3H), 7.61 (d, *J*=16.2 Hz, 1H), 6.90 (d, *J*=16.2 Hz, 1H), 3.75 (s, 3H), 1.38 (s, 3H).

The cinnamate derivative **51** from above (50.0 g, 0.162 mole) was charged into a flask that was immersed in an ice bath. Pre-cooled conc. H_2SO_4 (350 mL) was added, producing a dark red solution. After stirring in ice for 30 min, the clear solution was poured over ice (2.5 kg) to give a yellow solid. Hydroxycinnamate **52** was collected by filtration, washed with water and dried (43.6 g): ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.33 (s, 1H), 7.93 (d, *J*=8.6 Hz, 1H), 7.86 (d, *J*=16.4 Hz, 1H), 7.71 (d, *J*=2.4 Hz, 1H), 7.66 (dd, *J*=8.6, 2.4 Hz, 1H), 6.84 (d, *J*=16.4 Hz, 1H), 3.74 (s, 3H); FIA-MS *m/z* (M-H) 222.0.

Note: (E)-3-(2-Hydroxy-4-nitro-phenyl)-2-methyl-acrylic acid ethyl ester was prepared in a similar way replacing trimethylphosphonoacetate by triethyl 2-phosphonopropionate and used to prepare cinnamate derivatives containing α-alkyl substituents (e.g., **35**).

General procedure for the preparation of 2-alkoxycinnamates 53 from 2-hydroxycinnamate 52 using a Mitsunobu etherification protocol – Preparation of (E)-3-[2-(2-Methoxyethoxy)-4-nitrophenyl]acrylic acid methyl ester 53 and corresponding aniline 54 (R = CH_2CH_2OMe). Methyl-2-hydroxy-4-nitrocinnamate **52** (1.01 g, 4.5 mmol) was combined with 2-methoxyethan-1-ol (0.53 mL, 6.8 mmol) and diethylazodicarboxylate (1.07 mL, 6.8 mmol) in THF (20 mL). Triphenylphosphine (1.78 g, 6.8 mmol) was added and the solution was stirred 15 min at ambient temperature. The reaction mixture was diluted in EtOAc and washed with water and brine. The organic phase was dried with $MgSO_4$, filtered and concentrated under reduced pressure. The crude material was subjected to flash chromatography (1:9 EtOAc/Hex; 1:4; 1:3) and the desired ether **53** (R = CH_2CH_2OMe) was recovered as a light yellow solid (1.01 g, 80% yield): ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.02 (d, *J*=8.6 Hz, 1H), 7.82-7.90 (m, 3H), 6.89 (d, *J*=16.2 Hz, 1H), 4.33-4.41 (m, 2H), 3.72-3.78 (m, 5H), 3.34 (s, 3H).

The nitrocinnamate ester **53** from above (353 mg, 1.3 mmol) was combined with iron powder (209 mg, 3.8 mmol) in absolute ethanol (5 mL). Saturated aqueous ammonium chloride (1 mL) and distilled water (1mL) were added and the heterogeneous mixture was heated at 80 °C with stirring. After two hours the rust colored mixture was diluted in EtOAc and washed with water and brine. The organic phase was dried with $MgSO_4$, filtered and concentrated under reduced pressure. The residue was subjected to flash chromatography (1:2→1:1 EtOAc/Hex) to afford 223 mg (71%) of desired aniline **54** (R = CH_2CH_2OMe) as a yellow solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.72 (d, *J*=16.0 Hz, 1H), 7.33 (d, *J*=8.0 Hz, 1H), 6.27 (d, *J*=16.0 Hz, 1H), 6.14-6.22 (m, 2H), 5.85 (s, 2H), 3.99-4.11 (m, 2H), 3.70 (m, 2H), 3.64 (s, 3H), 3.34 (s, 3H); FIA-MS *m/z* = 252.1(MH^+).

