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Aza follow-ups to BI 207524, a thumb pocket 1 HCV NS5B polymerase inhibitor. Part 1: Mitigating the genotoxic liability of an aniline metabolite

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ABSTRACT

A series of heterocyclic aza-analogs of BI 207524 (**2**), a potent HCV NS5B polymerase thumb pocket 1 inhibitor, was investigated with the goal to reduce the liability associated with the release of a genotoxic aniline metabolite in vivo. Analog **4**, containing a 2-aminopyridine aniline isostere that is negative in the Ames test was identified, and was found to provide comparable GT1a/1b potency to **2**. Although the cross-species PK profile, poor predicted human liver distribution of analog **4** and allometry principles projected high doses to achieve a strong antiviral response in patients, this work has provided a path forward toward the design of novel thumb pocket 1 NS5B polymerase inhibitors with improved safety profiles.

In the last years, significant breakthroughs have occurred in the treatment and cure of disease conditions associated with chronic hepatitis C virus infection (HCV). The first Direct Acting Antivirals (DAAs) that enhance efficacy and tolerability of pegylated-interferon/ribavirin based therapies have reached the market and are providing patients with sustained viral responses (SVR) and cure in up to 80–90% of genotype 1 (GT1) treatment naïve patients.[1] More recently, IFN-free regimens combining two or three DAAs with complementary modes of actions have shown potential to further improve cure rates (SVR >90%) and tolerability and are showing improved efficacy in the harder-to-treat GT1a and cirrhotic populations.[2,3] To this end, we have been pursuing allosteric inhibitors of the HCV NS5B polymerase as partners in IFN-free combination therapy (e.g., with protease inhibitor faldaprevir).[4]

We have described the discovery and progression to the clinic of indole-based inhibitors that bind to the thumb pocket 1 allosteric site of NS5B and prevent inter-domain interactions between the thumb and finger regions of the protein, resulting in a replication-deficient enzyme. Proof of concept for this class of inhibitors was achieved with BILB 1941 (**1** Fig. 1) that produced up to 2.5 $\log_{10}$ reductions in viremia in HCV GT1 infected patients but was discontinued because of gastrointestinal intolerance at doses required to maintain a strong antiviral response (>450 mg TID).[5] BI 207524 (**2**) is a follow-up compound from this class with improved potency and a cross-species PK profile consistent with achieving a robust antiviral response at a reduced dose (400 mg BID), based on our recently published liver-corrected inhibitory quotient model (LCIQ).[6] In this model, inhibitor liver C_{trough} concentrations corresponding to $\geqslant$500-fold the EC_{50} are predicted to provide a strong antiviral response in infected patients.[7]

During preclinical development of **2**, a genotoxic aniline metabolite (**3**, X = Y = Z = CH) was detected in both human and rat liver microsome (~1 ppm/min in HLM in an NADPH-independent manner) and simulated gastric fluid (SGF) incubation studies and in vivo in rats, thus compromising further progression of this candidate.[6] A well established medicinal chemistry strategy to address such a liability consists of replacing the aniline with isosteres (e.g., heterocycles) that can modulate the binding affinity of the aromatic amine to the CYP1A2 enzyme cavity and stabilize anionic forms implicated in the formation and stability of the reactive nitrenium ion that is formed upon metabolic activation.[8] The suitability of the replacements can be assessed using computational methods and verified by testing in the Ames test. One of the strategies implemented in our program to resolve the genotoxic liability of **2**, involved replacement of the right-hand-side 4-amino-2-ethoxycinnamic acid moiety in **2** by nitrogen-containing isosteres

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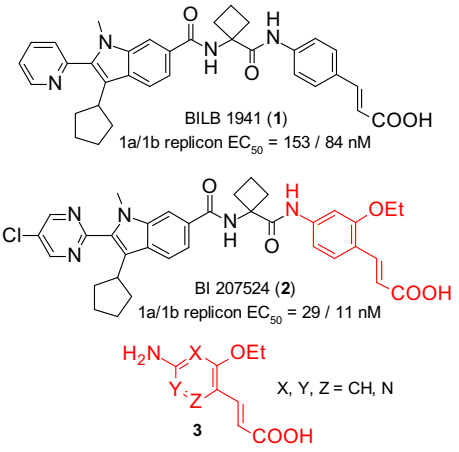


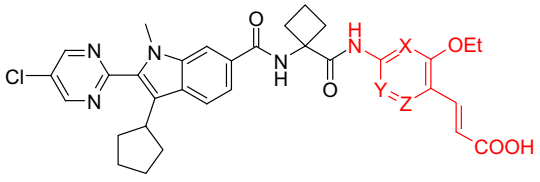
Figure 1. HCV NS5B polymerase thumb pocket 1 leads.

