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In vitro resistance development for RO-0335, a novel diphenylether nonnucleoside reverse transcriptase inhibitor

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ABSTRACT

Nonnucleoside reverse transcriptase inhibitors (NNRTIs) are important components of current combination therapies for the treatment of human immunodeficiency virus type 1 (HIV-1) infection. However, their low genetic barriers against resistance development, cross-resistance and serious side effects can compromise the benefits of the first generation compounds in this class (efavirenz and nevirapine). To study potential pathways leading to resistance against the novel diphenylether NNRTI, RO-0335, sequential passage experiments at low multiplicity of infection (MOI) were performed to solicit a stepwise selection of resistance mutations. Two pathways to loss of susceptibility to RO-0335 were observed, containing patterns of amino acid changes at either V106I/A plus F227C (with additional contributions from A98G, V108I, E138K, M230L and P236L) or V106I/Y188L (with a potential contribution from L100I, E138K and Y181C). Characterization of the observed mutations by site-directed mutagenesis in the isogenic HXB2D background demonstrated that a minimum of two or more mutations were required for significant loss of susceptibility, with the exception of Y188L, which requires a two-nucleotide change. Patterns containing F227C or quadruple mutations selected by RO-0335 showed a low relative fitness value when compared to wild-type HXB2D.

1. Introduction

Highly active antiretroviral therapy (HAART) has been successful in suppressing the disease symptoms associated with HIV-1 infection in patients. The nonnucleoside reverse transcriptase inhibitor class (NNRTI) of medicines is a key component in first line drug regimens for HIV-1 positive patients that meet the entry criteria for antiviral therapy (Hammer et al., 2006). First generation compounds of this class have a low genetic barrier to resistance development (i.e. single amino acid mutations can confer high-level resistance) and resistance develops rapidly during incomplete viral suppression (De Clercq, 2004). In addition, the mutations selected by the first generation compounds confer frequent cross-resistance to other molecules in the class (Bacheler et al., 2001; Delaugerre et al., 2001), compounding the disadvantage of the low genetic barrier and preventing sequential use of multiple NNRTIs for antiretroviral therapy in clinical practice (Antinori et al., 2002). Databases containing genotypic information on NNRTI mutation frequencies show high prevalence of K103N and Y181C containing patterns, and transmission of NNRTI resistance between sexual partners has been well documented (Grant et al., 2002; Richman et al., 2004; Weinstock et al., 2004). Therefore novel NNRTIs with potent antiviral activity against wild-type (Wt) and drug-resistant HIV-1 strains are needed to extend the armamentarium of therapeutic agents.

We have recently described the discovery of a series of diphenylether compounds (J. Dunn et al., 234th ACS National Meeting, Boston, MA, 2007) that were synthesized and screened with particular emphasis on identifying compounds with broad and potent antiviral activity against prevalent NNRTI-resistant HIV-1 strains. One representative of this series of compounds, RO-0335 (Fig. 1) showed potent inhibition of Wt HIV-1 with a 50% inhibitory concentration (IC_{50}) of 1.1 nM and retained activity (protein shifted IC_{50} < 100 nM) against 92% of a large number of NNRTI-resistant clinical isolates (K. Klumpp et al., XVI International HIV Drug Resistance Workshop, Barbados, West Indies, June 12–16, 2007).

To evaluate the potential clinical benefit of RO-0335 to patients infected with NNRTI-resistant HIV isolates, we sought to understand possible pathways to resistance against the molecule. *In*

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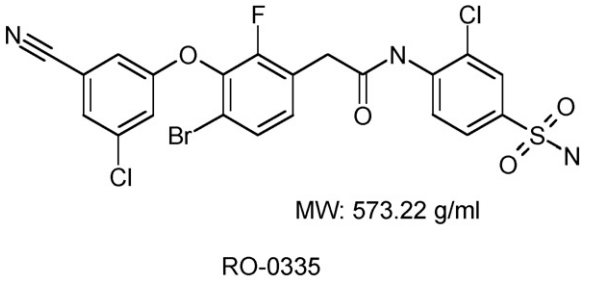

Fig. 1. Structure of the diphenylether compound RO-0335.

vitro selection or passaging experiments have been well established as tools to study potential resistance pathways. In this report we summarize multiple rounds of selection experiments using a low multiplicity of infection (MOI) and stepwise increase of drug pressure in the experimental design, aimed to generate rapid development of drug resistance and to understand the sequence of the resistance mutation pattern(s) for RO-0335. The selection experiments were started at 1 nM drug concentration which represents the IC_{50} and allows for incomplete viral suppression favoring rapid evolution of the replicating viral quasispecies. These conditions are optimized for rapid mutation selection and cannot be compared as such with the pharmacokinetic dynamics of drug metabolism in the human body. At the end of each experiment we analyzed the pool sequence of the virus resulting from the final passage step and determined the fold shift in IC_{50}. Subsequently, the virus pools were dissected by clonal analysis and prevalent mutation patterns were reconstructed by site-directed mutagenesis (SDM) to study the impact of specific mutation positions on the loss of susceptibility to RO-0335 and the relative fitness of the resulting viruses compared to wild-type.

