Identification and Characterization of a Novel HIV-1 Nucleotide-Competing Reverse Transcriptase Inhibitor Series

D. Rajotte,^a S. Tremblay,^a A. Pelletier,^a P. Salois,^a L. Bourgon,^{a*} R. Coulombe,^b S. Mason,^{a*} L. Lamorte,^a C. F. Sturino,^{b*} R. Bethell^{a*}

Department of Biological Sciences^a and Department of Chemistry,^b Boehringer Ingelheim (Canada) Ltd., Laval, Quebec, Canada

Several groups have recently reported on the identification of nucleotide-competing reverse transcriptase inhibitors (NcRTIs), a new class of RT inhibitors. NcRTIs reversibly inhibit binding of the incoming nucleotide to the RT active site but do not act as chain terminators, unlike the nucleos(t)ide reverse transcriptase inhibitor (NRTI) class. We identified a novel benzo[4,5]furo[3,2,*d*]pyrimidin-2-one NcRTI chemical series. Structure-activity relationship evaluation of this series with both RT and viral replication assays led to the identification of compound A, a new NcRTI. Compound A inhibited HIV-1 RT in a primer extension assay (50% inhibitory concentration, 2.6 nM) but had no measurable activity against human DNA polymerase γ at 10 μ M. It potently inhibited HIV-1 replication *in vitro* (50% effective concentration, 1.5 nM). The antiviral potency of compound A was unaffected by the presence of nonnucleotide RT inhibitor (NNRTI) mutations tested (L100I, K103N/Y181C, V106A, or Y188L). Notably, viruses encoding K65R were hypersusceptible to inhibition by compound A. Compound A also retained full activity against viruses encoding M184V. *In vitro* selection for resistant virus to compound A led to the selection of a single substitution within RT: W153L. A recombinant virus encoding the RT W153L was highly resistant to compound A (fold change, 160). W153 is a highly conserved residue in HIV RT and has not been previously associated with drug resistance. In summary, a novel NcRTI series with optimized antiviral activity, minimal cross-resistance to existing RT inhibitor classes, and a distinct resistance profile has been discovered. These results further establish NcRTIs as an emerging class of antiretroviral agents.

Human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) is an essential enzyme for retroviral replication (1–3). The RT enzyme catalyzes reverse transcription of viral RNA to proviral DNA. RT inhibitors are the cornerstone of current first-line HIV therapy (1, 4). Four nonnucleoside RT inhibitors (NNRTIs) and eight nucleoside or nucleotide RT inhibitors (NRTIs) are currently approved. However, the emergence of resistance represents a challenge that requires drug combinations with limited cross-resistance in multiple subsequent lines of therapies (5). Many NRTIs may cause mitochondrial toxicity (6). Thus, new agents with activity against strains of HIV-1 resistant to other reverse transcriptase inhibitors and devoid of activity against human DNA polymerases are still needed for optimal treatment of HIV infection.

Several groups have reported on the identification of a new class of HIV-1 RT inhibitors: nucleotide-competing reverse transcriptase inhibitors (NcRTIs) (7–10). The compound that defines this class is INDOPY-1 (7). This compound was first identified from a high-throughput screening campaign based on inhibition of HIV-1 replication *in vitro*. INDOPY-1 was reported to have a 50% effective concentration (EC_{50}) of 30 nM in a viral replication assay. Mode-of-action studies have pointed to RT as the target for INDOPY-1, while steady-state kinetic experiments have revealed that the compound is competitive with the deoxynucleoside triphosphate (dNTP) substrate (K_i , 0.16 μ M). Of note, the potency of INDOPY-1 was markedly increased (10- to 20-fold) in a standard RT inhibition assay when the mixture was supplemented with a physiological concentration of ATP (11). Time course experiments with heteropolymeric template showed a “hot spot” for inhibition following the incorporation of pyrimidines (T preferred over C). It was proposed that HIV RT forms a complex with INDOPY-1 at the N-site and the primer/template bound in its posttranslocation conformation (7, 12, 13). While the potency of INDOPY-1 was unaffected by mutations associated with NNRTI

or multidrug NRTI resistance, mutations M184V and Y115F were associated with decreased susceptibility (7, 9). Of note, K65R, which is associated with decreased susceptibility to tenofovir (14), confers increased susceptibility to INDOPY-1 (7, 9). The sum of the reported data clearly established INDOPY-1, which is not a nucleotide analogue, as a nucleotide-competing inhibitor of HIV RT. Other study groups also identified the same compound, using cell-based HIV-1 viral replication screens, and reported a nucleotide-competitive or mixed-type mode of action for INDOPY-1 (8, 10). To date, there has been no report of any additional chemical series with such properties.

