Novel Mechanism of Inhibition of HIV-1 Reverse Transcriptase by a New Non-nucleoside Analog, KM-1*

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Received for publication, June 4, 2004, and in revised form, June 30, 2004
Published, JBC Papers in Press, July 1, 2004, DOI 10.1074/jbc.M406241200

2-Naphthalenesulfonic acid (4-hydroxy-7-[[[5-hydroxy-6-[4-cinnamylphenyl]azo]-7-sulfo-2-naphthalenyl]amino]carbonyl]amino]-3-[[4-cinnamylphenyl]azo] (KM-1)) is a novel non-nucleoside reverse transcriptase inhibitor (NNRTI) that was designed to bind at an unconventional site on human immunodeficiency virus type 1 reverse transcriptase (RT) (Skillman, A. G., Maurer, K. W., Roe, D. C., Stauber, M. J., Eargle, D., Ewing, T. J., Muscate, A., Davioud-Charvet, E., Medaglia, M. V., Fisher, R. J., Arnold, E., Gao, H. Q., Buckel, R., Boyer, P. L., Hughes, S. H., Kuntz, I. D., and Kenyon, G. L. (2002) Bioorg. Chem. 30, 443–458). We have investigated the mechanism by which KM-1 inhibits wild-type human immunodeficiency virus type 1 RT by using pre-steady state kinetic methods to examine the effect of KM-1 on the parameters governing the single nucleotide incorporation catalyzed by RT. Analysis of the pre-steady-state burst phase of dATP incorporation showed that KM-1 decreased the amplitude of the reaction as previously shown for other NNRTIs, because of the slow equilibration of the inhibitor with RT. In the ternary enzyme-DNA-KM-1 complex (E-DNA-I), incorporation of the next nucleotide onto the primer is blocked. However, unlike conventional NNRTIs, the inhibitory effect was caused primarily by weakening the DNA binding affinity and displacing DNA from the enzyme. Wild-type RT binds a 25/45-mer DNA duplex with an apparent K_0 of 3 nM, which was increased to 400 nM upon saturation with KM-1. Likewise, the apparent K_0 for KM-1 binding to RT increased at higher DNA concentrations. We therefore conclude that KM-1 represents a new class of inhibitor distinct from nevirapine and related NNRTIs. KM-1 can bind to RT in both the absence and presence of DNA but weakens the affinity for DNA 140-fold so that it favors DNA dissociation. The data suggest that KM-1 distort RT conformation and misaligns DNA at the active site.

Since the introduction of antiretroviral therapy, the life span of patients infected with HIV\(^1\) or suffering from AIDS has been dramatically extended (1). However, the rate of infection is still on the rise (2), the current antiviral drugs do not eliminate HIV infection, and the high rate of mutation of the virus leads to rapid emergence of new variants of HIV resistant to each class of drugs (3–8). Thus, current treatments call for the frequent screening to look for increases in viral load indicative of new drug-resistant forms of the virus, which must be countered by a new combination of drugs. The current strategy for drug design is to address the changes in HIV by developing new drugs with a different resistance pattern. The purpose of this study is to investigate the properties of a new series of compounds to quantitatively evaluate their potency and to examine their mechanism of inhibition of HIV RT.

RT is responsible for replication of single-stranded viral RNA into double-stranded DNA, which is subsequently integrated into the host cellular DNA during the course of viral infection (9). Two types of anti-HIV medications target RT: the nucleoside analog RT inhibitors and non-nucleoside RT inhibitors (NNRTIs). Nucleoside analog RT inhibitors, such as 3'-azido-3'-deoxycytidine and 2',3'-dideoxycytidine, are nucleosides that become phosphorylated by cellular enzymes to their triphosphate form and are then incorporated by RT, acting as chain terminators to prevent further polymerization. To varying degrees, they also block cellular DNA polymerases, in particular the mitochondrial DNA polymerase (Pol \(\gamma\) ) (10), and their kinetics of incorporation are correlated with the toxic side effects of these drugs (reviewed in Ref. 11). NNRTIs are structurally diverse hydrophobic compounds that show fewer toxic side effects, but they are predominantly effective toward HIV-1 RT and not HIV-2 RT (12). NNRTIs bind in a hydrophobic pocket 10 Å away from the RT polymerase active site in the palm domain of the p66 subunit and distort the key residues that comprise the aspartic triad (D110, D185, and D186) so that the carboxyl groups of the side chains are out of alignment leading to slower rates of catalysis (13–15). Spence et al. (15) showed that the first generation NNRTIs, nevirapine and tetracyclimido[4,5,1-jk][1,4]-benzodiazepin-2(1H)-thione, are allosteric, slow-tight binding inhibitors that reduce the rate of the chemical reaction and increase the affinity of nucleotide binding to the inhibitor and DNA-bound RT (E-D-I) complex. As a result of a slower chemical reaction, the two steps of nucleotide binding entailing the initial ground-state binding and the subsequent conformational change reach equilibrium leading to tighter, albeit non-productive nucleotide binding.