2. Materials and methods

2.1. Compounds

RO-0335 (J. Dunn et al. Preparation of phenoxyphenylacetamides as nonnucleoside reverse transcriptase inhibitors. U.S. Pat. Appl. Publ. (2005), 61 pp. CODEN: USXXCO US 2005239881 A1 20051027 CAN 143:405690 AN 2005:1155549), efavirenz and etravirine were synthesized at Roche Palo Alto.

2.2. HIV-RT enzyme and substrates

The RNA-dependent DNA polymerase activity of HIV-RT was measured using recombinant, purified HIV-RT p66/p51 heterodimer. HXB2 p66 was co-expressed with HIV protease in *E. coli*, resulting in intracellular processing of p66 into p66/p51 dimers. The viral RNA template used for the polymerase reactions was derived from the sequences of the transcription initiation region of HIV-1 genome. The heteroduplex nucleic acid substrate was generated by annealing a 18-mer biotinylated primer to a 47-mer RNA template. Both of the oligonucleotides were chemically synthesized and HPLC-purified.

2.3. HIV-RT polymerase assay

HIV-1 RT polymerase activity was measured in a 96-well plate by quantification of tritiated dATP incorporation into DNA using biotinylated primer binding to scintillation proximity beads (SPA method). A similar SPA-based assay principle to measure HIV-RT polymerase has been described previously (Tillekeratne et al., 2002). Each 50 μL reaction contained 5 pM dCTP, dGTP, dTTP, 3 nM of RT enzyme, 103 nM titrated dATP, 32 nM of RNA template pre-annealed with 64 nM of Biotin-labeled DNA primer, 45 mM Tris–HCl, pH 8.0,45 mM NaCl, 2.7 mM magnesium acetate, 0.045% (v/v) Triton X-100 and 0.9 mM EDTA. 5 μL of serial compound dilutions in 100% DMSO were used for IC_{50} determination and the final concentration of DMSO in the reactions was 10%. The reactions were initiated by the addition of HIV-RT enzyme. After 30 min of incubation at 30 °C, the reactions were stopped by addition of 20 mM EDTA, pH 8.0 in 50 μL solution containing 2 mg/ml streptavidin scintillation proximity beads (SA-PVT beads). The radioactivity was quantified on a Packard Topcount@ instrument after overnight sedimentation of the SA-PVT beads.

Table 1
Mutations selected during *in vitro* passaging.

Passage step	Drug concentration (nM)	Mutations selected with RO-0335		
		Repeat 1	Repeat 2	Repeat 3
1	1	–	–	–
2	2.5	–	–	–
3	5	–	E138E/K	E138E/K
4	10	F227F/C, M230M/L	E138E/K, L214L/F	V106V/I, E138K
5	25	F227C	V106V/I, V108V/I, S162S/N, Y188L	V106V/I, E138K, L214L/F, H221H/Y, M230M/I
6	50	F227C	V106V/I, V108V/I, S162S/N, Y188L	V106I, E138K, L214L/F, H221H/Y, F227F/C, M230M/I
7	100	F227C, M230L/I	V106V/I, V108V/I, S162S/N, Y188L	V106I, E138K, L214L/F, F227F/C, M230M/I
8	250	V106V/A, F227C, M230M/L/I	V106V/I, V108V/I, S162S/N, Y188L	V106I, E138K, L214L/F, F227C, P236P/L, V365V/I
9	500	V106V/A, F227F/C, M230M/L/I	V106V/I, V108V/I, S162S/N, Y181Y/C, Y188L	V106I, E138K, F227C, P236L, V365V/I
10	1000	V106A, F227F/C, P236P/L	V106I, S162S/N, Y181Y/C, Y188L	V106I, E138K, F227C, P236L, V365V/I, E370E/Q
11	2500	V106A, F227F/C, M230M/L/I, P236P/L	L100L/I, V106I, E138E/K, S162S/N, Y181Y/C, Y188L	V106I, E138K, F227C, P236L, V365V/I, E370E/Q
12	5000	V106A, F227C, M230L	L100L/I, V106I, E138E/K, S162S/N, Y181Y/C, Y188L	A98A/G, V106I, V108V/I, E138K, F227C, P236L, E370E/Q
13	10,000	V106A, F227C, M230L	L100L/I, V106I, E138E/K, S162S/N, Y181Y/C, Y188L	A98A/G, V106I, V108V/I, E138K, F227C, P236L, E370E/Q