We performed a high-throughput screen against HIV-1 RT in the presence of millimolar concentrations of ATP in an effort to identify novel chemotypes with NcRTI-like properties. As a result, we identified several new chemotypes with such properties. In this report, we describe a benzofuranopyrimidone series with potent antiviral activity and a resistance profile distinct from NNRTIs and NRTIs.

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Address correspondence to D. Rajotte, daniel.rajotte@boehringer-ingelheim.com.

* Present address: S. Mason, Bristol-Myers Squibb, Wallingford, Connecticut, USA; L. Bourgon, Jubilant DraxImage, Kirkland, Quebec, Canada; C. F. Sturino, Vertex, Laval, Quebec, Canada; R. Bethell, Medivir AB, Huddinge, Sweden.

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MATERIALS AND METHODS

Chemical synthesis of compound A. A detailed description of the synthetic steps used to create compound A is provided in the methods section provided in the supplemental material.

HIV RT enzymatic assay. Reagents were purchased from Sigma-Aldrich unless otherwise mentioned. RNA-dependent DNA polymerase activity in the presence or absence of NcRTI was quantified on a poly(rA)/oligo(dT)₁₅ substrate in an assay buffer (40- μ l volume) containing 2 mM BH10 HIV reverse transcriptase enzyme, 50 mM Tris-HCl (pH 7.8), 60 mM NaCl, 2 mM MgCl₂ · 6H₂O, 0.02% 3-[(3-cholamidopropyl)-dimethylammonio]-2-propanesulfonate (CHAPS), 6 mM dithiothreitol (DTT), 2 mM glutathione (GSH, reduced form), 45 nM poly(rA) (GE Healthcare), 4.5 nM oligo(dT)₁₅ (Integrated DNA Technologies), 1 μ M dTTP, 1% dimethyl sulfoxide (DMSO), and 2 mM ATP. The reaction plates were incubated for 60 min at 37°C, followed by the addition of 40 μ l of the intercalating dye Quant-iT picogreen (Life Technologies; diluted 1:375 from commercial stock solution in 20 mM Tris-HCl–25 mM EDTA, pH 8.0). The plate was shaken for 10 s and incubated for 5 min at room temperature, and the fluorescence was measured using a Wallac Victor 2 plate reader (PerkinElmer) with wavelengths specific for fluorescein. A description of the assay automation for high-throughput screening is provided in the methods provided in the supplemental material.

Human DNA polymerase γ assay. Human DNA polymerase γ was expressed and purified as described previously (15, 16). The assay conditions to measure human DNA polymerase γ activity have been described previously (17). We adapted the protocol to use the same assay buffer and primer/template composition as the HIV-1 RT assay described above. We also adapted the assay to an Alphascreen (PerkinElmer) readout format. Briefly, DNA polymerase γ (2 μ M) and the G₂ cofactor (17 μ M) subunits were incubated in enzyme dilution buffer (50 mM Tris-HCl [pH 7.8], 60 mM NaCl, 2 mM MgCl₂, 0.02% CHAPS, 6 mM DTT) for 30 min on ice to allow enzyme reconstitution and activation. The mixture was then diluted 10-fold in enzyme dilution buffer prior to use. The assay was performed in HIV RT assay buffer (see above) that included 50 nM reconstituted DNA polymerase γ , 25 nM poly(rA) template, 1 nM biotin-triethylene glycol-oligo(dT)₁₅ primer, 0.8 μ M dTTP, 0.8 μ M digoxigenin-11-dUTP, and 2 mM ATP. The compounds diluted in DMSO were added to reach a final DMSO concentration in the assay mixture of 1%. The total assay mixture volume was 20 μ l (384-well AlphaPlate; catalog number 6005359; Perkin-Elmer). The reaction mixture was incubated for 2 h at 37°C. Then, 10 μ l of Alphascreen bead solution (30 μ g/ml each of streptavidin donor and antidigoxin/digoxigenin acceptor beads in 25 mM HEPES [pH 7.5], 100 mM NaCl, 50 mM EDTA, 0.1% bovine serum albumin, 0.25% Tween 20) was added to the reaction mixture and incubated for 2 h at room temperature before measuring the result on an EnVision 2103 multilabel reader (PerkinElmer) with an excitation filter of 680 nm and an emission filter of 570 nm.