The biggest barrier in the battle against AIDS is mutation, which confers resistance toward RT inhibitors. Drug-resistant RT mutants have been proposed to obstruct the binding of the drug (16, 17), reposition the template/primer (18–20), or facilitate scriptase; NNRTI, non-nucleoside reverse transcriptase inhibitor; E-D, enzyme-DNA complex; E-D-I, enzyme-DNA-inhibitor complex; Pol \(\gamma\), mitochondrial DNA polymerase.
Nucleotide Kinetics of a New Class of Inhibitors of HIV-1 Reverse Transcriptase

EXPERIMENTAL PROCEDURES

Materials—All chemicals were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted. Bacterial expression cell lines, dATP, T4 polynucleotide kinase, and kinase buffer were purchased from Invitrogen Corp. (Carlsbad, CA). Phosphate-buffered saline was purchased from Whatman (Chilton, NJ). The Sepharose resin was purchased from Amersham Biosciences. The non-nucleoside inhibitors were dissolved and stored in Me₂SO at −20°C and diluted by at least 700-fold to a stock solution with water. Controlled experiments verified that the final concentrations of Me₂SO did not affect the measured kinetic rates.

Buffers—All experiments were carried out at 37°C. RT was incubated with DNA in a reaction buffer containing 50 mM Tris acetate pH 7.5, 100 mM potassium acetate, 0.1 mM EDTA, and 0.1–1 mg/ml BSA. Nucleotides (such as dATP) were diluted into the same buffer with 4 mM magnesium acetate.

Protein Expression and Purification—Transformed DH5α cells producing the p66 and p51 subunits were grown separately in Lennox L broth supplemented with ampicillin (0.1 mg/ml) at 37°C. When the optical density reached 0.6, protein expression was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside. Cells were then grown for 16 h at 37°C and harvested by centrifugation. The crude cell lysates were purified with SDS-PAGE and Coomassie Blue staining. After the gel-staining intensities of the bands corresponding to the p66 and the p51 subunits, cell pellets of the large and small subunit were combined in a ratio of 3:9:1 to achieve a ratio of 1:2.5:1 at the end of the purification process. The procedures for purification of wild-type RT were described previously by Kati et al. (29, 30). The concentration of RT was determined by absorbance at 280 nm with an extinction coefficient of 260,450 M⁻¹ cm⁻¹. Active enzyme concentration was measured by active site titration as described below.

Synthetic Oligonucleotides—All experiments were performed with the same DNA duplex 25/45-bp as described by Kati et al. (29) in which the next base coded for incorporation was dATP. The sequence of the 25-mer primer was GCCGCGAGCCTGTCACAACCCACACAAGGC. The sequence of the 45-mer template was GGAGCGCATTGGATCGAGGTGGTGTTGGACGGCTGCGAGGC. Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) and purified by denaturing polyacrylamide gel electrophoresis. Concentrations of the oligonucleotides were determined by absorbance at 260 nm. Equimolar amounts of 25-mer and 45-mer were annealed by placing the solution in a heating block at 95°C and then allowing the heating block to gradually cool to room temperature.

5′-³²P Labeling of 25-bp Primer—Before annealing, the primer was 5′-radiolabeled with ³²P ATP by T4 polynucleotide kinase. The kinase was denatured after 1 h by placing the reaction at 95°C for 5 min. Un-reacted and contaminating nucleotides were removed by the labeled primer using a Micro Bio-Spin-30 column (Bio-Rad Laboratories, Inc.). The final concentration of labeled 25-mer was determined by thin layer chromatography of the samples taken before and after the Micro-Spin column.

Pre-steady-state Kinetics of Single Nucleotide Incorporation—Experiments were carried out at 37°C in a KinTek RQF-3 rapid quench flow (www.kintek-corp.com). The experiments were initiated by mixing a pre-equilibrated complex of enzyme-DNA, in the absence or presence of inhibitor, with an excess of dATP (15 μM each), and quenched with 80 μL of 0.5 M EDTA, pH 8.0. The reaction sample was collected into a microcentrifuge tube containing 30 μL of polyacrylamide gel loading buffer. We will refer to the concentrations of all reactants after 1-to-1 mixing in the quench-flow experiment.

Product Analysis—We quantified the extension of 5′-labeled 25-mer to 26-mer. Products were resolved on a denaturing gel (15% polyacrylamide, 0.8% bis-acrylamide, 7 M urea). The dried gel was exposed to a phosphor-screen then scanned with a Storm 860 PhosphorImager (Amersham Biosciences) and quantified using ImageQuant 5.0 software.

Data Analysis—Data were fitted by nonlinear regression using the program GraFit 5.0.1 (Erithacus Software). Data points from the pre-steady-state burst experiments were fitted to the burst equation $y = A \times (1 - e^{−kt}) + m \times t$, where $y$ represents the concentration of the 26-mer product, $A$ is the burst amplitude, $k$ is the observed burst rate, $m$ is the slope of the linear steady-state phase, and $t$ is the reaction time. The steady state rate was calculated by dividing the slope by the concentration of active enzyme. The observed burst rates were plotted against nucleotide (dATP) concentration and fitted to a hyperbolic equation, $y = \frac{a \times S}{1 + b \times S}$, where $k_{max}$ represents the maximum incorporation rate, $K_S$ the apparent equilibrium dissociation constant for the nucleotide, and $S$ the concentration of nucleotide. For single nucleotide incorporation experiments done in the presence of increasing concentrations of the inhibitor, the plot of the amplitude of each time course function of inhibitor concentration was fitted to a hyperbolic equation, $y = E - \frac{[E \times I \times (K_i + I)] + B}{B}$, where $E$ is the total enzyme concentration, $I$ is the total inhibitor concentration, $B$ is the background signal, and $K_i$ is the apparent equilibrium dissociation constant for the non-nucleoside inhibitor.

