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Inhibition of HIV-1 by non-nucleoside reverse transcriptase inhibitors via an induced fit mechanism—Importance of slow dissociation and relaxation rates for antiviral efficacy

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ABSTRACT

The importance of slow dissociation of non-nucleoside reverse transcriptase inhibitors (NNRTIs) for antiviral effect has been investigated. The kinetic characteristics of a series of NNRTIs interacting with wild type and drug resistant variants of HIV-1 RT (EC 2.7.7.49) were analyzed by SPR biosensor technology. The antiviral effect was determined in MT-4 and peripheral blood mononuclear cells. Due to extremely slow dissociation rates and a complex interaction mechanism, rate constants could not be quantified. Instead, interaction characteristics were qualitatively analyzed using simulated sensorgrams. The simplest model describing these interactions adequately was an induced fit mechanism, *i.e.* a mechanism involving the formation of an initial enzyme-inhibitor complex subsequently transformed into a more stable complex. Differences in rates of dissociation from the initial complex and rates of relaxation from the induced complex explained (1) the differences in the amounts of formed complex, (2) the stability of the complex and (3) the antiviral efficacies of the compounds. The effect of NNRTI binding site mutations also correlated with these kinetic characteristics. MIV-170 was the most effective inhibitor of wild type and mutant HIV-1 in cell culture, a property that was associated with the formation of the largest amount of complex and the slowest relaxation and dissociation rates. This study supports the hypothesis that the efficacy of anti-HIV drugs is dependent on slow dissociation from the target, thereby maximizing the duration of the inhibitory effect. It also illustrates the strength of simulating interaction data for qualitative analysis of tight-binding drugs and the importance of resolving the kinetic mechanism of drug–target interactions.

1. Introduction

The continued spread of HIV is a major threat to individuals and societies in many parts of the world and problems with resistance development due to the therapeutic use of current non-nucleoside reverse transcriptase inhibitors (NNRTIs) are increasing. All attempts to prevent transmission by the use of microbicides or vaccines have failed to date [1,2]. Many clinically evaluated microbicides lack preventive effect, which is not too surprising considering their non-specific mode of action, low potency, high protein binding and unselective irritating properties.

In the design of vaginal microbicides acting on a specific target, NNRTIs are the most promising compounds as active anti-HIV ingredients for a number of reasons [3]. These compounds are at least 1000-fold more efficient in inactivating virus than acid polymers, and much more efficient than nucleoside or nucleotide

Abbreviations: AIDS, acquired immunodeficiency syndrome; Ba-L, bronchoalveolar lavage; DA, detection area; DMSO, dimethylsulfoxide; DLV, delavirdine; EDC, 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide; EFV, efavirenz; HIV-1, human immunodeficiency virus type 1; MOI, multiplicity of infection; IL-2, interleukin-2; NHS, N-hydroxysuccinimide; NNRTI, non-nucleoside reverse transcriptase inhibitor; NVP, nevirapine; PBMC, peripheral blood mononuclear cell; PETT, phenylethylthioazolylthiourea; PHA, phytohaemagglutinin; RT, reverse transcriptase; RU, resonance unit; SPR, surface plasmon resonance.

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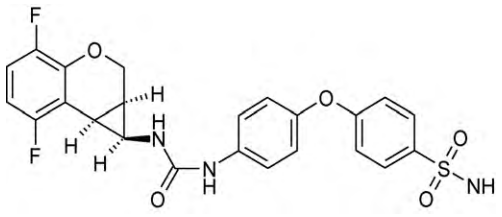

Fig. 1. Structure of MIV-170.

analogues. In addition, some NNRTIs may have the advantage of inhibiting both free virions and preventing infection of target cells by binding tightly to the reverse transcriptase [4]. Nevertheless, the usefulness of an NNRTI as a microbicide is expected to be dependent on its ability to rapidly and completely inactivate both wild type and mutant virus at a low concentration. MIV-170 (Fig. 1) is a novel high affinity NNRTI that we have hypothesized to possess such characteristics and that consequently may have the potential to be used in a topical microbicide for prevention of HIV-1 transmission. A rapid metabolism of MIV-170 in human hepatocytes makes the compound less suitable for oral use (unpublished observations).

To establish the characteristics of MIV-170 with respect to its interaction with its target, we have analyzed the interaction kinetics of the compound with wild type and mutant HIV-1 RT using a surface plasmon resonance (SPR) biosensor. We have previously used this technology to characterize the interaction mechanism for NNRTIs with their target and to determine the resistance profiles of NNRTIs [5–7]. Due to the tight-binding interaction of the clinically used NNRTIs, the initial characterization was performed with a drug resistant variant from which the compounds dissociated. In subsequent studies, we have also used a biosensor-based approach to identify novel scaffolds and leads for design of anti-HIV drugs or microbicides by screening both fragment and focused libraries for compounds with suitable kinetic characteristics and resistance profiles [8,9].