Viral stocks. The 2.12 plasmid (18) contains the HIV-1 proviral DNA derived from NL4.3 and was modified to harbor BH10 RT. Viral stocks were produced by transfection of p2.12 BH10 RT into 293T cells (American Type Culture Collection) using FuGENE 6 transfection reagent (Roche Applied Science). After an incubation period of 3 days at 37°C, supernatant was harvested and the 50% cell culture infective dose (CCID₅₀) was determined by monitoring the microscopic formation of syncytia in C8166 cells (obtained from J. Sullivan, University Massachusetts Medical Center). The construction of RT mutant viruses was performed using the QuikChange site-directed mutagenesis kit (Agilent Technologies). Mutations were confirmed by sequencing using the ABI Prism 3730 genetic analyzer (Life Technologies).

C8166 LTR-luciferase reporter viral replication assay. A C8166 stable long terminal repeat (LTR)-luciferase reporter cell line (C8166-LTR-Luc), containing firefly luciferase under the transcriptional control of the HXB2 LTR/*trans*-activating response region, was used to monitor viral replication. C8166-LTR-Luc cells were produced by stable transfection of C8166 cells with an HIV LTR-luciferase construct followed by selection

with 5 μ g/ml blasticidin S-HCl through three consecutive limiting dilutions. In preparation for viral replication assays, inhibitors were solubilized in 100% DMSO before being serially diluted into complete RPMI (RPMI supplemented with 10% fetal bovine serum [FBS], 10 μ M β -mercaptoethanol, and 10 μ g/ml gentamicin). C8166-LTR-luc cells were infected at a multiplicity of infection (MOI) of 0.005 with virus for 1.5 h at 37°C prior to seeding into 96-well plates at a density of 25,000 cells per well (final volume of 200 μ l). Plates were incubated for 3 days at 37°C in an incubator containing 5% CO₂. A volume of 50 μ l luciferase reagent (containing 50 μ M luciferin) was added to all wells, and the luminescence was measured using the EnVision plate reader (PerkinElmer). The EC₅₀ represents the concentration of inhibitor which reduces luciferase activity by 50%. EC₅₀s were determined using Symyx AssayExplorer software.

CC₅₀ determination. A volume of 200 μ l containing 50,000 C8166-LTR-Luc cells was added to individual wells of a 96-well plate containing diluted compound. Cells were incubated 3 days at 37°C in an incubator containing 5% CO₂. Reduction of the tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; Sigma-Aldrich) was used to assess mitochondrial function and cell viability. Thus, 40 μ l of a 5-mg/ml MTT solution prepared in complete RPMI 1640 medium lacking phenol red was added to each well. Assay plates were incubated for 3 to 5 h at 37°C. A total of 170 μ l of medium was removed, and the formazan product was solubilized using 100 μ l of 10% Triton X-100 in 0.01 N HCl. Formazan was quantified by measurement of the optical density at 570 nm. The 50% cytotoxic concentration (CC₅₀), represents the concentration of test compound that reduces the cell viability signal by 50%. CC₅₀ values were determined using Symyx Assay Explorer software.

p24 ELISA-based viral replication measurements. C8166 cells were infected with 2.12 BH10 RT virus at an MOI of 0.001 in complete RPMI for 1.5 h on a rotating rack at 37°C in an incubator containing 5% CO₂. The cells were then centrifuged for 5 min at 1,500 rpm and washed with complete RPMI medium. Cells were seeded into 96-well plates containing inhibitor at a density of 5,000 cells per well (in a final volume of 200 μ l). Plates were incubated for 3 days at 37°C in an incubator containing 5% CO₂. Aliquots (100 μ l) of supernatant from 8 replicates were pooled and centrifuged for 10 min at 1,500 rpm. The supernatant was used for the determination of extracellular p24 levels using the Alliance HIV-1 p24 enzyme-linked immunosorbent assay (ELISA) kit (PerkinElmer) according to the manufacturer's instructions.