Titration of Active Enzyme with DNA—The concentration of active enzyme and the $K_i$ for DNA in the presence of different concentrations of the inhibitor were determined by an active site titration experiment.
as described previously (29). RT was added to increasing concentrations of 5′-32P-labeled 25/45-mer DNA, in the presence or absence of inhibitor and incubated for 5 min. The sample was then mixed with an equal volume of dATP (100 μM) for 0.5 s and then quenched with 0.5 s × EDTA, pH 8. The amplitudes were obtained by fitting each time course to the burst equation. The amplitudes were then plotted as a function of DNA concentration. The results from experiments done in the absence of the inhibitor were fitted with a quadratic equation, A = (Kd + E + D × F) − √(Kd + E + D × F)2 − 4 × E × D × F/2, where A is the amplitude, E is the total enzyme concentration, D is the total DNA concentration, Kd is the equilibrium dissociation constant for DNA, and F is the binding factor. The binding factor is the fraction of E-D complexes that are productively bound. It corrects for the actual concentration of DNA in productive E-D complexes because the data from DNA titration experiments showed that both inactive and active enzymes were bound to DNA. The maximum amplitude obtained was ~50% of the protein concentration determined by the UV absorbance measurements at 280 nm. All enzyme concentrations reported here are calibrated via active concentration determined by the UV absorbance measurements at 280 nm. All enzyme concentrations reported here are calibrated via active concentration determined by the UV absorbance measurements at 280 nm.

DNA-directed single nucleotide incorporation in the presence of KM-1. A, wild-type RT (100 nM), 5′-32P-labeled 25/45-mer DNA (200 nM), and various concentrations of KM-1 were preincubated for 15–20 min at 37 °C in reaction buffer (see “Experimental Procedures”). Concentrations of 0 (○), 1 (●), 2 (□), 4 (■), 6 (▲), 8 (△), 12 (○), and 20 (▲) μM KM-1 were included in the initial solution in which the equilibrium between E-D and E-D-I complexes was established. The E-D complex was diluted by one half when mixed rapidly with an equal volume of 150 μM dATP (10 mM MgCl2) and allowed to react for 0.01–5 s then quenched with EDTA. The time interval of primer extension at each KM-1 concentration was fit to the burst equation to yield the amplitude and rate for each burst curve. B, the amplitudes of the burst phases were plotted against the KM-1 concentrations. The data were fitted to a hyperbolic equation to define a Kd value of 1.3 ± 0.2 μM.

**RESULTS**

Dose-dependent Inhibition of Single Nucleotide Incorporation by KM-1—We first examined the effect of KM-1 on the pre-steady state burst of single nucleotide incorporation catalyzed by HIV RT with a defined primer/template. In this experiment, the burst rate provides a direct measurement of the rate of polymerization at the active site and the amplitude defines the concentration of reactive enzyme-DNA (E-D) complexes, whereas steady state turnover is limited by the release of product DNA from enzyme (29, 32). We incubated the E-D complex with 0–20 μM inhibitor before mixing with Mg2+-dATP to start the reaction. The results in Fig. 2A show an initial burst of polymerization followed by a slow steady-state turnover. In the absence of KM-1, the burst amplitude for dATP incorporation catalyzed by 50 nM RT (with 100 nM DNA) was 43 ± 2 nm, the burst rate was 7.4 ± 0.9 s−1, and the steady state rate was 0.20 ± 0.03 s−1. Increasing the inhibitor concentration reduced the amplitude of the burst phase as well as the rate of the linear steady state phase to almost zero (Figs. 2A and 3A) but did not affect the burst rates. Reduction in the amplitude is presumably caused by the binding of KM-1 to E-D and/or E in a reaction that equilibrates slowly relative to the rate of the burst (15). The reduction in burst amplitude but not rate is consistent with the slow equilibration of the inhibitor so that the observed amplitude reflects only the reaction catalyzed by uninhibited enzyme. Therefore, plotting the amplitude of the burst as function of inhibitor concentration provides a direct titration to quantify inhibitor binding as shown in Fig. 2B. Fitting the curve to a hyperbolic function defines an apparent Kd of 1.3 ± 0.2 μM for the binding of KM-1 to the enzyme.

**Fig. 2.** DNA-directed single nucleotide incorporation in the presence of IOI-1. A, wild-type RT (100 nM), 5′-32P-labeled 25/45-mer DNA (200 nM), and various concentrations of KM-1 were preincubated for 15–20 min at 37 °C in reaction buffer (see “Experimental Procedures”). Concentrations of 0 (○), 1 (●), 2 (□), 4 (■), 6 (▲), 8 (△), 12 (○), and 20 (▲) μM KM-1 were included in the initial solution in which the equilibrium between E-D and E-D-I complexes was established. The E-D complex was diluted by one half when mixed rapidly with an equal volume of 150 μM dATP (10 mM MgCl2) and allowed to react for 0.01–5 s then quenched with EDTA. The time interval of primer extension at each KM-1 concentration was fit to the burst equation to yield the amplitude and rate for each burst curve. B, the amplitudes of the burst phases were plotted against the KM-1 concentrations. The data were fitted to a hyperbolic equation to define a Kd value of 1.3 ± 0.2 μM.