The present study was specifically designed to investigate how MIV-170 compared to the clinically used NNRTIs efavirenz, nevirapine, and delavirdine, as well as to TMC-120 (dapivirine), an NNRTI with in vitro anti-HIV properties superior to those of the compounds used in the clinic today [10]. Since efficacy against drug resistant virus is essential, the study involved analysis of the interaction with wild type RT and K103N, Y181C, L100I mutants, as well as of the inhibition of replication of the corresponding viral strains in cell culture. In this study we were particularly interested in identifying the interaction kinetic parameters that correlate with a long lasting antiviral effect, hypothesized to be primarily an effect of slow dissociation. We therefore also determined the effect of the different compounds on the inhibition of viral replication in two types of cell culture, using both MT-4 and peripheral blood mononuclear cells (PBMCs).

2. Materials and methods

2.1. Enzymes and inhibitors

The wild type and three drug resistant enzyme variants (Y181C, K103N and L100I) of reverse transcriptase (EC 2.7.7.49) of HIV-1 (BH10 isolate) were obtained as previously [5,11] described. All enzymes contained the additional substitution E478Q to abolish the RNase H activity. The purification procedure was simplified and only included three of the separation steps: anion exchange chromatography (Q Sepharose), affinity chromatography (Heparin Sepharose) and size exclusion chromatography (Superdex®). All chromatography media was from GE Healthcare Bio-Sciences, Uppsala, Sweden. The enzymes were diluted in 5 mM Hepes buffer (pH 7.6, 4 mM $MgCl_2$) to a final concentration of 5 μg/μL.

The NNRTIs (MIV-170, delavirdine, efavirenz, nevirapine and TMC-120) were synthesized according to published procedures and references cited therein [12]. They were dissolved in 100% DMSO (Riedl-de-Haën, Seelze, Germany) and prepared as 20 mM stock solutions in 96-well plates. For the viral replication assay in PBMC cultures, efavirenz, delavirdine and nevirapine were obtained through the NIH AIDS Research and Reference Reagent Program (Germantown, USA). TMC-120 was kindly provided by Tibotec BVBA, Mechelen, Belgium. Stock solutions of 10–50 mM were made in 100% DMSO. All compounds were used at non-toxic concentrations as determined by WST-1-tetrazolium based assays (data not shown).

2.2. Biosensor experiments

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

2.2.1. Immobilization and methodology

The four enzyme variants were immobilized in parallel detection areas (DAs) on the sensor chip surface with standard amine coupling chemistry [9]. The enzymes were immobilized in the same positions in all four flow cells of the instrument: the wild type in DA 1, Y181C in DA 2, L100I in DA 4 and K103N in DA 5, as illustrated previously [9]. DA 3 was used as a reference and was only activated and deactivated. The enzymes were injected at a concentration of 0.2 μg/μL with a contact time of 1.5 min. PBS-P (10 mM phosphate buffer pH 7.4, 2.7 mM KCl, 0.14 M NaCl and 0.05% surfactant P20) was used as running buffer.

Since the protein surface could not be regenerated after injection of the NNRTIs, each surface was only used once and each chip could consequently only be used for analysis of four compounds. Compounds were injected in parallel in the four different flow cells, each containing the same panel of immobilized enzymes. Five different NNRTIs were tested in duplicate and one of them was injected on all three chips (*i.e.* one time extra as a control) in order to enable comparisons of the data from the different chips. Each NNRTI was injected at 5.12 μM over the surface at a flow rate of 30 μL/min. The injection time was 1 min and the dissociation was monitored for 10 min, but only the first 3.5 min was used in the analysis. The running buffer included 3% DMSO and was used as negative control, injected once before and once after the sample.

2.2.2. Processing of data

The raw signal *vs.* time data (sensorgrams) from the screening was processed with the BIACORE™ A100 Evaluation 1.0 software (GE Healthcare Bio-Sciences, Uppsala, Sweden). The signals are reported in resonance units (RU). Non-specific signals were removed by subtraction of signals from the reference area. The data was normalized by dividing the signal by the molecular weight of the sample and then multiplied by 100 in order to get a scale from 0 to 100. Corrections for differences in DMSO concentrations between sample and running buffer (bulk refractive index calibration) were also performed. Since the five inhibitors were tested over different chip surfaces, the sensorgrams had also to be corrected for differences in immobilization levels. This was done using the BIAevaluation Software 4.1 (GE Healthcare Bio-Sciences, Uppsala, Sweden) by dividing the obtained signal by the immobilization level in RU, multiplied by a factor of 10 000 in order to get a scale in the same order of magnitude as the original data. Finally, the signal for a negative control was also subtracted.

2.2.3. Simulation of sensorgrams

Sensorgrams were simulated according to the principles and equations used in the simulation module of BIAevaluation software 4.1 (GE Healthcare Bio-Sciences, Uppsala, Sweden). Here, the model was defined as in Fig. 3 and the rate equations as: $dE_1I = (k_1 \times I \times E_1 + k_4 \times E_2I) - (k_2 \times E_1I + k_3 \times E_1I)$; $dE_2I = (k_3 \times E_1I) - (k_4 \times E_2I)$ and the simulations were performed with MatLab (The MathWorks, Inc., Natick, MA, United States).

