Inhibitors of DNA Strand Transfer Reactions Catalyzed by HIV-1 Reverse Transcriptase†

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ABSTRACT: The discovery and characterization of new inhibitors of HIV-1 reverse transcriptase (RT) is an important step toward understanding the mechanism of this multifunctional polymerase. We describe the identification of novel inhibitors of HIV-1 RT-catalyzed reactions utilizing a nucleic acid model system designed to mimic the essential features of DNA strand transfer reactions catalyzed by HIV-1 RT. This reaction requires the DNA polymerase and RNase H activities of RT, as well as the translocation of DNA from one template strand to another. In addition to the discovery of new inhibitors of DNA polymerase activity, two classes of inhibitors were identified that inhibit different steps of the DNA strand transfer reaction. One class of these, exemplified by actinomycin D, inhibits DNA strand transfer by interfering with the transfer of the DNA intermediate onto the acceptor template. The second class of strand transfer inhibitor, exemplified by the chlorophenylhydrazone of mesoxalic acid, was found to inhibit the ribonuclease H (RNase H) activity of HIV-1 RT under strand transfer conditions. This inhibitor is a potent and specific inhibitor of RNase H activity, which displays no inhibition of either DNA-dependent or RNA-dependent DNA polymerase activity. Together, these three inhibitors block different steps reverse transcription and will be valuable in studying the mechanism of multistep reactions such as DNA strand transfer. In addition, these new inhibitors of in vitro reverse transcription point to new strategies for the intervention of retroviral DNA replication and could be useful in the development of new HIV-1 therapeutic strategies.

HIV-1 reverse transcriptase (RT)[1] is responsible for the synthesis of double-stranded proviral DNA using the packaged viral RNA genome as the primary template in a complex series of reverse transcription events (Figure 1A). Two obligatory DNA strand transfer reaction events occur during reverse transcription (Figure 1A, I−III, IV−VI). During strand transfer, the nascent DNA synthesized by RT is translocated from one template to a second template. Since each viral particle is packaged with two copies of single-stranded RNA genome, the DNA could be transferred from one template onto the second copackaged template. These DNA strand transfers are known to occur frequently at internal regions of the viral genome during reverse transcription, resulting in recombinant proviral DNA products (*1*−*8*) (Figure 1B). As many as three crossovers per round of reverse transcription have been reported for HIV-1 (*8*), making DNA strand transfer reactions a central part of proviral DNA synthesis. This high rate of recombination during reverse transcription has been shown to contribute to the development of multidrug resistance (*9, 10*).

On the basis of mechanistic *in vitro* model systems and viral studies, a minimal mechanism for HIV-1 RT-catalyzed DNA strand transfer must include the polymerase and RNase H activities associated with RT, as well as the physical translocation of the nascent DNA strand from one RNA template to another. There is considerable biochemical and viral evidence for the requirement of RNase H activity during DNA strand transfer (*11*−*15*). For example, kinetic studies (*13, 14*) have shown that mutant HIV-1 RT lacking RNase H activity is incapable of supporting DNA strand transfer reactions. This has been confirmed in a variety of retroviral systems in culture (*11*). In addition, a second viral protein, the nucleocapsid protein (NC), is known to accelerate strand transfer reactions in vitro (*16*−*26*). The structural and kinetic natures of the intermediates of this complex reaction are largely unknown. Given the importance of this reaction in viral replication, a closer examination of the mechanism of this reaction is warranted.

The mechanistic characterization of DNA strand transfer catalyzed by HIV-1 RT would be greatly facilitated by the availability of RT inhibitors that function at different intermediate stages of the strand transfer process. For example, while many DNA polymerase inhibitors of HIV-1 RT are currently known (*27*−*30*), and their mechanisms of action have been extensively characterized, only very poor inhibitors of HIV-1 RT-associated RNase H activity are

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[1] Abbreviations: HIV-1, human immunodeficiency virus type-1; AIDS, acquired immunodificiency syndrome; RNase H, ribonuclease H; NC, nucleocapsid protein; RT, reverse transcriptase; EDTA, ethylenediaminetetraacetic acid; TBE, Tris−boric acid−EDTA buffer; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; DMSO, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis.