2.4. In vitro selection experiments

MT-4 cells were infected with HIV-1 wild-type strain HXB2D at an MOI of 0.01 in the presence of 1 nM compound for passage step 1. Cultures were monitored for virus breakthrough by MTS staining (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, Promega) and HIV-1 reverse transcriptase activity (Buckheit and Swanstrom, 1991) from day 3 through day 10 post-infection. Cultures were terminated and supernatant virus collected and stored as a new virus strain when the cell viability reached approximately <20% of the cell control. Upon completion of each passage, virus stocks were titered for standardization of MOI for the next passage, sequenced and amplified by acute infection of MT-4 cells for drug susceptibility testing. For the next passage step either a constant volume (experiments 1 and 2) or standardized MOI of 0.01 (experiment 3) of viral supernatant was used to infect fresh MT-4 cells. The drug concentration was increased with each passage as listed in Table 1.

2.5. Genotyping

Viral RNA was extracted from culture supernatant using the Dynal mRNA direct Micro kit or Qiagen QIAmp Viral RNA Mini kit. cDNA encompassing the reverse transcriptase coding region of HIV was generated with Superscript III (Invitrogen, Carlsbad, CA), followed by PCR using Expand high fidelity enzyme mix (Roche Applied Science, Indianapolis, IN) or using the Superscript III RT-PCR One-step system (Invitrogen, Carlsbad, CA). PCR products were genotyped using the Big Dye terminator kit on a 3730*xl* DNA analyzer (Applied Biosystems). Sequencing results are reported as amino acid changes compared to the wild-type HIV-1 HXB2D reference sequence.

PCR products from virus pools derived in passage step 13 were cloned into pCRBlunt (Invitrogen, Carlsbad, CA) and subjected to clonal analysis. Generally 50–96 clones were picked from the transformation LB plates and inserts were sequenced.

2.6. Phenotyping in MT-4 cell antiviral assay

Susceptibility testing was performed as previously described (Pauwels et al., 1987). The fold change in IC_{50} values were determined by dividing the IC_{50} values for the tested viruses by the average of IC_{50} values observed against wild-type $HIV\text{-}1_{HXB2D}$ when testing in parallel.

2.7. Construction of HIV-1 RT mutant virus

Mutation patterns were introduced into plasmid pLITMUS containing the HXB2 HIV-1 pol gene by standard site-directed mutagenesis using the Stratagene Quick Change kit according to Manufacturer's instructions. Recombinant viruses were generated by cotransfection of the modified pLitmus pol gene plasmids with a genomic construct carrying the whole HXB2 viral genome, which had been deleted for the pol gene region, into MT4 cells. Following observation of CPE and virus outgrowth, supernatant was harvested, sequence confirmed, amplified and titered for further use.

2.8. Competition assay

Viral fitness was measured using a real time Taqman quantitative PCR (QPCR) as described previously (Anastassopoulou et al., 2007). Reference Wt HXB2 (Wt_{Ref}) viruses were created by introducing silent mutation within the vif sequence of the Wt HXB2 genome which resulted into the following changes: 5′-AGC TTG CAA TAT CTA GCG TTA GCA-3′. Wt_{Ref} viruses retained the silent Vif mutations and did not revert to original sequence.

We constructed the standard for QPCR by PCR amplifying the Spe I-Sal-I fragment of HXB2 plasmid and cloning into the TOPO PCR2.1 plasmid which resulted into construction of the plasmid PCR2.0. The SpeI-Sal-I fragment of Wt_{Ref} was amplified with Spe I site introduced on both 5′ and 3′ of the sequence and subsequently cloned into the PCR2.0 plasmid. The final plasmid PCR2.2STD which contains both Wt and Wt_{Ref} Vif sequences were used as standard for all the subsequent QPCR experiments.

Viral infections were performed in 24-well plates with 10^6 MT4 cells in 2.0 ml of media. Competition assay were performed at a MOI of 0.0001. The assays were performed with three different ratios of viruses (1:1, 1:10, 10:1). After 7 days of infection, the cells were harvested and washed with phosphate-buffered saline (PBS), and were subjected to DNA extraction using QIAamp 96 DNA Blood Kit.

QPCR were performed using 5 μl (out of 50 μl) of the extracted DNA in a final volume of 25 μl. The ABI gene expression master mix was used with the following primers: Vif beta(5′-AGT TAG TCC TAG GTG TGA-3′) and Vif beta AS (5′-TCC ATC TGT CCT CTG TCA-3′). The reaction contained the reference dye ROX and probes for Wt Vif (5′-Cy5-TGC TAG TGC CAA GTA TTG TAG AGA TCC T-3′) and Wt_{Ref} Vif (5′-FAM-TGC TAA CGC TAG ATA TTG CAA GCT TCC T-3′). The reaction contained 500 nM of each primer and 250 nM of each probe. The QPCR reactions were performed on a ABI 4800 machine using the following setting: 50 °C for 2 min, 95 °C for 10 min, then 95 °C for 30 s and 55 °C for 1 min for 40 cycles. All reactions were performed in duplicate, including the serial dilutions of the standard DNA template. Each duplicated experiment was repeated at least three times.