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**Figure 3.** DNA-directed single nucleotide incorporation in the presence of KM-1 with a ratio of RT to DNA concentration at 1:3. A, wild-type RT (100 nM), 5'-32P-labeled 25/45-mer DNA (300 nM), and various concentrations of KM-1 were preincubated for 15–20 min at 37 °C in reaction buffer. For each titration experiment with 2 (–), 5 (△), or 10 (○) μM of KM-1, the inhibitor was combined with increasing concentrations of DNA at 37 °C before RT was added. The E-D or E-D-I complex was rapidly mixed for 0.5 s with an equal volume of 150 mM dATP in the reaction buffer containing 4 mM MgCl2. Each reaction was quenched with EDTA at the indicated time points. The individual time course of primer extension at each KM-1 concentration was fitted to the burst equation to obtain the amplitude for each burst curve. B, the amplitudes of the burst phases were plotted against the KM-1 concentrations. The data were fitted to a hyperbolic equation to define a $K_d$ value of 3.4 ± 1.0 μM.

**Figure 4.** Dependence of the DNA dissociation constant on KM-1 concentration. A, active site titration of wild-type RT with 25/45-mer duplex DNA was done by measuring the burst amplitude in the presence of different concentrations of KM-1. For the initial experiment without KM-1 (●), RT (at an 200 nM active concentration determined by the amplitude from the titration) and increasing concentrations of 5'-32P-labeled 25/45-mer DNA were incubated on ice for less than 5 min in reaction buffer. For each titration experiment with 2 (–), 5 (△), or 10 (○) μM of KM-1, the inhibitor was combined with increasing concentrations of DNA at 37 °C before RT was added. The E-D or E-D-I complex was rapidly mixed with 0.5 s with an equal volume of 150 mM dATP in the reaction buffer containing 4 mM MgCl2. Each reaction was quenched with 0.5 mM EDTA, pH 8.0. For each KM-1 concentration, the amplitudes were plotted as a function of the DNA concentrations. Each titration curve was fitted to a quadratic equation in the absence or a hyperbola in the presence of KM-1 to obtain the maximum amplitudes and apparent $K_{d,\text{DNA}}$. B, the values of apparent $K_{d,\text{DNA}}$ were plotted as a function of the DNA concentrations. Each titration curve was fitted to a quadratic equation in the absence or a hyperbola in the presence of KM-1 to obtain the maximum amplitudes and apparent $K_{d,\text{DNA}}$. C, the maximum amplitude obtained by extrapolation to infinite [DNA] was plotted as a function of KM-1 concentration and fit to a hyperbolic function to estimate the $K_d$ for KM-1 binding to the E-D complex.

Similar effects on the burst amplitude and steady state rates observed with EDC 11 and EDC 12 suggest identical inhibition mechanisms as KM-1. The modified moieties in EDC 11 and 12 (Fig. 1) affect the binding affinities to HIV-1 RT only slightly. The hyperbolic function of amplitude versus inhibitor concentration defined the apparent equilibrium dissociation constants for EDC-11 and EDC-12 of 1.9 ± 0.4 and 1.5 ± 0.6 μM, respectively.

Two modes of action were possible for this series of inhibitors. The reduction of burst amplitude with increasing concentrations of the inhibitor suggests that they can act like conventional NNRTIs and inhibit the chemistry step of polymerization pathway by binding to the E-D complex. On the other hand, they could bind to the free enzyme and prevent productive binding of DNA to HIV-1 RT. Their structural resemblance to DNA base pairs suggests possible competition between DNA and the inhibitor for binding to HIV-1 RT.

To examine whether KM-1 acted by competing with DNA binding, we repeated the titration of the reaction amplitude versus KM-1 concentration in the presence of a higher concentration of DNA. Increasing the total DNA concentration from 200–300 nM doubles the concentration of free DNA in the presence of the 100 nM enzyme during the pre-incubation step. The results showing the dependence of the burst kinetics on KM-1 concentration at the 300 nM DNA concentration are shown in Fig. 3. Doubling the concentration of free DNA led to an increase in the apparent $K_{d_{\text{KM-1}}}$ from 1.3 ± 0.2 μM (Fig. 2B) to 3.4 ± 1.0 μM (Fig. 3B). The correlation between the increase in DNA concentration and the increase in the apparent $K_{d_{\text{KM-1}}}$ suggests competition between the inhibitor and DNA such that $K_{d_{\text{KM-1}}}^{\text{app}} = K_{d_{\text{KM-1}}} (1 + [\text{DNA}] / K_{d_{\text{DNA}}})$. The slope of the DNA concentration dependence suggests that the ratio of $K_{d_{\text{KM-1}}} / K_{d_{\text{DNA}}}$ is ~40. Given a $K_d = 3 ± 1$ nM for DNA binding to RT, this suggests that KM-1 binds to free enzyme with a $K_d ≈ 80$ nM. We explored further this competition by examining the effect of KM-1 on observed DNA binding.