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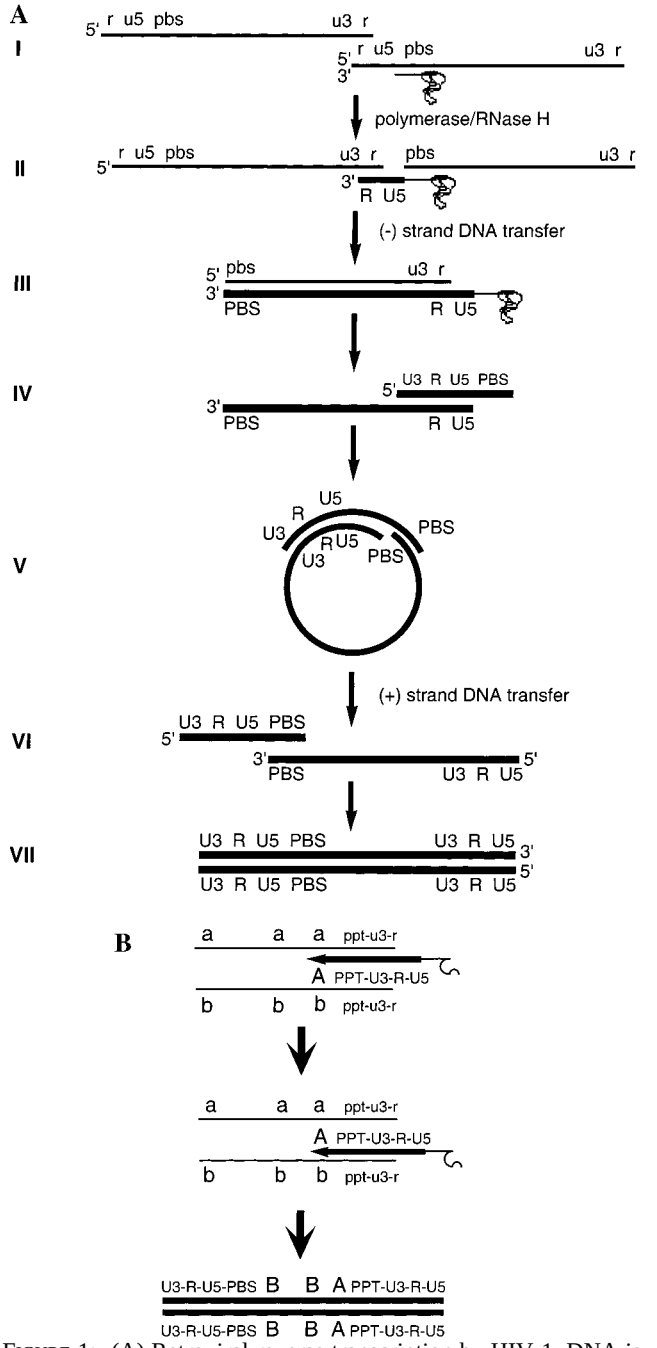


FIGURE 1: (A) Retroviral reverse transcription by HIV-1. DNA is shown in thick lines and capital letters, and RNA is shown in thin lines and lower case lettering. Reverse transcription is initiated using a $tRNA^{lys}$ annealed to the primer binding site (pbs) of the viral RNA genome. Two genomic RNAs are packaged per virion, both of which are competent for reverse transcription. DNA synthesis proceeds to the end of the RNA template with concomitant RNA hydrolysis by the polymerase and RNase H activities of RT (I, II). This strong-stop DNA fragment is translocated onto the same RNA or second copackaged RNA template (shown) guided by terminal repeat sequences located at the ends of the genome (r) (I−III). The remainder of the minus strand DNA is synthesized and plus strand DNA synthesis is initiated, followed by a second intramolecular DNA strand transfer event (IV−VI). DNA synthesis is completed to generate full length DNA destined for integration into the host chromosome. (B) DNA strand transfer reactions can also occur at internal genome regions during reverse transcription, resulting in the formation of recombinant provirus with genetic information provided from both copackaged RNA genomes. DNA strand transfer could occur at template termini resulting from genome breakage (forced copy-choice) or at polymerase pause sites as shown (copy-choice).

Table 1: DNA and RNA Substrates

oligo 1. DNA primer 5'-GCATCTGGGGCTCGCAAATTTG-3'
oligo 2. RNA template 5'-AGGUGAGUGAGAUGAUAACAAAUUUGCGAGCCCCAGAUGC-3'
oligo 3. DNA acceptor template 1 5'-CCCCCCCCCCCCCCCCAGGTGAGTGAGATGATAACA$_d$-3'
oligo 4. DNA acceptor template 2 5'-GAGCTGCTTGAATTCTGCGTACTAGGTGAGTGAGATGATAACA-3'

known (*31*−*35*). In addition, it would be of considerable interest to identify molecules that have the ability to interfere with the transfer of DNA from one template strand to another, a requisite for strand transfer. Inhibition of these various steps would allow for the detection of strand transfer intermediates and allow for the characterization of possible nucleic acid−protein intermediates (*14*, *36*, *37*)

In an effort to identify such inhibitors, we developed a chemical mass screening assay using a novel application of scintillation proximity technology that was specifically designed to detect inhibition of one or more steps of the DNA strand transfer process. From this assay we have identified novel inhibitors that target different intermediate stages of the DNA strand transfer process. The complete characterization of these new inhibitors will lead to a better understanding of the mechanism of DNA strand transfer. In addition, due to the central role of DNA strand transfer in reverse transcription and recombination, this reaction is an attractive target for inhibitor development and could provide new lead compounds and strategic targets for the development of therapeutic agents against HIV-1 replication.