Viral resistance. Drug resistance was determined as described previously (19). Briefly, C8166 cells were infected at an MOI of 0.1 with wild-type (WT) HIV-1, and cultures were maintained in complete RPMI (RPMI 1640 plus 10% FBS, 10 μ M β -mercaptoethanol, 10 μ g/ml gentamicin) containing an initial concentration of 20 nM inhibitor that represented the EC₉₀. After 3 to 4 days in culture (one passage), microscopic evaluation of the cytopathic effect (CPE) was assessed, and culture supernatant was used to infect fresh C8166 cells. The inhibitor concentration was maintained or increased based on the CPE level. For specific passages, genomic DNA was extracted from infected cells using the DNeasy tissue kit (Qiagen). RT gene sequences were amplified by PCR (IDProof DNA polymerase; ID Labs Biotechnology Inc.) with specific oligonucleotides. PCR fragments were cloned into the Zero Blunt TOPO PCR cloning kit (Invitrogen) and sequenced as previously described.

RESULTS

Screening for novel NcRTIs. We optimized the RT primer extension assay conditions to favor inhibition by putative NcRTIs, as described in Materials and Methods. Two key assay parameters that sensitized the assay to inhibition by NcRTI were (i) reduction of the dTTP concentration from 20 μ M to 1 μ M in order to use dTTP at its K_m value in our primer extension assay (data not shown) and (ii) addition of 2 mM ATP to the assay mixture (11). Using this optimized assay format, we performed a high-throughput screening campaign in which over 10⁶ compounds were evaluated. A total of 7,335 primary hits were identified. A series of

TABLE 1 Profile of Hit-1^a

Profiling assay	Concn (nM)
IC ₅₀ , WT RT (+ATP)	3,100 ± 1,900
IC ₅₀ , WT RT (−ATP)	33,000 ± 11,000
IC ₅₀ , K103N RT (+ATP)	470 ± 230
IC ₅₀ , Y188L RT (+ATP)	560 ± 160
EC ₅₀ , HIV (C8166/Luc reporter)	1,600
CC ₅₀ , C8166 cells	7,900

^a IC₅₀s represent averages (with standard deviations) from at least 3 independent experiments. Single experiments were conducted for the EC₅₀ and CC₅₀ assays.

subsequent profiling assays, including enzyme steady-state kinetics on a subset of these hits, were performed and led to the identification of 4 chemotypes that had both NcRTI-like properties and weak antiviral activity *in vitro* (data not shown). A benzofuranopyrimidone, Hit-1, which had the best properties with respect to inhibitory activity and cytotoxicity, was chosen for further study.

The biochemical and antiviral properties of Hit-1 are shown in Table 1. In the HIV-1 RT primer extension assay, Hit-1 had a 50% inhibitory concentration (IC₅₀) of 3,100 nM in the presence of ATP, while its IC₅₀ was 10-fold higher in the absence of ATP. Hit-1 could also inhibit HIV-1 RT with NNRTI resistance mutations, such as K103N (IC₅₀, 470 nM) or Y188L (IC₅₀, 560 nM). In the luciferase reporter-based HIV replication assay, Hit-1 showed an EC₅₀ of 1,600 nM with a selectivity index of 5. Note that the IC₅₀ measured in the RT primer extension assay in the presence of ATP matched the observed EC₅₀ in the HIV replication assay. This is in line with properties reported for INDOPY-1 (11).

Benzofuranopyrimidone series optimization leading to compound A. Structure-activity relationship analysis-driven lead optimization of the benzofuranopyrimidone series led to greater than a 1,000-fold improvement in intrinsic potency from Hit-1. Compound A (Table 2) is a representative from this series. It is an inhibitor of HIV-1 RT, with an IC₅₀ of 2.6 nM in the primer extension assay. Compound A is also highly selective for inhibition of HIV-1 RT over human DNA polymerase γ (IC₅₀, >10,000 nM). As expected, the potent and selective inhibition of HIV-1 RT translates into antiviral activity *in vitro* (EC₅₀, 1.5 nM) and a selectivity index greater than 10,000. Steady-state kinetic studies with compound A confirmed that it is a competitive inhibitor of RT with respect to the incoming nucleotide substrate. In a double-reciprocal plot of RT enzyme velocity as a function of dTTP concentration for 6 concentrations of compound A, all lines converged at the y axis, as shown in Fig. 1A. Using nonlinear regression models (Scientist 3.0), the competitive, noncompetitive, mix-type, and uncompetitive fits were evaluated. The data best fit to a competitive mode of inhibition with an r^2 of 0.997 and a predicted K_i of 1.1 nM. As shown in Fig. 1B, increasing the dTTP concentration from 1 μ M to 10 μ M in the RT primer extension