3 that unlike the parent aniline, were determined in silico to be devoid of genotoxic potential.[9]

The isosteric aza-BI 207524 analogs investigated in this study are listed in Table 1 and the synthesis of required right-hand-side heterocyclic aniline replacements is described in Scheme 1. For inhibitor **4**, 2-amino-6-bromopyridine **10** was treated with KO*t*Bu/EtOH and regioselectively iodinated to provide 5-iodopyridine **11** which was converted to 4-aminocinnamate **12** using a standard Heck Pd-catalyzed cross-coupling protocol with ethyl acrylate. Fragment **12** was then condensed with 1-aminocyclobutane carboxylic acid chloride hydrochloride and converted to inhibitor **4** as previously described.[6] 4-Ethoxypyridine *N*-oxide **13** was converted to 2-amino-4-ethoxypyridine **14** which was then iodinated and cross-coupled to ethyl acrylate as described for **12**, to provide building block **15** that was converted to inhibitor **5**. 2,3-Dihydroxypyridine **16** was converted to nitropyridine **17** and then to 2-amino-5-chloropyridine **18** as described in the literature for the corresponding methoxy analog.[10] Heck cross-coupling with *tert*-butylacrylate under the usual conditions provided cinnamate **19** that was used to prepare inhibitor **6**. Commercially available 2-aminopyrimidine **20** was acetylated and chlorinated to provide 4-chloropyrimidine **21**. Treatment with sodium ethoxide resulted in simultaneous deprotection of the amine function and displacement of the chloro group with ethoxy. The ester functionality was subsequently reduced to the corresponding benzylic alcohol that was then oxidized to provide aldehyde **22**. Aldehyde **22** was converted to cinnamate **23** using a Horner–Wadsworth–Emmons condensation and elaborated to inhibitor **7**. Chloropyridazine **24** was prepared in four steps following a literature procedure.[11] The ester function was reduced to the corresponding aldehyde using DIBAL which was homologated to cinnamate ester **25** through a Horner–Wadsworth–Emmons condensation. The chlorine atom was converted to an amine by sequential azide displacement and a two step reduction using triphenylphosphine followed by hydrolysis to give **26**. Aminopyridazine **26** was converted to analog **8** in the usual manner. Finally, 2-amino-6-chloropyrazine **27** was ethoxylated and iodinated under previously described conditions to provide iodopyrazine **28** that was converted to cinnamate **29** and subsequently to inhibitor **9**.

BI 207524 (compound **2**), was initially considered as an attractive follow-up candidate to BILB 1941 since it combined improved potency (7 to 8-fold; achieved through decreasing the measured acidity of the carboxyl function by introduction of the conjugated ethoxy group) with a predicted human PK profile consistent with lower dosing requirements (400 mg BID to achieve a liver-corrected IQ = 500, predictive of a strong antiviral response).[6] The success of our genotoxicity mitigating strategy relied on the identification of compounds that would maintain or improve potency relative to **2** (particularly against the less sensitive GT1a) as well as retain its favorable PK profile. Results from biological testing of BI 207524 and six aza-analogs are shown in Table 1.[12] In a polymerase inhibition assay using a C-terminally truncated NS5BΔ21 construct,[13] all compounds had comparable potency to **2** (IC_{50} = 60–110 nM), suggesting that introduction of nitrogen atoms in the aniline moiety was neither detrimental nor beneficial to interactions with the enzyme binding site. In a cell-based

Table 1
BI 207524 and aza-analogs containing non-genotoxic aniline replacements



Entry	X	Y	Z	IC_{50}[a] (nM)	GT1b EC_{50}[b] (nM)	GT1a EC_{50}[b] (nM)	TC_{50} (MTT, μM)	HLM/RLM[c] $t_{1/2}$ (min)	Caco-2[d] $\times 10^{-6}$ (cm/s)	Rat PK $C_{1h/2h}$[e] or C_{max}[f] (μM)	Liver/plasma ratio[h]	Log*D* (pH 7.4)
2	CH	CH	CH	84	13/11	29	38	102/104	13	3.2[g]	5.5 (6 h)	4.1
4	N	CH	CH	85	15/14	27	56	66/111	15	5.9[f]	2	>5.9
5	CH	N	CH	85	33/—	—	>10	145/104	15	1.8[f]	—	3.0
6	CH	CH	N	110	20/—	—	>10	275/143	7.5	0.4/0.1[e]	5	3.9
7	N	N	CH	63	40/—	89	>10	239/>300	4.3	0.1/0.03[e]	—	—
8	CH	N	N	80	360/—	—	>10	136/227	1.6	—	—	3.4
9	N	CH	N	100	20/—	34	>10	146/>300	13	2.1/0.9[e]	5	3.5