MATERIALS AND METHODS

Reagents. The sequences of oligonucleotides used in these studies are shown in Table 1. Biotin-phosphoramidite and 3′-dA-phosphoramidite were purchased from Glenn Research (Sterling, VA). The oligonucleotides were synthesized by the Parke-Davis Pharmaceutical Research DNA facility and were purified by denaturing PAGE through 20% acrylamide/8 M urea/TBE (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) gels. The eluted DNA was desalted using C18 Sep-Pak columns (Waters). DNA and RNA were quantitated by their absorbance at 260 nm on the basis of their calculated extinction coefficients.

HIV-1 RT was purified as described (*18*). Robotic screens were performed using a Biomek 2000 and a Wallac MicroBeta 96-well scintillation counter. The DNA substrates and the primary RNA template (Table 1) were synthesized using standard procedures (*38*). All other reagents used were of the highest quality available.

All inhibitors were determined to be >90% pure by HPLC, mass spectroscopy, and NMR analysis. Actinomycin D was purchased from Sigma. The Parke-Davis Pharmaceutical chemical library provided inhibitors PD029687 and PD126338.

Mass Screen Reactions. Strand transfer reactions contained the tested compound(s) (10 μM), 9.6 nM DNA primer (oligo 1, Table 1), 32 nM RNA template (oligo 2, Table 1), 96 nM DNA acceptor template (oligo 3, Table 1), dATP, dTTP, and dCTP at 0.6 μM each, 0.75 μM d[8-^{3}H]GTP (specific activity = ∼10 Ci/mmol), 10 nM RT, and reaction buffer (50 mM Tris-HCl (pH 8.0), 80 mM KCl, 10 mM $MgCl_2$, 10 mM

DTT, 0.5% w/v Nonidet P-40). In the initial screen each reaction contained 10 compounds, but in subsequent characterization inhibitors were tested individually (see below). The compound(s) were added as a 2-μL solution in dimethy sulfoxide (DMSO). Phosphoformic acid, a known inhibitor of HIV reverse transcriptase, was used as a positive control. Reactions were performed in 96-well plates and initiated by adding 10 μL of 100 μM RT into a 100-μL reaction volume using automated Biomek technology to accelerate the pipetting of a large number of reaction samples. Incubation was at 37 °C for 1 h. Reactions were quenched by adding 40 μL of 0.5 M EDTA (pH 8.0). Streptavidin scintillation proximity assay (SPA) beads (Amersham) (0.1 mg in 10 μL of 1× phosphate buffered saline) were added. The mixtures were incubated at 37 °C for an additional 10 min and diluted 2-fold with TBE buffer, and radioactivity was counted in a 96-well scintillation counter.

A collection of compounds (about 175 000), available at Parke-Davis Pharmaceuticals, was screened for strand transfer inhibitors. In the initial screening, each well contained a group of 10 compounds, each at a concentration of 10 μM. The compounds in a well showing inhibition for DNA strand transfer were rescreened to determine which individual compound within the mixture was responsible for the inhibition. Once an inhibitor was identified in this manner, its inhibition was confirmed by testing it individually in DNA strand transfer gel assays.

DNA Strand Transfer Gel Assay. Kinetic characterization of DNA strand transfer inhibitors was performed using a PAGE assay described previously (*18*). Briefly, a reaction mixture containing reaction buffer (50 mM Tris-HCl, pH 7.6, 60 mM KCl, 1.12 mM EDTA), 20 nM ^{32}P-5′-end-labeled 22-base DNA primer•40-base RNA template duplex, 400 nM DNA acceptor template (oligo 4, Table 1), 5 nM HIV-1 RT, and varying amounts of inhibitor as indicated was incubated at 37 °C for 5 min. Reactions were initiated by the addition of 0.1 mM of each dNTP and 10 mM $MgCl_2$ (final concentrations). Reaction samples were withdrawn at the indicated times, quenched into 50 mM EDTA/denaturing load buffer, and resolved by PAGE (20% acrylamide/8 M urea/TBE). Product bands were visualized and quantitated using a Molecular Dynamics phosphorimager and Imagequant 1.1 software.

RNase H Inhibition. To examine the inhibition of HIV-1 RT RNase H activity, the 40-base RNA primary template (oligo 2, Table 1) was ^{32}P-5′-end labeled, and the rate and pattern of RNA degradation were examined. The RNA was labeled using [γ-^{32}P]ATP as described previously (*18*). RNase H reactions were performed as described above for DNA strand transfer except that the RNA template was labeled. Products were resolved by PAGE (20% acrylamide/8 M urea/TBE) and visualized using a phosphorimager. RNase H inhibition was quantitated as described previously (*14, 18*) by both the rate of appearance of small RNA degradation products and the rate of consumption of RNA substrates.

