Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 507–515

BIOORGANIC & MEDICINAL CHEMISTRY

Template-Competitive Inhibitors of HIV-1 Reverse Transcriptase: Design, Synthesis and Inhibitory Activity†

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Received 8 March 2001; accepted 10 August 2001

Abstract—We report the design, synthesis and activity studies on a novel class of template-competitive reverse transcriptase inhibitors (TCRTIs). The TCRTIs are 1,N^6-etheno analogues of a series of dATP-based template-competitive DNA polymerase inhibitors synthesized in our laboratory (Moore, B. M.; Jalluri, R.; Doughty, M.B. *Biochemistry* **1996**, *35*, 11634). Thus, nucleotides 2-(4-azidophenacyl)thio-1,N^6-etheno-2′-deoxyadenosine 5′-triphosphate **1**, the tetrafluoro analogue 2-(4-azido-2,3,5,6-tetrafluorophenacyl)thio-1,N^6-etheno-2′-deoxyadenosine 5′-triphosphate **2** and its analogues were synthesized by alkylation of 2-thio-1,N^6-etheno-2′-deoxyadenosine 5′-monophosphate with the corresponding chloro- or bromo-alkyl halides and converted to the triphosphate. Kinetically, nucleotides **1** and **2** are both competitive inhibitors of reverse transcriptase versus template/primer with K_i's of 8.0 and 7.4 μM, respectively, and non-competitive inhibitors versus TTP with K_i's of 15 and 10 μM, respectively. Nucleotide **3**, which differs from **1** only in that it lacks the etheno group, non-complementary nucleotide triphosphates, and related monophosphates and nucleosides, are completely inactive as inhibitors of reverse transcriptase at concentrations up to 1 mM. Photoinactivation of RT by **1** was both time- and concentration-dependent, and protected by template/primer but not by dNTPs. The concentration-dependent inactivation data gave a $K_{D,app}$ of 17.2 μM and maximum inactivation of 90%, and radiolabeled [β,γ-^{32}P]-**1** photoincorporated specifically and covalently into the p66 subunit of RT. Thus the photoinactivation data support our main conclusion from the kinetic data that this class of RT inhibitors are non-substrate and template-competitive.

Introduction

HIV-1 reverse transcriptase (RT) is the enzyme necessary for the transformation of HIV genomic RNA into double-stranded proviral DNA and has been the object of extensive biochemical, structural, and mutagenesis studies. The p66/p51 heterodimer of RT is the mature form purified from virions and infected cells.[1] Crystal structures have been reported for heterodimer RT complexed with either a non-competitive inhibitor[2–4] or an 18-base pair double-stranded DNA and Fab antibody,[5] and RT complexed with covalently modified template/primer and dNTP.[6] The RT heterodimer is highly asymmetric, but the p66 subunit contains the three subdomains named the fingers, palm, and thumb that together constitute the polymerase domain and form a large template/primer binding cleft.

Two classes of RT inhibitors, including nucleoside (NRTIs) and non-nucleoside (NNRTIs) inhibitors, have been described. NRTIs, including 3′-azido-3′-deoxythymidine (zidovudine, AZT),[7,8] 2′,3′-dideoxyinosine (didanosine, ddI),[9] 2′,3′-dideoxycytidine (ddC),[10] and 2′,3′-dideoxy-3′-thiacytidine (3TC)[11,12] are an important class of drugs clinically available for AIDS treatment. These nucleoside RT inhibitors are classic anti-metabolites, needing activation by normal cellular kinases to the active 5′-triphosphates. The triphosphates are alternate substrates and are readily incorporated into the growing DNA chain. Kinetic studies have shown that AZT 5′-triphosphate is an alternate substrate of RT with a $K_{m,app}$ of 3.0 μM, which is similar to the K_m of 2.5 μM for TTP.[13] The binding site for the activated NRTIs is thought to be identical to that for normal dNTP substrates. In the crystal structure of an inactive ternary complex, TTP is oriented in a position where the α-phosphate is adjacent to the 2′-position on the primer by triphosphate ligation to two magnesium ions bound to the active site carboxylates of Asp^{110}, Asp^{185}, and Asp^{186} and by hydrogen bonding to a complementary base on the template.[6] AZT-5′-triphosphate as an alternate substrate also forms a dead-end complex which is a potent inhibitor of RT.[13]

†Taken in part from the PhD dissertation of Ke Li, The University of Kansas, 1996 and the PhD dissertation of Weiying Lin, The University of Kansas, 2000.

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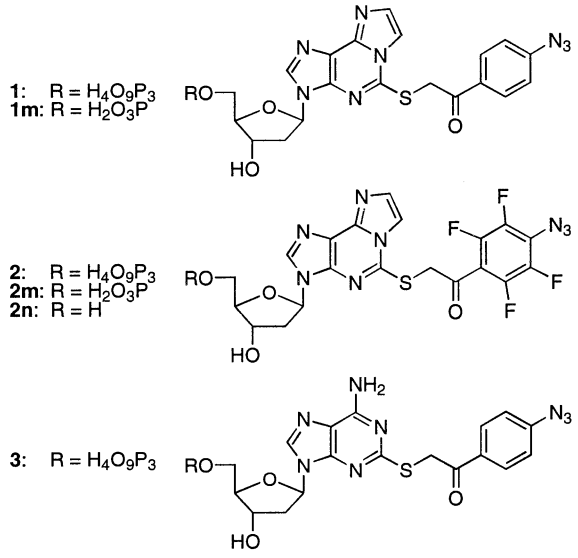
0968-0896/02/$ - see front matter
PII: S0968-0896(01)00297-8

Many HIV-1-specific non-nucleoside RT inhibitors have also been described.[14] These compounds belong to structurally distinct classes, and are non-competitive inhibitors with respect to the deoxynucleoside triphosphate substrate and show non-competitive or mixed type inhibition with respect to the template/primer. X-ray structure elucidation,[2–4] inhibitor photoaffinity labeling,[15,16] and mutagenesis studies[17] indicate that despite their structural diversity, non-nucleoside inhibitors share a common binding site in the RT p66 subunit involving amino acid residues surrounding Tyr^{181} and Tyr^{188}.[16] This allosteric, non-substrate binding site is near, but not coincident with, the primer binding site, and is distal from the template and dNTP binding sites.

