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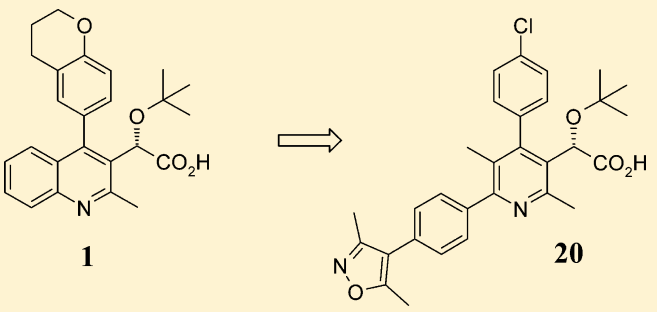
Minimizing the Contribution of Enterohepatic Recirculation to Clearance in Rat for the NCINI Class of Inhibitors of HIV

Lee D. Fader,*[,†] Rebekah Carson, Sébastien Morin, François Bilodeau, Catherine Chabot, Ted Halmos, Murray D. Bailey, Stephen H. Kawai,[‡] René Coulombe, Steven Laplante, Kevork Mekhssian, Araz Jakalian, Michel Garneau, Jianmin Duan, Stephen W. Mason,[§] Bruno Simoneau, Craig Fenwick, Youla Tsantrizos,[∥] and Christiane Yoakim

Research and Development, Boehringer Ingelheim (Canada), Ltd., 2100 Cunard Street, Laval, Québec H7S 2G5, Canada

Supporting Information

ABSTRACT: A scaffold replacement approach was used to identifying the pyridine series of noncatalytic site integrase inhibitors. These molecules bind with higher affinity to a tetrameric form compared to a dimeric form of integrase. Optimization of the C6 and C4 positions revealed that viruses harboring T124 or A124 amino acid substitutions are highly susceptible to these inhibitors, but viruses having the N124 amino acid substitution are about 100-fold less susceptible. Compound **20** had EC_{50} values <10 nM against viruses having T124 or A124 substitutions in IN and >800 nM in viruses having N124 substitions. Compound **20** had an excellent in vitro ADME profile and demonstrated reduced contribution of biliary excretion to in vivo clearance compared to BI 224436, the lead compound from the quinoline series of NCINIs.



KEYWORDS: *NCINI, enterohepatic recirculation, biliary excretion, integrase tetramer*

Since the discovery of HIV in 1983, it is estimated that about 36 million people have died from AIDS worldwide.[1] There has been a continuous search for antiretroviral (ARV) agents to combat HIV, which has led to the discovery of a number of mechanistic classes of replication inhibitors. These include nucleoside[2] and non-nucleoside[3] inhibitors of reverse transcriptase (RT), inhibtors of HIV protease,[4] strand transfer inhibitors of integrase (IN),[5,6] chemokine receptor 5 (CCR5) antagonists, and HIV entry inhibitors,[7] all of which have marketed drugs available to patients. Additionally, a number of investigational mechanisms of action have been identified,[8] including noncatalytic site integrase inhibitors (NCINIs), first disclosed in 2007.[9] Recent work has shown that this class of inhibitor binds to an allosteric pocket on the catalytic core domain (CCD) of IN, shifting the oligomerization equilibrium of IN toward the tetrameric state.[10−12] This aberrant multimerization negatively impacts viral maturation, which leads to the NCINI antiviral effect.[13,14]

Recently we disclosed our discovery of the NCINIs, culminating in the identification of BI 224436, the first compound from this mechanistic class to advance into clinical trials.[15] Compound **1** exemplifies the structural features of the prototypical quinolone series. The methyl substitution at C2 orients the C3 acetic acid group, favoring the bioactive form, and C4 bears an aromatic core. We demonstrated the importance of the C3 and C4 substituents in binding to IN CCD, which included hydrogen bonding interactions between the carboxylic acid and the backbone amide protons of residues E170 and H171 of IN. It was found that this interaction was critical to antiviral potency and that there was no tolerated isosteric replacement for the acid. The presence of a carboxylic acid can complicate the development of a lead series into a marketed drug for a number of reasons.[16,17] In the case of BI 224436, we found that the extremely low in vivo clearance of this molecule in rat is attributable to enterohepatic recirculation involving excretion of parent and the corresponding acyl glucuronide into the bile, reentry into the gastrointestinal tract, and reabsorption of parent back into the hepatic portal system. This phenomenon was common to all members of the NCINI class that were evaluated in rat pharmacokinetic experiments. The potential variability of the contribution of enterohepatic recirculation to in vivo clearance across species introduces uncertainty in allometric scaling and prediction of human PK parameters, which can complicate the development of drug candidates.[18] As BI 224436 progressed into clinical development, our medicinal chemistry back-up effort focused on biliary excretion as the key optimization parameter to mitigate the risk of attrition of the front runner in clinical trials.