RESULTS AND DISCUSSION

The discovery of compounds that inhibit different steps of the strand transfer reaction would serve as valuable tools for the mechanistic study of this important reaction by distinguishing what catalytic activities of RT and NC are important for the synthesis and turnover of DNA strand transfer intermediates. In particular, identification of DNA translocation and RT RNase H inhibitors would complement the function of known, as well as new, inhibitors of DNA polymerase activity.

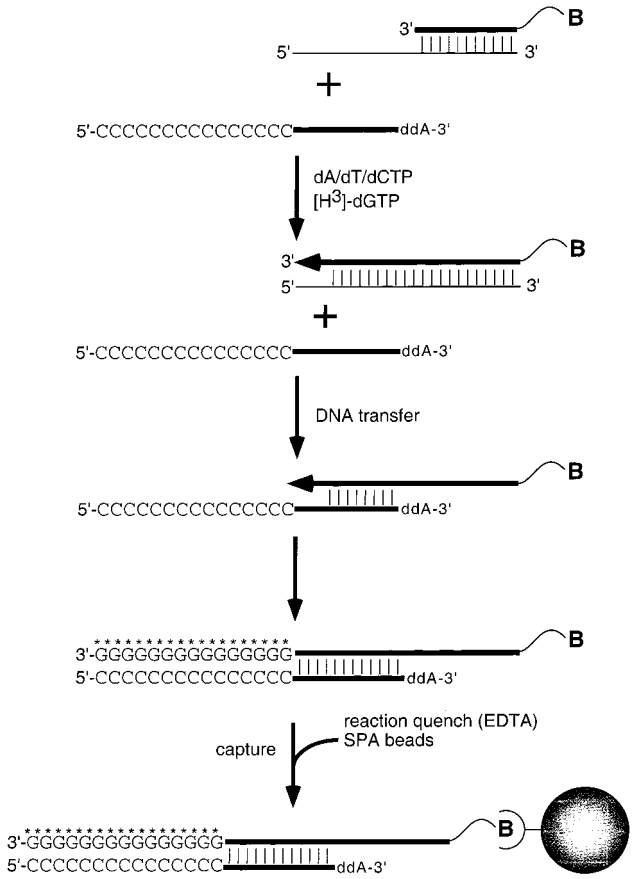


FIGURE 2: Screening assay for the identification of DNA strand transfer inhibitors. A 5′-end biotinylated DNA primer annealed to an RNA template was used. The 5′-end of the primary RNA template sequence was devoid of cytosine. The DNA acceptor template has a requisite 3′-end sequence homologous to the primary RNA template and also includes a polycytosine sequence on its 5′-end. The reaction is initiated by the addition of dATP, dTTP, dCTP, and [^{3}H]dGTP. Only dA, dT, and dCMP are incorporated prior to strand transfer because of the lack of C in the primary RNA template. After DNA strand transfer, radiolabeled dG is incorporated into the DNA product. DNA intermediates and products were captured with streptavidin-coated scintillation proximity beads. Solid-phase SPA beads contain a scintillant that is activated by weak β-emitting radionucleotides physically associated with the resin surface, while nonassociated radioligands produce no signal (*40*). Scintillation counting quantitates the amount of tritiated DNA strand transfer product bound to the SPA bead. The use of SPA technologies eliminated the need to resolve intermediate products by PAGE or recover radiolabeled products by labor-intensive precipitation or filtration methods.

We have developed an *in vitro* model system designed to characterize inhibitors of the strand transfer reaction catalyzed by HIV-1 RT (Figure 2), which permitted the screening of a large chemical library for new inhibitors of RT. In this assay, the RNA and DNA template sequences were chosen to facilitate the quantitation of DNA strand transfer reaction products using scintillation proximity methods (*39, 40*), without the need for purification and/or fractionation methods normally required to separate DNA strand transfer product from DNA intermediates and nucleotide starting materials. An important control experiment was performed that showed

