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Novel high throughput screen identifies an HIV-1 reverse transcriptase inhibitor with a unique mechanism of action

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Abstract

HIV-1 resistance to zidovudine (AZT) is associated with selection of M41L, D67N, K70R, L210W, T215F/Y and K219Q/E in reverse transcriptase (RT). These mutations decrease HIV-1 susceptibility to AZT by augmenting RT's ability to excise the chain-terminating AZT-monophosphate (AZT-MP) moiety from the chain-terminated DNA primer. Although AZT-MP excision occurs at the enzyme's polymerase active site, it is mechanistically distinct from the DNA polymerase reaction. Consequently, this activity represents a novel target for drug discovery, and inhibitors that target this activity may increase the efficacy of nucleoside analogs, and may help to delay the onset of drug resistance. Here, we developed a Förster resonance energy transfer based high throughput screening assay for the AZT-MP excision activity of RT. This assay is sensitive and robust, and demonstrates a signal to noise ratio of 3.3 and a Z' factor of 0.69. We screened 3 chemical libraries (7265 compounds) using this assay, and identified 3,3'-[(3-carboxy-4-oxo-2,5-cyclohexadien-1-ylidene)methylene]bis[6-hydroxy-benzoic acid] (APEX57219) as the most promising hit. APEX57219 displays a unique activity profile against wild-type and drug-resistant HIV-1 RT, and was found to inhibit virus replication at the level of reverse transcription. Mechanistic analyses revealed that APEX57219 blocked the interaction between RT and the nucleic acid substrate.

Keywords

reverse transcriptase; inhibitor; zidovudine; excision; high throughput screen

INTRODUCTION

HIV-1 reverse transcriptase (RT) catalyzes the conversion of the viral single-stranded RNA into double-stranded DNA, which then serves as a substrate for integration into the host cell's genome. RT is multifunctional and possesses two distinct activities: (i) a DNA polymerase activity that can use either RNA or DNA as a template; and (ii) a ribonuclease H (RNase H) activity that degrades the RNA strand in RNA/DNA duplexes [1]. Due to its essential role in virus replication, RT is a major target for antiretroviral drug development.

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AUTHOR CONTRIBUTION

N.S.-C. planned the experiments. C.-W.S. and O.A. performed all of the experiments and analyzed data. N.S.-C. wrote the manuscript.

To date, two distinct therapeutic groups of inhibitors have been approved by the US Food and Drug Administration (FDA) that target RT. These include the nucleoside and nucleotide RT inhibitors (NRTIs) such as zidovudine (AZT), and the nonnucleoside RT inhibitors (NNRTIs), which include nevirapine (NVP) and efavirenz (EFV). NRTIs are dNTP analogs that lack a 3'-hydroxyl group on the ribose sugar. Once metabolized to their active diphosphate or triphosphate forms, they inhibit viral reverse transcription by acting as chain-terminators of nucleic acid synthesis [2]. In contrast, the NNRTIs constitute a group of amphiphilic compounds that bind to a hydrophobic pocket in RT that is near but distinct from the polymerase active site, and inhibit DNA polymerization via an allosteric mechanism of action [3]. Both NRTIs and NNRTIs are routinely used in first-line and second-line combination antiretroviral therapies. However, although their use has profoundly reduced morbidity and mortality associated with HIV-1 infection, their long-term efficacy is limited by the selection of drug-resistant variants of HIV-1 and cross-resistance among RT inhibitors [4]. Consequently, there is an ongoing need to identify and/or develop inhibitors that are active against drug-resistant HIV-1.

