Screening for NNRTIs with Slow Dissociation and High Affinity for a Panel of HIV-1 RT Variants

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A lead optimization library consisting of 800 HIV-1 nonnucleoside reverse transcriptase inhibitors (NNRTIs) was screened in parallel against 4 clinically relevant variants of HIV-1 RT (Wt, L100I, Y181C, and K103N) using a surface plasmon resonance–based biosensor. The aim was to identify inhibitors suitable in specific topical microbicides efficient for preventing the transmission of a range of clinically significant strains of HIV-1. The authors hypothesized that such compounds should have high affinity and slow dissociation rates for multiple variants of the target. To efficiently analyze the large amount of real-time data (sensorgrams) that were generated in the screening, they initially used signals from 3 selected time points to identify compounds with high affinity and slow dissociation for the complete panel of enzyme variants. Hits were confirmed by visually inspecting the complete sensorgrams. Two structurally unrelated compounds fulfilled the hit criteria, but only 1 compound was found to (a) compete with a known NNRTI for binding to the NNRTI site, (b) inhibit HIV-1 RT activity, and (c) inhibit HIV-1 replication in cell culture, for all 4 enzyme variants. This novel screening methodology offers high-resolution real-time kinetic data for multiple targets in parallel. It is expected to have broad applicability for the discovery of compounds with defined kinetic profiles, crucial for optimal therapeutic effects. (*Journal of Biomolecular Screening* 2009:395-403)

Key words: HIV-1 reverse transcriptase, screening, surface plasmon resonance (SPR)-biosensor, NNRTIs, microbicides

INTRODUCTION

TRANSMISSION OF THE HUMAN IMMUNODEFICIENCY VIRUS (HIV) remains a major problem in the developing world, where women, because of socioeconomic, cultural, and biological disadvantages, are at a substantially higher risk of infection than men.[1] Topical strategies that women can use to protect themselves from HIV infection has therefore emerged as a prioritized field in AIDS prevention research. Although topical microbicides have not yet been successful in the clinic, the challenges this strategy is facing are of a different magnitude than those faced in HIV vaccine research, where serious setbacks during the past years severely cloud the hopes for a vaccine to become available during the next decades.[2,3]

Topical microbicides are self-administered prophylactic agents that kill or disable the disease-causing organism through specific or unspecific mechanisms of action. Several unspecific agents have failed in the clinic, and hope therefore lies in the use of specific and more potent agents, such as HIV-1 nonnucleoside reverse transcriptase inhibitors (NNRTIs).[4] This class of compounds is especially promising as they are known to inactivate both free viral particles, by binding to reverse transcriptase (RT) in the virus, and cell-bound virus, by inhibiting replication in cells. A topical microbicide must be safe, effective and cheap, and should ideally confer sustained protection from HIV after application. An active agent that binds with high affinity and dissociates slowly from its target is likely to be effective at a low concentration and offer a long-lasting protective effect in a microbicide delivery system, such as a sponge or a ring. Because HIV mutants are getting increasingly common because of the use of anti-HIV drugs, the active agent in a new topical microbicide must confer protection also against the most common mutant strains.

The aim of this study was to identify inhibitors suitable for use in topical microbicides against HIV-1. We used a surface plasmon resonance (SPR) biosensor to screen 800 NNRTIs against 4 clinically relevant variants of HIV-1 RT (Wt, L100I, Y181C, and K103N). A hypothesis stating that inhibitors with high affinity and slow dissociation would provide long-lasting anti-HIV effect led us to use an approach where interaction kinetic thresholds could be used for hit identification. Although SPR biosensor analysis is still often seen as a low-throughput technique that is useful for detailed characterization of leads and

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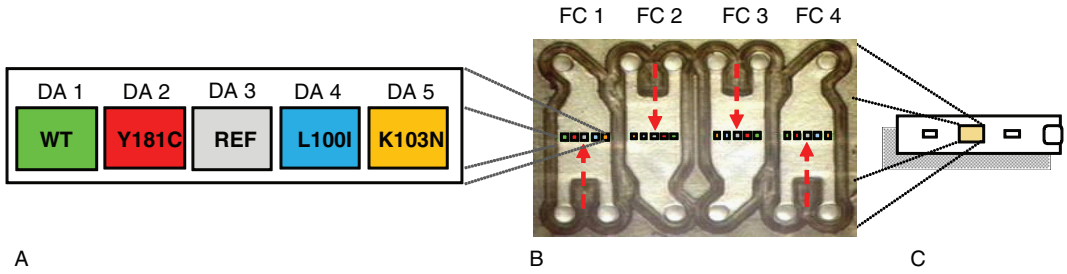

FIG. 1. Position of (**A**) immobilized enzymes and (**B**) layout of sensor surfaces on the (**C**) biosensor chip. The arrows show the flow direction in each individual flow cell.

for secondary screening, it is now emerging as an efficient and cost-effective tool for primary screening of compound libraries.[5] The detailed real-time kinetic information (sensorgrams) that is obtained for each interaction is an advantage of this type of screening, as compared to conventional high-throughput screening (HTS) and equilibrium-based interaction assays (e.g., enzyme inhibition assays).[6] However, because rigorous evaluation of thousands of complete sensorgrams is not a realistic option, mathematical/statistical methods for extracting and simplifying relevant information contained in the sensorgrams are needed for a primary identification of hits. Because such methods have not yet been established, this study developed a novel strategy for the extraction of kinetic information from screening data and for verifying the hits by retrieving the original data representing the complete sensorgrams. This was complemented by a competition assay exploring if hits competed with NNRTIs for binding to the enzyme and by enzyme inhibition and HIV replication assays.