2.3. Viral replication inhibition assays

Inhibition of HIV replication was measured in PBMC (kindly provided by the Antwerp Blood Transfusion Centre, Belgium) as a representative in vivo target of HIV-1 and in MT-4 cells as previously described [9]. The virus strain for the MT-4 assay was HIV-1IIIb (R.C. Gallo) and resistant virus containing the L100I, K103N or Y181C amino acid substitutions were isolated by in vitro selection from HIV-1IIIb, replicating in the presence of increasing concentrations of NNRTIs.

For the PBMC analysis, a wild type HIV-1 subtype B strain (Ba-L) was used. The inhibition was determined by pre-incubating 50 μL of the compound with 50 μL of cell-free virus at 10^{-3} MOI in a transparent 96-well plate at 37 °C and 5% CO_2 for 30 min. The compound had been prepared as a serial dilution in interleukin-2 (IL-2) medium (RPMI 1640 medium) containing 15% heat-inactivated fetal bovine serum (FBS) and 50 mg/mL gentamicine (Lonza, Verviers, Belgium) supplemented with 1 ng/ml IL-2 (Gentaur, Brussel, Belgium), 2 μg/mL polybrene (Sigma–Aldrich, Bornem, Belgium) and 5 μg/mL hydrocortisone (Calbiochem, Leuven, Belgium), with delavirdine and nevirapine used at 50 000–10 000–1000–100–10–1 nM, and TMC-120, MIV-170 and efavirenz used at 10000–1000–100–10–1–0.1 nM. Seventy five thousand PHA/IL-2 stimulated PBMCs (cultured for 48 h in RPMI-1640 containing 10% FBS and 50 mg/ml gentamicin supplemented with 2 μg/ml phytohemagglutinin (PHA) (Remel, Kent, United Kingdom) following activation for 24 h in IL-2 medium) in 100 μL IL-2 medium were then added to each well and incubated for 24 h. Subsequently, excess virus was removed and the cells were resuspended in fresh IL-2 medium with the same concentration of compound as used in the incubation. Medium and compound were refreshed once more after one week of culture. On day 14, the p24 antigen concentration in the supernatant of these cultures was measured using an in-house ELISA [13]. The experiment contained six replicates and was performed three times using PBMCs from different donors.

The compound mediated-inhibition of viral replication (as the percentage of the value for the untreated control) was plotted against the compound concentration. Non-linear regression analysis was performed using GraphPad Prism 5.02 (GraphPad Software, Inc., La Jolla, USA). The results were expressed as EC_{50} values, defined as the concentration of drug at which there was 50% viral replication as compared to the drug-free control.

3. Results

3.1. Interaction analysis

The immobilization levels for the enzyme variants varied between 17 300–18 500 RU for the wild type, 5400–14 400 RU for Y181C, 12 300–14 900 RU for L100I and 17 500–18 900 RU for K103N. All tested NNRTIs formed stable complexes with the proteins and regeneration of the sensor surface could therefore not be achieved without damaging the enzyme. Hence, only one concentration of each inhibitor could be injected over each enzyme surface. Since data from a series of concentrations of each inhibitor is needed for a reliable determination of maximal binding levels and kinetic constants, an experimental design that reduced the consumption of chips but still provided an adequate data set for qualitative kinetic information to be extracted from single compound injections was devised. A total of 11 injections were required for two replicates of each compound with one of these serving as a reference sample in another experiment, thus consuming 3 (2 3/4) chips. The total consumption of each enzyme variant was 115.5 μg (10.5 μg per detection area, *i.e.* 42 μg per full chip.)

The sensorgrams in Fig. 2 show that all of the studied inhibitors except nevirapine formed stable complexes with the wild type enzyme. The sensorgrams differed qualitatively with respect to the signal level at the end of the injection (*i.e.* at 60 s) and after dissociation (*i.e.* at 200 s), as well as to the slope of the sensorgram during the dissociation phase, seen most easily at the end (*e.g.* 150–