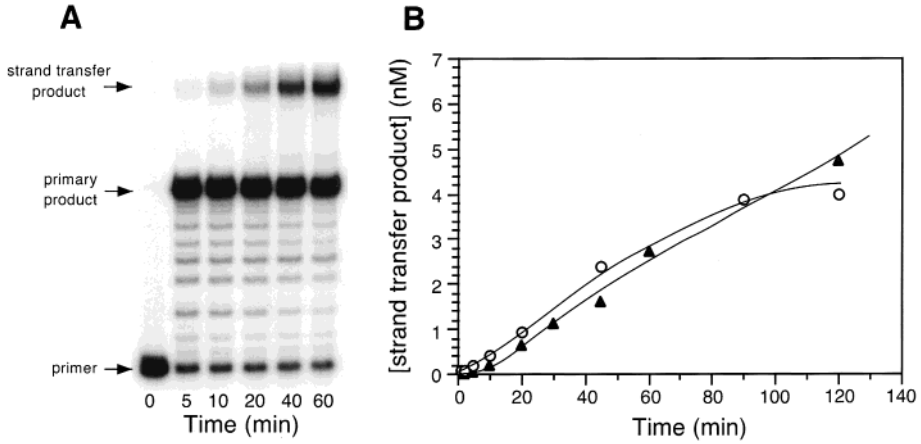


FIGURE 3: DNA strand transfer catalyzed by HIV-1 reverse transcriptase. (A) Analysis of DNA strand transfer by PAGE. The DNA strand transfer reaction assay shown in Figure 2 was monitored by [^{32}P]-end labeling of the DNA primer. Reaction substrates, intermediates (primary product) representing DNA synthesis using the RNA template are resolved from DNA strand transfer product by PAGE. (B) Comparison of PAGE and SPA DNA strand transfer assays. Reactions were performed using the same concentration of RT and substrates (see Materials and Methods). The SPA assays (circles) were performed in the presence of [^{3}H]dGTP and were quantitated using scintillation proximity beads, while the PAGE assay (triangles) was performed and quantitated as in (A).

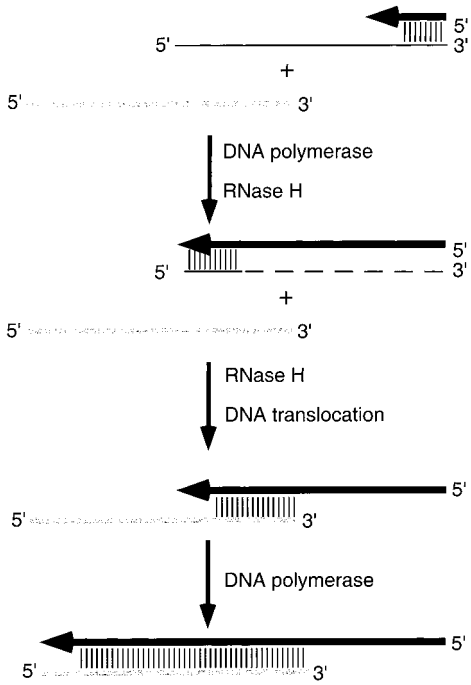


FIGURE 4: Minimal steps involved in DNA strand transfer. DNA is synthesized using by the RNA-dependent DNA polymerase activity of RT. RNase H activity is required for strand transfer by removing the primary RNA template, allowing the DNA intermediate to transfer onto the acceptor template. After DNA transfer, the intermediate duplex is extended by the DNA polymerase activity of RT. Three principle catalytic events are required: DNA synthesis, RNase H, and DNA transfer activities.

that the kinetics of DNA strand transfer monitored by the scintillation proximity assay were comparable to those obtained by the standard method (*14*, *18*) where the reaction products were separated by gel electrophoresis and the 5′-^{32}P-end labeled DNA product was quantified using phosphorimager technologies (Figure 3).

We identified 115 compounds that displayed >70% inhibition at the screen concentration of 10 μM. Several inhibitors could be eliminated from further consideration because of structural considerations (i.e. polymeric or polyanionic), or they contained biotin within their structures and were interfering with the SPA detection method rather than RT-catalyzed strand transfer. A secondary screen was performed by constructing IC_{50} titration curves for the remaining 68 compounds, with 49/68 inhibitors displayed IC_{50} values for DNA strand transfer inhibition of less than 10 μM, while 18 compounds showed IC_{50} values of 3 μM or less.

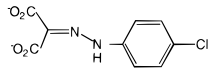
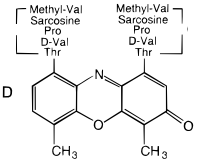
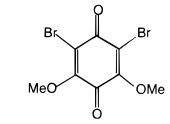
To further characterize the mechanism of inhibition, DNA strand transfer kinetics were monitored using ^{32}P-labeled DNA and RNA substrates (Figure 4). Resolution of the reaction intermediates and products by high-resolution PAGE provides information on which step(s) of strand transfer are inhibited. Radiolabeling of the DNA primer allows for the examination of DNA polymerase activity and the synthesis of both strand transfer product and the accumulation of strand transfer intermediates (Figures 3 and 4). Alternatively, the RNA template can be labeled and its degradation by the RT RNase H activity can be monitored. The acceptor template used in the inhibitor screen contained a homopolymeric stretch of guanines. To avoid possible inhibitor sequence dependence or bias, we replaced this homopolymeric region with a random sequence in the acceptor template for all subsequent mechanistic analysis (Table 1, oligo 4).