TABLE 2 Profile of compound A^a

Profiling assay	Concn ^b (nM)
IC ₅₀ HIV RT	2.6 ± 0.2
IC ₅₀ human DNA polymerase γ	>10,000
EC ₅₀ (C8166; p24 ELISA)	1.5 ± 0.5
CC ₅₀ (C8166 cells)	>15,000

^a Compound A's molecular weight is 513.6.

^b Values represent averages (with standard deviations) from at least 3 independent experiments.

assay led to a proportional linear decrease in the potency of compound A. This is in sharp contrast with the noncompetitive NNRTI nevirapine. The IC₅₀ of nevirapine was not affected by varying the dTTP concentration in the HIV-1 RT primer extension assay.

Cross-resistance profile of compound A. We assessed the phenotypic susceptibility of a panel of NRTI^r and NNRTI^r mutant viruses to compound A (Table 3). For comparison, we used INDOPY-1 as the prototypic NcRTI. As reported previously by Jochmans et al. (7), the antiviral potency of INDOPY-1 was not affected by the NNRTI resistance mutations tested. The observed fold change (FC) ranged from 0.6 to 1.9. Compound A showed similar properties; its antiviral potency was not affected by the NNRTI resistance mutations tested (FC, 0.3 to 1.4). The major resistance mutations reported for INDOPY-1 are M184V and Y115F (9). While viruses with M184V, Y115F, or M184V/Y115F RT mutations were less susceptible to INDOPY-1 (3.0-, 4.8-, and 15-fold, respectively), these mutations did not affect the antiviral potency of compound A. The potency of compound A was not affected by thymidine-associated mutations (TAMs). Of note, viruses with mutations K65R, K65R/M184V, or Q151M complex were hypersusceptible to inhibition by compound A (FC, 0.1). In sum, the potency of compound A was not affected by the NNRTI resistance mutations tested, while the NRTI resistance mutants tested were either fully susceptible or hypersusceptible to inhibition by compound A.

Selection of resistance against compound A. *In vitro* selection for virus resistant to compound A was performed (Fig. 2). HIV-1 2.12 virus was used to infect C8166 cells at an MOI of 0.1 in the presence of inhibitor at an initial concentration of 20 nM (corresponding to the EC₉₀). The CPE was monitored at each passage. If an increase in the CPE was observed, which is an indicator of reduced susceptibility to an inhibitor, proviral DNA was amplified and cloned, and the RT coding region was sequenced. Additionally, the concentration of compound was increased on subsequent passages of virus. On day 20, 70% of the clones contained a tryp-

FIG 1 Compound A is competitive with a dTTP substrate. (A) Lineweaver-Burk plot. The velocity of HIV-1 RT polymerase activity was evaluated at various dTTP substrate concentrations for each concentration (in nM) of compound A indicated. Linear regression for each data set is shown. Data points shown are averages of two independent measurements. (B) Inhibitor titration curves were performed for compound A and nevirapine in an HIV-1 RT assay with 1, 2, 4, 6, 8, and 10 μ M dTTP. The fold change in IC_{50} observed with increasing substrate concentration (compared to the IC_{50} measured at 1 μ M) is shown. Data points shown are averages of two independent measurements.

tophan-to-leucine substitution at position 153 of the p66 subunit of RT. The remaining 30% of the clones were WT or harbored either R83G/K219E or V406I mutations. At the end of the study (day 45), the virus pool was fully resistant to compound A, and all sequenced clones had only the W153L substitution.