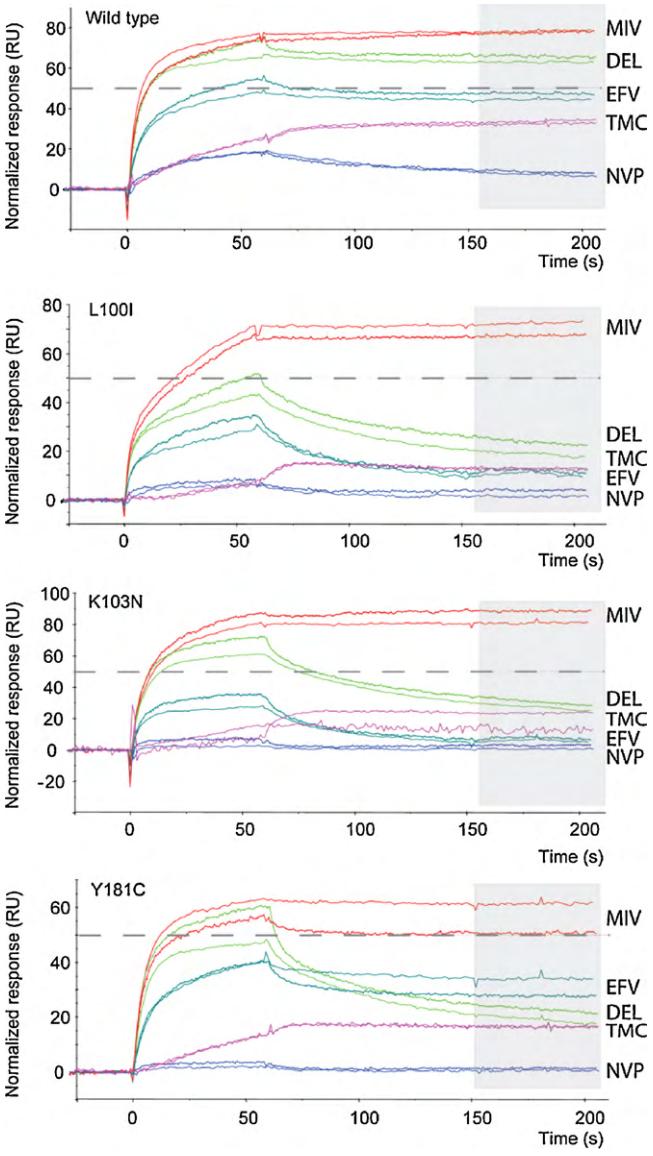

Fig. 2. Interaction profiles for 5.12 μM MIV-170 (MIV, red), delavirdine (DEL, green), efavirenz (EFV, cyan), TMC-120 (TMC, violet) and nevirapine (NVP, blue) with four different variants of HIV-1 RT (WT, K103N, Y181C and L100I). Sensorgrams from the two replicate experiments are shown overlaid. They are reference subtracted, solvent corrected, molecular weight adjusted and normalized with respect to the immobilization levels. The grey horizontal dashed lines and the grey boxes are inserted to facilitate visual analysis of the data. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

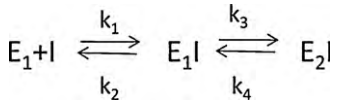

Fig. 3. The scheme for an induced fit interaction mechanism. E_1 represents the free enzyme in its low energy ground state, I the free inhibitor, E_1I the enzyme-inhibitor complex in its high energy state and E_2I the low energy induced complex. The rate constants are designated k_1, k_2, k_3 and k_4.

200 s, shaded area). The sensorgrams for the two replicates for each compound were very similar. For most of the inhibitors, the kinetics of the interactions (primarily seen in the dissociation phase) was significantly influenced by single amino acid substitutions in the NNRTI binding pocket. MIV-170 was most resilient to these changes.

Due to the complex interaction and slow dissociation between HIV-1 RT and the NNRTIs [5], regression analysis using a 1:1 interaction model was unsatisfactory in describing the interactions and extracting the kinetic parameters (data not shown). More complex models were also unsatisfactory and unsuitable for the regression analysis of the limited data set that could be obtained for MIV-170, the compound of interest. Even if larger data sets, allowing for a regression analysis, in principle could be obtainable for some of the faster dissociating reference compounds and the mutants, it would not provide the information required to understand the characteristics of MIV-170. In addition, the absence of a regeneration procedure also prevented normalization of surfaces with a positive control and determination of the maximal signals for each compound, also of importance for quantification of parameters. The comparison of the interactions of NNRTIs with their target and the effect of the amino acid substitutions in the NNRTI binding pocket on the interaction was consequently restricted to a qualitative analysis of the sensorgrams.

3.1.1. Qualitative analysis of sensorgrams

In order to establish the kinetic mechanism of the interaction and qualitatively determine the difference in the kinetics for the different inhibitors, sensorgrams were simulated with a set of mechanistic models representing steps of the complex mechanism previously found to describe the interactions of NNRTIs with the K103N variant of HIV-1 RT [5]. An induced fit mechanism was the simplest model that captured the basic features of the experimental data (Fig. 3). In this model, the equilibrium for the initial encounter complex, is governed by the association and the dissociation rate constants (k_1 and k_2, respectively) while the second equilibrium, representing the conformational change between the primary enzyme-inhibitor complex (E_1I) and the induced complex (E_2I), is balanced by k_3 and k_4 (Fig. 3).