Calculation of virus growth was performed as previously described (Anastassopoulou et al., 2007; Quiñones-Mateu et al., 2000). Initially, for every experiment the DNA copy numbers of each virus were determined from the duplicate QPCR reactions. The copy numbers of individual virus (f_o) in the dual infection was corrected for its initial proportion in the inoculums (i_o) to determine the copy number (ws) value for a single virus ($ws = f_o/i_o$). The resulting copy numbers of the three infection conditions (1:1, 1:10, 10:1) were averaged. *p*-Values were calculated by testing the null hypothesis that the mean of the mutant equals the mean of the wild-type. We used ANOVA followed by Tukey test for multiple comparisons to suggest that *p*-values smaller than 0.05 indicate significant relative fitness values compared to wild-type.

The selection coefficient (s) and the relative fitness ($1+s$) was calculated as previously explained (Marée et al., 2000) using the following formula:

$$s = \frac{\ln[H(T)/H(0)]}{\ln[W(T)/W(0)]}$$

where W and M are the proportions of the reference and tested virus, respectively. In this formula $W(T)/W(0)$ is the fold expansion of the reference virus and $H(T)/H(0)$ is the fold change in the genotypic ratio $H = M/W$ over the T days of the experiment. In our experiments, the number of cells that can be infected do not represent a limitation and we observed high replication rates, hence we assumed a decreased effect of δ and calculated with the modified equation from Marée et al. (2000) where $\delta = 0$.

3. Results

3.1. Inhibition of HIV-1 reverse transcriptase by RO-0335 in biochemical and cell based assays

The polymerase activity of recombinant HIV-1 RT was measured as incorporation of labeled dNTP into DNA using a heteroduplex substrate of RNA template annealed to a 5′ biotin-labeled

Table 2
In vitro susceptibility of RO-0335 passage 13 virus pools to NNRTIs.

Experiment	Genotype of virus pool from passage 13 (at 10 uM)	Phenotype IC_{50} (nM)		
		RO-0335	Efavirenz	TMC-125
Wt HXB2D virus		1.1 (1)	1.9 (1)	3.4 (1)
Repeat 1	V106A, F227C, M230L	>400 (>363)	>400 (210)	>400 (118)
Repeat 2	L100L/I, V106I, E138E/K, S162S/N, Y181Y/C, Y188L	>400 (>363)	>400 (210)	>400 (118)
Repeat 3	A98A/G, V106I, V108V/I, E138K, F227C, P236L, E370E/Q	>400 (>363)	97 (51)	195 (57)

Fold changed compared to Wt.

DNA primer. The RNA template sequence was derived from the HIV genomic primer binding site. The concentration of R0-0335 required to reduce DNA synthesis by 50% was determined from five independent experiments. The mean IC_{50} value of RO-0335 was 8.1 ± 1.9 nM. For comparison, the mean 50% inhibitory concentrations (IC_{50}) value for the inhibition of HIV replication by RO-0335 in the cell line used in the selection experiments was 1.3 ± 0.4 nM.

3.2. Characteristics of in vitro escape from RO-0335

We performed three rounds of *in vitro* selection experiments using the Wt HIV-1 strain HXB2D. The set up of the experimental conditions allowed for a steady, stepwise selection of resistance mutations under drug pressure spanning a range of compound concentrations from 1 nM to 10 μM (Table 1). A passage step was defined as the time in culture required for ongoing virus replication to overcome the drug pressure and manifest as cytopathic effect leading to less than 20% cell viability (per MTS readout) compared to the cells only (uninfected) control. At this point the viral supernatant was harvested, titered and population sequenced. For the next passage step the drug concentration was increased (as shown in Table 1) and the process was repeated. Typically, one experiment would be carried out over a 3–4 month time period, with 60–90 cumulative assay days to reach passage 13 at a final drug concentration of 10 μM. Experimental controls included the parallel passaging of virus with efavirenz and/or etravirine, together with a no drug control (NDC) to monitor viral replication dynamics.

In a representative experiment we obtained the final passage 13 virus pool for RO-0335 in 83 days, compared to 63, 74 and 68 days for efavirenz, etravirine and the NDC, respectively (data not shown). Viral breakthrough titers were monitored and varied between approximately 1000 and 3,000,000 $CCID_{50}$/mL with no direct correlation to any given passage step or drug compound used (data not shown).