Susceptibility of W153L virus and RT to inhibition. In order to confirm that W153L confers resistance to compound A, the mutation was introduced into both 2.12 virus and RT expression constructs. First, recombinant W153L RT mutant enzyme was expressed and purified to homogeneity. The enzyme was active in the RT assay, with only an approximately 25% reduction in activity compared to the wild-type enzyme (data not shown). Compound A had an IC_{50} of 37 nM against W153L RT, compared to 2.6 nM for WT RT, or a 14-fold shift in inhibitory activity. Next, we evaluated the RT W153L virus. The W153L mutant virus was shown to have 50% of the replication capacity of WT virus in a

Jurkat cell *in vitro* replication assay (data not shown), suggesting reduced viral fitness *in vitro*. Table 4 summarizes the FC in potency observed for W153L compared to WT virus with compound A, INDOPY-1, nevirapine, and four different NRTIs. Compound A showed a 160-fold loss in potency against the W153L mutant compared to WT, while INDOPY-1 had an FC of 8. Interestingly, the potency of nevirapine was not affected by the W153L mutation. In contrast, W153L was hypersusceptible (5- to 10-fold) to inhibition by all four NRTIs tested.

Tryptophan at residue 153 in RT is highly conserved in sequences from treatment-naïve and treatment-experienced HIV-infected patients, as determined by searches of the HIV database at the Los Alamos National Laboratory and the Stanford University HIV drug resistance database. This suggests that it plays a critical role in RT enzyme structure or function. Figure 3A highlights the position of W153 in a ternary complex structure composed of RT, the primer/template, and incoming dATP (generated using the deposited structure [Protein Data Bank code 2KK2] of the HIV RT ternary complex and published previously by Lansdon et al. [20]). The W153 residue resides on the loop between β 8 and α E

TABLE 3 EC_{50} fold changes caused by various resistance mutations

Resistance group and mutation(s)	FC^a in EC_{50} (mutant/WT)	
	INDOPY-1	Compound A
NNRTI^r		
Y188L	0.7 $\pm$ 0.2	0.3 $\pm$ 0.1
K103N/Y181C	1.8 $\pm$ 0.5	1.3 $\pm$ 0.4
L100I	1.9 $\pm$ 0.4	1.4 $\pm$ 0.3
E138K	0.6 $\pm$ 0.3	0.5 $\pm$ 0.2
V106A	0.6 $\pm$ 0.2	0.4 $\pm$ 0.2
NRTI^r		
M184V	3.0 $\pm$ 1.5	0.9 $\pm$ 0.6
K65R	0.2 $\pm$ 0.1	0.1 $\pm$ 0.1
K65R/M184V	0.5 $\pm$ 0.3	0.1 $\pm$ 0.1
Q151 M complex ^b	0.7 $\pm$ 0.2	0.1 $\pm$ 0.1
TAM2 ^c	1.5 $\pm$ 0.5	2.1 $\pm$ 2.3
K219E	1.1 $\pm$ 0.3	1.3 $\pm$ 0.2
Y115F	4.8 $\pm$ 3.1	0.4 $\pm$ 0.4
Y115F/M184V	15 $\pm$ 6.0	1.3 $\pm$ 0.8

^a Values represent averages (with standard deviations) from at least 2 independent experiments.

^b A62V/V75I/F77L/F116Y/Q151M.

^c M41L/D67N/K70R/T215F/K219E.

FIG 2 Viral resistance to compound A. C8166 cells were infected at an MOI of 0.1 with HIV-1. Infected cells were cultured with compound A starting at the EC_{90} . Cells were passaged every 3 to 4 days, and the CPE was evaluated. Proviruses from the breakthrough point were sequenced. The RT mutation and its prevalence in the recovered pool is shown.

TABLE 4 Activity of RT inhibitor classes against the W153L RT mutant virus^a

RT inhibitor	EC ₅₀ (nM)		FC (W153L/WT)
	WT	RT W153L	
Compound A	7.5 ± 0.9	1,200 ± 330	160 ± 60
INDOPY-1	170 ± 60	1,300 ± 320	8.1 ± 3
Nevirapine	22 ± 10	14 ± 4	0.9 ± 0.7
Abacavir	2,900 ± 570	260 ± 7	0.1 ± 0
TFV	5,900 ± 3,500	1,300 ± 860	0.2 ± 0.1
FTC	420 ± 370	48 ± 36	0.1 ± 0
3TC	1,700 ± 1,025	140 ± 57	0.1 ± 0

^a Values represent averages (± 1 standard deviation) from at least 2 independent experiments. TFV, tenofovir; FTC, emtricitabine; 3TC, lamivudine.

and is surrounded by a number of other hydrophobic residues within a radius of 4 Å, including V10, T84, L120, F124, P150, F77, and I159. W153, together with these residues, forms a strong hydrophobic core near the catalytic pocket. Notably, W153 is also located in the vicinity of resistance mutations reported for INDOPY-1, such as A62, Y115, and M184.