General procedure for the preparation of 2-alkoxycinnamates 53 from 2-hydroxycinnamate 52 using an alkylative etherification protocol – Preparation of 4-amino-2-cyclobutyloxycinnamate methyl ester 54 (R = cyclobutyl). Methyl 2-hydroxy-4-nitro cinnamate **52** (0.30 g, 1.3 mmol, 1 equiv) and cesium carbonate (0.55 g, 1.7 mmol, 1.3 equiv) were suspended in DMF (2.5 mL) and bromocyclobutane (0.13 mL, 1.3 mmol, 1 equiv) was added. The mixture was stirred at 60 °C for 2 days, after which the mixture was poured into water (30 mL) and extracted with EtOAc (2 X 20 mL). The extract was washed with water (30 mL) and brine (30 mL) and dried ($MgSO_4$). Removal of volatiles under reduced pressure gave the desired 2-cyclobutyloxy derivative as an orange gum (0.35 g, 94% yield) that was used without further purification: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.97-8.07 (m, 1H), 7.79-7.92 (m, 2H), 7.62 (d, *J*=2.1 Hz, 1H), 6.86 (d, *J*=16.0 Hz, 1H), 4.98 (m, 1H), 3.75 (s, 3H), 2.55-2.49 (m, 2H), 2.06-2.20 (m, 2H), 1.77-1.90 (m, 1H), 1.65-1.77 (m, 1H); FIA-MS *m/z* MH^+ not observed.

The crude nitrocinnamate from above (0.35 g, 1.25 mmol, 1 equiv) was suspended in EtOH (4.5 mL) and water (5 mL) and the suspension stirred vigorously while adding potassium carbonate (1.04 g, 7.5 mmol, 6 equiv) and sodium hydrosulfite (85%, 1.31 g, 7.5 mmol, 6 equiv) were added. The mixture was stirred for 16 h at RT, diluted with water (50 mL) and extracted with EtOAc (2 X 25 mL). The extract was washed with saturated $NaHCO_3$ solution (2 X 50 mL) and brine (50 mL). The extract was dried ($MgSO_4$) and concentrated to give the title compound as a yellow-brown syrup (115 mg) that was used without further purification: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.70 (d, *J*=16.0 Hz, 1H), 7.31 (d, *J*=8.4 Hz, 1H), 6.24 (d, *J*=16.0 Hz, 1H), 6.15 (dd, *J*=1.8, 8.4 Hz, 1H), 6.08-6.09 (m, 1H), 6.02-6.10 (m, 1H), 5.83 (br. s, 2H), 4.64-4.57 (m, 1H),

3.65 (s, 3H), 2.38-2.46 (m, 2H), 2.00-2.15 (m, 2H), 1.81 (m, 1H), 1.55-1.73 (m, 1H); FIA-MS *m/z* = 248.3 (MH^+).

(E)-3-{4-[(1-Amino-cyclobutanecarbonyl)-amino]-2-ethoxy-phenyl}-acrylic acid methyl ester. aniline **46** (7.64 g, 34.5 mmol) was added portion wise over 3 min to a suspension of 1-amino cyclobutane acid chloride hydrochloride[11] (6.80 g, 40 mmol) in MeCN (150 mL). The suspension was stirred for 20 h at room temperature. Solid K_3PO_4 (18.50 g, 87 mmol) was added and after 15 min, the suspension was poured into water. The oily product was extracted into EtOAc, washed with brine and the solution dried (Na_2SO_4). The material was purified by flash chromatography on silica gel using 90% EtOAc in hexane as eluent. The title compound was obtained as a white solid: mp 120-122.5 ^oC; ^1H NMR (400 MHz, DMSO-d_6) δ 7.81 (d, *J*=16.4 Hz, 1H), 7.62 (d, *J*=8.2 Hz, 1H), 7.59 (d, *J*=1.9 Hz, 1H), 7.33 (dd, *J*=1.9, 8.2 Hz, 1H), 6.54 (d, *J*=16.0 Hz, 1H), 4.10 (q, *J*=7.0 Hz, 2H), 3.70 (s, 3H), 2.55-2.45 (m, 2H), 1.82-1.97 (m, 3H), 1.69-1.82 (m, 1H), 1.40 (t, *J*=7.0 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 175.5, 167.3, 157.8, 142.6, 139.3, 129.6, 117.0, 115.6, 111.2, 102.8, 63.7, 59.4, 51.2, 33.7, 14.5, 13.8; HRMS calcd. for $C_{17}H_{23}N_2O_4$ (M+H): 319.1652; found: 319.1667.