Table 1 summarizes the mutation pattern progression observed in the three experiments as the result of passaging virus in increasing concentrations of RO-0335. As controls, efavirenz selected for a mutation pattern containing L100I and G190S and etravirine for a pattern containing E138K, V179F and Y181I/C (data not shown). These findings are consistent with previously published reports for these compounds (Bacheler et al., 2000, 2001; Vingerhoets et al., 2005).

3.3. Virus pool genotypes at passage 13 and resulting IC_{50}

Analysis of HIV-1 reverse transcriptase from the passage 13 virus pools for RO-0335 from three separate passaging experiments yielded two major pathways containing multiple mutated amino acid positions as listed in detail in Table 2. Both pathways contain mutation of position V106 to either A or I. Pathway one shows an additional change at position F227 to C, with possible contributions of mutations at other positions associated with NNRTI resistance (Johnson et al., 2008) A98G, V108I, M230L and polymorphism at E138K, P236L and E370Q. The second pathway contains mutations at Y181C and Y188L, with possible contribution from L100I and polymorphisms at E138K and S162N. All passage 13 virus pools lost susceptibility to RO-0335 as expected (Table 2). Due to multiple, well characterized NNRTI resistance-associated mutations contained in the passage 13 virus pools, cross-resistance was generally observed with efavirenz and etravirine.

3.4. Clonal analysis

The final passage 13 virus pools for RO-0335 contained mixtures at several amino acid positions. To better understand the contribution of the positions that were still observed as mixtures in the final passage 13 pools and to study which mutation patterns would arise co-linearly within one RT gene, we performed clonal analysis of the final virus pools. Viral RNA was extracted, amplified and cloned, with sequence evaluation of 50–96 individual clones. Clones containing inserts with premature stop codons were discarded from the analysis. Similarly, mutation patterns occurring at low frequencies containing permutations of mutations found in the majority of species listed are not shown in Table 3.

The virus pool from experiment 1 showed the most restricted clonal diversity with 82% of the clones containing the triple mutation pattern of V106A, F227C and M230L; 4% of the clones contained the double mutation F227C and M230L. Examples of the minor populations we also observed include individual clones containing only V106A or the triple mutation at V106, F227 and M230 in combination with either V179I or Y318V. The virus pool from experiment 2 showed a high clonal diversity with patterns revolving around various double, triple and quadruple mutation patterns including mutations L100I, V106I, E138K, Y181C and Y188L. The majority patterns are listed in Table 3 with all other clones occurring at 4% frequency or below. Experiment 3 repeated the primary mutation pattern seen in experiment 1, with mutations at positions V106 and F227, this time in various combinations with A98G, V108I, E138K and P236L.

Table 3
Clonal analysis of passage 13 virus pools.

	Mutations	Frequency
Repeat 1		
	V106A, F227C, M230L	37 of 45 (82%)
	F227C, M230L	2 of 45 (4%)
	Related patterns	6 of 45 (14%)
Repeat 2		
	L100I, V106I, E138K, Y188L	13 of 48 (27%)
	L100I, V106I, Y181C, Y188L	4 of 48 (8%)
	L100I, V106I, E138K, Y181C, Y188L	4 of 48 (8%)
	V106I, E138K, Y188L	3 of 48 (6%)
	Related patterns	24 of 48 (50%)
Repeat 3		
	A98G, V106I, V108I, E138K, F227C, P236L	22 of 46 (48%)
	V106I, V108I, E138K, F227C, P236L	6 of 46 (13%)
	Related patterns	18 of 46 (22%)

Table 4
In vitro susceptibility of SDM constructs to NNRTIs.

Virus construct	IC_{50} (nM) (FC)		
	RO-0335	Efavirenz	Etravirine
Wt	1.3	2.4	3.5
L100I	0.3 (<1)	1.3 (<1)	4.6 (1)
K103N	2.2 (1)	65.2 (27)	1.8 (<1)
V106A	1.6 (1)	6.5 (3)	2.9 (1)
E138K	2.3 (2)	2.1 (1)	6.7 (2)
Y181C	1.4 (1)	3.5 (2)	8.5 (2)
Y181I	0.8 (<1)	2.5 (1)	2.2 (1)
Y188L	34.0 (26)	>100 (42)	3.1 (1)
G190A	0.7 (<1)	6.0 (3)	1.1 (<1)
M230L	0.6 (<1)	1.0 (<1)	1.2 (<1)
L100I/K103N	0.9 (1)	>100 (>50)	9.5 (3)
K103N/Y181C	1.2 (1)	82.8 (35)	9.2 (3)
V106A/F227C	173 (133)	79 (33)	4.7 (1)
V106A/F227C/M230L	>400 (>308)	>400 (>167)	160 (46)
L100I, V106I, E138K, Y188L	>400 (>308)	>400 (>167)	>400 (>114)
L100I, V106I, Y181C, Y188L	>400 (>308)	>400 (>167)	>400 (>114)
E138K/Y181I	2.0 (2)	1.7 (1)	179 (51)
V179F/Y181C	0.8 (<1)	5.8 (2)	325 (93)
Y181I/M230L	12 (9)	25 (10)	>400 (>114)

FC = fold changed compared to Wt.