Our laboratory has synthesized a series of nucleoside 5′-triphosphate photoprobes to explore the structural regions of DNA polymerases important in substrate binding and catalysis. One such photoprobe, 2-(4-azidophenacyl)thio-2′-deoxyadenosine 5′-triphosphate (**3**), binds to the DNA polymerase Klenow fragment (KF) with a K_d in the low μM range and is an efficient photoprobe of this enzyme.[18,19] In the present communication we demonstrate that its 1,N^6-etheno bridged analogue **1** and its etheno and tetrafluoro analogue **2** are poor inhibitors of the Klenow fragment but show surprisingly good activity as inhibitors of HIV-1 reverse transcriptase. In addition, their inhibition is competitive with respect to template-primer and non-competitive with respect to nucleoside 5′-triphosphate. The kinetic, photochemical, and structural data provided evidence to differentiate the TCRTIs from both the nucleoside and non-nucleoside inhibitors.



Results

The monophosphates **1m**, **2m**, and **5m**–**8m** were synthesized by alkylation of 2-thio-1,N^6-etheno-2′-deoxyadenosine 5′-monophosphate[20] **4** with substituted phenyl or benzyl acylhalides at pH 7–8 in aqueous DMF (Scheme 1). The 5′-monophosphates **1m**, **2m**, and **5m**–**8m** were then converted to the 5′-triphosphates **1**, **2**, and **5**–**8** using the diphenylphosphoryl anhydride method.[21] The products were >95% pure based on elution as a single peak from a C_{18} reverse-phase HPLC column, elution as a single peak on mono-Q FPLC ion-exchange chromatography, and ^{31}P and ^{1}H NMR spectroscopy

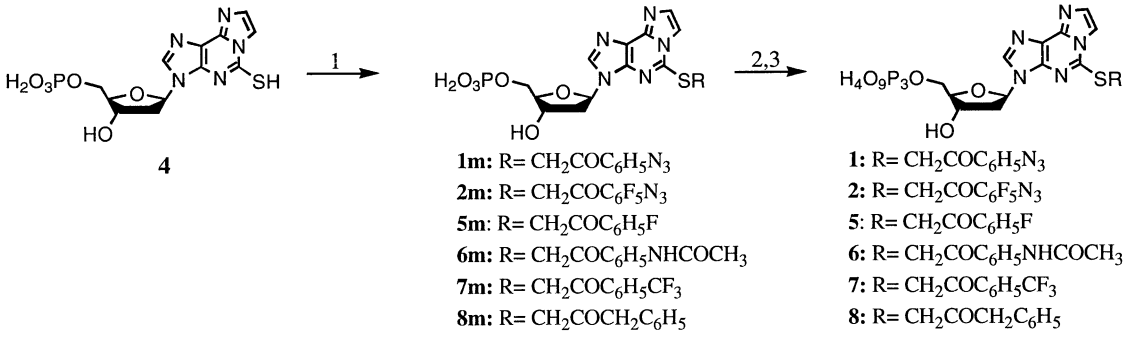
The reversible inhibition of HIV-1 RT and the Klenow fragment by nucleotides **1** and **2** was investigated in order to ascertain if the nucleotides specifically interact with the substrate binding sites and to provide information on the binding modes of these inhibitors. Table 1 summarizes the kinetic analysis of the inhibition of RT and KF by nucleotide **1**, and on the inhibition of only RT by **2**. Both KF and RT were inhibited competitively by **1** relative to template/primer as the varying substrate with K_i's of 8.0 and 96 μM, respectively, and non-competitive relative to TTP with K_i's of 15 μM for RT and 78 μM for KF. TCRTI **2** was evaluated only as a reverse transcriptase inhibitor; this analogue had activity similar to that of **1** with competitive inhibition relative to varying template/primer and non-competitive inhibition relative to varying TTP with K_i's of 7.4 and 10 μM, respectively.

To further define the interaction of this inhibitor class with reverse transcriptase, we studied the photoinactivation and photoincorporation of **1** into RT in the absence and presence of substrates. RT was rapidly inactivated by 100 μM **1** with 83 and 87% inactivation after 6 and 12 min, respectively (Table 2). This inactivation was protected (11% inactivation relative to control) by template/primer, but not by TTP, a result consistent with the poor binding of dNTPs to RT in the absence of template/primer (see Discussion). The concentration-dependent photoinactivation data displayed saturation, reaching 90% maximum inactivation with IC_{50} of 17 μM when fit to a binding model where photoinactivation occurs from an enzyme-inhibitor binary complex (Fig. 1). As suggested in the time-dependent inactivation data, the concentration-dependent inactivation was protected by template-primer; at lower concentrations of template/primer the dose-response curve was shifted to the right without changes in total photoinactivation (not shown). Additionally, [^{32}P]-**1** incorporated specifically into the p66 subunit (>98%) at 100 μM (Fig. 1).

For structure–activity studies, a series of nucleotide triphosphates and their monophosphate and nucleoside analogues were tested for RT inhibition based on their IC_{50} at substrate concentrations equal to the K_m, and the data were analyzed for K_i using the method of Chou.[22] The results given in Table 3 first demonstrate that both the etheno group and the triphosphate are necessary requirements for RT inhibition. Second, the series in Table 3 demonstrates that the 2-position side chain is sensitive to steric interaction at the *para* position (either electron donating or releasing), but the side chain can be lengthened by addition of a carbon to the side chain.

Discussion

Our approach to the design of nucleotide inhibitors of DNA polymerases was based on a thermodynamic



Scheme 1. (1) alkyl halides, pH 7–8; (2) diphenyl chlorophosphate, pyridine; (3) tributylammonium pyrophosphate, pyridine.

model of Klenow fragment-substrate interactions developed by Doronin and Kolocheva.[23,24] In this model, the enzyme interacts with about seven nucleosides in the primer strand and about 12 nucleosides in the template strand. Numerous interactions contribute to formation of the enzyme-template/primer complex, interactions which include: (1) hydrogen bonds with phosphoryl oxygens (−4.7 kcal/mol), (2) ionic interactions with phosphate anions (−1.2 kcal/mol), (3) hydrophobic interactions with the deoxyribose sugar and bases (−0.32 kcal/mol) and (4) interactions with the primer 3′ terminus (−5.8 kcal/mol). The incoming dNTP binds to the active site of the binary E-template/primer complex near the 3′ end of the primer and base-pairs with the complimentary nucleoside in the template strand. In contrast to the extensive binding interactions between the enzyme and the template/primer in which no single interaction dominates, the binding energy for the dNTP substrate is mainly contributed from the interaction of the enzyme with the magnesium-complexed triphosphate moiety (about −5.9 to −6.7 kcal/mol) and by hydrogen bonding (about −0.35 kcal/mol) to the complementary template.