**DNA Dissociation Constant Dependence on KM-1 Concentration**—The competition between DNA and KM-1 was checked by measuring the apparent $K_{d_{\text{DNA}}}$ for DNA binding in the presence of various concentrations of KM-1. The apparent $K_d$ for DNA binding was obtained by measuring the DNA concentra-
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**Scheme 1. Pathway of inhibition by KM-1.** The inhibitor binds to enzyme in the presence and absence of DNA as shown but weakens DNA by 140-fold.

\[
K_{d,\text{KM-1}} = 35 \pm/\pm 16 \text{nM}
\]

**burst equation** to obtain the rate and amplitude of the fast product formation. The burst rate was plotted against dATP concentration (Fig. 5A). Estimates were inserted into the equations along with the known values. The solved \(K_d\) values were used to simulate the data for the DNA titration experiments. The simulations were superimposed with real data to check the accuracy of the calculations.

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d\text{ATP} \text{Concentration Dependence of the Nucleotide Incorporation Rate in the Presence of Different Amounts of KM-1—}
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Next, we examined the kinetics of single nucleotide incorporation (15, 29, 33). In this experiment, we measured the maximum incorporation rate (\(k_{\text{pol}}\)) and the dissociation constant of dATP (\(K_d,\text{dATP}\)) in the presence of 0–5 \(\mu\text{M}\) of KM-1. During the quench flow operation, equal volumes of the pre-incubated mixture (consisting of RT, DNA, and KM-1) and dATP-Mg\(^{2+}\) were rapidly mixed and quenched after various times. Product concentration versus time (Fig. 5A) was fitted to a burst equation to obtain the rate and amplitude of the fast product formation. The burst rate was plotted against dATP concentration (Fig. 5B) and fitted to a hyperbola to obtain \(k_{\text{pol}}\) (Fig. 5C) and \(K_d,\text{dATP}\) (Fig. 5D). The \(k_{\text{pol}}\) was approximately constant and the \(K_d,\text{dATP}\) remained in the low micromolar range. These observations are consistent with the conclusion that the observed product formation was catalyzed only by uninhibited E-D complex.
concentrations were 0.025 (1), 0.05 (2), 0.075 (3), and 0.1 (4) mM. A, the E-DNA solution was 2-fold diluted when rapidly mixed with KM-1 at a concentration of either 5 (1), 10 (2), 15 (3), or 20 μM for the designated times, then mixed with an equal volume of dATP (50 μM) for 200 ms. The reaction was quenched with about 80 μl of 0.5 M EDTA, pH 8.0. The listed concentrations of RT, DNA, and KM-1 are the concentrations before the addition of dATP. The concentration listed for dATP is that after mixing with the RT-DNA-KM-1 solution. The amount of product drops inversely with the amount of KM-1. Fitting the curves to single exponential decays rendered apparent binding rates of 0.093 ± 0.009, 0.51 ± 0.02, 1.6 ± 0.2, and 1.7 ± 0.2 s⁻¹ for 5, 10, 15, and 20 μM KM-1, respectively. B, association and dissociation rate constants for formation of the E-D-I complex were obtained by analysis of the KM-1 concentration dependence of the observed rate constant of binding, where $k_{obs} = k_{on} \times [I] + k_{off}$. An independent estimate of $k_{off}$ was not possible because of the large errors in the extrapolated intercept; therefore, the binding rate was calculated by linearly fitting the first two data points at KM-1 concentrations of 5 and 10 μM and fixing the intercept to zero, such that $k_{on}$ was constrained to agree with both the $K_i$ value ($= k_{on}/k_{off}$) and the observed binding rate. The linear fit rendered a slope of 0.044 ± 0.009 (dashed line). The estimated on rate was $(4.4 ± 0.9) \times 10^4$ M⁻¹ s⁻¹ and the off rate was 0.06 ± 0.01 s⁻¹.

**KM-1 Binding Rates**—Our data indicated that the equilibration of inhibitor with the E-D-I complex is slow relative to the rate of polymerization. We measured the rates of KM-1 binding by a rapid quench-flow method consisting of two sequential mixing steps. First, KM-1 was mixed with a solution of E-D and allowed to react for 0.1–30 s, then Mg²⁺-dATP was added to initiate the reaction. Polymerization was then allowed to take place for 0.2 s, which is sufficient for approximately a single turnover. The reactions were terminated by mixing with EDTA. The product concentration was normalized to the maximum amount of turnover detected in 0.2 s without the inhibitor. The fraction of product formation was plotted as a function of time allowed for the binding of KM-1 to E-D (Fig. 6A). Four KM-1 concentrations were tested: 5, 10, 15, and 20 μM. Each curve of the product formation was fitted to a single exponential decay function to calculate the binding rates, $K_{obs}$, which were in turn linearly dependent on the KM-1 concentration to define the values for the association and dissociation rate constants (Fig. 6B). For reasons that are unclear to us, the observed binding rates at 15 and 20 μM KM-1 were ~2-fold higher than expected from the linear relationship established at the lower KM-1 concentrations. Because the latter two concentrations are beyond the inhibition concentration range observed in