The simulation was performed with a single concentration of inhibitor (5.12 μM, *i.e.* the one used in the current experiments) and a value for k_1 ($1 \times 10^4\ M^{-1}\ s^{-1}$) similar to that previously determined for nevirapine and delavirdine and the K103N variant and a value for k_3 ($5 \times 10^{-2}\ s^{-1}$) that was found to give sensorgrams similar to the experimental sensorgrams (this parameter was not quantified in the former study). The rate constant k_2 for the dissociation of the encounter complex was varied from 0.0001 to $1\ s^{-1}$ (covering the range found in [5]), but with two different values for k_4, *i.e.* $1 \times 10^{-5}\ s^{-1}$ (Fig. 4A) or $1 \times 10^{-2}\ s^{-1}$ (Fig. 4B), representing "fast" and "slow" relaxation of the induced complex (relative both k_2 and k_3). A series of simulations with other parameter values, that support the selected values and show the effect of variations in the rates, is presented in Supplementary Material. Simulation of data using a selected fit model (*i.e.* with a pre-equilibrium between two forms of the free enzyme) was also tried, but did not give sensorgrams resembling the experimental sensorgrams, with any combinations of parameters tried (data not shown). A model including both the selected fit and induced fit steps was also used and resulted in sensorgrams very similar to the experimental data (Supplementary Material, Fig. SM3). However, as the aim was simply to identify the primary steps and the order of magnitude of the rate constants of the interaction it was not meaningful to extract any details from this simulation considering the large number of variables involved.

The simulation shows that the maximal signal level is predominantly influenced by k_2, *i.e.* the rate by which the primary enzyme-inhibitor complex dissociates into free enzyme and inhibitor (Fig. 4A). A change in the rate of relaxation (k_4) from the induced complex into the primary enzyme-inhibitor complex does not affect the maximal signal level, but indeed the slope of the dissociation curve (Fig. 4B). In other words, a compound can reach high complex levels due to a low k_2, but show fast dissociation due to a high k_4, or only reach low complex levels despite little dissociation. Thus, the overall signal levels and the mere shape of the interaction curves revealed important pieces of information about the interaction mechanism and the different contributions of the dissociation and association rates to the overall affinity.

3.1.2. MIV-170

The sensorgrams showed that, of all tested compounds, MIV-170 blocks all variants of HIV-1 RT most efficiently over time. This is a result of the highest level of complex concentration formed (maximum signal level at the end of the injection) and the slowest

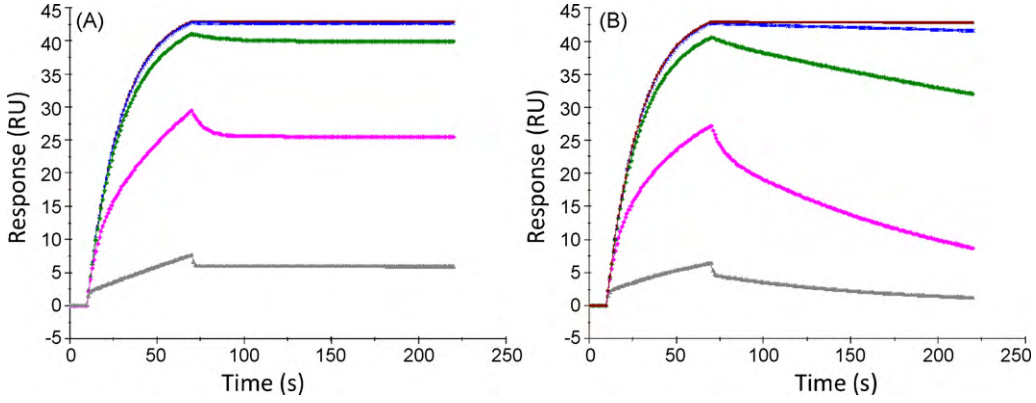

Fig. 4. Simulated sensorgrams representing interactions according to an induced fit mechanism for varying magnitudes of k_2. All sensorgrams were simulated with $k_1 = 1 \times 10^4\ M^{-1}\ s^{-1}$, $k_3 = 5 \times 10^{-2}\ s^{-1}$ and the same concentration of enzyme (5.12 μM). The dissociation rate constant k_2 was varied from 0.0001 (top) to 0.001, 0.01, 0.1 and $1\ s^{-1}$ (bottom). The values for k_4 were different in the two simulations, with A) $k_4 = 1 \times 10^{-5}\ s^{-1}$ and B) $k_4 = 1 \times 10^{-2}\ s^{-1}$ (additional simulated sensorgrams with other combinations of rate constants are shown in Supplementary Materials).

Table 1
Anti-HIV effect of NNRTIs against WT and mutant HIV-1 in MT-4 cells.

Strain[a] HIV-1IIIb	EC_{50} (nM)				
	MIV-170	Delavirdine	Efavirenz	TMC-120	Nevirapine
WT	0.97	110	1.6	1.2	170
L100I	9	7900	88	40	1200
K103N	3.2	6400	20	5.5	>10 000
Y181C	5.3	5000	3.9	16	>10 000

[a] The strain (HIV-1IIIb) is defined with respect to the reverse transcriptase sequence.

apparent dissociation rate (Fig. 2). The compound appeared to interact essentially irreversibly since no dissociation was detected during the dissociation phase, measured for up to 10 min (not shown). The experimental sensorgrams corresponded to the simulation with very slow k_2 and k_4 values (exemplified in Fig. 4A by 1×10^{-4} and 1×10^{-5} s^{-1}, respectively).