DNA strand transfer involves DNA polymerase activity, RNase H activity, and the physical translocation of the nascent primary DNA product from one RNA template to a second acceptor template (Figure 4). If any one or more of these reaction steps were inhibited, DNA strand transfer product formation would be inhibited.

Our screen should, in principle, be capable of identifying a wide range of inhibitors for HIV-1 RT. Initial kinetic and mechanistic characterization of inhibitors identified from this screen showed that we were able to identify distinct classes of new RT replication inhibitors (Table 2).

Perhaps the most important compound identified in our experiments was the inhibitor PD126338. This dicarboxylic acid hydrazone inhibits DNA strand transfer by affecting the activity of HIV-1 RT RNase H activity (Figure 5B). No significant DNA polymerase inhibition was observed under conditions of DNA strand transfer with PD126338 up to inhibitor concentrations of ~50 μM (Figure 5A), ap-

Table 2: Inhibitors of HIV-1 RT-Catalyzed DNA Strand Transfer[a]

Compound	Structure	DNA Strand Transfer IC_{50} (μM)	DNA Polymerase IC_{50} (μM)	RNase H IC_{50} (μM)
PD126338		4.5	>75	3.0
Actinomycin D		2.7 [b]	>30	ND
PD029687		23	3.6[c]	

[a] DNA strand transfer, DNA polymerase and RNase H activities were determined as described in Materials and Methods. [b] Data on actinomycin C inhibition of DNA strand transferis from ref *18*. [c] K_I determined using steady-state single nucleotide incorporation. ND: not detected under conditions tested.

proximately 10-fold higher concentration than the IC_{50} for DNA strand transfer (4.5 μM, Table 2). Under DNA strand transfer reaction conditions, the specificity of RNase H inhibition over DNA polymerase inhibition is >20-fold (on the basis of an inhibitor concentration of 75 μM, the highest inhibitor concentration tested). Also, PD126338 had no detectable effect on the annealing of single-stranded DNA intermediates (see below). The RNase H activity of HIV-1 RT in the presence of inhibitor was examined by 5′-end labeling the primary RNA template. Examination of the cleavage products upon initiation of the DNA strand transfer reaction shows that PD126338 inhibits RNase H activity, resulting in a change in the distribution of accumulated RNase H cleavage intermediates. Longer length RNA cleavage products (>18 bases) accumulate in the presence of PD126338 which are highly resistant to cleavage by the RNase H activity of RT (data not shown). Quantitation of the inhibition of small RNA cleavage products (<17-bases) gives an IC_{50} for RNase H inhibition of 3 μM, in good agreement with the observed IC_{50} of 4.5 μM for DNA strand transfer inhibition (Figure 5B, Table 2).

The discovery of a specific RNase H inhibitor that shows little or no DNA polymerase inhibition activity is significant because of surprising lack of RNase H-specific inhibitors of HIV-1 RT described to date. Reagents that modify cysteine sulfhydryl groups such as *N*-ethylmaleimide and iodoacetamide at millimolar concentrations have been shown to selectively inhibit RNase H activity without affecting DNA polymerase activity (*41*). Naphthalenesulfonic acid derivatives are inhibitors of both DNA polymerase and RNase H activity of HIV-1 RT (*35*). Borkow et al. have reported that the metal chelator *N*-(4-*tert*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH) is an inhibitor of HIV-1 RT. RNase H inhibition was observed with homopolymeric template·primer substrates with an IC_{50} of approximately 3 μM (*31*). However, BBNH also displayed inhibition of both RNA-dependent and DNA-dependent polymerase activities. BBNH appears to bind to the well-characterized nonnucleoside inhibitor binding pocket of HIV-1 RT and inhibit DNA polymerase activity. While the mechanisms of polymerase and RNase H inhibition by BBNH appear to be distinct, the mechanism of RNase H inhibition is not well understood. The natural product illimaquinone shows inhibition of the RNase H activity of HIV-1 RT with no observable effect on DNA polymerase activity (*32*−*34*) but with IC_{50} values significantly higher (∼20 μM) than those reported here for PD126338. Thus, the inhibitor PD126338 represents the most potent specific inhibitor of HIV-1 RT RNase H activity to date.

While the mechanism of inhibition by PD126338 has not been determined, it is intriguing to speculate that the dicarboxylic acid may participate in metal chelation. Both the DNA polymerase and RNase H activities of HIV-1 RT are dependent on divalent metal cations (Mg^{2+}), and the X-ray structure of HIV-1 RT clearly shows these metals bound to both the polymerase and RNase H active sites (*42*−*44*). If metal chelation to active site metal cations is involved in RNase H inhibition, the fact that DNA polymerase activity is only very weakly inhibited suggests that other structural features of this compound give rise to the high specificity for RNase H inhibition.