One approach widely used by medicinal chemists to improve the properties of a lead series is scaffold hopping.[19] During the

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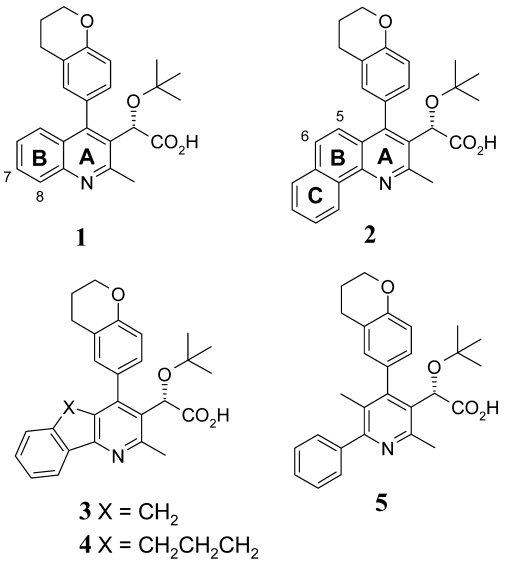
course of lead optimization efforts, it was recognized that replacement of the quinoline core of the NCINI scaffold impacted properties other than potency. Herein we describe our scaffold replacement effort that led to the discovery of the pyridine series of NCINIs, whose salient feature is a reduced contribution of enterohepatic recirculation to in vivo clearance.[20]

During our investigation of B-ring substitution of compound **1**, we synthesized compound **2** and noted that the additional ring fused to the C7 and C8 positions was well tolerated in antiviral assays, in spite of a 7-fold loss in binding affinity in our displacement assay (Table 1).[21] The antiviral assays used to profile NCINIs utilized a panel of wild-type and recombinant viruses that differ in the amino acid substitutions at positions 124 and 125 on IN since these residues are naturally polymorphic in the HIV patient population.[15] In our profiling panel, we included six variants comprising an estimated 93% of the potential patient population, according to an analysis of 1532 sequences from the Los Alamos database. Table 1 shows data from three of these IN variants intended to exemplify general trends. The displacement assay we used to profile compounds measures apparent dissociation constants of inhibitors from IN.[22] Throughout the course of our effort to optimize the quinoline series of NCINIs, the displacement assay correlated with the antiviral assays (i.e., R^2 = 0.80 for the TT variant). On the basis of these results, we began modifying the B ring of compound **2** by replacing the linker between the A- and C-rings made up of C5 and C6 methines. Compounds **3** and **4** introduced different length linkers between the A- and C-rings, and while compound **3**, having the shorter linker, had similar antiviral potency to compound **2**, compound **4** was about 10-fold less potent in terms of antiviral potency and had 6-fold less affinity for IN. We speculated that one difference between these molecules might be the degree of coplanarity between the A- and C-rings. Pyridine analogue **5** was made to test this hypothesis since we believed the A- and C-rings would be close to orthogonal. To our surprise, compound **5** was about 8-fold more potent than compound **3** in the antiviral assays using viruses having the T124 IN substitution, in spite of having similar binding affinity for IN. Equally surprising was the lack of antiviral potency against viruses having the N124 substitution. We later found that these were general trends with all pyridine-based NCINIs. This prompted us to explore the biophysical interaction of these inhibitor series with integrase and modifications of the displacement assay. Isothermal titration calorimetry and NMR revealed that the pyridine series of inhibitors did not show significant binding to the dimeric CCD form of integrase, while the quinoline series bound in a manner that reflected the antiviral potency of the inhibitor. Both series bound tightly to full length integrase at micromolar protein concentrations. Chemical cross-linking experiments followed by SDS-PAGE revealed that monomer and dimer forms of integrase could be captured at the 100 nM His_6-IN concentrations used in the displacement assay. In contrast, cross-linking studies with a thioredoxin (Trx)-tagged IN construct also gave bands associated with trimer and tetramer forms of integrase. This indicates that the Trx-tagged IN construct exists to a greater extent in the higher order tetramer form of integrase compared to the His_6-IN construct under assay conditions. When this construct was used in a modification of the displacement assay, compound **5** bound to IN with 25-fold more affinity than to His-IN. The level of affinity was also similar in magnitude to antiviral potency against viruses having the T124 substitution on IN, highlighting the importance of the tetrameric form to the antiviral potency of NCINIs.