Nucleotide-based photoaffinity probes of DNA polymerases which rely only on the triphosphate for binding interaction generally show low incorporation and weak affinity. Examples include TTP,[25,26] 8-azido-dATP,[25,27,28] and the 2-azido-dATP and 2-azido-1,N^6-etheno-dATP synthesized in our laboratory.[20] We concluded that these photoprobes are poor polymerase labels since in binding to the free enzyme they lack the stabilizing interactions with the template and primer. To overcome this inherent instability of the binary enzyme-inhibitor complex, we appended 2-alkyl substituents in order to stabilize binding interactions. We reasoned that a minor groove substituent would be in position to stabilize the modified dNTP by interaction with a lipophilic site in the template binding domain (as predicted from the thermodynamic model; Figure 2). Although stabilizing this dead-end complex in DNA polymerase has to date no clinical relevance, the stabilized complex provides a method for exploring polymerase structure and function. Indeed, nucleotide **3** is an efficient inhibitor and photolabel of KF. With photolysis at 3000 Å, probe **3** inactivates >91% of KF polymerase activity, and [8-^{3}H]-**3** covalently incorporates into the Klenow fragment with an IC_{50} of 0.75 μM, an incorporation which is protected competitively by template/primer, at residue Asp^{732} in the template-binding finger subdomain.[18,19] Thus, the azidophenacyl side chain in **3** does stabilize a binary complex between the Klenow fragment and modified dNTPs as proposed in the model.

Nucleotides in this series are inactive as reverse transcriptase inhibitors. For example, nucleotide **3** shows no inhibition of reverse transcriptase at concentrations up to 1 mM (the highest concentration tested), indicating that its K_i must be >5 mM (Table 2). This lack of binding of nucleotide is consistent with other studies

Table 1. Reverse transcriptase and Klenow fragment steady-state inhibition patterns and constants for TCRTIs **1** and **2** as reversible inhibitors

Inhibitor	Enzyme	Variable substrate	Inhibition Pattern	K_i (μM)
1	RT	poly(dA)·$(dT)_{10}$[a]	Competitive	8
1	RT	TTP[b]	Non-competitive	15
1	KF	poly(rA)·$(dT)_{10}$[c]	Competitive	92
1	KF	TTP[b]	Non-competitive	78
2	RT	poly(dA)·$(dT)_{10}$[a]	Competitive	7.4
2	RT	TTP[b]	Non-competitive	10

[a]TTP was maintained at 50 μM.
[b]Template/primer was maintained at 20 nM.
[c]TPP was maintained at 100 μM.

Table 2. Time-dependent photoinactivation of RT by **1** and substrate protection[a]

Time (min)[b]	Additions	% Act[c]
0	100 μM **1**	100
6.0	100 μM **1**	17
12.0	100 μM **1**	13
6.0	100 μM **1** + 500 nM poly(dA)·$(dT)_{10}$	76.3
6.0	(none; control[d])	87.5
6.0	100 μM **1** + 5 mM TTP[e]	17

[a]Standard reaction conditions: 2 μM RT, 3 mM $MgCl_2$, and 2 mM 2-mercaptoethanol as scavenger.
[b]Photolysis time at 25 °C.
[c]Percent activity remaining after photolysis as measured by initial rate data after dilution of the photolysis mixture.
[d]Photolysis control used standard reaction conditions with pre-photolyzed 100 μM **1**.
[e]Magnesium chloride was increased to 50 mM to account for the high triphosphate concentration.

showing very weak binding of dNTPs to free RT.[29,30] Thus our model of modified dNTP interaction to KF in a dead-end complex in the dATP series was not directly applicable to reverse transcriptase.

We subsequently added the 1,N^6-etheno group into **3** with the expectation that increased lipophilicity might increase binding to the free forms of both DNA polymerases and reverse transcriptases. But in the Klenow fragment the etheno analogue is significantly less active than the corresponding adenosine analogue (by a factor of 50–100), a result consistent with the general activity of other 1,N^6-ethenoadenosine analogues.[31,32] In addition to its poor inhibition, nucleotide **1** is also not an effective photoprobe of DNA polymerase I Klenow fragment since it photoincorporates by only 35%.[33] Nevertheless, the etheno modification had a dramatic and positive effect on the inhibition of HIV-1 reverse transcriptase. Thus both nucleotides **1** and **2** are competitive with template/primer with K_i's in the low micromolar range, which is a >500-fold increase over the adenosine congener, suggesting the etheno group is a key modification in this series. The exact role of the etheno group is not clear, but preliminary molecular modeling studies suggest that the etheno group shifts the conformational equilibrium of the 2-position side chain due to etheno-H to side chain CH_2 steric interactions.[33] Whether the etheno group shifts the conformational equilibrium or makes direct contact with the enzyme in the binary E-I complex remains to be determined.

In addition to being competitive relative to template-primer, the inhibitors also show non-competitive inhibition relative to the nucleotide triphosphate substrate (TTP in this case). Since dNTP binds to the E-template/primer binary complex in the kinetic mechanism,[34,35] a noncompetitive inhibition is fully consistent with binding of the inhibitor to the free enzyme form and not to the binding site occupied by dNTP's in the kinetic mechanism.[36] Thus, the TCRTIs, unlike the triphosphate activated NRTIs, are not RT substrates. The K_i's from the two sets of kinetic experiments show a good correlation, indicating similar binding modes to the free enzyme in the two sets of experiments.