Preparation of amino acid derivative 56. Methyl 4-amino-2,6-dimethoxybenzoate[17] (2.00 g, 9.5 mmol) was dissolved in DMSO (20 mL). N-Boc-1-aminocyclobutane-1-carboxylic acid (2.07 g, 9.6 mmol) was added followed by triethylamine (4.18 mL, 30 mmol, 3.2 equivalents) and TBTU (3.69 g, 11.5 mmol, 1.2 equiv). The mixture was stirred for 24 h at room temperature after which it was added dropwise to a solution of AcOH (15 mL) in water (150 mL). The white

precipitated **56** was collected by filtration, washed with water and dried in vacuum (3.50 g, 71% yield): ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.47 (br. s., 1H), 7.47 (br. s., 1H), 7.15 (br s, 2H), 3.68-3.74 (m, 9H), 2.52-2.60 (m, 2H), 2.00-2.19 (m, 2H), 1.87 (m, 1H), 1.70-1.84 (m, 1H), 1.38-1.24 (m 9H); MS *m/z* 409.3 (MH^+).

Preparation of aldehyde 57. Methyl ester **56** from above (1.84 g, 4.5 mmol) was dissolved in THF (20 mL) and the solution cooled in ice under an inert atmosphere. $LiAlH_4$ (1M in ether, 27 mL, 27 mmol) was added dropwise and the reaction mixture allowed to warm up to room temperature. After stirring for 3.5 h, the reaction was judged complete (HPLC analysis). It was quenched by careful addition of AcOH, diluted with CH_2Cl_2 (250 mL) and 150 mL of 1M sodium-potassium tartrate solution (Rochelle's salt) was added. After stirring vigorously for 90 min, the mixture was filtered to remove solids and the organic phase was separated, dried (Na_2SO_4) and concentrated to a white solid. The residue was triturated with TBME (25 mL) and the white solid collected and dried in air to give the desired benzylic alcohol intermediate (0.65 g, 38% yield): ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.28 (br. s., 1H), 7.43 (br. s., 0.6H), 7.03 (br. s., 2.4H), 4.37 (d, *J*=5.5 Hz, 2H), 4.20 (t, *J*=5.5 Hz, 1H), 3.71 (s, 6H), 2.43-2.58 (m, 2H), 2.08 (m, 2H), 1.86 (m, 1H), 1.68-1.82 (m, 1H), 1.37 (br. s., 6H), 1.23 (br. s., 3H); MS *m/z* 379.2 (M-H).

The benzylic alcohol from above (656 mg, 1.72 mmol) was suspended in THF (50 mL) and activated MnO_2 (85%, 2.12 g, 20 .7 mmol) was added. The mixture was refluxed for 24 h, cooled and diluted with additional THF (50 mL). The mixture was filtered through a small pad of silica gel using THF for washings and the filtrate concentrated to give a brown solid. The solid was suspended in TBME (10 mL), filtered, washed with fresh TBME and dried in air to give benzaldehyde **57** (450 mg, 69% yield): ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.23 (s, 1H), 9.69 (br

s, 1H), 7.52 (br. s., 1H), 7.21 (br. s., 2H), 3.78 (s., 6H), 2.52-2.59 (m, 2H), 2.11 (m, 2H), 1.88 (m, 1H), 1.69-1.82 (m, 1H), 1.12-1.47 (m, 9H); MS m/z 379.2 (MH^+).