Binding of the quinoline and pyridine NCINIs to Trx-tagged IN was also compared using ^{19}F NMR spectroscopy. To do this, we titrated the same full length construct used in the new displacement assay with fluoro analogues **6** or **7** (Figure 1). Under these conditions, resonances for bound inhibitors are extremely broad and are not observable, consistent with slow binding on the NMR time scale to a very large biomolecular complex. Thus, after the protein is saturated with ligand, the resonance for free inhibitor will appear in the ^{19}F NMR spectrum as excess inhibitor is added. When this titration is performed with compound **6**, saturation was observed at a 1:1 ratio of IN to inhibitor, consistent with binding of the quinoline NCINI to each available pocket on the IN CCD, in the context of full length IN. In contrast, saturation was only observed at a 1:2 ratio of pyridine analogue **7** to full length IN. One possible explanation for these results is that the pyridine NCINI's bind selectively to tetrameric IN, while quinoline NCINIs bind to both dimeric and tetrameric IN forms.

Having identified the pyridine scaffold as a possible replacement for the quinoline core of compounds such as **1**, the process of optimizing potency and in vitro ADME properties was initiated. Initial focus was placed on establishing SAR around the C6 position with the expectation that much of the SAR from the rest of the molecule would parallel what was known from the quinoline series. Simple substitutions at the

Table 1. Scaffold Modification of NCINIs



	$K_{d\text{-app}}$ (nM) His-IN	$K_{d\text{-app}}$ (nM) Trx-His_6-IN	EC_{50} (nM) TT[a]	EC_{50} (nM) TA[b]	EC_{50} (nM) NT[c]
1	24		51	30	77
2	180		62	63	
3	160		72	110	34
4	1100		560		
5	1000	40	68	78	800

[a]Determined with NL4.3 virus (T124/T125). [b]Determined with recombinant NL4.3 virus (T124/A125). [c]Determined with recombinant NL4.3 virus (N124/T125).

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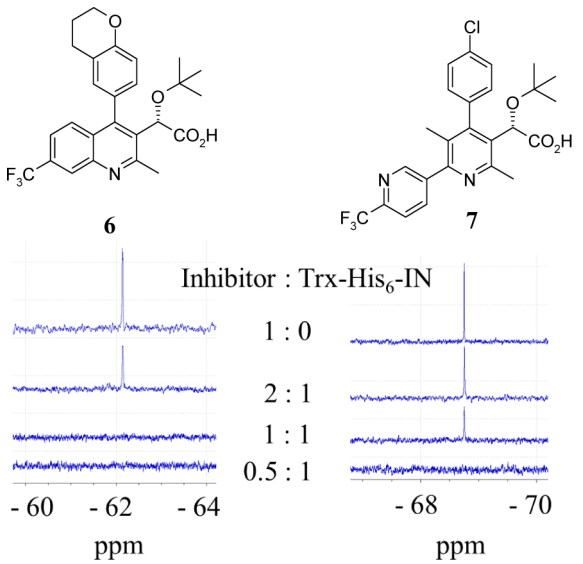
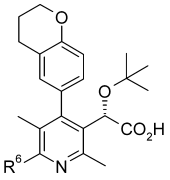
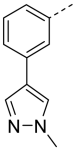
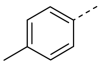
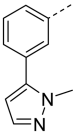
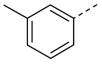
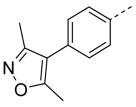
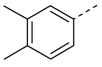
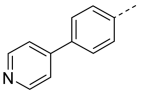
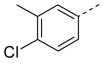
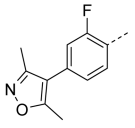


Figure 1. ^{19}F NMR titration of Trx-His$_6$-IN with inhibitors **6** and **7**.