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**Fig. 6.** The apparent binding rate of KM-1. RT (100 nM) was pre-incubated with 5'-³²P-labeled DNA (100 nM). A, the E-DNA solution was 2-fold diluted when rapidly mixed with KM-1 at a concentration of either 5 (1), 10 (2), 15 (3), or 20 μM for the designated times, then mixed with an equal volume of dATP (50 μM) for 200 ms. The reaction was quenched with about 80 μl of 0.5 M EDTA, pH 8.0. The listed concentrations of RT, DNA, and KM-1 are the concentrations before the addition of dATP. The concentration listed for dATP is that after mixing with the RT-DNA-KM-1 solution. The amount of product drops inversely with the amount of KM-1. Fitting the curves to single exponential decays rendered apparent binding rates of 0.093 ± 0.009, 0.51 ± 0.02, 1.6 ± 0.2, and 1.7 ± 0.2 s⁻¹ for 5, 10, 15, and 20 μM KM-1, respectively. B, association and dissociation rate constants for formation of the E-D-I complex were obtained by analysis of the KM-1 concentration dependence of the observed rate constant of binding, where $k_{obs} = k_{on} \times [I] + k_{off}$. An independent estimate of $k_{off}$ was not possible because of the large errors in the extrapolated intercept; therefore, the binding rate was calculated by linearly fitting the first two data points at KM-1 concentrations of 5 and 10 μM and fixing the intercept to zero, such that $k_{on}$ was constrained to agree with both the $K_i$ value ($= k_{on}/k_{off}$) and the observed binding rate. The linear fit rendered a slope of 0.044 ± 0.009 (dashed line). The estimated on rate was $(4.4 ± 0.9) \times 10^4$ M⁻¹ s⁻¹ and the off rate was 0.06 ± 0.01 s⁻¹.

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**Fig. 5.** Nucleotide concentration dependence of the incorporation rate in the presence of KM-1. A, a solution of RT (200 nM), 5'-³²P-labeled duplex DNA (400 nM) mixed with 0, 1, 2, and 5 μM KM-1, respectively, was incubated at 37 °C for 10–15 min. The reaction was initiated by mixing equal volumes of Mg²⁺-dATP and E-D-KM-1 complex solutions, and was quenched with 0.5 x EDTA, pH 8.0. The dATP concentrations were 0.025 (1), 0.05 (2), 0.075 (3), 0.25 (4), 1 (5), 2 (6), 4 (7), 8 (8), 12 (9), and 20 (10) μM. Each of the time courses was fitted to a burst equation, $y = A \times [1 - e^{-kt}] + m \times t$. Shown here is a plot of time courses of product formation in the presence of 2 μM KM-1. B, the single nucleotide incorporation rates were plotted as a function of the dATP concentration. The plot of the burst rate dependence on dATP concentration with 1 μM KM-1 is shown here. The curves were fitted to a hyperbola that yielded the maximum incorporation rates (C) and the apparent $K_{d,ATP}$ values (D), which were plotted against the KM-1 concentrations.
prior experiments, and because it is possible that KM-1 aggregated at the higher concentrations, we used only the first two concentrations to estimate the binding rate.

Compared with nevirapine, with a $k_{on}$ of $9.9 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ and a $k_{off}$ of 0.0019 s$^{-1}$ (15), KM-1 displayed a similar $k_{on}$ of $(4.4 \pm 0.9) \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ and a 30-fold higher $k_{off}$ of 0.06 ± 0.01 s$^{-1}$. The differences in the on and off rate are not surprising because the apparent $K_{d,KM-1}$ (1.3 $\mu M$) is about 100-fold greater than $K_{d,nevirapine}$ (0.019 $\mu M$). KM-1 displayed binding rates in the same order of magnitude as nevirapine and tetrahydroimindazolo[4,5,1,jk][1,4]-benzodiazepin-2(1H)-thione within the range of inhibition concentrations. However, this simple analysis may not be entirely valid for KM-1, because the inhibition seems to be a function of binding to both E and E-D and leads to the dissociation of the E-D complex. Because these experiments were performed by mixing the preformed E-D complex with inhibitor, the time dependence of inhibition reflects the rate of binding to form the E-D-I complex, and it is possible that the DNA dissociates more rapidly after inhibitor binding. Given the complexities in performing this experiment, it was not possible to evaluate whether the time dependence was biphasic, reflecting a two-step process. Nonetheless, these data demonstrate that the binding and equilibration of KM-1 is slow relative to the rate of the burst reaction, thereby justifying the assumption inherent in the analysis of the burst amplitude as a measurement of the concentration of un-inhibited E-D complex, poised for rapid catalysis.