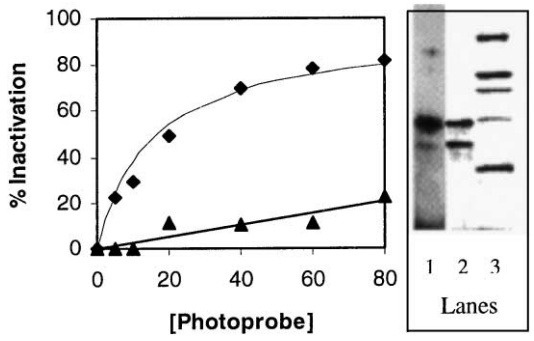
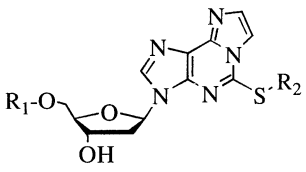
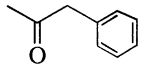
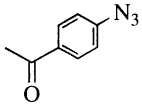
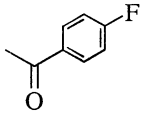
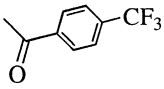
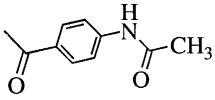
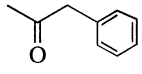
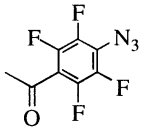


Figure 1. Evaluation of nucleotide **1** as an RT photoprobe. Left: Photoinactivation of RT by **1**. Standard reaction conditions included 2 μM RT, 3 mM $MgCl_2$, 2 mM 2-mercaptoethanol as scavenger, and varying concentrations of **1** (0–80 μM), in the absence (diamonds) or presence (triangles) of 500 μM template primer. The photolysis mixture was photolyzed for 6 min at 3500 Å, and residual polymerase activity was measured after dilution. The solid line is the best fit to eq (1). Right: Photoincorporation of **1** into RT. Standard reaction conditions included 2 μM RT, 3 mM $MgCl_2$, 2 mM 2-mercaptoethanol as scavenger, and 100 μM **1**. The photolysis mixture was subjected to SDS–PAGE, and analyzed by autoradiography (lane 1) or coomassie stain (lane 2). Lane 3 contains coomassie stain of MW standards of (top to bottom) 200, 116, 97, 66, and 45 kD.

Reverse transcriptase is irreversibly photoinactivated by **1** at 3500 Å photolyis in a time- and concentration-dependent manner. The photoincorporation of [^{32}P]-**1** is >98% into the catalytically active p66 subunit, a result consistent with an active site dependent binding. Additionally, the photoinactivation of **1** into RT saturates with a K_d nearly identical to that determined from kinetic studies, and the photoinactivation is protected competitively by template/primer. Thus the photo-

Table 3. Reverse transcriptase inhibition by 2′-deoxynucleoside 5′-triphosphates and analogues



Compd	R_1	R_2	K_i (μM)[a]
8	Triphosphate		6.0
1	Triphosphate		10
2	Triphosphate		10
5	Triphosphate		10
7	Triphosphate		55
6	Triphosphate		75
8m	Monophosphate		>5000
2n[b]	H		>5000
3[b]	—	—	>5000

[a]K_i's are calculated from concentration-dependent inhibition initial rate data as described in the Experimental.
[b]Assays performed in 10% DMSO.

inactivation and photoincorporation data indicate that this class of inhibitors occupy a site on the free enzyme which is competitive to template/primer binding.

Our limited SAR studies shed some light on the nature of the binding interactions between RT and the TCRTIs. First, the monophosphates of all analogues and the nucleoside analogue of **8** are inactive, clearly establishing that the triphosphate is a required substituent. This structural prerequisite distinguishes this group of RT inhibitors from the lipophilic NNRTIs,[14] and is our only current evidence that the triphosphate binding site of dNTP substrates, which in the crystal structure of an inactive ternary complex is composed of three active site carboxylates in the palm subdomain of the p66 subunit,[6] is an important recognition site for TCRTIs. Second, the combination of both the side chain and the 1,N^6-etheno group are required for RT inhibition. Analogue **3** lacking the etheno group is inactive, as are 1,N^6-etheno nucleotide triphosphates lacking a 2-position side chain.[33] This requirement structurally distinguishes this class of RT inhibitors from NRTIs, which all have heterocyclic bases which can form complementary hydrogen bonds to template. The side chain shows some preference for small substituents (e.g., -F or -H) at the *para* position, and does not tolerate larger substituents, either electron donating or withdrawing. Finally, the most active analogue in this series is the 3-phenyl-2-propanone analogue **8**, indicating that some lengthening of the side chain is permissible or preferred.

The thermodynamic model used for design of these inhibitors (see Fig. 2) cannot fully explain the increases in inhibitory activity observed for the binding of **1** and **2** to reverse transcriptase relative to related dNTPs. The steric/electronic interactions of the side chain of **3** stabilized the dead-end complex with KF by about 150 fold relative to dNTP binding, so it is unlikely that the steric/electronic effects of the side chain/etheno group combination of **1** and **2** alone can account for a >500-fold increase in binding affinity at RT. One possible explanation is that the combined substrate (or bisubstrate) nature of the TCRTIs, with binding interactions in both the dNTP and template binding sites (Fig. 2), causes a more than additive, or synergistic, inhibitory

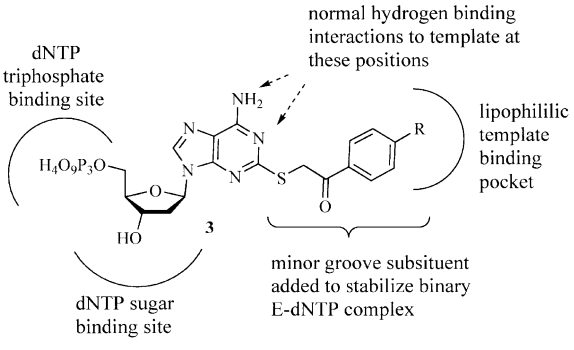


Figure 2. Binding model for the interaction of template-competitive inhibitors with DNA polymerase. Potential sites of hydrogen bonding to template which are not available in the binary enzyme–inhibitor complex are marked with arrows.

response.[37] In any case, given that TCRTIs **1** and **2** do not support the polymerization reaction, and that the inhibition patterns and structural requirements are distinct from that observed for the nucleoside and non-nucleoside inhibitors of RT, the data support our conclusion that nucleotides **1** and **2** are unique template-competitive inhibitors of HIV-1 reverse transcriptase. We are currently mapping the binding site for inhibitors **1** and **2** in order to gain further insights into this unique class of RT inhibitors.