In addition, our screen predictably identified DNA polymerase inhibitors. While numerous DNA polymerase inhibitors are known against HIV-1 RT, the quinone PD029687 falls into a structurally distinct class of RT inhibitors. As shown in Figure 6, the presence of PD029687 causes a decrease in DNA strand transfer product production, which correlates with the inability of RT to extend the DNA template·primer. At high concentrations of inhibitor, HIV-1 RT polymerase activity can be completely abolished. Steady-state kinetic experiments examining single nucleotide polymerase incorporation show that PD029687 displays uncompetitive inhibition versus nucleotide substrate with a K_i = 3.6 μM (not shown). This value is significantly lower than the observed IC_{50} for DNA strand transfer (23 μM, Table 2). This apparent disparity arises because of the different reaction conditions used. The DNA strand transfer reactions are performed in the presence of high concentrations of HIV-1 RT enzyme (20 nM, stoichiometric or excess over the DNA template·primer substrate). Under these reaction conditions, DNA synthesis is fast and not the rate-limiting step of strand transfer (*14*). Therefore, high concentrations of inhibitor are required to detect inhibition of RT polymerase activity during the time course of the strand transfer assay. Under reaction conditions designed to monitor steady-state enzyme turnover, the enzyme concentration is kept low, and the resulting K_i represents the true dissociation constant for the enzyme·inhibitor complex. The uncompetitive nature of the inhibition suggests that PD029687 binds at a location distinct from the dNTP binding site. In addition, inhibition of polymerase activity by PD029687 is independent of the DNA template·primer concentration (not shown).

Finally, our strand transfer inhibition screen identified molecules which block DNA strand transfer by targeting the substrates rather than binding to the HIV-1 RT enzyme. This type of inhibition is exemplified by the cytotoxic, antibiotic drug actinomycin D (*45*). The inhibition of retroviral replication by actinomycin D has been known for some time (*54*, *55*), although the mechanism of inhibition has only recently been characterized in detail for HIV-1. We (*18*), and others (*22*, *46*), have shown that actinomycin D inhibits