[a] GT1b NS5BΔ21 ($n \geqslant 2$).
[b] Luciferase reporter and RT-PCR replicon assay values are reported; see Refs. 6 and 13 ($n \geqslant 2$).
[c] Human and rat microsomal stability at 2 μM initial concentration.
[d] Apical to basolateral permeability.
[e] Following oral administration to rats as mixtures of four compounds at a dose of 4 mg/kg each in 0.5% Methylcellulose and 0.3% Tween-80 + 1% *N*-methylpyrrolidone (NMP).[14]
[f] Administered orally as a single compound at a dose of 5 mg/kg in 0.5% Methylcellulose and 0.3% Tween-80 + 1% *N*-methylpyrrolidone (NMP).
[g] Normalized to 5 mg/kg from a 10 mg/kg dose.
[h] Determined at 2 h for cassettes or 8 h for single compound PK.[14]

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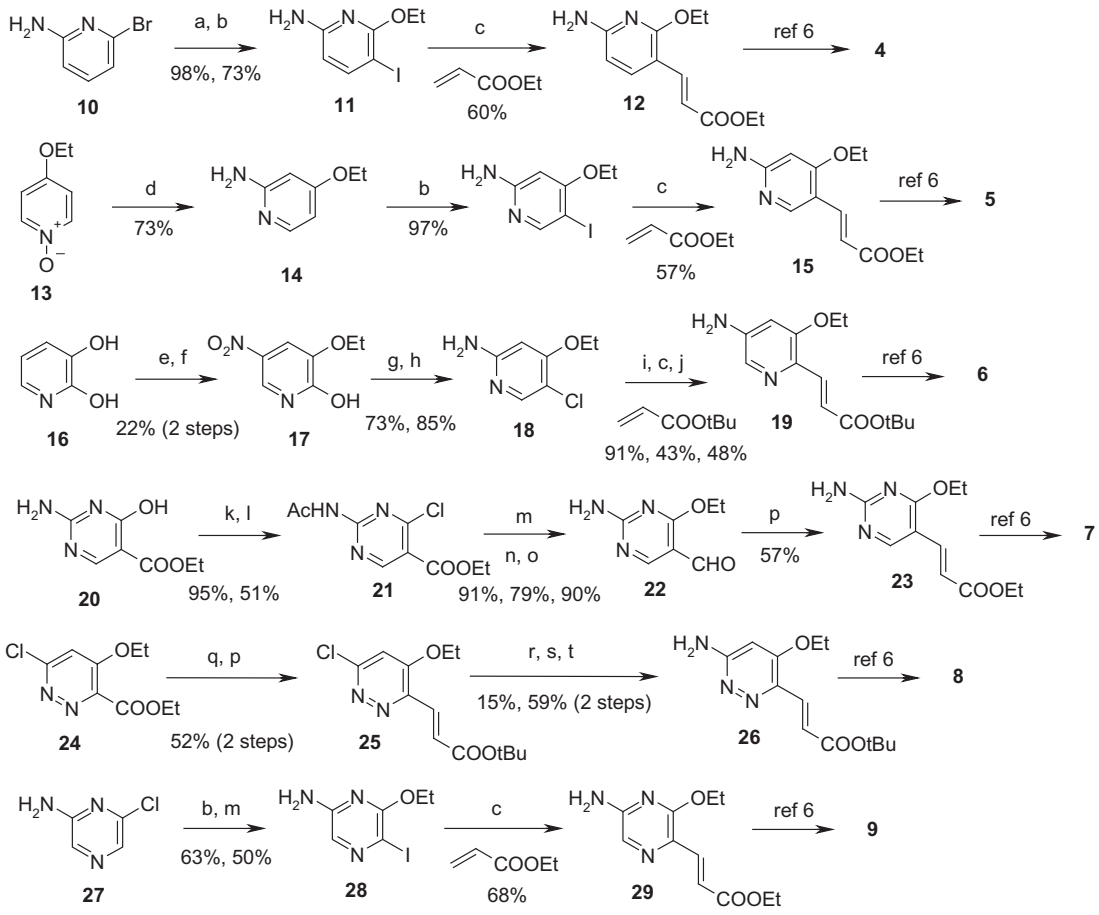