MATERIALS AND METHODS

Enzymes and inhibitors

The wild type and 4 drug-resistant variants (Y181C, K103N, L100I, and double mutant K103N/Y181C) of HIV-1 RT (BH10 isolate) were obtained as previously described.[7,8] The purification procedure was simplified and included only 3 of the separation steps: anion exchange chromatography (Q-Sepharose), affinity chromatography (Heparin Sepharose), and size exclusion chromatography (Superdex®). The enzymes were diluted to at least 0.2 µg/µL in 5 mM HEPES buffer (pH 7.6, 4 mM $MgCl_2$) before freezing.

The NNRTIs, including the references MSC-197 and MIV-170, were synthesized according to published procedures and references cited therein.[9] They were dissolved in 100% DMSO and prepared as stock solutions of 100 µM in 96-well plates.

Biosensor experiments

Experiments were performed with a BIACORE™ A100 instrument (GE Healthcare Bio-Sciences, Uppsala, Sweden) at 25 °C. Reagents and BIACORE™ Series S Sensor Chip CM5 research grade were from GE Healthcare Bio-Sciences.

Immobilization

All HIV-1 RT variants were covalently immobilized to the sensor chip surface by amine coupling at a concentration of 0.2 µg/µL in HEPES buffer with PBS-P (10 mM phosphate buffer [pH 7.4], 2.7 mM KCl, 0.14 M NaCl, and 0.05% surfactant P20) as running buffer. The CM5 surface was activated with a 10-min injection of EDC/NHS (200 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride/50 mM *N*-hydroxysuccinimide). The contact time for the enzyme injection was 1.5 min, and the remaining active succinimide esters on the chip surface were deactivated by an injection of 1M ethanolamine. The 4 enzyme variants were immobilized in the same positions in all 4 flow cells of the instrument (**Fig. 1**): the wild type in detection area (DA) 1, Y181C in DA 2, L100I in DA 4, and K103N in DA 5 (**Fig. 1A**). L100I was substituted by the double-mutant K103N/Y181C in 3 experiments. DA 3 was used as a reference and was only activated and deactivated with EDC/NHS and ethanolamine.

Screening

A library of 800 compounds was obtained in 96-well plates, each containing 80 compounds at a concentration of 100 µM in 100% DMSO. MSC-197 (5 µM) was used as a reference, and running buffer was used as a negative control. Samples were diluted to a final concentration of 5 µM and 5% DMSO in PBS-P, using a MultiPROBE II^{HT} automated liquid-handling system (PerkinElmer, Waltham, MA), except for the controls that were pipetted manually. The plates were immediately sealed and used in the screening.

The samples were injected over the chip surface for 1 min, and dissociation was monitored for 15 min. The flow rate was 30 µL/min. Because regeneration was not possible for all enzyme variants,[7] each cycle ended with a 30-s injection of 15% DMSO in PBS-P to refresh the surface. This was followed by an injection of running buffer to monitor carryover, and finally by an injection of 50% DMSO in purified water to wash

the flow system. Each sensor surface was used for 160 substances (2 plates). Thus, 5 sensor chips were required for the complete set of compounds. The procedure was repeated with the samples injected in reverse order (the order of the 2 plates was switched and the orientation turned 180 degrees), as compared to the original (forward) experiment.

Data analysis

The raw signal versus time data (sensorgrams) from the screening were processed with the BIACORE™ A100 Evaluation 1.0 software (GE Healthcare Bio-Sciences). Nonspecific signals were removed by subtraction of signals from the reference area, and the data were normalized by dividing the signal with the molecular weight of the sample. Corrections for differences in DMSO concentrations between sample and running buffer (bulk refractive index calibration) were not needed because only data from the dissociation phase were used. In this phase, the nonspecific signals that can arise from differences in the DMSO concentration are not seen.

Sensorgrams for the negative control (buffer) were standardized by using a nominal molecular weight of 300 Da. The normalized scale was defined by setting the signals for the negative and the positive controls to 0 and 100 RU, respectively. Report points (i.e., time points at which signal data were extracted) were defined at 5, 40, and 440 s after the end of the injection. To function as an appropriate quantitative measure of complex formation, the E values had to be corrected for changes in the binding capacity of the surface during the course of an experiment. By normalizing the response signal of the sample with respect to the response signals obtained from repeated positive and negative control samples, a corrected E value (E_{corr}) was obtained. Reference subtracted values for the report points and the E_{corr} values for all samples were exported from the BIACORE™ A100 Evaluation software to Excel (Microsoft). A complete data set with all samples from the 10 experiments was then generated using Matlab software (MathWorks, Natick, MA). Spotfire Decision Site-9.1.1 (TIBCO, Palo Alto, CA) was used to get an easily interpreted overview of the data and a combined visualization of compounds relative to the selection criteria.