para-position generally led to improvements in antiviral potency (Table 2). For example, the addition of a methyl group to give compound **8** resulted in a 4-fold improvement in antiviral potency against the virus harboring the TT variant of IN, but no change in potency against the NT variant. Similarly, substitution at the meta-position led to improvements in antiviral potency against the TT variant, with no appreciable change against the NT variant, as exemplified by comparison of compound **9** to compound **5**. In general, gains made by substitution at the meta- and para-positions could be combined, although the gains were not completely additive (compare increase from compound **10** to those of **8** and **9**). Heterocyclic rings were also explored at the meta- and para-positions. In the case of the meta-ring biaryl, gains in antiviral potency against the TT variant could be realized with introduction of 5-membered heterocyclic rings (i.e., compounds **12** and **13**), with no appreciable change in potency against the NT variant. When the *para*-biaryl motif was explored, excellent gains in potency against the TT variant were accompanied for the first time by gains in potency against the NT variant. It was noted that introduction of the *ortho*-fluoro modification to the *para*-biaryl motif had a modest, negative impact on antiviral potency against the NT variant (cf. compounds **14** and **16**). Taken together, these results indicated that optimization of antiviral potency is possible through structural changes at the R^6 position. In addition to the difficulty encountered in improving antiviral potency against the NT variant of IN, it was noted that all of the pyridine compounds made to this point suffered from modest to poor metabolic stability when incubated with human liver microsomes. In our earlier work, we discovered that the presence of a saturated ring on the C4 substituent was generally met with poor metabolic stability. It was also discovered that

Table 2. SAR at the R^6 Position



	R^6	$K_{d\text{-}app}$ (nM)	EC_{50} (nM) TT[a]	EC_{50} (nM) NT[b]	HLM $t_{1/2}$ (min)		R^6	$K_{d\text{-}app}$ (nM)	EC_{50} (nM) TT[a]	EC_{50} (nM) NT[b]	HLM $t_{1/2}$ (min)
5		40	68	800	32	**12**		59	24	920[c]	-
8		31	18	1400	43	**13**		52	10	1000[c]	-
9		-	23	1100	-	**14**		-	5.5	390	-
10		27	11	1400	34	**15**		24	23	150[c]	-
11		17	5.8	960	43	**16**		35	7.3	830[c]	60

[a]Determined with NL4.3 virus (T124/T125). [b]Determined with recombinant NL4.3 virus (N124/T125). [c]Denotes n = 1.

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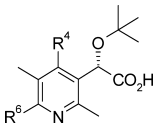
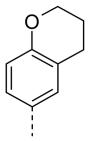
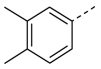
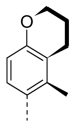
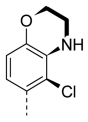
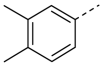
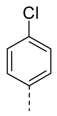
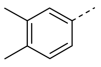
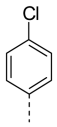
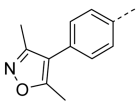
Table 3. SAR at the R^4 Position



	R^4	R^6	$K_{d\text{-}app}$ (nM)	EC_{50} (nM) TT[a]	EC_{50} (nM) NT[b]	HLM $t_{1/2}$ (min)	RLM $t_{1/2}$ (min)
10			27	11	1400	34	110
17			26	2.7	620	68	90
18			28	3.3	830	102	56
19			40	14	2700	77	51
20			50	7.7	810[c]	230	160

[a]Determined with NL4.3 virus (T124/T125). [b]Determined with recombinant NL4.3 virus (N124/T125). [c]Denotes $n = 1$.

the introduction of polarity into the C4 substituent was one successful strategy toward balancing antiviral potency with good metabolic stability. To evaluate this approach in the pyridine series, a range of C4 modified analogues of compound **10** were made (Table 3). Stable atropisomers **17** and **18** were prepared and showed similar potency compared to compound **10**, improved HLM stability, but decreased RLM stability. Our goal was to identify compounds with balanced potency and metabolic stability not only in human but also in rat microsomal preparations in order to more accurately evaluate the relative contribution of enterohepatic recirculation to in vivo clearance. Although this strategy to improving metabolic stability showed signs of being successful, it was also noted that simplifying the C4 substituent showed similar promise. An unanticipated result was that the simple 4-ClPh group at C4 gave compounds with similar virological profiles to analogues with more complex C4 substituents (cf. compounds **10** and **19**). We explored combinations of this simplified C4 group with the SAR summarized in Table 2 and discovered compound **20**, a compound with excellent antiviral potency against the TT variant and improved metabolic stability in HLM and RLM assays.