As shown on Fig. 3B, W153 forms a hydrogen bond with the hydroxy group of T84, while the loop and residues on each side (G152 and K154) interact with the ribose (backbone) of the RNA template. Thus, the size change of the residue 153 side chain (tryptophan-to-leucine resistance mutation) may directly affect the conformation or position of the loop backbone atoms. This would impact the template orientation at the active site.

DISCUSSION

INDOPY-1 has been reported to be a reversible inhibitor of HIV-1 RT, exhibiting either a competitive (7) or mixed-type (8) mode of RT inhibition. We screened for compounds that would be competitive with the incoming nucleotide. The assay conditions and our compound triage strategy led to the identification of the benzofuranopyrimidone series. The steady-state kinetics analysis we performed for compound A, and analogues within the same series, clearly demonstrated that they are competitive with the incoming nucleotide (Fig. 1 and data not shown). Prior to our report, the only known NcRTI was the INDOPY-1 compound. Our discovery now establishes NcRTI as a novel class of RT inhibitors comprised of at least two chemical classes, the indolopyridones and benzofuranopyrimidone. It should be noted that a 4-dimethylamino-6-vinylpyrimidine (DAVP) series was proposed to belong to the NcRTI class (21, 22). The DAVPs were shown to be competitive with dNTPs, yet representatives from this series show a >100-fold loss in potency against NNRTI-resistant mutants K103N and Y181I. This is in sharp contrast to the cross-resistance profile observed for INDOPY-1 and the benzofuranopyrimidone series described herein. Recently, Freistz et al. reported that DAVPs do not bind to the NNRTI binding pocket or to the active site but rather to a hinge region at the interface between the thumb and palm subdomains of the p66 RT subunit (23).

Jochmans et al. discovered that physiological concentrations of ATP dramatically increase the *in vitro* activity of INDOPY-1 (11) against RT. This effect is opposite to the ATP-dependent excision of incorporated nucleotides that leads to reduced NRTI potency (24). In our study, we also observed that a millimolar concentration of ATP in the HIV-1 RT primer extension assay can increase the potency of NcRTIs from the benzofuranopyrimidone series

(Table 1 and data not shown). Based on biochemical data and *in silico* binding simulations, Ehteshami et al. recently proposed a model where ATP and INDOPY-1 bind to the polymerase active site of the RT-primer-template complex at the same time to form a quaternary complex (25). Based on a recent crystal structure analysis of ATP-bound RT (26), we propose another potential mechanism for the contribution of ATP to NcRTI binding. Surprisingly, when Das et al. (26) crystallized HIV-1 RT (L103N/Y181C mutant) in the presence of ATP, they found that ATP bound in the polymerase active site. This binding mode is distinct from the expected mode of binding of ATP for excision. The ATP binding was found to involve primarily metal coordination, a hydrophobic interaction with Y115, and hydrogen bonding with Q151. The resulting complex provides a geometry at the active site that would mimic a catalytic reaction intermediate leading to pyrophosphate hydrolysis and nucleotide incorporation. Thus, ATP binding at the RT polymerase site provides a reaction intermediate (without pyrophosphate hydrolysis) that may favor its displacement by NcRTIs. The low-affinity ATP would allow its dis-

FIG 3 (A) NcRTI resistance mutations map to the active site of the ternary complex. The X-ray structure of HIV RT ternary complex shows the location of the NcRTI resistance mutations. (B) The X-ray structure of HIV RT ternary complex near Trp153. The β8-αE loop (in cyan) makes direct interactions with the template (green). The Trp153 hydrophobic core is shown by the orange surface. A hydrogen bond to Thr84 is shown as a green dotted line. Representations in panels A and B were generated using the deposited structure (Protein Data Bank code 2KK2) of the HIV RT ternary complex (20).

placement by NcRTI. The binding of NcRTI to the RT-DNA/RNA complex would then trap this ternary complex in the posttranslocation state, as documented previously using DNA footprinting experiments (7; reviewed in reference 13). More definitive biochemical and structural evidence will be needed to better understand the role of ATP in potentiating the activity of NcRTIs.