Preparation of *N*-Boc-4-amino-2,6-dimethoxycinnamate 58. Triethylphosphonoacetate (0.92 mL, 5.68 mmol) was added dropwise to a suspension of NaH (60% in oil, 0.227 g, 5.68 mmol) in dry THF (25 mL). The resulting thick-white suspension was stirred for 30 min at room temperature and a solution of aldehyde derivative **57** from above (0.43 g, 1.14 mmol) in THF (5 mL + 2 mL rinse) was added. The reaction mixture was stirred for an additional 30 min at room temperature and then quenched by addition of AcOH (0.5 mL). THF was removed under reduced pressure and the residue was dissolved in EtOAc (50 mL). The solution was washed with 10% $NaHCO_3$ (20 mL), dried ($MgSO_4$) and concentrated. The residue was purified by flash chromatography on silica gel using 0-20% EtOAc in $CHCl_3$ as eluent. The *N*-Boc protected form of cinnamate **58** was obtained as a white solid (452 mg, 91% yield): ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.57 (br. s., 1H), 7.93 (d, *J*=16.2 Hz, 1H), 7.49 (br. s., 1H), 7.22 (br. s., 2H), 6.67 (d, *J*=16.2 Hz, 1H), 3.83 (s, 6H), 3.68 (s, 3H), 2.52-2.67 (m, 2H), 2.10 (m, 2H), 1.88 (m, 1H), 1.70-1.84 (m, 1H), 1.38-1.23 (m, 9H); MS *m/z* 435.2 (MH^+).

The Boc-protected derivative **58** from above (120 mg) was suspended in 4M HCl in dioxane (4 mL) and the mixture stirred at room temperature for a few minutes until homogeneous. Reversed-phase HPLC analysis indicated complete conversion to the amine hydrochloride along with some decomposition. Volatiles were removed under reduced pressure and the residue was dissolved in $CHCl_3$ (2 mL). While stirring, TBME (10 mL) was added slowly to produce a white precipitate that was stirred overnight. The solid was then collected, washed with TBME and

dried to provide 92 mg of impure material that was used directly to prepare inhibitor **14** by condensation with 6-indolecarboxylic acid **38** in the usual manner.

General procedure for the preparation of final inhibitors - (E)-3-{4-[(1-{[2-(5-Chloro-pyrimidin-2-yl)-3-cyclopentyl-1-methyl-1H-indole-6-carbonyl]-amino}-cyclobutanecarbonyl)-amino]-2-ethoxy-phenyl}-acrylic acid methyl ester (27 ethyl ester). 2-(5-Chloro-pyrimidin-2-yl)-3-cyclopentyl-1-methyl-1H-indole-6-carboxylic acid (13.40 g, 37.7 mmol), (E)-3-{4-[(1-amino-cyclobutanecarbonyl)-amino]-2-ethoxy-phenyl}-acrylic acid methyl ester (12.00 g, 37.7 mmol) and TBTU (13.00 g, 40.5 mmol, 1.1 equiv) were dissolved in 300 mL DMSO and triethylamine (16 mL, 115 mmol, 3 equiv) was added. The yellow reaction mixture was stirred at RT for 2 h after which point conversion was complete as judged by HPLC. The reaction mixture was poured into vigorously stirred 5% aqueous Na_2CO_3 (2.5 L) yielding a white-yellowish suspension that was allowed to stand for 30 min before the solids were collected by filtration. The white solid was washed with water (3 x 500 mL) and the wet cake was dissolved in warm EtOAc (2.2 L). The solution was filtered to remove insoluble particulates and cooled to 5 °C overnight to yield after filtration and drying in vacuum the desired **27** methyl ester as a white solid (22.5 g, 91% yield): mp 213-215.5 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.70 (s, 1H), 9.13 (s, 2H), 8.87 (s, 1H), 8.25 (s, 1H), 7.73-7.84 (m, 2H), 7.67 (dd, *J*=1.2, 8.61 Hz, 1H), 7.60 (d, *J*=8.6 Hz, 1H), 7.55 (d, *J*=1.6 Hz, 1H), 7.24 (dd, *J*=1.6, 8.6 Hz, 1H), 6.51 (d, *J*=16.0 Hz, 1H), 4.08 (q, *J*=7.0 Hz, 2H), 3.90 (s, 3H), 3.69-3.78 (m, 1H), 3.68 (s, 3H), 2.64-2.86 (m, 2H), 2.27-2.44 (m, 2H), 1.80-2.07 (m, 8H), 1.53-1.74 (m, 2H), 1.26-1.47 (t, *J*=7.0 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.3, 167.2, 166.7, 157.8, 157.7, 155.9, 143.1, 139.3, 137.4, 135.1, 129.4, 128.6, 128.1, 127.1, 121.4, 120.1, 118.7, 116.9, 115.5, 111.6, 110.8, 103.0, 63.7, 59.9,