3.5. Antiviral activity of RO-0335 against SDMs

A panel of SDM RT plasmids was constructed to understand the role of the specific mutations selected by RO-0335 during the passaging experiments. The constructs were used to generate recombinant, isogenic viruses and drug susceptibility assays were carried out with RO-0335, efavirenz and etravirine. Table 4 summarizes the IC_{50} value and fold change in susceptibility for each SDM virus to RO-0335, efavirenz and etravirine. As previously demonstrated, none of the single mutations conferred an $IC_{50} > 50$ nM. Multiple mutations such as V106A/F227C with and without M230L or the quadruple patterns with L100I, V106I, E138K, Y188L or L100I, V106I, Y181C, Y188L were required to confer high IC_{50} fold changes.

M230L alone did not confer a loss of susceptibility to any of the compounds tested (fold change values of less than 1), however, addition of this mutation to both the V106A/F227C and the Y181I background increased the overall fold change by a factor of 3–50 depending on the compound evaluated. The mutation patterns observed in selection experiments with etravirine, such as E138K/Y181I, V179F/Y181C and Y181I/M230L, did not confer high-fold change in susceptibility to RO-0335 (fold change of 1–9) or efavirenz (fold change of 1–10).

After treatment with efavirenz or nevirapine viruses carrying L100I, K103N, Y181C, G190A or combinations of these are frequently selected (Bacheler et al., 2001; Richman et al., 1994; Winslow et al., 1996; Young et al., 1995). While not all of these mutants appeared during our *in vitro* selection, we tested RO-0355 and the control compounds additionally against constructs carrying these patterns, as listed in Table 4. None of these mutations conferred a measurable fold shift in the IC_{50} to our compound; in comparison, K103N containing viruses showed the expected shift against efavirenz. Additional mutation patterns containing e.g. V106I in combination with others were explored in a large panel of clinical isolates as presented previously (K. Klumpp et al., XVI International HIV Drug Resistance Workshop, Barbados, West Indies, June 12–16, 2007).

Interestingly, we attempted repeatedly and independently at Roche Palo Alto and Southern Research Institute to generate the single mutation F227C in the HXB2D background by SDM and we were unable to generate a virus containing this single mutation. Sequence confirmed mutated plasmids were transfected into cells to recombine the mutation into the isogenic background. However, when all outgrowing viruses were sequenced they were confirmed to harbor other mutations or polymorphisms in the absence of the F227C change. These data suggest that this mutation alone might not be viable in the HXB2D genetic background and highlights the necessity to sequence any recombinant viral supernatants to confirm the presence of the desired change. To study the effect of the F227C mutation on the viral reverse transcriptase we were able to successfully produce mutant recombinant RT protein and perform enzyme assays. The F227C mutation conferred a 5-fold change to the IC_{50} for RO-0335 in the enzyme assay, compared to a 4- and 2-fold change for efavirenz and etravirine, respectively (G. Su et al., XVI International HIV Drug Resistance Workshop, Barbados, West Indies, June 12–16, 2007).

3.6. Replication competition assays

The relative fitness values of RO-0335 resistant mutants from our *in vitro* passaging experiments were measured in virus competition assays using a real time TaqMan quantitative PCR method, as previously described (Anastassopoulou et al., 2007). This method involves dual infection of cells with a test virus and Wt HXB2D reference virus (Wt_{Ref}). Wt_{Ref} virus contains sequence tags between nucleotide positions 5466 and 5493 in the Vif coding region of the HXB2D viral sequence. The changes create a stretch of synonymous mutations that do not affect the Vif protein sequence, do not overlap the central termination sequence (Charneau et al., 1994), and do not significantly interfere with RNA secondary structure. This allows for the detection of two competing virus species without further altering the HIV-1 genome. Our results showed reproducibility and replication capacity of the tagged Wt virus (Wt_{Ref}) similar to previous reports (Anastassopoulou et al., 2007; Koval et al., 2006). The results from the competition assays performed are summarized in Table 5. The virus containing the sequence tag in vif is indicated as Wt HXB2 reference. To validate the assay the tagged virus was compared in multiple experiments against the parent HXB2 without a tag. As expected there is a selection coefficient 's' of 0 and the relative fitness of the tagged virus equals the untagged Wt parent.

Among the single mutant patterns tested, V106A, Y188H or L and M230L showed slight variability from the 50/50 ratio at the end of the growth cycles but none of the differences were statistically significant, also visualized in very small selection coefficients. In contrast, V179F, F227L and P236L as a single mutations had much lower mutant proportions at the end of the replication cycle and these differences reached statistical significance

Table 5
Relative fitness difference of SDM constructs compared to Wt HXB2.