HIV-1 resistance to AZT is associated with selection of the thymidine analog mutations (TAMs) M41L, D67N, K70R, L210W, T215F/Y and K219Q/E in RT [5]. Two or more TAMs typically results in high-level resistance to AZT, however these substitutions also confer cross-resistance to other NRTIs including stavudine, abacavir, didanosine, tenofovir, emtricitabine and lamivudine [6-7]. Pioneering work in the laboratory of Dr. Walter Scott revealed that TAMs decrease HIV-1 susceptibility to AZT, and other NRTIs, by augmenting RT's ability to excise a chain-terminating NRTI-monophosphate (NRTI-MP) from a prematurely terminated DNA chain using ATP as a phosphorolytic donor [8-9]. Removal of the chain-terminating NRTI-MP can also be achieved by pyrophosphorolysis [10], although this reaction does not appear to be physiologically relevant. Although the ATP-mediated NRTI-MP excision reaction occurs at the polymerase active site of the enzyme, it is mechanistically distinct from the DNA polymerase reaction: the substrates (ATP/GTP vs. dNTP) and leaving products (A-(P)4-NRTI vs. pyrophosphate) are different, as are the rate-limiting steps of the reaction (chemistry vs. conformational change) (Figure 1) [8-11]. Consequently, it has been proposed that the NRTI-MP excision activity of RT represents a novel target for drug discovery and that inhibitors that target this activity may increase the efficacy of NRTIs, and may delay the onset of drug resistance. Indeed, multiple studies have suggested that the observed synergy between NRTIs and NNRTIs is due, in part, due to the NNRTIs ability to inhibit NRTI-MP excision [12-14]. Additionally, a series of bisphosphonate inhibitors have been reported to inhibit NRTI-MP excision without impacting DNA polymerization, although none of these compounds were found to exhibit antiviral activity [15]. The discovery of additional inhibitors of NRTI-MP excision has been hampered by the fact that ATP-mediated excision assays are not well-suited for high throughput screening. As such, the primary goal of this study was to develop a high throughput screening (HTS) assay for NRTI-MP excision, and to identify small molecules (which did not belong to the NNRTI class) that inhibited this activity.

EXPERIMENTAL

Materials

Wild-type (WT) and mutant HIV-1 RT was over-expressed and purified to homogeneity as described previously [16]. RT concentration was determined spectrophotometrically at 280 nm using an extinction coefficient of $260,450 \text{ M}^{-1} \text{ cm}^{-1}$. The NCI Natural Product Set and the NCI Diversity Library were requested from the Developmental Therapeutics Program NCI/NIH. The ApexScreen library was purchased from TimTec (Newark, DE). EFV and NVP were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. AZT-triphosphate (AZT-TP) was from Trilink BioTechnologies (San Diego, CA). DNA and RNA oligonucleotides were synthesized, chemically modified, and purified by Integrated DNA Technologies (Coralville, IA, USA). Alexa Fluor® 555-aha-dUTP (AF555-dUTP) and Alexa Fluor® 555 C₂ maleimide were purchased from Life Technologies (Grand Island, NY). All other reagents were of the highest quality available and were used without further purification.

Template/Primer (T/P) Substrates

The following template/primer (T/P) substrate was used in the NRTI-MP excision assay: a 30 nucleotide (nt) DNA template (5'-CCTAAAGTCTCTAGCAGTGGCGCCCGA ACA-3') annealed to 22 nt AZT-MP terminated primer (5'-TCGGGCGCCACTGCTAGAG ACZ-3'; where Z is AZT-MP). The template was synthesized with AlexaFluor® 666 (AF666) attached to the 5'-terminus. The primer was chain-terminated with AZT-MP, as described previously [14]. For the ensemble Förster resonance energy transfer (FRET) assays for RT-T/P interactions, the primer was synthesized with AF666 attached to the 5'-terminus. This same T/P substrate was used in the gel shift assays, however in these experiments neither the template nor primer was covalently labeled with a fluorescence dye. For the DNA polymerase-independent RNase H assays, we used an 18 nt template (5'-GAUCUGAG CCUGGGAGCU-3') annealed to an 18 nt primer (5'-AGCTCC CAGGCTCAGATC-3'), as described previously [17]. For DNA polymerase-dependent RNase H cleavage assays we used a 26 nt DNA primer (5'-CCTGTTCGGGCGCCACT GCTAGAGAT-3') annealed to a 35 nt RNA template (5'-GAAUGGAAAAUCUCUAGCA GUGGCGCCCGAACAG-3') [17]. Both RNase H assays were FRET based and fluorescein and IowaBlack FQ™ were covalently attached to the 3'- and 5'-termini of the RNA and DNA oligonucleotides, respectively.

NRTI-MP Excision Assays

Assays were carried out in a total volume of 50 μL (96 well) or 30 μL (384 well) in solid black, round bottom 96 well or 384 well microplates. The typical reaction contained 100 nM AZT-MP terminated (T/P), 2 mM ATP, 1 μM AF555-dUTP, 1 μM dATP and 1 μM dGTP in 50 mM Tris-HCl pH 7.5 containing 50 mM KCl and 10mM MgCl_2 . Reactions were initiated by the addition of 600 nM WT or mutant HIV-1 RT. All inhibitors were dissolved in dimethylsulfoxide (DMSO); the final concentration of DMSO in the reactions did not exceed 2.0 %. Fluorescence was measured at 37 °C with a SpectraMax M2 microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA) using an excitation wavelength of

515 nm and an emission wavelength of 668 nm. A 530 nm cutoff filter was used in all assays. Data was analyzed using SoftMax Pro software (Molecular Devices).