Competition analysis

Competition between MIV-170 (M. Elinder et al., manuscript in preparation) and 2 hits (MV026340 and MV026119) was analyzed by immobilizing enzymes as described above and injecting MIV-170 for 1 min at a flow rate of 30 µL/min to block the allosteric NNRTI site. The sample was immediately injected for 1 min, and the dissociation was followed for 10 min.

HIV reverse transcriptase inhibition assay

The inhibition of HIV-1 RT activity was analyzed using a scintillation proximity assay (SPA). The incorporation of ^{3}H-dGMP into newly synthesized DNA was measured using a heterogeneous biotinylated RNA-template/DNA-primer and a streptavidin-coated SPA FlashPlate (PerkinElmer Life and Analytical Sciences, Waltham, MA). The reaction was performed in a total volume of 100 µL reaction buffer (50 mM Tris [pH 8.0], 80 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol [DTT], 0.05% Nonidet P40) containing 10 nM RNA oligo (5′-CG UCU GGC AUU GCG AGC GGA UAA CAA UUU CAC ACA GGA AAC AGC UAU GAC-3′) annealed with a biotinylated oligo-DNA (biotin 5′-GTC ATA GCT GTT TCC TG-3′; Eurogentec, Seraing, Belgium), 1.5 nM HIV-1 RT (wild type, L100I, K103N, or Y181C), 100 nM dNTPs (dATP, dCTP, dGTP), 50 nM ^{3}H-dGTP (GE Healthcare Bio-Sciences), and the test compounds in 5-fold serial dilutions from an initial concentration of 10 mM. The final concentration of DMSO was 1% in all wells. The reactions were stopped after 2 h at room temperature by the addition of 100 µL 0.25 M EDTA solution (pH 8.0), and the samples were transferred into FlashPlates. After another 4 h, the incorporated radioactivity was quantified using a liquid scintillation counter (Wallac Microbeta Trilux), and the percent inhibition was calculated using $[1 - (CPM_{compound} - CPM_{background})/(CPM_{enzyme} - CPM_{background})] \times 100$, where CPM stands for counts per minute.

Viral replication inhibition assay

Inhibition of HIV replication was measured in MT4 cells, a human HTLV-I-transformed T cell line obtained from Harada and Yamamoto.[10] The wild-type virus strain was HIV-1_{IIIb} (R.C. Gallo), and resistant virus containing the L100I, K103N, or Y181C amino acid substitutions was isolated by in vitro selection from HIV-1_{IIIb} replicating in the presence of increasing concentrations of NNRT inhibitors.

Anti-HIV replication activity was measured in a 96-well microplate in which 2×10^4 MT4 cells/well were infected with 20-50 TCID (tissue culture infectious dose) of wild-type or mutant HIV-1_{IIIb}. The test compounds were dissolved to a concentration of 10 mM in DMSO and further 5-fold serially diluted to a lowest concentration of 128 nM in RPMI medium (GIBCO, Carlsbad, CA) containing 10% fetal calf serum (FCS) and added to the plates. After 5 days of incubation in 37 °C and 5% CO_2, the cell viability was determined using the XTT formazan assay.[11] EC_{50} values were calculated by comparing the viability in treated HIV-infected cells with uninfected, untreated control cells.

RESULTS

Experimental design and strategy for data analysis and hit selection

An experimental design for the parallel screening of a compound library against 4 different variants of HIV-1 RT was devised. The method was set up so that the consumption of compounds and enzyme, as well as the time for screening, was

minimized. The screening was divided into 2 series with 5 separate experiments, each monitoring 640 interactions. The second screening series was performed with the compounds in a different order to that in the initial experiment. Each experiment (160 compounds) took 18 h and consumed 42 µg of each enzyme variant (10.5 µg per detection area) and 64 to 302 ng of compound, depending on the molecular weight. A total of 10*42 µg of each enzyme variant was thus used for the complete forward and reverse screening of 800 compounds.

A new biosensor chip was used for each experiment. The 4 enzymes were immobilized in the same order and position in each of the 4 flow cells, leaving the central detection area (DA 3) as a reference surface (**Fig. 1A**). The chip formed 4 flow cells, each comprising 5 detection areas (**Fig. 1B**). Immobilization levels varied between 13,000 and 19,000 RU.

The complete screening of 800 compounds against 4 targets in duplicate generated a total of 6400 sensorgrams for the compounds and 1280 sensorgrams for the controls. To enable an efficient analysis of this large amount of data, we designed a data reduction method based on a strategy in which the information contained in a sensorgram was reduced into a unique combination of 2 values.

Interaction kinetic variables (E, DR)

Each sensorgram was translated into 2 numerical values by extracting the response signal at 3 defined time points (report points) in the dissociation phase (**Fig. 2**). The first report point (E, early), taken 5 s after the end of the injection, represents the amount of enzyme-inhibitor complex formed within the injection time. The 2 subsequent report points, taken 40 and 440 s after the end of the injection, were combined into a dissociation ratio (DR = $response_{440s}/response_{40s}$) that represents the dissociation rate. The 2 variables E and DR describe the basic kinetic characteristics of the interaction between a compound and the enzyme and formed the basis for the subsequent data analysis and evaluation. A high E value is obtained for interactions that result in a high complex concentration on the sensor chip at the end of the injection, whereas a DR value close to 1 indicates slow dissociation of the complex. Sensorgrams with negative values for any of the 3 report points (60%) were excluded from the data set *before* DR was calculated for 2 reasons: First, sensorgrams with negative report point values represented interactions where the compound did not bind to the target enzyme and hence were of no interest. Second, the DR for 2 negative values would give a misleading positive value. A numerical data set containing all interactions with positive report point values was thus obtained, with each sensorgram represented by the 2 values for E_{corr} and DR.