Virus construct	% i_o	% f_o	SD	p-Value	s	$(1+s)$
Wt HXB2 reference		–	–		0	1.0
Wt	50	51	3.6	1	0.00	1.0
V106A	50	60	5.6	0.89	0.03	1.03
V179F	50	16	5.9	<0.0001	−0.13	0.87
Y188H	50	36	1	0.19	−0.04	0.96
Y188L	50	57	2.7	0.99	0.02	1.02
M230L	50	65	0.6	0.24	0.04	1.04
F227L	50	12	5.0	<0.0001	−0.14	0.86
P236L	50	29	9.1	0.005	−0.06	0.94
V106A/F227L	50	52	7.0	1	0.00	1.0
V106A/F227C	50	6	2.3	<0.0001	−0.18	0.82
V106I/Y188L	50	39	5.5	0.58	−0.03	0.97
F227C/M230L	50	5	3.8	<0.0001	−0.21	0.79
Y181CM230L	50	56	0.2	0.99	0.03	1.03
E138K/Y181I	50	62	4.4	0.58	0.05	1.05
V179F/Y181C	50	62	12	0.67	0.04	1.04
V106A/F227C/M230L	50	20	9.7	<0.0001	−0.09	0.91
L100I, V106I, E138K, Y188L	50	21	5.3	<0.0001	−0.08	0.92
L100I, V106I, Y181C, Y188L	50	5	4	<0.0001	−0.22	0.78

Relative fitness of the SDM recombinant viruses relative to the Wt HXB2. % i_o and % f_o refers to the initial and the final percentage of the tested virus during the competition assay, respectively. Standard deviation (SD) was derived from three independent experiments. The coefficient of selection (s) and the relative fitness ($1+s$) was calculated as explained in material and methods. p-Values were calculated by testing the null hypothesis that the mean of the mutant equals the mean of the wild-type. We used ANOVA followed by Tukey test for multiple comparisons to suggest that p-values smaller than 0.05 indicate significant relative fitness values compared to wild-type.

compared to Wt. Our findings on P236L reproduce well with a previously described study, where this mutation also had a lower relative fitness value when tested in the NL4-3 viral background (Koval et al., 2006). Both the double mutant patterns V106A/F227C and F227C/M230L constructed to investigate the relative fitness of mutations generated in the presence of RO-0335 showed very small percentages of mutant virus remaining and selection coefficients '*s*' of −0.18 and −0.21, respectively. Similar results were found with the triple and quadruple patterns selected during the RO-0335 passaging (Table 5). Interestingly addition of the M230L to F227C or V106A/F227C results in a moderate gain of relative fitness. In detail the F227C single mutation does not seem to be viable versus F227C/M230L has a low but measurable selection coefficient of −0.21 and similarly for V106A/F227C with '*s*' of −0.18 versus the improvement in V106A/F227C/M230L with '*s*' of −0.09. Probing of SDM constructs resembling mutation pathways observed with etravirine, such as Y181C/M230L, E138K/Y181I and V179F/Y181C, demonstrated equal relative fitness compared to Wt with no statistical significant differences.

4. Discussion

RO-0335 is a novel NNRTI compound, as was demonstrated by inhibition of HIV-1 recombinant reverse transcriptase in biochemical experiments. The compound showed potent inhibition of the enzyme with an IC_{50} of 8.1 nM.

In vitro experiments generating resistance against a potential novel antiviral compound have been used to anticipate patterns that might be observed during a failing antiviral regimen against HIV-1 in the clinic. Clearly the experimental conditions, such as viral strains, level of drug pressure relative to the IC_{50}, cell lines and MOI, influence the speed and mutation patterns that arise. However, for example with the NNRTI efavirenz, the results from independent *in vitro* resistance development studies suggested loss of susceptibility with mutations at position 100, 108, 179, 181 and the double mutants L100I/K103N (Winslow et al., 1996; Young et al., 1995). From the phase II clinical studies, it was confirmed that patients failed the efavirenz containing regimen with mutations at positions 100, 103, 108 and 188 (Bacheler et al., 2001). We performed classic, low MOI *in vitro* selection experiments aimed at selecting resistance via viral escape due to a steady accumulation of mutations facilitated by initially sub-optimal and then increasing concentrations of the inhibitor. To highlight possible differences in the viral growth dynamics, the experimental set up was repeated three independent times and at two laboratory sites. To ensure that the mutation patterns described for RO-0335 reflect comparable results with previous work, we included efavirenz and etravirine as positive controls. The patterns observed for the control compounds agree well with previously published literature (Bacheler et al., 2001; Vingerhoets et al., 2005; Winslow et al., 1996).