Experimental

Chemicals and solvents of analytical or reagent grade were purchased from commercial suppliers and used without purification. Recombinant HIV-1 RT was kindly provided by Professor John V. Schloss or purchased from Worthington Biochemical Corporation (Freehold, NJ, USA). The Klenow fragment of DNA polymerase I was purchased from United States Biochemical Corporation (Cleveland, OH, USA) and dialyzed against phosphate buffer (50 mM, pH 7.4) containing 1 mM 2-mercaptoethanol and 50% glycerol to remove dithiothreitol. Polynucleotides, oligonucleotides, and nonradioactive nucleotides were purchased from Pharmacia (Piscataway, NJ, USA). Tritiated thymidine ([^{3}H]TTP) was purchased form DuPont New England Nuclear (Boston, MA, USA).

Inhibitor precursor **4** was purified as described earlier,[20] α-bromo-4-azido-2,3,5,6-tetrafluoroacetophenone was prepared by a standard literature procedure,[38] α-bromoacetophenones were prepared by bromination of the corresponding ketone with bromine and toluenesulfonic acid catalyst, and 3-chloro-1-phenyl-2-propanone was prepared by acidification of the corresponding α-diazoketone.[39] All manipulations of phenylazides were conducted in the dark or under a red light. Proton (^{1}H) nuclear magnetic resonance (NMR) spectra were recorded on General Electric QE-300 and Varian VXR-500S 500 MHz instruments. Chemical shifts are reported as parts per million (ppm) relative to HOD peak ($\delta = 4.80$) in D_2O. Negative fast atom bombardment mass spectra (−FABMS) were obtained on a VG Analytical ZAB spectrophotometer, and ultraviolet spectra were recorded on a Hewlett-Packard 8450A diode array spectrophotometer. FPLC chromatography was performed using a Pharmacia FPLC system. Reverse-phase high pressure liquid chromatography (HPLC) was conducted on a Beckman dual pump gradient HPLC system with System Gold software on a Vydac C-18 analytical or semi-preparative column. Solvent flow rates were 2.5 and 1 mL/min for semi-preparative and analytical columns, respectively. The purity of the nucleotides was $>95\%$ as determined by analytical HPLC using a 30 min linear gradient from 0 to 50% acetonitrile in 10 mM triethylammonium acetate, analytical FPLC on a Mono-Q column using a linear gradient from 0.2 to 2 M NH_4OAc, pH 4, and by ^{1}H NMR analysis.

Photolysis was conducted in a Rayonet Photochemical Mini-Reactor chamber Model RMR-600 purchased

from the Southern New England Ultraviolet Company (Branford, CT, USA). Samples were photolyzed in 1.0 mL water jacketed quartz cells. Liquid scintillation counting was conducted in a Packard 1900 TR liquid scintillation counter (Meridian, CT, USA). SDS gel electrophoresis was performed on a Protean II unit and gels were dried on a Model 543 gel drier, both from BioRad (Richmond, CA, USA). X-ray films were developed using an X-Omat developer (AFP Imaging, Elmsford, NY, USA).

Ammonium 2-(4-azidophenacyl)thio-1,N^6-etheno-2′-deoxyadenosine 5′-monophosphate (1m). The tetrabutylammonium salt of 2-thio-1,N^6-etheno-dAMP (120 mg, 0.29 mmol) in water (2 mL) was added to a solution of *para*-azidophenacyl bromide (78 mg, 1.1 equivalent) in acetonitrile (2 mL) in a brown reacti-vial. The pH of the solution was adjusted to 8 using KOH (6 N); the resulting clear yellow solution was purged with nitrogen and stirred at room temperature for 3 h. The reaction mixture was then diluted with water (10 mL), acidified to pH 4 with HCl (1 N), and extracted with three volumes of ethyl ether. The aqueous phase was neutralized with 1 N NaOH, and the product was purified by preparative HPLC on a C_{18} column using a 30 min linear gradient from 0 to 50% acetonitrile in 10 mM ammonium acetate. Collection and lyopholization of the major peak with a retention time of 29 min afforded 52.3 mg (50%) of **1m**: IR (KBr) 2116 (N_3); UV λ_{max} ($\varepsilon\times10^{-4}$): H_2O, 297 (1.61); ^{1}H NMR (D_2O) δ 2.08 (m, 1H, C2′H1), 2.62 (m, 1H, C2′H2), 3.86 (br s, 2H, C5′H), 4.33 (br s, 1H, C4′H), 4.78 (br s, 1H, C3′H), 5.70 (t, $J=6.3$ Hz, 1H, C1′H), 7.10 (d, $J=8.4$ Hz, 2H, PhH), 7.71 (s, 1H, C10H), 7.53 (s, 1H, C11H), 7.98 (d, $J=8.4$ Hz, 2H, PhH), 8.25 (s, 1H, C8H). High-resolution FABMS (glycerol) *m*/*e* 545.0772, calcd for $C_{20}H_{18}N_8O_7PS$ ($M-H^+$), 545.0757.