The in vitro ADME profile of compound **20** was supportive of a further in vivo PK experiment in bile duct cannulated rat allowing the assessment of the biliary excretion and contribution of enterohepatic recirculation to PK parameters such as $t_{1/2}$. In addition to having good metabolic stability in the RLM assay, the compound showed good permeability in the caco-2 assay, moderate to low inhibition of the CYP450 isozymes, acceptable logD7.4, and excellent solubility at pH 6.8. The comparison of BI 224436 and compound **20** is shown in Table 4 and shows that the in vitro ADME profiles are very similar. The main difference between these two compounds is their virological profiles, where viruses harboring all six variants of IN are susceptible to BI 224436, while viruses harboring the NT and NA variants of IN, which are estimated to be present in about 12% of the patient population, are far less susceptible to compound **20** than the TT, TA, AT, and AA variants of IN. Additionally, BI 224436 has been optimized for low serum shift, but serum shift remains as an optimization parameter for compound **20**. Compound **20** was evaluated in a rat PK study and showed an in vivo half-life of 8.9 h, very similar to that observed for BI 224436. However, in a bile duct cannulated rat experiment 60% of BI 224436 was excreted into the bile, while only 22% of compound **20** was excreted into the bile, as measured at the 3 h time point. This translated into an in vivo half-life in the bile duct cannulated rat of 1.6 h for BI 22436, compared to a 5.4 h half-life for compound **20**. These results provided the proof of concept for our optimization strategy by

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Table 4. Comparison of BI 224436 and 20

	BI 224 436	20
EC_{50} range,[a] nM	11−27	6.5−810
serum shift (50% HS), fold-change[b]	2.1	9.9
HLM/RLM ($t_{1/2}$), min	210/>300	230/160
Caco-2 (P_{app}), $\times 10^6$, cm/s	14	17
CYP450 inh. (IC_{50}, 3A4/2D6), μM	23/>30	17/>30
$logD_{7.4}$	0.44	3.1
solubility[c] (pH = 6.8), mg/mL	>0.85	0.25
% excreted into bile @ T_{3h}	60	22
rat in vivo CL (%QH)	0.7	0.6
rat in vivo $t_{1/2}$ (h)	8.8	8.9
bile duct cannulated rat in vivo $t_{1/2}$ (h)	1.6	5.4

[a]Determined with HxB2 virus (A124/T125 IN variant), NL4.3 virus (T124/T125), or recombinant NL4.3 virus (A124/T125, A124/A125, N124/T125, or N124/A125 IN variants) as previously described. [b]Determined by measurement of EC_{50} values ±50% human serum. [c]For the amorphous powder.

demonstrating a reduced contribution of biliary excretion and enterohepatic recirculation to in vivo clearance in rat.

In conclusion, a scaffold hopping strategy within the NCINI class of HIV replication inhibitors has led to the discovery of the pyridine series of NCINIs exemplified by compound **20**. These compounds bind with greater affinity to tetrameric IN than to the dimeric IN, which differentiates them from the original quinoline series of NCINIs. Additionally, NMR titration experiments indicate that the pyridine based NCINIs bind the IN tetramer at a 1:2 ratio, whereas the quinoline series occupies all available pockets on the IN tetramer. Further work is required to understand what the implication of this finding will be on the progression of pyridine-based NCINIs. These studies provide proof of concept for the hypothesis that scaffold modification might have an impact on the contribution of enterohepatic recirculation of carboxylic acid-containing NCINIs. Our effort to incorporate this finding into pyridine-based NCINIs that show excellent potency against viruses harboring all variants of IN will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic schemes for preparation of NCINIs and characterization of key compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*(L.D.F.) E-mail: lee.fader@boehringer-ingelheim.com. Phone: +1 (203) 791-6766.

Present Addresses

[†](L.D.F.) Boehringer Ingelheim Pharmaceuticals, Inc., 900 Ridgebury Road, Ridgefield, Connecticut 06877, United States.
[‡](S.H.K.) Department of Chemistry and Biochemistry, Concordia University, 7141 Sherbrooke Street West, Montreal, QC H4B 1R6, Canada.
[§](S.W.M.) Virology, Bristol-Myers Squibb, 5 Research Parkway, Wallingford, Connecticut 06492, United States.
[∥](Y.T.) Department of Chemistry, McGill University, 801 Sherbrooke Street West, Montreal, QC H3A 0B8, Canada.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CCD, catalytic core domain; CyP450, cytochrome P450; NCINI, noncatalytic site integrase inhibitor; HLM, human liver microsomes; RLM, rat liver microsomes; PK, pharmacokinetic

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