For example, experimental repeat 3 shown in Table 1, RO-0335 displays a complex, stepwise evolving pattern of mutation selection that resulted in mutations A98A/G, V106I, V108V/I, E138K, F227C, P236L and E370E/Q in the final virus pool sequence. Among these mutations, changes at amino acid position 98, 106, 108, 138, 227 and 236 have been previously described as arising under NNRTI pressure (Demeter et al., 2000; Sato et al., 2006) and several of these are listed in the IAS guidelines for antiviral resistance to this class of compounds (Johnson et al., 2008). The presence of mixtures in the virus pool sequence is indicative of ongoing viral evolution under compound pressure, which is also supported by the analysis of individual clones (Table 3) where 22% of the clones had various combinations of mutations (with a minimum of 2 mutations) at the positions listed. This observation suggests that the viral evolution is forced to explore combinations of various mutations to provide a balance between robust viral replication and discrimination against the compound pressure in the compound binding pocket of the reverse transcriptase enzyme. Based on the results observed, we conclude the behavior of RO-0335 is similar to etravirine in the display of a complex final virus pool mutation pattern that may constitute a higher genetic barrier to antiviral resistance.

As shown in Table 2, the selection of multiple NNRTI resistance-associated mutations by the final passage of virus at 10 μM RO-0335 (passage 13) was repeated throughout all three replicate experiments. Experiment 2 highlighted a possible alternate escape pathway consisting of a combination pattern with L100L/I, V106I, E138E/K, S162S/N, Y181Y/C and Y188L. Mutation at V106 to I or A seemed to be a common thread in all three experiments. Exploring the susceptibility change conferred by e.g. V106A does not explain the need for this mutation, as there is no significant change in IC_{50} as compared to the Wt (Table 4). As this mutation does not seem to impact the relative fitness of the virus, the selection coefficient '*s*' can be barely measured, it could provide an acceptable background for further mutations under higher drug pressure.

The mutation Y188L arises through a two-nucleotide change and one of the pathways to this mutation is via the intermediate Y188H. In our competition studies we could demonstrate that Y188H showed a smaller proportion of mutant virus (36%) remaining after the competition growth cycles; however, the difference did not reach statistical significance, compared to Y188L. The single mutation Y188L does confer a sizable loss in susceptibility to RO-0335 (FC = 26), but could not be isolated as a virus with only this single mutation in the pool; all clones contained more complex patterns than the Y188L mutation alone. The clonal viruses that we identified containing L100I, V106I, E138K and Y188L or L100I, V106I, Y181C and Y188L grow very poorly compared to Wt (Table 5). The final passaging pool that contained these constructs as distinct clones had a high degree of variability in various permutations of these mutations, suggesting again an ebb and flow pattern between overcoming the drug pressure and the replication capacity contribution of reverse transcriptase on the viral life cycle.

The predominant path to loss of susceptibility for RO-0335 appears to be patterns containing the V106/F227 double mutation. The double mutation V106A/F227C for example confers a 133-fold shift in IC_{50} despite growing very poorly compared to Wt, with only 6% of mutant remaining after culture and a selection coefficient of −0.18. This mutation pathway had been previously described for *in vitro* resistance development of capravirine (CPV) (Sato et al., 2006), a second generation NNRTI that did not reach the market. As was suggested for CPV, compounds with this binding mode generally do not follow the K103N mutation pathway that is so common for the first generation NNRTI compound class. Similar to CPV, RO-0335 retains full activity against virus with the single mutation at K103N (Table 4). Query of the Stanford HIV Drug Resistance Database (http://hivdb.stanford.edu/) for occurrence frequencies for mutations V106A or I and F227C or L in HIV-1 Subtype B isolates resulted in very low percentages of 1–2% occurrence within this data set. Similar percentages can be commercially acquired from the Virco and Monogram Biosciences database. Pre-existing viral populations that would already be resistant to RO-0335 are therefore expected to exist at very low frequency in the patient population.

In summary, we present results from the *in vitro* selection and characterization of HIV-1 virus pools resistant to RO-0335. Our results suggest a requirement for multiple mutations at different amino acid positions in reverse transcriptase in order for the virus to develop resistance to RO-0335, with several of the mutation combinations showing reduced relative fitness values compared to Wt *in vitro*. The studies presented demonstrate possible escape pathways for HIV-1 against RO-0335, but confirmation of these pathways would have to occur in the clinical use of the compound. The mutation patterns observed are currently occurring at very low frequencies in the HIV-1 sequence databases. Clonal dissection of high drug level passaging virus pools showed ongoing evolution to avoid the drug pressure—suggesting a need to compromise between a replication competent virus and escape from the drug pressure. These data taken together suggest that RO-0335 may have a higher genetic hurdle compared to first generation NNRTIs such as nevirapine and efavirenz. Together with the very robust antiviral profile of RO-0335 and promising pharmacokinetics in animal species, the existing data support further clinical development of this compound.

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