2-(2,3,5,6-Tetrafluoro-4-azidophenacyl)-thio-1,N^6-etheno-2′ deoxyadenosine 5′-monophosphate (2m). Triethylammonium 2-thio-1,N^6-etheno-2′-deoxyadenosine 5′-monophosphate (**4**, 276 mg, 0.4 mmol) in water (10 mL) was added to a solution of α-bromo-4-azido-2,3,5,6 tetrafluoroacetophenone (136 mg, 0.44 mmol) in acetonitrile (2 mL). The pH of the solution was adjusted to 7.0 using 0.2 N HCl. The reaction mixture was stirred at room temperature for 1 h. The solvents were removed under vacuum, and the resulting residue was dissolved in water (10 mL). The aqueous solution was washed with methylene chloride. The aqueous layer was separated and lyophilized to dryness to give the crude product. The crude product was purified on a reverse-phase C_{18} HPLC column using a 30 min gradient from 0 to 50% acetonitrile in 0.1% TFA. Collection and lyopholization of appropriate fractions afforded 50.3 mg (50%) of the product as a off-white solid: IR (cm^{-1}, KBr) 2112 (N_3); UV λ_{max} ($\varepsilon\times10^{-4}$): H_2O, 310 (2.01); ^{1}H NMR (DMSO) δ 2.35 (m, 1H, 2′H1), 2.56 (m, 1H, C2′H), 3.94 (t, 2H, C5′H), 4.35 (m, 1H, C4′H), 4.89 (m, 1H, C3′H), 6.15 (t, 1H, C1′H), 7.70 (d, 1H, C10H), 8.02 (d, 1H, C11H), 8.42 (s, 1H, C8H); ^{19}FNMR (DMSO) δ −141.38 (dd, 2F), −151.75 (dd, 2F); High-resolution MS (+FAB, glycerol) *m*/*e* 619.0519; calcd. for $C_{20}H_{16}N_5O_7SF_4P$ ($M+H^+$), 619.0536.

2-(4-Fluorophenylacyl)-thio-1,N^6-etheno-2′-deoxyadenosine 5′-monophosphate (5m). This was prepared from ammonium 2-thio-1,N^6-etheno-2′-deoxyadenosine 5′-monophosphate (**4**, 120 mg, 0.29 mmol) and α-bromo-4-fluoroacetophenone (69 mg, 0.32 mmol) as described above for the synthesis of **2**, yielding 140 mg (92%) of pure **5m**. ^{1}H NMR (DMSO) δ 1.95 (m, 1H, 2′H1), 2.52 (m, 1H, C2′H), 3.80 (t, 2H, C5′H), 4.10 (m, 1H, C4′H), 5.10 (m, 1H, C3′H), 6.00 (t, 1H, C11′H), 6.90 (m, 2H, Ph H), 7.30 (m, 2H, PhH), 7.60 (d, 1H, C10H), 7.92 (s, 1H, C11H), 8.30 (s, 1H, C8H). High resolution MS (+FAB, glycerol) *m*/*e* 524.0804; calcd for $C_{20}H_{20}N_5O_7SFP$ ($M+H^+$), 524.0805.

2-(4-*N*-acetylamino-phenylacyl)-thio-1,N^6-etheno-2′-deoxyadenosine 5′-monophosphate (6m). This was prepared from triethylammonium 2-thio-1,N^6-etheno-2′-deoxyadenosine 5′-monophosphate (**4**, 100 mg, 0.26 mmol) and α-bromo-4-*N*-acetylamino-acetophenone (73.2 mg, 0.28 mmol) using the method described above for the synthesis of **2**, yielding 95 mg (66%) of pure **6m**. ^{1}H NMR (DMSO) δ 2.02 (s, 3H, CH of amide), 2.12 (m, 1H, 2′H1), 2.52 (m, 1H, C2′H), 3.85 (t, 2H, C5′H), 4.15 (m, 1H, C4′H), 4.78 (s, 2H, CH of ketone), 5.10 (m, 1H, C3′H), 6.10 (t, 1H, C1′H), 7.60 (d, 1H, C10H), 7.80 (m, 2H, Ph H), 7.92 (s, 1H, C11H), 8.10 (m, 2H, PhH), 8.40 (s, 1H, C8H). High-resolution MS (+FAB, glycerol) *m*/*e* 563.1103; calcd for $C_{22}H_{24}N_6O_8SP$ ($M+H^+$), 563.1114.

2-(4-Trifluoromethylphenylacyl)-thio-1,N^6-etheno-2′-deoxyadenosine 5′-monophosphate (7m). This was prepared from triethylammonium 2-thio-1,N^6-etheno-2′-deoxyadenosine 5′-monophosphate (**4**, 100 mg, 0.26 mmol) and α-bromo-4-trifluoromethylacetophenone (86.1 mg, 0.32 mmol) using the method described above for the synthesis of **2**, yielding 53 mg (36%) of pure **7m**. ^{1}H NMR (DMSO) δ 1.92 (m, 1H, 2′H1), 2.50 (m, 1H, C2′H), 3.45 (t, 2H, C5′H), 4.10 (m, 1H, C4′H), 5.10 (m, 1H, C3′H), 5.25 (s, 2H, CH_2), 6.00 (t, 1H, C1′H), 7.60 (m, 2H, Ph H), 7.95 (m, 2H, PhH), 8.12 (d, 1H, C10H), 8.33 (s, 1H, C11H), 8.85 (s, 1H, C8H). High resolution MS (+FAB, glycerol) *m*/*e* 574.0776; calcd for $C_{21}H_{20}N_5O_7SF_3P$ ($M+H^+$), 574.0773.

2-(3-phenyl-2-propanonyl)-thio-1,N^6-etheno-2′-deoxyadenosine 5′-monophosphate (8m). This was prepared from triethylammonium 2-thio-1,N^6-etheno-2′-deoxyadenosine 5′-monophosphate (**4**, 138 mg, 0.2 mmol) and benzyl chloromethyl ketone (36.9 mg, 0.22 mmol) as described above for the synthesis of **2**, yielding 41.8 mg (41%) of pure **8m**. ^{1}H NMR (DMSO) δ 2.35 (m, 1H, 2′H1), 2.68 (m, 1H, C2′H), 3.88 (t, 2H, C5′H), 3.98 (m, 1H, C4′H), 4.06 (s, 2H, CH of benzyl), 4.42 (m, 1H, C3′H), 6.12 (t, 1H, C1′H), 7.18 (d, 3H, PhH), 7.62 (d, 1H, C10H), 7.95 (s, 1H, C11H), 8.40 (s, 1H, C8H); MS (-FAB, glycerol) *m*/*e* 517 ($M-H^+$).