Statistical Analyses of NRTI-MP Excision Assay

Statistical analyses of the signal-to-noise ratios of the assay were carried out by calculating the mean (M) and standard deviations (SD) for the signal and background reactions and then determining the signal-to-noise ratio (S/N) using the following equation: $S/N = (M_{\text{signal}}/2M_{\text{background}})/[(SD_{\text{signal}})^2 + (SD_{\text{background}})^2]^{1/2}$ [18]. The Z' value, which is a relative indication of the separation of the signal and background populations, was calculated from data from five 96-well plates, which were filled with both positive (signal) and negative (background) controls, using the following equation: $Z' = 1 - 3(SD_{\text{signal}} + SD_{\text{background}})/|M_{\text{signal}} - M_{\text{background}}|$ [19].

Inhibition of DNA Polymerase and RNase H Activity

The concentration of drug required to inhibit 50 % of the RNA-dependent DNA polymerase, DNA-dependent DNA polymerase and RNase H activities of HIV-1 RT (i.e., IC_{50}) was determined, as described previously [20]. IC_{50} values were calculated using nonlinear regression analyses (SigmaPlot Software Version 11, Systat Software, Inc., San Jose, CA, USA), from at least three independent experiments.

Antiviral Activity and Cytotoxicity

Antiviral activity was assessed in P4/R5 cells, as described previously [21]. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide based assay. For quantitative analyses of reverse transcripts, HIV-1 replication used VSV-g pseudotyped HIV-1 virions that undergo a single cycle of replication. The virions were generated by co-transfecting 293 cells with pNL4-3HSAR^{-E-} and pHCMV-g, as described previously [22]. Quantitative real-time PCR analyses of reverse transcriptase products were performed as described previously [23].

Ensemble FRET Assays for RT-T/P Interactions

The application of FRET for quantifying the binding interaction between RT and T/P has been described previously [24,25]. Here, we used a modified assay as described below. Briefly, C38V/C280S/L560C p66RT was co-expressed with C38V/C280S p51 RT in bacteria using the pDUET prokaryotic vector. The resultant chimeric p66/p51 RT was purified, as reported previously. The purified recombinant RT was then chemically modified Alexa Fluor® 555 C₂ maleimide. Stoichiometry of labeling was determined using the appropriate extinction co-efficient values for RT (260, 450 cm⁻¹.M⁻¹) and AF555 (150, 000 cm⁻¹.M⁻¹), respectively. Typically, we found the stoichiometry of labeling to be between 0.7-1.0 mol of AF555 per mol of RT. Of note, the mutant RT described above possesses comparable DNA polymerase specific activity to the wild type HIV-1 RT (data not shown). The chemically-modified RT retains about 80% of the DNA polymerase and RNase H activity of the unmodified protein, but remains equally sensitive to inhibition by APEX57219, EFV and NVP. Thus, the genetic and chemical manipulations of RT required for these assays have little effect on the response of the enzyme within the parameters of the

assay protocols. Labeled RT (400nM) was then equilibrated with varying concentrations of T/P (0-2 μ M) for 2 hours in 50mM Tris-HCl pH7.5, 50mM KCl, and the amount of T/P-bound RT present in an equilibrium solution is measured by quantification of the energy transfer from AF555 to AF647. Appropriate control experiments were carried out to correct for background signal and inner filter effect. Data were analyzed using a fraction saturation plot and are fitted to a one site saturation equation: $y = (B_{\max}x)/(K_d + x)$, where y is the fraction of RT saturated with T/P at a specific concentration (x). $B_{\max}$ is the saturation value at which all RT is bound with T/P.

Gel mobility shift assays

The amount of T/P-bound RT present in an equilibrium solution was assayed by native gel electrophoresis. RT (0-900 nM total) was equilibrated with 1 μ M of 32 P-labeled T/P for 2 hours in the absence or presence of NVP or APEX57219 in 50 mM Tris pH7.5, 50 mM KCl. Samples were loaded on a 4% polyacrylamide gel in 40 mM Tris-acetate, pH 8.0, and 1 mM EDTA. Gels were run at room temperature for 1 h (100 V constant voltage), dried, and quantified as described previously [14].