Kinetic thresholds for hit identification

The strategy for hit identification was based on the hypothesis that a high affinity combined with a slow dissociation rate would be a suitable interaction profile for a microbicide based on NNRTIs. In addition, for the compounds to be efficient also against resistant viral strains of clinical importance, they should exhibit these interaction characteristics for the complete panel of enzymes. A desired interaction profile was hence defined with 2 kinetic thresholds: DR ≥ 0.8, based on the reasoning that a ratio of 0.8 to 1 represents very slow (k_{off} ~$5.3*10^{-4}$ s^{-1}) to no dissociation, and E_{corr} ≥ 100 RU, which equals the normalized value for the affinity of the positive control MSC-197.

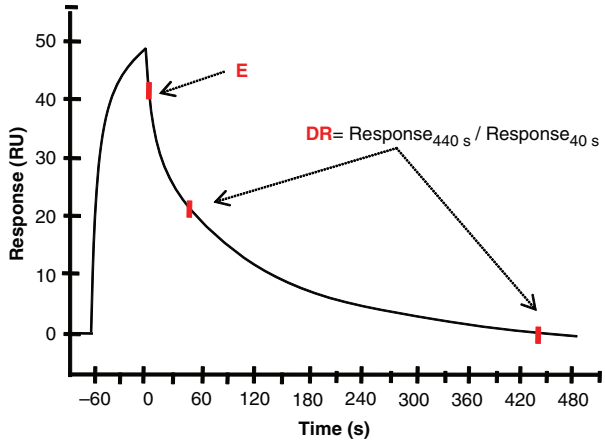

FIG. 2. Schematic illustration of a simulated sensorgram showing the positioning of the report points 5 s (E), 40 s, and 440 s after the end of the 60-s injection. The last 2 report points form the basis for the dissociation ratio (DR).

DR values higher than 1 were sometimes observed, although theoretically not possible. Ratios *slightly higher* than 1 can be attributed to compounds that bind to and slowly dissociate from the reference detection area, resulting in a positive slope for the reference-subtracted dissociation signal. Compounds with ratios *substantially higher* than 1 are likely to be erroneous, and the sensorgrams of these compounds were therefore visually inspected and discarded, unless the invalid value could be attributed to experimental errors (e.g., local signal spikes).

Screening

Identification of hits to individual enzyme variants. The screening and evaluation procedure identified 720 compounds in 3211 interactions with positive report point values (5 s, 40 s, and 440 s). The E_{corr} and DR values for all identified compounds were displayed in a hit identification plot (**Fig. 3**) where the kinetic thresholds (E_{corr} ≥ 100 RU and DR ≥ 0.8) are shown as solid (blue) lines. The plot illustrates how the 2 thresholds help in identifying 28 interactions, corresponding to 12 compounds (1.5% of all screened substances), as hits to individual enzyme variants. The sensitivity of 0.8 as a hit threshold was analyzed by lowering DR to 0.6 (k_{off} ~$1.3*10^{-3}$ s^{-1}), which resulted in the identification of an additional 18 compounds, that is, a total of 30 compounds (3.8% of all screened substances) in 56 interactions.

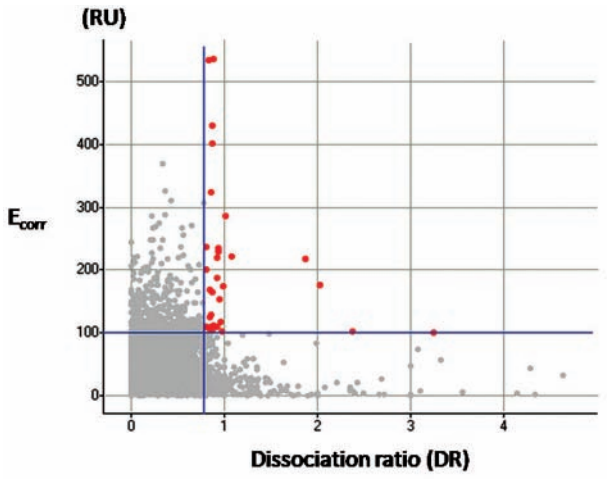

FIG. 3. Identification of hits for individual enzyme variants. Interaction kinetic variables (E_{corr} and DR) for all samples (3211) with positive report point values. The 28 samples in red are those selected on the basis of the hit identification thresholds (solid blue lines) for E_{corr} (100 RU) and DR (0.8).

Identification of hits to multiple enzyme variants. Resilience to amino acid substitutions in the NNRTI binding pocket is an important property for NNRTIs that should be used in HIV microbicides. Therefore, the 12 compounds that had been identified as hits to single-enzyme variants were only identified as *final hits* if the interactions with *all 4 enzyme variants passed the kinetic thresholds*. An evaluation showed that 2 compounds (17%) of hits to individual enzyme variants passed the kinetic thresholds for all 4 enzyme variants, 3 compounds (25%) were hits to ≥3 enzyme variants, and 4 compounds (33%) were hits to ≥2 enzyme variants. A sensitivity analysis showed that the same final hits were obtained even when the threshold value for DR was reduced to 0.6.