2-(2,3,5,6-Tetrafluoro-4-azidophenacyl)-thio-1,N^6-etheno-2′-deoxyadenosine 5′-triphosphate (2). The triphosphate **2** was prepared from 2-(2,3,5,6-tetrafluoro-4-azidophenacyl)-thio-1,N^6-etheno-2′-deoxyadenosine 5′-monophosphate (**2m**) by a previously published procedure.[18]

Briefly, the trioctylammonium salt of **2m** (10 mg, 16.2 μmol) was activated with diphenyl chlorophosphate (3.7 μL, 1.1 equiv) and tributylamine (3.8 μL, 1 equivalent) in anhydrous dioxane, and after removal of excess activating reagent, the mixed anhydride (in pyridine) was treated with a solution of tributylammonium pyrophosphate (8.5 mg, 1.1 equiv) in pyridine (1 mL). The crude product was precipitated with ether and purified by FPLC ion exchange chromatography on a Mono-Q column using a gradient of 2 M ammonium acetate containing 30% acetic acid (pH 4). The appropriate fractions were collected, pooled, and lyophilized. The resulting residue was further purified on a reverse phase C_{18} HPLC column using a 30 min gradient from 0 to 50% acetonitrile in 5 mM triethylammonium bicarbonate. After HPLC purification, 1.2 mg (9.6%) of highly pure product as a light yellow solid was obtained: R_f 0.13 (20:12:1 propanol/NH_4OH/H_2O); UV λ_{max} ($\varepsilon\times10^{-4}$): H_2 O, 309 (2.00); ^{31}P NMR (D_2O) δ −6.83 (d, γP), −10.41 (d, αP), −21.71 (t, βP); ^{1}H NMR (D_2O) δ 2.23 (m, 2′H1), 2.47 (m, 1H, C2H), 4.04 (t, 2H, C5′H), 4.41 (m, 1H, C4′H), 4.91 (m, 1H, C3′H), 6.21 (t, 1H, C1′H), 7.73 (d, 1H, C10H), 8.10 (d, 1H, C11H), 8.45 (s, 1H, C8H).

Ammonium 2-(4-azidophenacyl)thio-1,N^6-etheno-2′-deoxyadenosine 5′-triphosphate (1). Compound **1m** (30 mg, 42.5 μmol) was converted to the free acid by HPLC purification using a semi-preparative C_{18} column, eluting with a 30 min linear gradient from 0 to 50% acetonitrile in 0.1% trifluoroacetic acid. The appropriate fractions were pooled and lyophilized to dryness. The resulting solid (20 mg, 37 μmol) was converted to the triphosphate according to the method used for the preparation of **2**: MS (−FAB, glycerol) *m*/*e* 705 ($M-H^+$).

Ammonium [β,γ-^{32}P]-2-(4-azidophenacyl)thio-1,N^6-etheno-dATP. [β,γ-^{32}P]-**1** was prepared from **1m** and ^{32}P-tributylammonium pyrophosphate as described above for the synthesis of **2**. Tributylammonium [^{32}P]-pyrophosphate was prepared immediately before use by addition of tetrasodium [^{32}P]-pyrophosphate (103 μl, 0.365 mCi) to a solution of tributylammonium pyrophosphate (7.4 mg, 16 μmol, 200 equiv) in 50% aqueous ethanol (2 mL). The mixture was stirred at room temperature for 10 min, and after removing the solvent by roto-evaporation, the residue was resuspended in ethanol (2 mL) and dried by evaporation under vacuum. Anhydrous pyridine (2 mL) was then added and evaporated to remove traces of water. The residue was dissolved in anhydrous pyridine (0.5 mL) and added to the P^1-nucleotide-5′ P^2-diphosphate. The final product was then purified on an analytical HPLC C_{18} column as described for **1**. The fractions were collected every 1 min and the radioactivity of each fraction was measured by scintillation counting. The appropriate radioactive fractions were pooled and lyophilized, and the residue was dissolved in water (86 μL), yielding a 1 mM solution of [β,γ-^{32}P]-**1**.

2-(4-Fluorophenylacyl)-thio-1,N^6-etheno-2′ deoxyadenosine 5′ triphosphate (5). This was prepared from 2-(4-fluorophenylacyl)-thio-1,N^6-etheno-2′-deoxyadenosine 5′-monophosphate (**5m**, 20 mg, 0.038 mmol) as described above for the synthesis of **2**, yielding 7.8 mg (30%) of pure **5** as a light-yellow solid: R_f 0.13 (20:12:1 propanol/NH_4OH/H_2O); MS (−FAB, glycerol) *m*/*e* 684 ($M-H^+$).

2-(4-*N*-Acetylamino-phenylacyl)-thio-1,N^6-etheno-2′-deoxyadenosine 5′-triphosphate (6). This was prepared from 2-(4-*N*-acetylamino-phenylacyl)-thio-1,N^6-etheno-2′-deoxyadenosine 5′-monophosphate (**6m**, 20 mg, 0.035 mmol) as described above for the synthesis of **2**, yielding 7.8 mg (31%) of pure **6** as a light-yellow solid: R_f 0.10 (20:12:1 propanol/NH_4OH/H_2O); ^{1}H NMR (DMSO) δ 1.98 (m, 1H, 2′H1), 2.14 (s, 3H, CH of amide), 2.55 (m, 1H, C2′H), 3.75 (t, 2H, C5′H), 4.05 (m, 1H, C4′H), 4.68 (s, 2H, CH of ketone), 4.95 (m, 1H, C3′H), 5.85 (t, 1H, C1′H), 7.58 (d, 1H, C10H), 7.85 (m, 2H, Ph H), 7.90 (s, 1H, C11H), 8.11 (m, 2H, PhH), 8.25 (s, 1H, C8H); MS (−FAB, glycerol) *m*/*e* 721 ($M-H^+$).

2-(4-Trifluoromethylphenylacyl)-thio-1,N^6-etheno-2′-deoxyadenosine 5′-triphosphate (7). This was prepared from 2-(4-trifluoromethylphenylacyl)-thio-1,N^6-etheno-2′-deoxyadenosine 5′-monophosphate (**7m**, 20 mg, 0.038 mmol) as described above for the synthesis of **2**, yielding 5.6 mg (20%) of pure **7** as a light-yellow solid: R_f 0.11 (20:12:1 propanol/NH_4OH/H_2O); MS (−FAB, glycerol) *m*/*e* 723 ($M-H^+$).