RESULTS AND DISCUSSION

Development of an HTS assay to identify inhibitors of the ATP-mediated AZT-MP excision activity of HIV-1 RT

In an effort to identify inhibitors of the AZT-MP excision activity of RT, we developed a FRET based HTS assay (Figure 1a). In this assay, an AZT-MP terminated primer is annealed to a complementary DNA template that has the fluorescent dye AF647 conjugated to its 5'-terminus. AZT-MP excision is initiated by the addition of recombinant (WT or mutant) HIV-1 RT, ATP, dATP, dGTP and dUTP that is modified at the C-5 position with a hexylacrylamide linker attached to the fluorescent dye AF555. Once RT has excised the AZT-MP moiety, it rapidly incorporates AF555-dUTP (adTTP analog), resulting in energy transfer from the donor (AF555) to the acceptor (AF647) which can be measured continuously (Figure 1b). If the AZT-MP moiety is not excised, then dUTP cannot be incorporated into the primer strand, and consequently there is no change in the fluorescence signal over time (Figure 1b). Importantly, fluorescence readings can be taken as frequently as every 10 s, which generates excision isotherms with multiple time points and therefore increases the correlation coefficient (R^2) when data are fit to the appropriate equations. Consistent with previously published data [11-27,28], we found that RTs containing M41L/L210W/T215Y or D67N/K70R/T215F/K219Q increased the enzyme's apparent affinity for ATP (K_M) and the rate of excision (k_{excision}) (Table 1). In contrast, the D67N/K70R enzyme only displayed an increase in the rate of ATP-mediated AZT-MP excision (Table 1). Pyrophosphate (PPi) can also be used as a substrate for AZT-MP excision in this assay (Figure 1e) [10]. However, while the rates of PPi-mediated excision were found to be ~ 20-fold greater than the rates of ATP-mediated excision, no significant differences in the AZT-MP excision activities were observed between the WT and M41L/L210W/T215Y RTs, as published previously [11-27]. NNRTIs have been shown to inhibit AZT-MP excision [12-14]. Therefore, we also determined the concentration of EFV required to inhibit the AZT-MP excision reaction catalyzed by WT and TAM containing RTs (Figure 1f, Table 3).

Collectively, these data demonstrate that our FRET assay faithfully recapitulates previously published findings. Most importantly, the assay is sensitive and robust and demonstrates a signal to noise (S/N) ratio of 3.3 and a Z' factor of 0.69 in reactions using 2 mM ATP. The S/N ratio compares the level of the desired signal to the level of background noise [18]. The Z' factor is a relative indication of the separation of the signal and background populations [19]. It is a dimensionless parameter that ranges from 1 (infinite separation) to <0 . Signal and background populations start to overlap when $Z'=0$. In general, the acceptance criteria for a quality assay is one in which the $Z'>0.4$. In this regard, our *in vitro* AZT-MP excision assay meets and exceeds this criterion.

Chemical library screen to identify novel inhibitors of RT

To identify inhibitors of AZT-MP excision, we screened 3 chemical libraries (7265 compounds in total) using M41L/L210W/T215Y HIV-1 RT and 2 mM ATP. A summary of the screening results are provided in Table 2. Excision isotherms from one of the twenty five 96-well plates screened in the NCI Diversity Set is illustrated in Figure 2a. In this set, each plate contained 80 structurally diverse compounds, 8 control reactions without inhibitor, 4 control reactions that lacked ATP (data not shown), and 4 reactions that contained 100 nM EFV (data not shown). It is clearly evident in this Figure that there is very little spread in the isotherms for 87 of the reactions (79 compounds + 8 controls), which allows for the facile identification of the one compound which inhibited the AZT-MP excision reaction. Like all fluorescence-based assays, our assay is also subject to “false-positives”; i.e. compounds that appear to inhibit (or stimulate) activity but show little or no activity in follow-up assays. This can be due to either the compound absorbing the excitation energy or by emitting its own fluorescent signal. Fortunately, we are able to identify such compounds as their kinetic traces differ significantly from the controls (Figure 2b). In this regard, compounds that absorb the excitation energy yield “flat” isotherms with fluorescent values lower than the no ATP control. By contrast, compounds that are fluorescent themselves generate isotherms with values much higher than the control reaction. It should be noted that of all the hits we have identified to date, none were found to be false-positives in follow-up assays.