3.1.3. TMC-120

TMC-120 was the only other compound showing essentially irreversible interactions with the targets. But this compound gave atypical sensorgrams with all four protein surfaces, with very slow apparent rates of association. This type of sensorgram is characteristic for interactions with compounds that are barely soluble under the experimental conditions (see Section 4). Even the qualitative analysis therefore becomes difficult for this compound. But it can be concluded that, although the magnitude of k_2 cannot be estimated, the magnitude of k_4 must be very slow, *i.e.* similar to that for MIV-170.

3.1.4. Delavirdine, efavirenz and nevirapine

Two of the NNRTIs in clinical use (delavirdine and efavirenz) also formed stable complexes with the wild type enzyme, but the complexes with the resistant variants were clearly weaker (Fig. 2). The amount of complex formed was also lower than for MIV-170. Delavirdine appeared to have a faster k_4 than the other compounds, especially with the substituted variants, as suggested by the simulation. Efavirenz appeared to have a faster k_2 than MIV-170 and delavirdine, explaining why it did not reach the same levels as the other compounds.

The third NNRTI in clinical use, nevirapine, interacted only weakly with the wild type, as indicated by the low signal levels, but the simulation indicated that this could be a result of a relatively fast k_2 rather than a slow k_1. Despite the low signal levels, it was possible to see that nevirapine, at least for wild type HIV-1 RT, had a relatively slow k_4.

3.2. Antiviral efficacy

In order to determine how the interaction kinetics correlate with antiviral efficacy, the anti-HIV effect of the inhibitors was also analyzed. It was initially tested in a standard assay with MT-4 cells infected with wild type virus and three mutant viral strains with resistance to NNRTIs, as shown in Table 1. MIV-170 was the most potent inhibitor, with nM EC_{50} values for all tested mutants. Nevirapine and delavirdine showed little effect, essentially only with the wild type virus. TMC-120 and efavirenz were effective with the wild type virus, and to varying degrees with the mutant virus strains.

The effect of the compounds against a wild type Ba-L virus (subtype B, R5) was also tested in PBMCs (Table 2) as these cells represent a real HIV-1 target cell type. The compounds were constantly present in the growth medium during the 14 days long experiments in order to mimic in vivo microbicide use, where an intra-vaginal ring secretes inhibitor constantly or a gel is applied on a regular basis. The assay duration was established to 14 days in order to detect possible breakthrough infections that were barely, or not at all, detectable at earlier time points. Again, analysis revealed that MIV-170 had the highest efficacy of all tested compounds.

3.3. Correlation between interaction kinetic profile and antiviral efficacy

A quantitative correlation analysis of the interaction data and the cell-based data was not possible since the kinetic rate constants for the interactions could not be determined. Nevertheless, the comparison between experimental and simulated sensorgrams revealed important kinetic characteristics of the inhibitors that could be used in a qualitative correlation analysis.

It was clear that the best antiviral efficacy was achieved when both k_2 and k_4 were very slow, as exemplified by MIV-170. The poor antiviral effect by delavirdine was attributed to a relatively fast k_4, while the poor antiviral efficacy of nevirapine was interpreted to be primarily an effect of a relatively fast k_2. Efavirenz differed from MIV-170 by having a faster k_2. Thus, the qualitative kinetic analysis allowed the explanation of critical kinetic features that correlated with antiviral efficacy.

4. Discussion

One of the aims of the present study was to investigate if the novel NNRTI MIV-170 has kinetic properties suitable for use as a microbicide against HIV-1 transmission. Since the long-term effect of an antiviral drug and a microbicide is expected to be dependent on the stability of the inhibitor-target complex, *i.e.* the rate of dissociation, we were particularly interested in studying the importance of slow dissociation for antiviral effect. This was evaluated in a physiological context by analyzing the correlation between the interaction kinetic parameters and antiviral activity in cells.

The choice of biosensor-based interaction analysis for this study was based on our experience that it can provide detailed information on the interaction between NNRTIs and the enzyme [5–7], although not as mechanistically revealing as the pre-steady-state kinetic analysis of the enzyme activity [14] previously used to determine the mechanism of NNRTI inhibition. However, there were several experimental challenges to overcome in the present study. One concerned the very tight complexes formed by most of the inhibitors with the enzyme, especially with the wild type enzyme form. In the previous studies, drug resistant enzyme variants and inhibitors that allowed regeneration of the sensor surfaces and repeated

Table 2
Anti-HIV effect of NNRTIs against WT HIV-1 in PBMCs.

	EC_{50} (nM) (95% CI)[a]				
Strain[b]	MIV-170	Nevirapine	Delavirdine	Efavirenz	TMC-120
WT HIV-1	2.1 (0.2–25)	370 (244–558)	190 (72–533)	4.9 (3.1–7.7)	3.8 (2.8–5.1)

[a] The calculation of the EC_{50} values and their 95% confidence intervals (CI) was based on data of three independent experiments using non-linear regression analysis.
[b] The virus strain is subtype B HIV-1 Ba-L.

injections over the same surface were used. The sensorgrams could then be analyzed by regression analysis and some parameters quantified. In this study it was essential to perform experiments also with inhibitors and enzyme forms where regeneration was not possible, *e.g.* wild type. For these interactions the dissociation rate was too slow for the estimation of kinetic constants or affinities even when a simple 1:1 model had been considered an acceptable approximation [15]. Simulation of sensorgrams was therefore found to be very useful (see below).