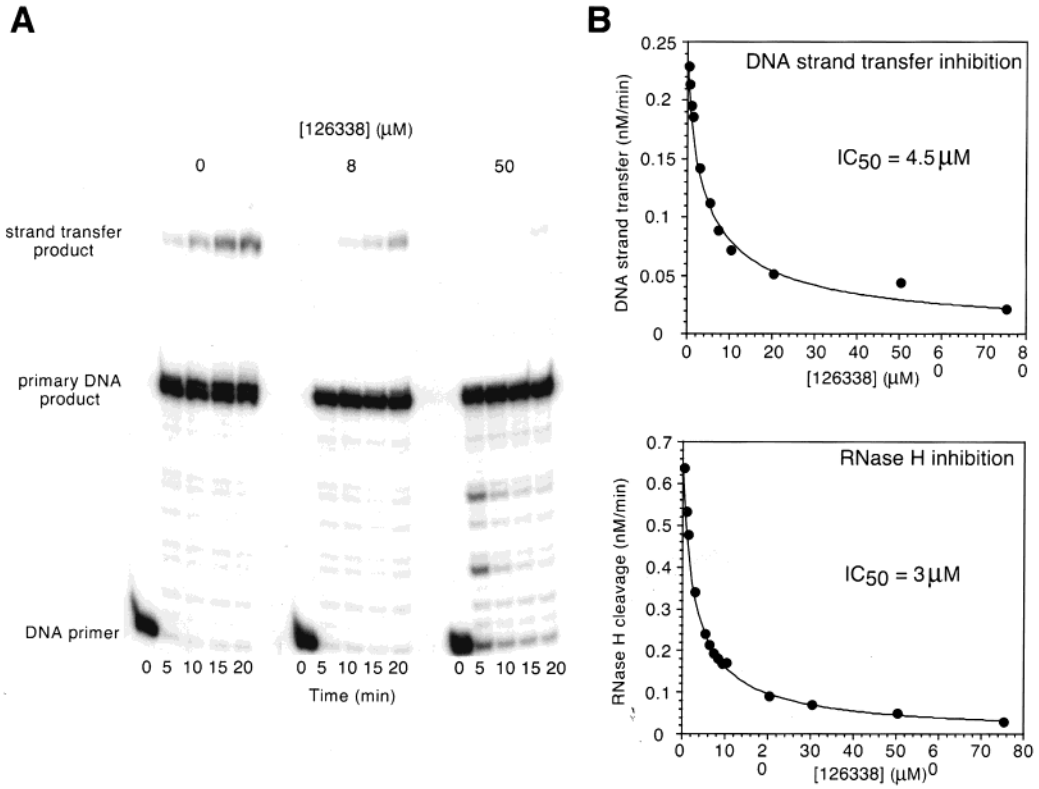


FIGURE 5: Inhibition of DNA strand transfer by PD126338. Reactions were performed as described in Materials and Methods with increasing amounts of inhibitor. (A) Gel analysis of DNA strand transfer products in the presence of PD126338. Representative autoradiograms are shown for three reaction time courses at different concentrations of PD126338 (0, 8 and 50 μM). The amount of DNA strand transfer was quantitated by phosphorimager analysis, and initial rates were determined. (B) The concentration dependence of DNA strand transfer (upper) and RNase H (lower) inhibition by PD126338. The rates of DNA strand transfer or RNase H cleavage quantitated as in (A) were plotted as a function of the final concentration of inhibitor.

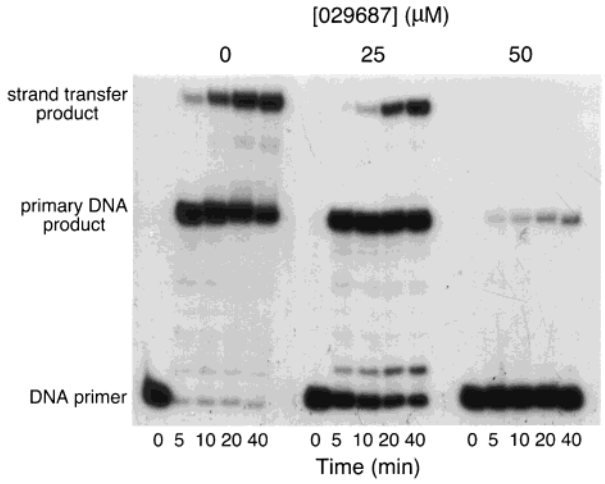


FIGURE 6: Inhibition of DNA strand transfer by the polymerase inhibitor PD029687: Representative gel analysis of DNA synthesis in the presence of increasing amounts of PD029687. The concentrations of strand transfer and primary DNA products were quantitated by phosphorimager analysis. At increasing concentrations of inhibitor, the DNA·RNA primer template extension was inhibited as indicated by the loss of primary DNA product and the persistence of the DNA primer substrate.

DNA strand transfer by inhibiting the translocation of the nascent DNA primary product onto the acceptor template (Figure 4). Actinomycin D was only a very weak inhibitor of DNA polymerase activity under the conditions of DNA strand transfer, and little or no RNase H inhibition by actinomycin D was detected (*18*, *22*, *46*). The current evidence suggests that actinomycin D binds to the single-stranded DNA and acceptor template intermediates and prevents the efficient annealing of these complementary strands. Without the formation of the double-stranded DNA strand transfer intermediate (Figure 4), genomic replication would halt shortly after initiation of minus strand DNA synthesis, producing a stalled DNA strong-stop intermediate (Figure 1). This was found to be the case in both *in vitro* model systems and endogenous HIV-1 RT reactions (*18*, *22*, *46*). A unique feature of this inhibitor of DNA strand transfer is that its primary inhibitory effect is not a result of binding to the enzyme but rather to the substrates. In effect, this is similar to the mechanism of inhibition by antisense DNA inhibitors of HIV-1 RT (*47*−*52*) which bind to complementary sequences on the viral genome. However, antisense strategies have primarily focused on inhibiting DNA synthesis by designing oligonucleotides that serve as blocks to RT function. The strand transfer inhibition mechanism of actinomycin D suggest that blocking the annealing of nascent DNA products to acceptor templates during strand transfer could offer an alternative approach for antisense strategies. In addition, the discovery of small, nonnucleotide DNA-binding inhibitors offers the potential for better delivery and stability than that normally associated with DNA oligonucleotide therapeutics.

The majority of anti-HIV drugs under current use target the DNA polymerase activity of HIV-1 RT. Currently, these reverse transcriptase inhibitors can be assigned to two broad classes termed nucleoside analogues, exemplified by AZT-triphosphate (*28*), and nonnucleoside inhibitors, exemplified

by the benzodiazapine TIBO (*30*, *53*). Both of these classes of inhibitors target the DNA polymerase activity of HIV-1 RT. The usefulness of these inhibitors in mono-therapy regimens is limited by the rapid development of drug resistance.

We present the identification of inhibitors targeting DNA polymerase and RNase H activities of HIV-1 RT, and inhibitors of DNA translocation activities, all of which are required for DNA strand transfer. Each of these mechanistically distinct classes of inhibition display the ability to inhibit DNA strand transfer in vitro. Elucidation of the complete mechanism of inhibition of each of these novel inhibitors could point to new strategies for the development of therapeutic agents against HIV-1 replication. The discovery of a broad range of inhibitors against DNA strand transfer suggests that this complex reaction could be an important target for drug discovery and development.

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