51.2, 36.3, 32.8, 31.8, 30.8, 26.0, 15.0, 14.5; HRMS calcd. for $C_{36}H_{39}ClN_5O_5$ (M+H): 656.2634; found: 656.2642.

(E)-3-{4-[(1-{[2-(5-Chloro-pyrimidin-2-yl)-3-cyclopentyl-1-methyl-1H-indole-6-carbonyl]-amino}-cyclobutanecarbonyl)-amino]-2-ethoxy-phenyl}-acrylic acid 27. The methyl ester of **27** from above (22.20 g, 33.8 mmol) was dissolved in DMSO (500 mL) by heating to 75 °C. The solution was cooled down to 28 °C and 2.5N NaOH (60 mL, 4 equiv) was added dropwise over a period of 15 min. Reaction progress was followed by HPLC and saponification was complete after 2.5 h. Water (300 mL) was added and the solution filtered to remove insoluble particulates. The filtrate (approximately 1 L) was slowly added to 0.5N HCl (3 L) at RT resulting in a fine yellowish suspension that was then allowed to stand for 20 min before filtration and copious washing with water. The resulting wet cake was then slurried with diethyl ether (2 L) to produce a white suspension that was filtered, rinsed with additional diethyl ether (2 X 500 mL) and then again with water (2 X 500 mL) and finally air-dried overnight to give a pale-yellowish solid (17.3 g, 77% yield): mp 170-175 ^{o}C. dec.; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.03 (br. s., 1H), 9.69 (s, 1H), 9.14 (s, 2H), 8.89 (s, 1H), 8.26 (br. s., 1H), 7.71-7.80 (m, 2H), 7.67 (dd, *J*=1.6, 8.6 Hz, 1H), 7.58 (d, *J*=8.6 Hz, 1H), 7.54 (d, *J*=2.0 Hz, 1H), 7.23 (dd, *J*=1.8, 8.4 Hz, 1H), 6.41 (d, *J*=16.0 Hz, 1H), 4.08 (q, *J*=7.0 Hz, 2H), 3.90 (s, 3H), 3.72 (m, 1H), 2.58-2.83 (m, 2H), 2.32-2.44 (m, 2H), 1.85-2.01 (m, 8H), 1.66 (br. s., 2H), 1.39 (t, *J*=7.0 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.3, 172.0, 168.1, 166.7, 157.8, 157.5, 155.9, 142.8, 138.6, 137.4, 135.1, 129.0, 128.7, 128.2, 127.1, 121.4, 120.2, 118.7, 117.2, 117.0, 111.6, 110.8, 103.1, 63.7, 59.9, 36.3, 32.8, 31.8, 30.8, 26.0, 21.0, 15.0, 14.6; HRMS calcd. for $C_{35}H_{37}ClN_5O_5$ (M+H): 642.2478; found: 642.2483.