2-(3-phenyl-2-propanon-1-yl)-thio-1,N^6-etheno-2′-deoxyadenosine 5′-triphosphate (8). This was prepared from 2-(benzylacyl)-thio-1,N^6-etheno-2′-deoxyadenosine 5′-monophosphate (**8m**, 19 mg, 0.036 mmol) as described above for the synthesis of **2**, yielding 13.8 mg (57%) of pure **8** as a light yellow solid: R_f 0.11 (20:12:1 propanol/NH_4OH/H_2O); MS (−FAB, glycerol) *m*/*e* 677 ($M-H^+$).

KF and RT inhibition assays

The reverse transcriptase activity of HIV-1 reverse transcriptase was assayed using freshly prepared poly(rA)·$(dT)_{10}$ as template/primer and TTP as the nucleoside triphosphate substrate. The assays were performed using 0.5 units of RT in 50 mM Tris–HCl buffer (pH 7.4) containing 3 mM of $MgCl_2$ and 1 mg/mL of BSA. For kinetic analysis, the enzyme was assayed at 12.5–100 nM of the template/primer and 50 μM of TTP or at 5–25 μM of TTP and 10 nM of the template/primer; for IC_{50} determinations, both substrates were held at their K_m concentrations (10 nM for template/primer and 3.3 μM for TTP) and inhibitor concentration was varied. The enzyme and substrates (with or without the inhibitor) were diluted separately in 50 mM Tris–HCl buffer (pH 7.4) containing 3 mM magnesium chloride, and the reaction was initiated by addition of 20 μL of the enzyme solution to 30 μL of substrate solution. The reaction was incubated at 37 °C for 30 min and terminated by addition of an aliquot (40 μL) of the reaction mixture to an equal volume of 30 mM EDTA, and the quenched solution was immediately mixed by vortexing. Aliquots (20 μL) were spotted on DEAE cellulose filter paper (swatches of 2×2 cm^2) in triplicate. The filter

papers were washed in ammonium formate solution (0.3 M, pH 8, 3×200 mL) with gentle stirring, dehydrated in ethanol (150 mL, 95%) and ethyl ether (100 mL) and air-dried. The radioactivity on each filter paper was then measured by scintillation counting in 7 mL of Scintiverse II. The initial rate of the polymerization was determined as the picomoles of [^{3}H]TTP incorporated into DNA per min. The initial velocity versus substrate data were fit to competitive, noncompetitive and uncompetitive models using non-linear regression analysis using the program KINETICS as previously described.[18]

The Klenow fragment polymerase assays were performed at 1 unit/assay of the Klenow fragment, varying concentrations of TTP (10–100 μM) and a fixed concentration of the poly(dA)·$(dT)_{10}$ (20 nM) or at varying concentrations of poly(dA)·$(dT)_{10}$ (5–50 nM) and a fixed concentration of TTP (100 μM) in the absence or presence of varying concentrations of inhibitor **2** (5–100 μM). The enzyme and substrates (with or without the inhibitor) were diluted separately in 50 mM Tris–HCl buffer (pH 7.4) containing 3 mM magnesium chloride, and the reaction was initiated by addition of 20 μL of the enzyme solution to 30 μL of substrate solution. The assay procedure and kinetic analyses were performed as described above for the polymerase assay of RT.

Irreversible photoinactivation of reverse transcriptase

A standard irradiation mixture contained 2 μM HIV-1 reverse transcriptase, 2 mM 2-mercaptoethanol, 3 mM magnesium chloride and various concentrations of the photoprobe (5–100 μM) in 100 mM Tris–HCl buffer (pH 7.4) in a total volume of 40 μL. The photolysis mixture was incubated at 37 °C for 10 min in the dark and irradiated at 3500 Å at 25 °C. Aliquots of 5 μL were removed at indicated times and diluted 10-fold in assay buffer. An aliquot (5 μL) of the diluted sample was assayed for polymerase activity as described above. The % photoinactivation at each concentration of the photoprobe was calculated as the percentage loss of enzyme activity relative to the enzyme activity of the control in which the enzyme was photolyzed in the absence of the photoprobe. The concentration-dependent photoinactivation data were fit to eq (1):

$$\frac{[I_i]}{[I_t]} = \frac{[I]^* I_{max}}{[I] + K_{D,app}} \quad (1)$$

where $[I_i]/[I_T]$ is the fraction of photoinactivation of the enzyme by photoprobe I, I_{max} is the maximum inactivation at saturation, [I] is the photoprobe concentration, and $K_{D,app}$ is the concentration of the photoprobe which causes 50% of maximum photoinactivation of the enzyme.[18] $K_{D,app}$ values were calculated by nonlinear regression using the software MINSQ.

For substrate protection experiments, 5 mM TTP or 500 nM template-primer poly(rA)·$(dT)_{10}$ was added to the photolysis mixture. The photolysis and enzyme assays were performed using the same procedures as described for enzyme photoinactivation in the absence of substrate except that the enzyme and the substrate were preincubated for 4 min prior to addition of the photoprobe.

Photoincorporation of [β,γ-^{32}P]-1 into reverse transcriptase

HIV-1 reverse transcriptase (158 pmol) was incubated in the presence of various concentrations of [β,γ-^{32}P]-1 (5–100 μM) in 50 mM Tris–HCl buffer (pH 7.4) (0.78 mL) containing $MgCl_2$ (3.0 mM) and 2-mercaptoethanol (2 mM or 10 times the concentration of the photoprobe) at 37 °C in the dark for 10 min. The solutions were irradiated at 3500 Å at 25 °C for 5 min. For the control experiment, the photoprobe was preirradiated before addition to the enzyme solution. The photolysis mixtures were diluted with 2-times treatment buffer (50 mM Tris–HCl, pH 6.8, containing 100 mM dithiothreitol, 2% SDS, 10% glycerol, and 1% bromophenol blue) and denatured on a boiling water bath for 3 min. The samples were subjected to denaturing gel electrophoresis using the methodology of Laemmli.[40] For autoradiography, gels were fixed in destaining solution (10% acetic acid, 5% methanol, and 85% water) for 10 min and dried at 80 °C under vacuum for 2 h. The dried gel was exposed to Kodak XRA-5 X-ray film in a cassette with an intensifying screen. After 5 days at −70 °C, the film was developed, and band intensities were measured by densitometry scanning.

Acknowledgements

This work was supported in part by grants from the U.S. Public Health Service (GM38722) and The University of Kansas General Research Fund.

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