Discovery of APEX57219

As described in Table 2, we identified 125 hits from 7265 compounds (1.7% hit rate). To evaluate these hits, we adopted a systematic approach to identify those with a unique structure-activity-resistance profile (Figure 3a). Briefly, the activity of each hit was verified in a gel-based assay for AZT-MP excision. Thereafter, all were evaluated to determine their ability to inhibit AZT-MP excision, RNA- and DNA-dependent DNA polymerase activity and RNase H activity. Of note, all of the hits identified in the screen were found to inhibit the DNA polymerase activity of RT (data not shown), suggesting that we did not identify compounds that exhibited specificity toward the AZT-MP excision reaction. Because NNRTIs inhibit the excision activity of RT [12-14], we next screened each hit for their capacity to inhibit RTs containing the NNRTI resistance mutations K103N, V106A, V179D, Y181C, Y188C or G190A. We found that 114 of the hit compounds exhibited reduced activity against one or more of these mutant RTs (data not shown). These compounds were excluded from further analysis. Of the remaining eleven hits, 5 were found to share a common anthracycline pharmacophore structure (e.g. see NSC301739 in Figure 3c).

Antacycline analogs are known to inhibit DNA and RNA synthesis by intercalating between base-pairs of the DNA/DNA or DNA/RNA strand [29]. Consequently, we were interested to understand how intercalators, such as NSC301739, impacted the kinetics of AZT-MP excision compared to an inhibitor that binds to RT (i.e., NVP). Dose response curves for both NVP and NSC301739 are illustrated in Figures 3b and 3c. The excision isotherm for each drug concentration was fitted to an exponential equation to yield the apparent rate of excision (k_{excision}) as well as the amplitude (i.e. extrapolated maximum fluorescence value corrected for background signal). This analysis revealed that k_{excision} decreased as a function of NVP concentration, yielding an IC_{50} value of 869 ± 17 nM (Figure 3d). In comparison, the amplitudes of the excision isotherms for each NVP concentration were largely similar (Figure 3d). By contrast, NSC301739 exerted only a moderate effect on k_{excision} , but significantly decreased the amplitude in a concentration dependent manner to yield an IC_{50} value of 6.3 ± 3.5 μM . We also determined the dependence of each inhibitors IC_{50} value on ATP concentration (Figure 3e). This analysis revealed that the IC_{50} value for NVP (based on k_{excision}) was dependent on the ATP concentration used in the assay, whereas the IC_{50} value for NSC301739 (based on amplitude) was largely insensitive to ATP concentration. Using this kinetic approach, we excluded an additional 3 hits that were also found to show similar kinetic profiles to NSC301739. Finally, to complete the follow-up characterization we assessed the antiviral activity and cytotoxicity of the remaining 3 hits.

The most potent hit identified from these analyses was 3,3'-[(3-carboxy-4-oxo-2,5-cyclohexadien-1-ylidene)methylene]bis[6-hydroxy-benzoic acid] (APEX57219; Figure 4). APEX57219 was found to inhibit the AZT-MP excision, RNA-dependent and DNA-dependent DNA polymerase, and RNase H activities of WT and mutant HIV-1 RT (Table 3). APEX57219 was also found to be a potent inhibitor of HIV-1 replication in cell culture ($EC_{50} \sim 250$ nM), and no cellular toxicity was observed at the highest drug concentration tested (50 μ M). To verify that the observed antiviral activity was due to APEX57219 inhibiting HIV-1 reverse transcription, and not other steps of viral replication, we used real-time PCR to quantify the amount of full-length (–) strand DNA synthesis that occurred in HOS cells infected with VSV-g pseudotyped HIV-1 virions. NVP was included as a positive control because its antiviral potency is comparable to that of APEX-57219. The data (Figure 4) clearly shows that APEX-57219 inhibits viral reverse transcription in a dose-dependent manner.