The possibility of quantitatively analyzing interactions with very slow dissociation by use of simulation is expected to be very useful for lead–target interaction where ranking of compounds with respect to significant kinetic parameters is very difficult. Previous studies have shown that the use of a higher temperature than 25 °C for the experiments increases the dissociation rate [7]. However, we did not expect that a rise in temperature to *e.g.* physiologically relevant 37 °C, would have such an influence on the very slowly dissociating MIV-170 to resolve the regeneration problems experienced.

Another problem was the low solubility of at least one of the compounds, TMC-120. Poor solubility can result in a significantly lower effective free concentration of the analyte in the flow cell than the nominal concentration. Since the observed rate of association is directly correlated with the concentration of analyte, even small deviations in the concentration can be detected in the association phase. The observed rate of dissociation, on the other hand, is not directly influenced by the solubility of the analyte since it is only dependent on the dissociation rate constant (k_2) and the concentration of the protein–ligand complex. However, if the concentration of analyte is very low, the system may not reach steady-state during the injection time. The amount of protein–ligand complex will then easily be underestimated in comparison to the amounts of complex formed with compounds that reach steady-state during the injection, thereby indirectly affecting the observed dissociation rate.

TMC-120 is known to have a very low solubility (<0.3–1.2 μM) [16] and did not reach steady-state for any of the interactions studied. The compound showed an atypical behavior with all four enzymes, clearly indicating an inherent property independent of the interaction with the proteins, in accordance with a very low solubility. Although the TMC-120 sensorgrams do not accurately reflect the interaction mechanism, they clearly show that the compound dissociates very slowly (if at all). The other NNRTIs lacked the solubility problems of TMC-120, with MIV-170 being readily soluble up to 20 μM (unpublished data). The possibility that solubility was a problem for other compounds was taken into consideration when analyzing the data, but in practice it only influenced the interpretation of the data for TMC-120.

The focus on kinetics in the present study was based on an expectation that the antiviral effect of NNRTIs is dependent on the kinetics of interactions rather than the equilibrium behavior, *e.g.* as described by K_D, K_i or IC_{50} values. It has previously been proposed that a tight-binding mode of inhibition is essential for anti-HIV-1 virucidal activity of NNRTIs [17] and NNRTIs have been described as tight-binding (efavirenz) or rapid equilibrium (nevirapine and delavirdine). This terminology is, however, founded on an equilibrium-based analysis of reversible interactions rather than the time-resolved analysis that has been performed in the present study. As shown here, these terms are misleading for this type of inhibitors since an equilibrium-based approach is not appropriate for compounds that are truly "tight-binding", in the sense that they dissociate very slowly rather than simply having high affinities (low K_D-values) [18]. The use of enzyme inhibition data that does not account for irreversible inhibition mechanisms is therefore not relevant.

This study also illustrates the importance of determining the mechanisms of protein–ligand interactions as they are often more complex than simple 1:1 interactions. HIV-1 RT has previously been shown to interact with NNRTIs in a complex mechanism involving (1) a rate limiting pre-equilibrium between two unbound forms of the enzyme (*i.e.* selected fit [19]), (2) a slow conformational change (*i.e.* induced fit [19]), as well as (3) a heterogeneity in the immobilized protein [5,6]. However, the simulation showed that an induced fit mechanism alone could describe the dominating features of the current interactions. This may appear inconsistent seeing that the previous study concluded that the interaction was primarily selected fit, even if slow conformational changes also were inferred. But two datasets for the same target can be described primarily by different mechanisms if the mechanisms can be seen as parts of the same overall mechanistic model and that the dominant mechanism is defined by which steps are rate limiting [19]. The previous dataset, performed with the K103N variant and with inhibitors tested in concentration series, was best described by a selected fit mechanism for three out of four studied inhibitors, while the fourth compound (nevirapine) was adequately described by a simple 1:1 model [5]. Here we show that changes in the target (*i.e.* resistance mutations) may also influence the mechanism. Also the experimental conditions can have an impact as they may influence the relative rates of binding and conformational transitions in proteins.

Consequently, for NNRTI interactions with HIV-1 RT there are multiple equilibria that contribute to the "apparent dissociation rate". In the induced fit mechanism there is only one true dissociation rate constant (k_2). This makes it clear that a good understanding of the interaction mechanism is essential before the critical kinetic features of a certain protein–ligand interaction can be defined. Our interest in resolving the mechanism and the kinetics for NNRTIs was to correlate these features with the efficacy of the compounds in cell culture. Although it was frustrating that the rate constants could not be quantified, the simulations were very powerful in revealing the critical kinetic and mechanistic features for efficacy.