**KM-1 Specificity**—Recent reports on nonspecific inhibition by drugs via aggregation (34–37) raised our concerns about the specificity of KM-1. Shortly after Skillman et al. (28) discovered KM-1 founded in part on structure-based design, Shoichet et al. (34–37) reported promiscuous inhibition by aggregation of small molecules designed by high throughput screening or molecular docking. These ligands typically display three properties: 1) they innately bind to many targets; 2) they react chemically with proteins; and 3) they aggregate in solution at low micromolar concentrations. One of the first-generation NNR-TIs, delavirdine, displayed indiscriminate and aggregation-based inhibition of non-targeted enzymes (37). Because KM-1 was designed based on similar molecules (28) and consists of many aromatic rings, which are stereotypical for aggregating inhibitors, we used three approaches to examine the aggregation of KM-1. First, we examined the structures of KM-1 by electron microscopy at concentrations of 5, 50, 250, and 500 $\mu M$. In comparison with the electron microscopy images published by McGovern et al. (35), no large aggregated particles were detected in the samples of KM-1 at any concentration. Small particles (~30 nm) were observed only at the highest concentration (data not shown). Second, we examined aggregation by classic light scattering, showing no significant changes in the particle size as a function of concentration. Finally, we tested the sensitivity of the inhibition by KM-1 to nonionic detergent (34) by repeating the DNA-directed single nucleotide incorporation experiments with the addition of 0.1% Triton-X. Prior work had shown that Triton-X reversed nonspecific inhibition by disrupting aggregates so that they could not interact with arbitrary surface residues on an enzyme (34). In our experiments, the presence of Triton-X did not change the inhibition mechanism of KM-1. The burst amplitude and the steady state rate decreased, whereas the burst rate remained the same in response to the increase in KM-1 concentration (Fig. 7). The apparent $K_{d,KM-1}$ was raised $4$-fold, from $1.3 \pm 0.2$ to $5.2 \pm 1.4 \mu M$. A similar effect was observed when we repeated the experiment with an RNA template of the same sequence: the apparent $K_{d,KM-1}$ was increased from $3.1 \pm 1.3$ to $13 \pm 3 \mu M$ (data not shown). Triton-X weakened the binding of KM-1 to RT although it did not eliminate the inhibitory effect of KM-1. These data argue against a model in which the aggregation of KM-1 was responsible for the observed inhibition. Moreover, the observed competition by DNA implies that KM-1 is interacting with the primer/template binding site. Aside from the likelihood of aggregation, the aromatic structure of KM-1 also allows for possible intercalation with DNA. KM-1 resembles chemotherapeutic drugs such as daunomycin and nogalamycin, which inhibit cell replication by inserting themselves between two planar or stacked aromatic rings, such as adjacent bases in a genetic sequence. The interaction results in a distortion of DNA helix structure. We scanned the emission spectra of three mixtures of $10 \mu M$ KM-1 with 100, 150, and 200 nM DNA, respectively, by fluorescence spectroscopy, but we observed no change in the fluorescence intensity. We also examined changes in fluorescence anisotropy of 2 $\mu M$ KM-1 when titrated with a high concentration of linearized DNA plasmid pUC18 (data not shown). The data fit an apparent $K_{d}$ of $400 \text{ nM}$ plasmid. Given a theoretical binding site size of 20 bp for the 2686-bp plasmid, this translates to a $K_{d} \sim 50 \text{ nM}$ per site. Although the site size is not known, these data indicate that the binding of KM-1 to DNA is very weak and do not support a model in which KM-1 intercalates with DNA to inhibit polymerization by HIV-1 RT.

**KM-1 Also Inhibits the Mitochondrial DNA Polymerase**—Further testing of KM-1 selectivity was carried out by inspecting its effect on the human mitochondrial DNA polymerase, Pol.

Fig. 7. The presence of 0.1% Triton-X slightly attenuates the efficacy of KM-1. DNA-directed single nucleotide incorporation in the presence of KM-1 and 0.1% Triton-X. A, wild-type RT (100 nM), 5'-32P-labeled 25/45-mer DNA (200 nM), and various concentrations of KM-1 were preincubated for 15–20 min at 37 °C in reaction buffer (see "Experimental Procedures"). Concentrations of 0 (●), 1 (□), 2 (□), 4 (□), 6 (□), 8 (■), 12 (○), and 20 (●) $\mu M$ KM-1 were included in the initial solution where the equilibrium between E-D and E-D-I complexes was established. The E-D complex was diluted by half when mixed rapidly with an equal volume of 150 $\mu M$ DTT (in 10 mM MgCl$_2$) and allowed to react for 0.01–3 s. The reactions were quenched with EDTA at the indicated time points. The individual time course of primer extension at each KM-1 concentration was fitted to the burst equation to yield the maximum amplitude for each curve. B, the amplitudes of the burst phases were plotted against the KM-1 concentrations. The data were fitted to a hyperbolic equation that defined a $K_{d}$ value of $5.2 \pm 1.4 \mu M$.
The ternary E-D-I complex can form but is unable to catalyze incorporation. Taken together, these results suggest that the KM-1 binding site overlaps with the primer/template binding site and KM-1 displaces DNA so that it cannot access the polymerase active site. The ternary complex forms only weakly. The binding of DNA to KM-1-bound RT is 140-fold weaker than to the free enzyme, as summarized in Scheme I.

The amplitude of the burst phase represents the amount of uninhibited E-D complex available for fast nucleotide incorporation in a single turnover. Therefore, the reduction in the burst amplitude in the presence of the inhibitor is caused by a reduction in the concentration of the reactive E-D complex as a result of formation of E-D-I and E-I. The reduced burst amplitude and unchanged burst rate suggest that the rate of equilibration of KM-1 with RT (dissociation and rebinding) is slower than the rate of polymerization catalyzed by uninhibited E-D complex in a single turnover. The unchanged rate of polymerization and constant $K_d$ for dATP indicate that the observed incorporation is catalyzed by the uninhibited E-D complex.