To get an easily interpreted overview of the data set, we plotted E_{corr} versus DR for the 12 compounds identified as hits to individual enzyme variants and organized them as panels in a larger plot (**Fig. 4A**). The 12 compounds could thus be visualized simultaneously and evaluated on the basis of all 3 hit identification variables: the 2 kinetic thresholds and the interaction with multiple-enzyme variants.

Because each compound was screened twice against the 4 enzyme variants, each panel of the plot ideally contained 8 hits, represented as different shapes referring to the target enzyme (**Fig. 4A**). All compounds that were identified as hits to all 4 enzyme variants, either only in one or in both experiments, merited further analysis. Two structurally different compounds, the thiourea derivative MV026340 (**Fig. 5A**) and the urea derivative MV026119 (**Fig. 5B**), were identified from the overview plot as forming a stable complex with all 4 enzyme variants. The plot showed that both compounds fulfilled the identification criteria for 7 of 8 interactions (**Fig. 4B**). Consequently, each substance failed to pass one or both thresholds for one enzyme variant in one of the experiments. For MV026340, the disqualifying factor was a DR of 0.78 when interacting with L100I in the forward screening, whereas for MV026119, it was an E_{corr} of 85 when interacting with the wild type in the forward screening (shaded in gray in **Fig. 4B**).

Visual inspection of the sensorgrams confirmed that both identified compounds exhibited interaction profiles with a high relative response at the end of the injection (high affinity) and slow dissociation for all 4 enzyme variants, both in the forward and reverse experiment (**Fig. 5C-D, E-F**). However, the 2 compounds showed marked differences in their association phases.

A sensitivity test for use of the DR ($response_{440s}/response_{40s}$) value in hit identification was performed by observing if a small change in position of one of the report points would result in identification of different hits. Four alternative DR values were tested: $response_{420s}/response_{40s}$, $response_{460s}/response_{40s}$, $response_{440s}/response_{20s}$, and $response_{440s}/response_{60s}$. The same hits against multiple-enzyme variants were identified irrespective of the DR values used.

Competition analysis

A compound that binds equally well to all 4 enzyme variants either is insensitive to changes in the NNRTI binding pocket (containing residues 100, 103, 181) or binds to another site. To explore if MV206119 and MV026340 compete with NNRTIs, we performed a competition analysis in which the allosteric site was occupied by the tight binding NNRTI MIV-170 (**Fig. 6**; M. Elinder et al., manuscript in preparation). The signal did not increase when MV026340 was injected over the enzyme with the blocked NNRTI site (**Fig. 7**), indicating direct competition between MV026340 and MIV-170. In contrast, injection of MV026119 (**Fig. 7**) resulted in a signal of approximately 40 RU, irrespective if MIV-170 had been injected prior. Thus, MV026119 and MIV-170 did not compete for binding to the enzyme, indicating that MV026119 did not bind to the NNRTI binding pocket.

Enzymatic assay

To verify that MV026340 was an inhibitor of HIV-1 RT, we determined the effect of the compound on the enzymatic activity of HIV-1 RT. MV026340 was confirmed to be an inhibitor and had lower IC_{50} values for all tested enzyme variants than efavirenz, nevirapine, and MV026119, the latter showing no inhibition, confirming the conclusion from the competition experiment (**Table 1**).

Inhibition of wild-type and mutant HIV-1 in MT4 cells

MV026340 was found to be a potent inhibitor of replication of both the wild-type virus and the 3 mutant strains (**Table 1**).