APEX57219 prevents RT from binding the T/P substrate

To elucidate the mechanism by which APEX57219 inhibits RT, we initially used the steady-state kinetic approach to determine how the inhibitor impacted the Michaelis-Menten constants (i.e., K_M , V_{max}) for dNTP and T/P binding. These analyses revealed that APEX57219 was a competitive inhibitor with respect to T/P binding, and a non-competitive inhibitor with respect to dNTP binding (data not shown). Surprised by these findings, we further investigated the effect of APEX57219 on RT-T/P interactions using two complementary assays. First, we established an ensemble FRET assay that directly monitors nucleic acid binding to RT. In this assay, RT is labeled with AF555 on the C-terminus of the enzyme, and the T/P substrate is labeled with AF647 on the 5'-end of the DNA primer. Specific interaction between RT and T/P results in energy transfer between donor and

acceptor (Figure 5a), which can be used to calculate the binding affinity constant (K_d) for the RT-T/P complex (Figure 5b). Importantly, when APEX57219 was included in the assay, we observed a loss of energy transfer between donor and acceptor molecules indicating that the inhibitor disrupted RT-T/P interactions (Figure 5c, 5d). Prior studies have suggested that NNRTIs can induce RT to “flip” orientation on the T/P substrate [24]. Consequently, we used gel shift mobility assays to verify, and further demonstrate, that APEX57219 abrogates the RT-T/P interactions (Figure 5E).

Conclusions

In this study, we describe the development of an HTS assay for the NRTI-MP excision activity of HIV-1 RT. By using this assay to screen 7265 small molecules we identified APEX57219. APEX57219 exhibits a novel mechanism of HIV-1 RT inhibition: it abrogates the interactions between enzyme and the nucleic acid substrate. Consistent with this finding, APEX57219 inhibits all of the enzymatic activities of RT (i.e., DNA polymerase, RNase H and NRTI-MP excision) and retains activity against common NNRTI resistance mutations. Importantly, APEX57219 inhibits virus replication and the level of reverse transcription. Ongoing studies are focused on identifying the APEX57219 binding site in RT.

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Abbreviations used

RT	reverse transcriptase
RNase H	ribonuclease
NRTI	nucleoside reverse transcriptase inhibitor
AZT	zidovudine
MP	monophosphate
TAMs	thymidine analog mutations
NNRTI	non-nucleoside reverse transcriptase inhibitor
EFV	efavirenz
NVP	nevirapine
WT	wild-type
T/P	template/primer
HTS	high throughput screen
APEX57219	3,3'-[(3-carboxy-4-oxo-2,5-cyclohexadien-1-ylidene)methylene]bis[6-hydroxy-benzoic acid]

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SUMMARY STATEMENT

We developed a FRET-based high throughput screening assay for the AZT-MP excision activity of HIV-1 RT. Using this assay we screened 7265 compounds, and identified 3,3'-[(3-carboxy-4-oxo-2,5-cyclohexadien-1-ylidene)methylene]bis[6-hydroxy-benzoic acid] (APEX57219). APEX57219 inhibits the interaction between RT and the nucleic acid substrate.

Figure 1. Kinetic pathways for the RT catalyzed dNTP incorporation and NRTI-MP excision reactions. The substrates, leaving products and rate limiting steps are different for these two mechanistically distinct reactions.

Figure 2. Overview of HTS AZT-MP assay.

(A) Schematic of HTS assay for AZT-MP excision activity. AZT-MP is designated by a “Z”. Assay conditions are defined in Experimental.

(B) Isotherms for excision of AZT-MP by M41L/L210W/T215Y RT in the absence and presence of 2 mM ATP.

(C) Isotherms for excision of AZT-MP by M41L/L210W/T215Y RT in the presence of varying concentrations of ATP.

(D) Plot of k_{excision} (determined from Figure 2C) versus ATP concentration. Data were fit to a hyperbolic plot, and calculated kinetic parameters are provided in Table 1.

(E) Dependence of k_{excision} of WT and M41L/L210W/T215Y RT on pyrophosphate concentration

(F) Inhibition of the AZT-MP excision activity of M41L/L210W/T215Y RT by EFV.

Reactions were carried out using 2 mM ATP. The IC_{50} value is provided in Table 3.

Figure 3. AZT-MP excision isotherms acquired during the HTS campaign.

(A) Excision isotherms from 1 plate in the NCI Diversity Set that contained 80 different compounds and 8 control reactions.

(B) Examples of false positives in which the compound absorbs the excitation energy (green $\square$) or is fluorescent itself (red $\circ$). Control reactions in the presence (?) and absence ($\diamond$) of ATP are also shown.

Figure 4. Characterization of “hit” compounds

(A) Schematic illustrating the systematic approach used to characterize the hit compounds.