It was clear that the antiviral effect of the compounds was dependent on the dissociation rate constant for the encounter complex (k_2) and the rate of relaxation from the induced complex (k_4). The least effective compound in cell culture, nevirapine, was apparently compromised by a fast k_2 rate, preventing the efficient formation of enzyme-inhibitor complex. The poor antiviral efficacy of delavirdine was more puzzling, as it clearly formed large amounts of complex which appeared to be stable. However, careful scrutiny of the sensorgrams showed that the apparent rate of dissociation was faster than for MIV-170. It can be speculated that small differences in rate constants (also inferred from the simulation in Fig. 4A) can have significant effects during the much longer experiment performed when measuring antiviral efficacy in a cell culture based assay. This is supported by comparing the effect of mutations on the interactions with the compounds. Interestingly, also the value of k_2 was important, and correlated with the antiviral effect of the compounds, with MIV-170 being the most effective compound in cell culture, and nevirapine the least effective. Consequently, slow apparent dissociation alone, represented by the inability to detect dissociation, was not enough to explain the antiviral efficacy of the compounds.

The simulations also clearly show how differences in rate constants influence the amount of complex formed. In the case of viral enzymes, it is critical to block any enzyme present. This is dependent on the kinetics as well as the inhibitor concentration. Of the studied inhibitors MIV-170 resulted in the largest amount of complex, and consequently allows the least amount of enzyme to

be active (everything else being equal). We have already shown that the amount of blocked target is a critical parameter for HIV-1 protease, correlating better with antiviral efficacy than dissociation rate alone [20]. The focus on residence time of drugs can obviously be misleading for cases where residual enzyme activity can be problematic.

There are obvious limitations in using in vitro data to predict antiviral effects in vivo since in vivo data are influenced by more complex mechanistic features that cannot be reproduced in vitro. In addition, it is generally difficult to compare viral efficacy data with in vivo studies due to differences in viral strains and experimental conditions. A recent inhibition study with TMC-120 (dapivirine), delavirdine, efavirenz and nevirapine showed the same relative efficacy as in this study, qualitatively supporting the current results [10]. The correlation between biosensor kinetics and cell culture efficacy of NNRTIs are further supported by previous biosensor studies performed on etravirine (TMC-125). These show that etravirine, which is a potent inhibitor of viral replication in cell culture, dissociated slowly for the majority of the HIV-1 RT variants (9).

The compound of interest, MIV-170, did not dissociate with a measurable rate from any of the enzyme variants during the dissociation used in these experiments (10 min). Although it was not possible to detect and quantify the dissociation of MIV-170 it is not excluded that it does in fact dissociate, but it clearly does so very slowly. The only other compound with slow dissociation from all enzyme variants, TMC-120, gave considerably lower signal levels due to solubility problems. But despite these problems, the dissociation rate is interpreted to be realistic. Although delavirdine and efavirenz interact tightly with wild type HIV-1 RT, they are greatly affected by the substitutions in the binding site, as also reflected in the resistance towards these drugs by viruses with these mutations.

With respect to the use of NNRTIs as topical microbicides, it is important to use a compound that can block the target essentially irreversibly over time. This should apply to both wild type and mutant strains. Although side-effects are expected to be less of a problem for microbicides as compared to a systemically delivered drug, it is still desirable to be able to use as low concentrations as possible. MIV-170 is clearly the most suitable compound of those tested here, at least from a kinetic perspective. In addition to the advantages of MIV-170 for use as a microbicide revealed by the present studies, other data suggests that systemic levels of the inhibitor will be small as a result of a rapid liver metabolism (unpublished observations). This will lower the risk of resistance development towards MIV-170 upon topical administration. A low protein binding and high chemical stability (unpublished observations) are further properties making MIV-170 suitable for a vaginal microbicide. Obviously other factors also need to be considered, but they were outside the scope of this study. The next steps would be additional tests with HIV-1 subtypes A and C and an evaluation of the ability of MIV-170, in a vaginal formulation, to prevent Simian-Human immunodeficiency virus (SHIV) infection in monkeys.

5. Conclusion

This study illustrates that a biosensor-based method for studying the direct interaction between the NNRTI and its target can be used to identify compounds with suitable kinetic features for antiviral efficacy. Simulation of sensorgrams was useful for both establishing the interaction mechanism and identifying critical parameters for formation of large amounts of stable inhibitor-target complex. It is clear that the cell culture efficacy is an effect of the kinetics of both equilibria involved in an induced fit mechanism, emphasizing the importance of resolving the kinetic mechanism for drug–target interactions. It also reveals that long residence time (the time during which the target is blocked by the inhibitor) is not a good parameter for judging the efficacy of NNRTIs as the amount of complex is also a critical parameter. Finally, the results confirm that the kinetics, resulting in essentially irreversible binding of MIV-170 to wild type and NNRTI-resistant mutants of HIV RT, makes MIV-170 very interesting for development as a microbicide.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.06.035.

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