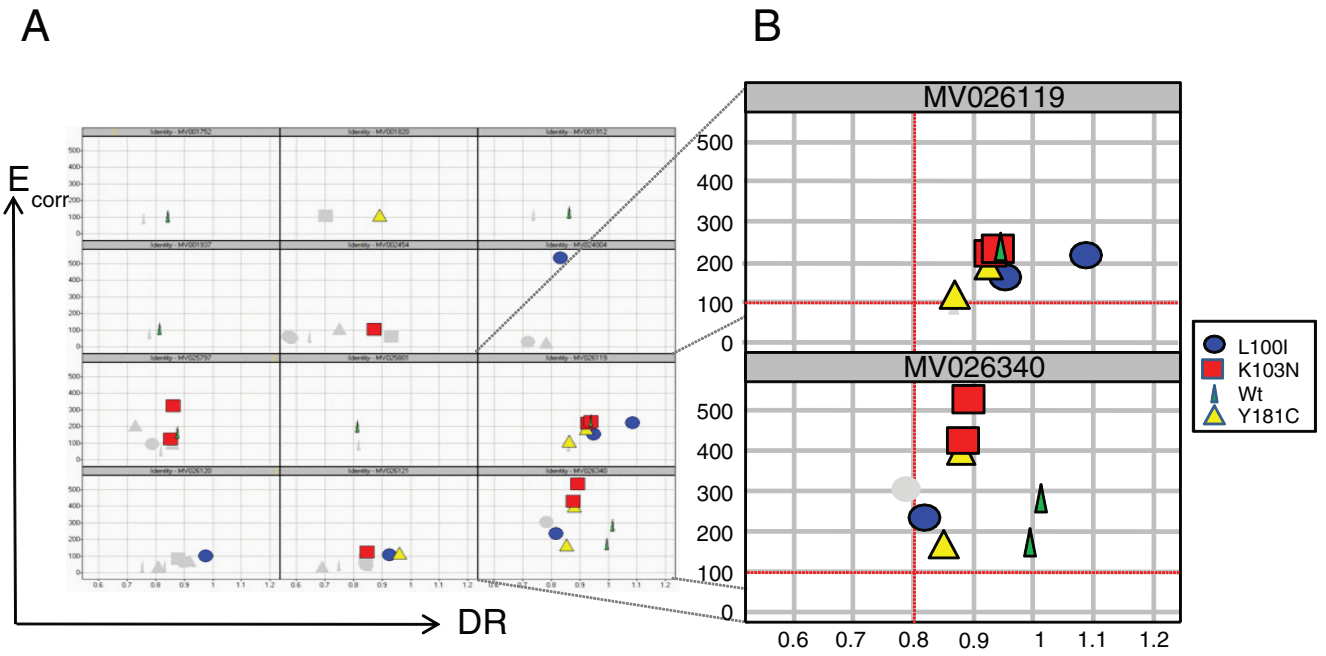

FIG. 4. Identification of hits against multiple-enzyme variants: (**A**) overview plot with all 12 compounds identified as hits to individual enzyme variants and (**B**) close-up of MV026340 and MV026119, the compounds interacting with all 4 enzyme variants. Hit identification thresholds are marked with dotted lines. Shaded shapes represent samples that failed to pass the hit thresholds.

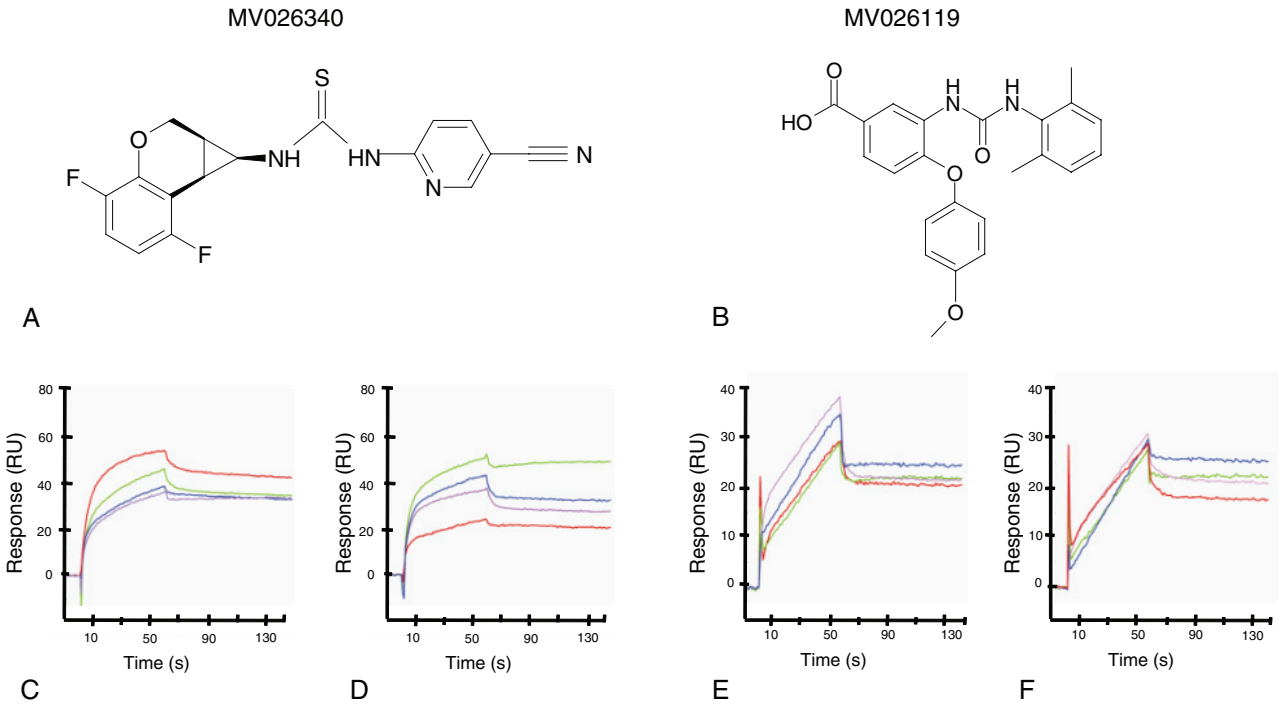

FIG. 5. Sensorgrams and structures for the (**A**) thiourea derivative MV026340 and (**B**) the urea derivative MV026119. The 4 HIV reverse transcriptase (RT) variants (L100I, blue; wild type, green; Y181C, violet; and K103N, red) in the forward (**C**, **D**) and reverse (**E**, **F**) screening series. Curves are corrected with respect to different immobilization levels, but because the responses are not surface activity adjusted, they may appear low.