Previously characterized non-nucleoside inhibitors form a ternary E-D-I complex that is still capable of nucleotide incorporation, but at a reduced rate in that there is a slow but significant continuing reaction even at saturating concentrations of tetrahydroimidazo[4,5,1-\(jk\)][1,4]-benzodiazepin-2(1H)-thione and nevirapine (15). KM-1, however, inhibits the polymerization catalyzed by RT to a greater extent because it abolishes the reaction seen during the slower phase. This suggests that the E-D-I complex is non-reactive or at least extremely slow relative to the rate seen in the presence of nevirapine. Because the only observed reaction was caused by the activity of the uninhibited E-D complex, we cannot ascertain whether KM-1 blocks the nucleotide incorporation at the binding step, conformational change, or chemistry. In the case of previous analysis of inhibition by nevirapine, analysis of the incorporation kinetics of the fully inhibited enzyme allowed us to establish that NNRTIs block chemistry without interfering with nucleotide binding or the conformational change step (15). A similar analysis is not possible with KM-1 because no reaction is seen that is not attributable to the uninhibited fraction of the enzyme.

In contrast to nevirapine and tetrahydroimidazo[4,5,1-\(jk\)][1,4]-benzodiazepin-2(1H)-thione, KM-1 leads to dissociation of the DNA from the E-D-I to favor the E-I complex. Although the E-D-I complex may form, as suggested by the observation that saturating DNA cannot completely overcome the inhibition, the E-D-I complex seems to be non-reactive. It is reasonable to suppose that the binding sites for DNA and KM-1 overlap substantially but that the binding site for DNA is much larger. At high concentrations, KM-1 and DNA both may bind, but the DNA may be dislodged from the catalytic site in such a way as to prevent nucleotide incorporation. Increasing DNA concentration cannot overcome the inhibition because the DNA site is already occupied. We conclude that KM-1 binds to RT at a different site than that for the incoming nucleotide or conventional NNRTIs.

A clear picture of KM-1 selectivity awaits further experiments. KM-1 seemed to inhibit human mitochondrial DNA polymerase as effectively as HIV-1 RT. However, broader cellular toxicity assays are necessary because it is not known whether KM-1 will effectively inhibit the replication of DNA in cells. Our studies test only whether KM-1 can inhibit the mitochondrial polymerase in vitro and do not address questions regarding cellular and mitochondrial uptake. Moreover, it is possible that structure/activity studies based upon KM-1 as a lead compound could succeed in altering the relative affinities for HIV RT versus other cellular polymerases to overcome the enzyme. The ternary E-D-I complex can form but is unable to catalyze incorporation. Taken together, these results suggest that the KM-1 binding site overlaps with the primer/template binding site and KM-1 displaces DNA so that it cannot access the polymerase active site. The ternary complex forms only weakly. The binding of DNA to KM-1-bound RT is 140-fold weaker than to the free enzyme, as summarized in Scheme I.

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undesirable side reactions. This study and our prior work (15) outline a series of experiments that can be used to define the mechanism of inhibition by non-nucleoside inhibitors and quantify the affinity.

Like nevirapine, KM-1 binds slowly to the E-D complex, with an apparent second-order rate constant of $4 \times 10^8$ M$^{-1}$ s$^{-1}$ and a dissociation rate of 0.04 s$^{-1}$. The rate of dissociation of the DNA from the E-D-I complex was not resolved in these studies. Parniak has described another NNRTI that he claims is unique in that it is a slow-tight binding inhibitor, unlike other NNRTIs (38). However, his claim of uniqueness is without merit, in that all NNRTIs characterized thus far are slow-binding inhibitors with binding rates on the order of $10^4$ M$^{-1}$ s$^{-1}$, and his rough estimates, using slow-onset inhibition kinetics produce similar rates. All data support the conclusion that Parniak’s inhibitor is simply another NNRTI binding to the well established and exploited conventional NNRTI binding site.

It is likely that KM-1 interferes with the proper alignment of DNA at the polymerase active site with its bulky shape. The characterization of this non-nucleoside inhibitor may help in the design of more effective drugs that are potent toward wild type and drug-resistant strains of RT. Given the high affinity of KM-1 for the free enzyme, it is possible that further structure/activity studies on compounds related to KM-1 could lead to a more potent and selective inhibitor of a new class.

Acknowledgments—We are grateful to Dr. Elisabeth Davioud-Charvet for synthesizing the napthylurea drugs; Dr. Stephen Hughes and Roger Goody for providing the DNA plasmids for the expression of HIV-1 RT; Jeff Bartron for providing the human mitochondrial DNA and Roger Goody for providing the DNA plasmids for the expression of Charvet for synthesizing the napthylurea drugs; Drs. Stephen Hughes and Marc Charvet for synthesizing the napthylurea drugs; Drs. Stephen Hughes and Roger Goody for providing the DNA plasmids for the expression of Davis for providing the human mitochondrial DNA polymerase; Jeremaiah Hanes, Harold Lee, Vi Dougherty, and Dr. David Humphrey for reading this manuscript; and Dr. Jarle Lillemoen for technical assistance with the QuantaMaster spectrofluorometer.

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Novel Mechanism of Inhibition of HIV-1 Reverse Transcriptase by a New Non-nucleoside Analog, KM-1

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J. Biol. Chem. 2004, 279:38424-38432.
doi: 10.1074/jbc.M406241200 originally published online July 1, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M406241200

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