(B) Isotherms for excision of AZT-MP by WT RT in the absence or presence of NVP. A control reaction that lacked ATP is also shown.

(C) Isotherms for excision of AZT-MP by WT RT in the absence or presence of NSC301739. A control reaction that lacked ATP is also shown.

(D) Dependence of k_{excision} for WT RT on the concentration of NVP or NSC301739.

(E) Dependence of the excision amplitude for WT RT on the concentration of NVP or NSC301739.

(F) Dependence of the IC_{50} for inhibition of AZT-MP excision by WT RT by NVP or NSC301739 on the concentration of ATP used in the assay.

Figure 5.

Real-time PCR analysis of intracellular reverse transcription in the absence (control) or presence of NVP or APEX57219. The amount of full-length (–) strand DNA synthesis was evaluated at defined times after infection and quantified as described in Experimental. The structure of APEX57219 is also shown.

Figure 6. APEX57219 prevents HIV-1 RT from binding the T/P substrate.

(A) FRET assay for RT-T/P interactions. Figure illustrates the transfer of fluorescence energy from RT (labeled with AF555) to T/P (labeled with AF667). Data have been corrected for background signals and acceptor fluorescence in the absence of donor.

(B) Binding isotherm for RT to the T/P substrate determined from the sensitized emission data acquired in Figure 6A. The K_d for the interaction was calculated to be 0.8 ± 0.1 nM.

(C) The sensitized fluorescence of AF667-T/P in complex with AF555-RT is lost upon addition of APEX57219.

(D) Dependence of the sensitized fluorescence of AF667-T/P in complex with AF555-RT upon the concentration of APEX57219.

(E) Gel mobility shift analyses of WT RT in complex T/P in the absence or presence of NVP or APEX57219. The amount of RT-T/P complex formed is decreased with increasing concentrations of APEX57219. By contrast, NVP has no effect on the RT-T/P equilibrium.

Table 1

Steady-state kinetic parameters determined for the ATP-mediated AZT-MP excision activity of WT and mutant HIV-1 RT.

RT	K_M (mM)	k _{excision} (min ⁻¹)	k _{excision} / K_M (mM ⁻¹ .min ⁻¹)	Fold-resistance
WT	7.0 ± 1.5	0.004 ± 0.001	6.3×10 ⁻⁴	-
D67N/K70R	5.7 ± 1.1	0.063 ± 0.007	0.011	18
D67N/K70R/T215F/K219Q	2.2 ± 0.4	0.045 ± 0.006	0.021	33
M41L/L210W/T215Y	2.3 ± 0.4	0.049 ± 0.005	0.021	33

Table 2

Summary of HTS to identify inhibitors of AZT-MP excision

Chemical Library	Library Size	Screen Format	Concentration in HTS	Hits	Hit Rate (%)
NCI Natural Product Set	235	96-well (3 plates)	20 μ M	7	3
NCI Diversity Set	1990	96-well (25 plates)	20 μ M	13	0.65
TimTec ApexScreen	5040	384-well (15 plates)	15 μ M	105	2

Table 3

Inhibitory activity of APEX57219 and EFV against different activities of WT and mutant HIV-1 RT

Assay	RT/Virus	APEX57219 (IC ₅₀ /EC ₅₀ ; nM)	EFV (IC ₅₀ /EC ₅₀ ; nM)
	WT	140 ± 19	43 ± 7
AZT-MP excision assay (2 mM ATP)	M41L/L210W/T215Y	220 ± 17	85 ± 6
	D67N/K70R	207 ± 22	77 ± 6
	D67N/K70R/T215F/K219Q	242 ± 31	80 ± 4
	WT	330 ± 14	48 ± 1
	TAM41	355 ± 23	31 ± 3
	K103N	383 ± 44	> 1000
RNA-dependent DNA polymerase assay	V106A	325 ± 31	144 ± 11
	V179D	345 ± 22	106 ± 9
	Y181C	312 ± 17	215 ± 22
	Y188C	309 ± 28	700 ± 65
	G190A	290 ± 26	222 ± 31
DNA-dependent DNA polymerase activity	WT	455 ± 27	171 ± 13
DNA-polymerase independent RNase H activity	WT	314 ± 30	enhanced activity
DNA-polymerase dependent RNase H activity	WT	430 ± 42	enhanced activity
Antiviral activity	WT	250 ± 34	1.3 ± 0.2
Cytotoxicity	not applicable	> 50, 000	> 10, 000