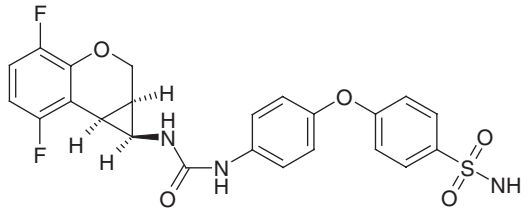

FIG. 6. Structure of MIV-170, a tight-binding nonnucleoside reverse transcriptase inhibitor (NNRTI).

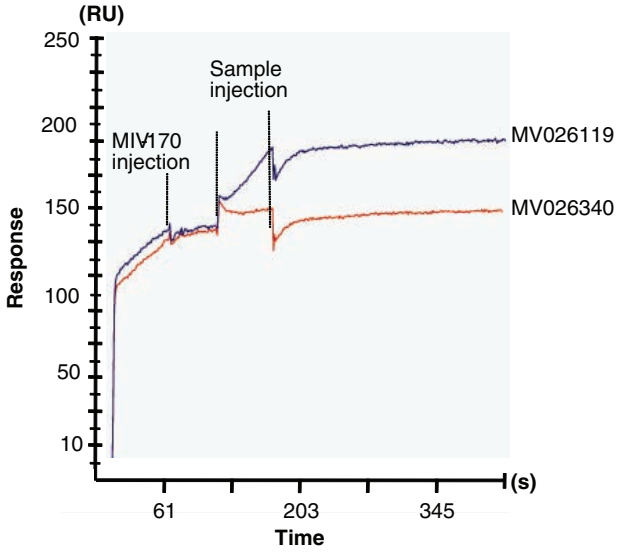

FIG. 7. Sensorgram showing the different interactions of MV026119 (blue) and MV026340 (red) with wild-type HIV-1 reverse transcriptase (RT) after blocking of the nonnucleoside reverse transcriptase inhibitors (NNRTI) pocket with MIV-170.

For the mutants L100I and K103N, the EC_{50} values were substantially lower than for the clinically approved NNRTI control substances efavirenz and nevirapine. For the wild type and the Y181C mutant, MV026340 showed substantially lower values than nevirapine and similar values as efavirenz. MV26199 showed no inhibition toward the wild-type virus and was not tested against the resistant viral strains.

DISCUSSION

The method developed for screening for HIV-1 RT inhibitors was based on the hypothesis that a certain interaction kinetic profile could be used as criteria for identifying compounds of interest. In the present study, the ideal interaction profile was defined as one with high affinity combined with slow dissociation. This definition was based on the overall aim to identify NNRTIs suitable for use in an HIV microbicide and, more specifically, our hypothesis that effective binding combined with slow dissociation would result in a long-lasting protective effect of a microbicide. In view of the problems with resistant HIV, it was also considered important that the hits showed similar interaction profiles against both wild-type and variants of the enzyme resistant to clinically used NNRTIs.

Screening strategy

The design of a screening strategy is a multivariate problem that aims at identifying hits in a robust and efficient manner. In the present study, the strategy was based on a duplicate screening with the samples in reverse order to minimize the risk of false positives and negatives. This is a particularly suitable strategy for screening with a method that uses SPR for detection and direct binding of samples to immobilized targets, where carryover and blocked surfaces can give rise to misleading results. The experimental setup with a 15-min dissociation time minimized problems with slowly dissociating compounds and lack of regeneration. The throughput and enzyme consumption is dependent on the number of compounds that can be screened with the same sensor chip. In the current study, 160 compounds were analyzed on each chip, but with an effective surface regeneration, this number could be increased substantially. Despite the relatively large number of chips (5) used in this study, only about 200 µg of each enzyme variant was consumed.

A high number of false positives is a well-known problem for many conventional hit discovery approaches[12] and should be avoided if possible. The same obviously applies to false negatives, but the reasons behind the 2 problems are unrelated. An advantage of the procedure used in this study is that the thresholds for hit identification can be defined to match the aim of the screening. High hit thresholds result in fewer hits but also entail a higher risk for false negatives and vice versa. It is a fine balance that needs to account for the number of hits that can be brought forward into the next stage of the drug discovery process and for the chances of identifying novel scaffolds rather than a series of analogs.

Identification of hits in SPR-based screening is typically based on the response signal reached at a defined sample concentration (e.g., as described by Nordström et al.).[13] In the present study, we have refined the hit identification criteria into a combination of 3 identification criteria: response magnitude (E_{corr}) as a measure of affinity, dissociation rate (in the form of DR), and the requirement of binding to all of 4 selected enzyme variants. This methodology resulted in 2 hits in the library used (i.e., a hit rate of 0.25%). This can be compared to using a single hit threshold of ≥100 RU for E_{corr}, which would have resulted in the identification of 139 hits, a hit rate of 17%. Even when the hit threshold of ≥100 RU for E_{corr} was combined with the requirement of binding to all 4 enzyme variants, it resulted in a high hit rate.