Scheme 1. Reagents and conditions: (a) KO*t*Bu (2.4 equiv), EtOH, 130 °C (sealed bomb for 3 days or microwave for 30 min) (98%); (b) *N*-iodosuccinimide (1 equiv), DMF, 0 °C then rt 4 h (73%); (c) acrylate (5 equiv), P(*o*-Tol)$_3$ (0.2 equiv), Et$_3$N (1.5 equiv), Pd(OAc)$_2$ (0.1 equiv), dry DMF, 100 °C, 18 h (60%); (d) *t*BuNH$_2$ (9 equiv), DCM/α,α,α-trifluorotoluene (1:1), TsCl (4 equiv in DCM added dropwise over 3 h), 0 °C then rt 4 h (73%); (e) Et$_2$SO$_4$, NaOH (1 equiv), 5 °C, 20 h; (f) HNO$_3$/H$_2$SO$_4$, CHCl$_3$, 10–15 °C; (g) PCl$_5$/POCl$_3$, 100 °C, 3 h (73%); (h) SnCl$_2$ (4 equiv), concd HCl 80 °C, 1 h (85%); (i) AcCl (1.5 equiv), Et$_3$N (2 equiv), CH$_2$Cl$_2$, rt (91%); (j) 2 N NaOH, *t*BuOH, reflux, 36 h (48%); (k) Ac$_2$O (2 equiv), pyridine, rt, 1 h (95%); (l) POCl$_3$ (4 equiv), 60 °C, 4 h (51%); (m) NaOEt (21% solution in EtOH, 4 equiv), EtOH, 40 °C, 14 h (91%); (n) LiAlH$_4$ (2.4 M in THF, 1.8 equiv), THF, 0 °C to rt overnight (79%); (o) MnO$_2$ (10 equiv), CH$_2$Cl$_2$, rt, overnight (90%); (p) triethylphosphonoacetate (1.6 equiv)/NaH (1.5 equiv) in THF, 0 °C, 30 min, then add aldehyde, 0 °C, 30 min (57%); (q) DIBAL, CH$_2$Cl$_2$, −78 °C, 2 h; (r) NaN$_3$, DMF (15%); (s) PPh$_3$, benzene, reflux, 6 days; (t) 10% AcOH, 70 °C, 5 h.

luciferase reporter GT1b replicon assay,[13] potency of pyridine (**4**–**6**), pyrimidine **7** and pyrazine **9** analogs remained within 2 to 3-fold of **2** but pyridazine analog **8** suffered a 25-fold loss in potency (EC_{50} = 360 nM). This result is not consistent with the measured lipophilicity of this analog whose measured Log*D* of 3.4 at pH 7.4 was comparable to others in this series. The lower Caco-2 permeability value for **8** (1.6×10^{-6} cm/s) could be an indication of the reduced ability of this derivative to cross cell membranes. Comparable values were obtained for compounds **2** and **4** when tested in a replicon assay using RT-PCR for RNA quantification. Thumb pocket 1 NS5B inhibitors are typically 2- to 3-fold less potent in GT1a replicon assays, as previously reported for compound **2**.[6] This was also observed in the case of aza-analogs. Both pyridine **4** and pyrazine **9** retained potency comparable to **2** in the GT1a assay (EC_{50} = 27, 34 nM, respectively, vs 29 nM), while pyrimidine **7** was significantly less potent (EC_{50} = 89 nM) and was eliminated from further consideration. None of the analogs tested in the cell-based assays displayed appreciable cytotoxicity in the Huh-7 cell line (TC_{50} >10 μM). Aminopyridine **12** and aminopyridazine **29** (as the free carboxylic acid derivatives) were tested in the Ames test (multi-strain and TA100 strain of *Salmonella typhimurium*) at concentrations ranging from 315–5000 μg per plate with and without S9 metabolic activation. Both fragments were found to be non-mutagenic, confirming in silico predictions.