Preparation of azalactone 61. Indolecarboxylic acid **59**[11] (3.52 g, 11 mmol) and 1-aminocyclobutane carboxylic acid ethyl ester hydrochloride (1.72 g, 12 mmol, 1.1 equiv) were dissolved in DMSO (40 mL) and TBTU (4.49 g, 14 mmol, 1.3 equiv) and triethylamine (5 mL, 36 mmol, 3.3 equiv) were added. The resulting amber solution was stirred overnight at RT. The solution was then heated to 60 °C and 5N NaOH (11 mL) was added dropwise over 3 min. Saponification was complete by HPLC analysis after stirring for 20 min. The reaction mixture was then poured in water (300 mL) containing AcOH (60 mL) and the beige precipitate that formed was collected by filtration, washed with water and dried. Carboxylic acid **60** was obtained as a beige solid (4.26 g, 93% yield): ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.22 (br s, 1H), 8.87 (s, 1H), 8.80 (d, *J*=4.1 Hz, 1H), 8.12 (s, 1H), 7.98 (dt, *J*=1.8, 7.7 Hz, 1H), 7.66-7.74 (m, 1H), 7.54-7.65 (m, 2H), 7.48 (dd, *J*=4.9, 6.8 Hz, 1H), 3.70 (s, 3H), 3.15 (m, 1H), 2.55-2.66 (m, 2H), 2.21-2.43 (m, 2H), 1.75-2.05 (m, 8H), 1.62 (br. s., 2H); FIA-MS *m/z* 418.2 (M+H).

Carboxylic acid **60** from above (3.50 g, 8.0 mmol) was added to acetic anhydride (30 mL) and the suspension stirred at 100 °C. The material slowly dissolved and after 40 min, TLC analysis showed complete conversion of starting material. Volatiles were removed under vacuum and the gummy residue was triturated with diethyl ether to provide after filtration and washing with ether, azalactone **61** as a beige solid (3.30 g, 85% yield): ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.22 (br. s., 1H), 8.87 (s, 1H), 8.80 (d, *J*=4.1 Hz, 1H), 8.12 (s, 1H), 7.98 (dt, *J*=1.8, 7.7 Hz, 1H), 7.66-7.74 (m, 1H), 7.54-7.65 (m, 2H), 7.48 (dd, *J*=4.9, 6.8 Hz, 1H), 3.70 (s, 3H), 3.15 (m, 1H), 2.55-2.66 (m, 2H), 2.21-2.43 (m, 2H), 1.75-2.05 (m, 8H), 1.62 (br. s., 2H); FIA-MS *m/z* 400.1 (M+H).

General procedure for coupling of 4-aminocinnamates with azalactone 61. Preparation of inhibitor 13. Azalactone **61** from above (25.5 mg, 0.063 mmol, 1 equiv) and methyl 4-amino-2-methoxycinnamate (11.0 mg, 0.053 mmol, 0.8 equiv) were dissolved in glacial acetic acid (0.25 mL) and the mixture was stirred overnight at room temperature. The solution was then taken-up in EtOAc (20 mL) and washed with saturated aqueous $NaHCO_3$ (2 X 20 mL). The organic layer was dried ($MgSO_4$) and evaporated to provide crude **13** methyl ester as a yellow solid (26 mg) that was used without further purification.

The crude ester from above (26 mg) was dissolved in DMSO (0.5 mL) and 2.5N NaOH (172 μL, 10 equiv) was added followed by MeOH (0.3 mL). After stirring at RT for 2 h, saponification of the methyl ester was complete. AcOH (10 drops) were added to the reaction mixture that was then purified directly on a reversed-phase preparative HPLC to provide inhibitor **13** trifluoroacetate salt (8.5 mg) as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.70 (s, 1H), 8.70-8.87 (m, 2H), 8.21 (s, 1H), 8.01 (dt, *J*=1.5, 7.7 Hz, 1H), 7.63-7.78 (m, 3H), 7.44-7.63 (m, 4H), 7.18-7.28 (m, 1H), 6.39 (m, *J*=16.0 Hz 1H), 3.83 (s, 3H), 3.73 (s, 3H), 3.07-3.23 (m, 1H), 2.68-2.81 (m, 2H), 2.28-2.43 (m, 2H), 1.77-2.05 (m, 8H), 1.62 (m, 2H). FIA-MS *m/z* 593.3 (M+H).