The presence of M184V and Y115F mutations in RT is associated with a decreased susceptibility to INDOPY-1. Binding studies have shown that M184V reduces the affinity for INDOPY-1, while Y115F facilitates the binding of natural dNTP substrates (9). This is in sharp contrast to the resistance profile of compound A, which is not affected by M184V, Y115F, or M184V/Y115F (Table 2). However, some other compounds from the benzofuranopyrimidone series were found to have decreased susceptibility (3- to 10-fold) to M184V (data not shown), indicating some cross-resistance between compounds from this series and the indolopyridones. Given the prevalence of the M184V mutation in the treatment-experienced HIV-1 patient population, maintaining potency against M184V is a desirable feature of a novel RT inhibitor. Our data with the benzofuranopyrimidone series described herein indicate that this can be achieved within the NcRTI class.

The K65R HIV-1 RT mutation is a multidrug resistance mutation (27). It can develop in response to therapy with tenofovir, abacavir, stavudine, zalcitabine, or didanosine (14) and occurs more readily in HIV-1 subtype C than subtype B (28). K65R RT HIV-1 is hypersusceptible to both indolopyridone and the benzofuranopyrimidone series (Table 2). In fact, we observed that all the NcRTI chemotypes we have characterized so far (at least 4 distinct chemotypes) share this feature of hypersusceptibility to K65R RT. We also observed that the increased NcRTI potency against the K65R RT virus compared to WT virus correlated with the increased potency of the compound in the presence of ATP *in vitro* in RT polymerase assays (data not shown). Biochemically, K65R RT decreases the rate of incorporation of all natural substrates and appears to affect a rate-limiting conformational change required for nucleotide incorporation (29). We hypothesize that this may favor competition of nucleotide binding by the NcRTI class. Of note, compound A also showed increased potency against other multidrug resistance mutations involved in substrate discrimination, such as the Q151M complex (Table 3).

We observed a tryptophan-to-leucine mutation at position 153 of HIV-1 RT to be the major pathway for development of resistance to compound A (Fig. 2). The RT W153L virus showed >150-fold decreased susceptibility to compound A compared to WT virus. The same mutant virus also discriminated against INDOPY-1, but to a lesser extent (8-fold). The W153 residue of HIV RT resides on the loop between $\beta 8$ and αE and is surrounded by a number of other hydrophobic residues within a 4-Å radius at the polymerase active site. In the crystal structure of the ternary complex, W153 is at the center of the hydrophobic core, at the junction of the palm and fingers subdomains, in the vicinity of the catalytic center (30). It has been proposed that residues within this hydrophobic core may play an important role in enhancing substrate reactivity by facilitating the exclusion of water molecules, which would lead to higher polymerase fidelity (31) (Fig. 3B). All substitutions at W153 that have been studied were more error prone than WT RT (30). The proposed lower enzyme fidelity of W153L may account for the hypersusceptibility of the W153L RT mutant virus to all four NRTIs tested (Table 4). Thus, the impact

of the highly conserved W153 residue on the geometry of the polymerase active site and the fidelity of the enzyme are in line with our observations with the W153L mutant virus. In addition, the reduced susceptibility of the W153L RT mutant to NcRTIs of the indolopyridones and benzofuranopyrimidone classes may be attributed to a conformational change in the binding site that might include the primer/template itself.

It should be noted that compound A is predicted to have poor intestinal permeability *in vivo*. In a Caco-2 cellular permeability assay, it showed an efflux ratio of 39 (data not shown). However, related compounds within this benzofuranopyrimidone series were shown to have improved the absorption, distribution, metabolism, and excretion properties while maintaining antiretroviral potency *in vitro* (32).

In summary, we identified a novel benzofuranopyrimidone chemical series with NcRTI activity. Compound A is a potent and selective inhibitor of HIV-1 RT with an attractive resistance profile distinct from NNRTIs, NRTIs, and INDOPY-1. The sum of our data supports the previous observation that the NcRTI binds to the RT active site in a highly specific manner and most likely recognizes a catalytic transition state of the polymerase active site. Our data expand on the initial observations for the indolopyridone series and demonstrate that different chemical classes of NcRTIs can be identified. Further work in this area may lead to the identification of novel RT inhibitors that can be used therapeutically.

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