Bioavailability of analogs **4**–**7** and **9** in rats was evaluated following oral administration either as single compounds (5 mg/kg) or within a mixture of 4 compounds dosed at 4 mg/kg each.[14] Plasma levels are reported in Table 1 (either as C_{max} values for single compound administration or at 1 and 2 h time points for compound cassettes). Pyridine analog **4** displayed the best profile overall, with potency, ADME and preliminary rat PK comparable to **2**, albeit with lower partitioning to the liver in this species (liver/plasma ratio = 2 vs 5). Compound **4** was advanced into cross-species PK profiling to provide human pharmacokinetic predictions and estimate the dose required to achieve a strong antiviral response, based on its potency as well as in vitro hepatocyte and in vivo liver partitioning as previously described.[7] Overall, compound **4** exhibited a similar PK profile to **2** in preclinical animal species with low clearance and improved exposure and bioavailability in rats and dogs (Table 2). However, the lower in vitro partitioning of **4** in human compared to rat hepatocytes (Kp = 3.5 vs 1.5) coupled with a low in vivo liver/plasma ratio in rats (Kp = 2.6 vs 5.5) resulted in lower predicted human plasma/liver partitioning compared to **2** (human Kp = 0.9 vs 3.5).[7] It is interesting to note that **4** is significantly more lipophilic than other compounds in this series (Log*D* >5.9), yet it retains a low V_{ss} across all species tested, in the range expected for highly protein bound carboxylic acid derivatives. While compound **4** was also predicted

Table 2
Cross-species PK parameters for compounds **2** and **4** at an oral dose of 5 mg/kg[a] and IV dose of 2 mg/kg[b]

	Species	LM $t_{1/2}$[g] (min)	C_{max} (μM)	AUC (μM h)	$t_{1/2}$ (h)	V_{ss} (L/kg)	CL (mL/min/kg)	%F	in vivo liver Kp[h]	Estimated dose for C_{trough} LCIQ$_{500}$
2[6]	Rat[c]	104	3.2	9.6	2.4	0.38	2.6	19	5.5	
	Monkey[d,c]	96	4.8	26	3.0	0.60	3.6	72		
	Dog[c]	175	4.1	38	4.5	0.38	1.0	29		
	Human[e]	102			8.6	0.5	0.7	37	3.5[i]	400 mg BID
4	Rat	111	5.8	17	1.5	0.28	3.1	42	2.6	
	Monkey[f]	32	2.6	8.9	2.0	0.06	1.5	11		
	Dog	219	10	56	6.1	0.28	0.9	40		
	Human[e]	66			2.3	0.1	0.5	16	0.9[i]	>4000 mg TID

[a] Dosed as an oral suspension in 0.5% Methylcellulose and 0.3% Tween-80 + 1% *N*-methylpyrrolidone (NMP).
[b] Bolus injection prepared in 70% PEG-400:30% water.
[c] Normalized from a 10 mg/kg oral dose.
[d] Cynomolgous monkeys.
[e] Predicted values derived from allometry principles as described in Ref. 15.
[f] Rhesus monkeys.
[g] Liver microsome half-life at 2 μM starting concentration.
[h] Ratio of compound concentration in liver/plasma at 6–8 h.
[i] Estimated value.[6]

to be a low clearance compound in human (IV CL = 0.5 vs 0.7 mL/min/kg for **2**), the significantly lower volume of distribution of **4** in man led to a short predicted human $t_{1/2}$ = 2.3 h. As a result of the moderate GT1a potency (EC_{50} = 27 nM) and short predicted human $t_{1/2}$, a strong antiviral response in human would necessitate administration of >4000 mg three times a day to maintain the required liver C_{trough} concentrations = 500-fold GT1a EC_{50} (i.e., C_{trough} ~13.5 μM).[15] Due to these unreasonable exposure and dosing requirements, compound **4** was not considered for further progression.

In summary, we have identified analogs of BI 207524 in which the liability associated with the release of a genotoxic aniline metabolite has been reduced by replacing the aromatic amine by 6-membered ring nitrogen heterocycles. Although the potency of pyridine analog **4** was comparable to that of BI 207524, the overall preclinical ADME-PK profile and lower human plasma to liver partitioning predicted for this compound, predicted unacceptably high dosing requirements to achieve a strong antiviral response. Subsequent optimization efforts focusing on improving liver partitioning and the cross-species ADME-PK profile of this class of NS5B thumb pocket 1 inhibitors is reported in the accompanying Letter (Part 2).[16]

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12. Inhibitors had >95% homogeneity as determined by reversed-phase HPLC analysis and had ^{1}H NMR and mass-spectral data consistent with their structures.
13. Inhibition of HCV polymerase activity in a biochemical assay was performed as previously described using a C-terminal truncated NS5BΔ21 construct. Reported values are the average of duplicate measurements. EC50 determinations using the cell-based 1b luciferase reporter assay or replicon assays using RT-PCR for RNA quantification were performed in duplicates as described elsewhere.[5a,6]
14. 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 1% NMP, as additional solubilizer ($N = 3$ 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).
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