Biological testing. Inhibition of HCV polymerase activity in a biochemical assay was performed as previously described using full-length NS5B or a C-terminal truncated NS5BΔ21 construct.[14a] Reported values are the average of duplicate measurements. EC_{50} determinations using the cell-based 1b luciferase reporter assay[14c,d] or replicon assays using RT-PCR for RNA quantification[14b] were performed in duplicates as described elsewhere.

Pharmacokinetic Experiments. All rat PK studies were performed at Boehringer Ingelheim (Canada) Ltd. PK studies in dogs and monkeys were performed at LAB Pre-Clinical Research International Inc., Laval, QC. All protocols involving animal experimentation were reviewed and approved by the respective Animal Care and Use Committee of each test facility. In-life procedures were in compliance with the Guide for the Care and Use of Laboratory Animals from the Canadian Council of Animal Care. All chemicals used were reagent grade or better.

Animals were fasted overnight prior to dosing. IV dose administration (2 mg/kg) was performed using a 70% PEG400: 30% water dosing solution. Oral dose administration (10 mg/kg) was performed using a suspension containing 0.3% Tween-80 and 0.5% methylcellulose, with or without addition of 1% NMP, as additional solubilizer for poorly soluble compounds (N=3 per dose and per route). In the cassette screen experiments, each "cassette" containing 4 compounds at 4 mg/kg for each compound was dosed to 2 rats. Blood samples collected from all time points were placed on ice, and then centrifuged at 4°C. The plasma was separated and stored frozen at approximately -20°C until analysis.

Plasma samples were extracted by solid phase extraction. Samples were analyzed by HPLC using either a UV diode array detector between 200 and 400 nm with quantitative determination made by peak height at the wavelength representing the best signal to noise ratio or a LC/MS systems using the appropriate retention time and m/z+. Calibration standards were prepared in blank plasma. The calibration curve was linear to cover the time-concentration curve with a r^2 values > 0.99, and a limit of quantification (LOQ) at 7 ng/mL. The temporal profiles of drug

concentrations in plasma were analyzed by non-compartmental methods using WinNonlin (version 3.1; Scientific Consulting, Inc., Cary, NC).

ASSOCIATED CONTENT:

Supporting Information Available. Mass spectral and ^{1}H NMR data for building blocks are provided. Mass spectral and HPLC homogeneity data are provided in Table format, along with a copy of the ^{1}H NMR for all inhibitors. This material is available free of charge via the internet at http://pubs.acs.org.

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ABBREVIATIONS USED

HCV, hepatitis C virus; DAA, direct acting antiviral; HCC, hepatocellular carcinoma; PegIFN, pegylated interferon; RBV, ribavirin; gt1, genotype 1; SVR, sustained viral response; SoC, standard of care; RdRp, RNA-dependent RNA polymerase; ADME, Absorption-distribution-metabolism-excretion; PK, pharmacokinetics; SAR, structure-activity relationship; HLM, human liver microsomes; RLM, rat liver microsomes; BSA, bovine serum albumin; CYP, cytochrome P-450; IV, intravenous; TFA, trifluoroacetic acid; TBME, *tert*-butyl methyl ether; THF, tetrahydrofuran; DMF, *N,N*-dimethylformamide; HATU, *O*-(7-azabenzotriazol-1-yl)-*N,N,N'-N'*-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole.

ANCILLARY INFORMATION

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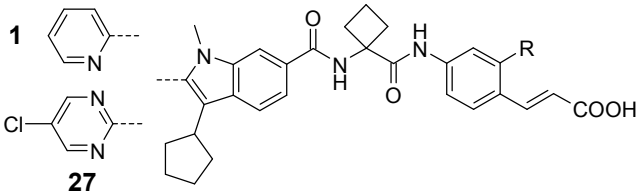
TABLE OF CONTENT GRAPHICS



1 R = H; HCV gt1a/1b EC_{50} = 84 / 153 nM (pKa = 4.5)

27 R = OEt; HCV gt1a/1b EC_{50} = 11 / 29 nM (pKa = 6.5)