The rationale behind choosing MSC-197 as a positive control was that the substance shows similar interaction profiles to all 4 mutant enzymes as the clinically used NNRTIs,

Table 1. Inhibition of the Enzymatic Activity of 4 HIV-1 RT Variants (IC_{50}) and Inhibition of the Replication of Wild-Type and 3 Mutant Strains of Virus in MT4 Cells (EC_{50})

	IC_{50} (nM)				*EC_{50} (nM)*			
	Wt	*L100I*	*K103N*	*Y181C*	*Wt*	*L100I*	*K103N*	*Y181C*
Efavirenz	7.5	340	340	35	1.6	88	20	3.9
Nevirapine	1900	ND	180,000	ND	170	1200	>10,000	>10,000
MV026119	>5000	ND	>5000	ND	>10,000	ND	ND	ND
MV026340	5.7	5.2	8.7	16	2.5	7.1	6.5	8.2

ND, not determined; RT, reverse transcriptase.

efavirenz and delavirdine, do with the wild-type enzyme.[7] At the concentration used in this screening (5 μM), MSC-197 binds ≥80% to both the wild-type and all 3 enzyme variants, making the normalized positive control response (100 RU) a reasonable threshold value for E_{corr}.

Report points

To extract data at 3 report points and let these variables represent each interaction served as an efficient and accurate method to make the extensive data set easier to evaluate. Some information retained in the original sensorgrams is temporarily ignored, but the information contained in the report points is sufficient for hit identification. The report points were positioned to give the information needed to identify compounds that corresponded to the defined kinetic interaction profile. Once a number of hits had been identified, the sensorgrams for these compounds could be retrieved and further analyzed. In other words, the extraction of report point data from each interaction only functioned as a filter to enable an efficient selection of compounds whose kinetics were considered worth analyzing more in detail.

There is an advantage in using report points 40 s and 440 s after the start of the dissociation phase, in comparison to extracting data from time points closer to the end of the injection, because these later report points are less influenced by rapid secondary interactions, which in most cases have dissociated during the start of the dissociation. The identification of 2 hits whose sensorgrams showed the desired interaction kinetic profile confirmed that the identification strategy could be used with success, and a sensitivity analysis of the exact positioning of the report points showed that small changes in choice of time did not alter the final hit identification. Correction for differences in DMSO concentrations between sample and running buffer was not needed using this evaluation method because only data from the dissociation phase were used.

Multivariate visualization

By visualizing the screening data in a plot with E_{corr} versus DR, the evaluator could instantly view the effect of a change in hit identification thresholds on the number of hits. Furthermore, it facilitated a flexible interpretation of the data relative to the hit identification criteria, something that can be of value when a definite and optimal threshold value is lacking and the limitation is the number of compounds that can be brought further in the drug discovery process.

Anomalies in the data set could easily be recognized in the plot, and a visual inspection of the sensorgrams behind these patterns could confirm or reject the suspicions. Making a plot with many panels representing E_{corr} versus DR for individual compounds (**Fig. 4**) facilitated evaluation of 3 variables at the same time: 2 threshold variables and hits toward multiple enzymes. A fourth variable would be possible to visualize in the same type of plots by using different colors of the symbols. A look at the sensorgrams for the 2 final hits revealed a marked difference in the association kinetics, which could be added as a fourth selection variable.

The hits

Of 800 compounds, we identified 2 structurally unrelated compounds (MV026340 and MV026119) that exhibited the defined interaction kinetic characteristics: high affinity and slow dissociation with all 4 enzyme variants. A compound that binds equally well to all 4 variants of HIV RT is either insensitive to changes in the NNRTI binding pocket or interacts with another site. In the case of the urea derivative MV026119 (406 Da), the competition experiment indicated that the substance interacts unspecifically with the enzyme. The lack of inhibitory effect on purified enzymes as well as on viral replication in MT4 cells supports that the compound interacts in a nonproductive manner with the protein.

When one of the prerequisites for a hit is interaction with several resistant variants of an enzyme, there will be an inherent bias toward identification of compounds that bind outside the site where the mutations are located. The identification of MV026119 is an example of this. If specificity to a particular site is required, this bias increases the risk for false positives; however, if there is a search for novel binding sites, this could be an efficient strategy. The magnitude of the problem may also vary depending on how focused the compound library is

because the risk of false positives is lowered with a focused library. A competition screening as implemented by Nordström et al.[13] and a structure-based biophysical analysis as described by Huber[5] are rapid ways to verify target site specificity for many substances.

For the thiourea derivative MV026340 (358 Da), it can be speculated that the resilience to mutations stems from an interaction with conserved residues within the NNRTI binding site. When the compound is co-crystallized with HIV-1 RT (T. Unge, personal communication, 2008), it is found to bind to the NNRTI pocket, which is consistent with the competition analysis. This makes MV026340 the most interesting hit, a view that is strengthened both by enzyme assay data and cell inhibition data.

NNRTIs are known to have low thresholds against viral resistance and could hence rapidly become inefficient in preventing HIV transmission. Because topical microbicides are nonsystemic, the risk for development of resistance is reduced. Still, NNRTIs used in microbicides should nevertheless have a high threshold for resistance development. The mechanisms behind resistance to NNRTIs are diverse and not yet fully elucidated, but factors such as reduced interactions with Tyr181 and ability to undergo conformational changes within the mutated drug site may contribute to improved resistance profiles.[14] Early screening against a panel of clinically relevant enzymes can facilitate the identification of inhibitors such as MV026340 with improved resistance profiles.

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