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# Steady state kinetics and inhibition of HIV-1 reverse transcriptase by a non-nucleoside dipyridodiazepinone, BI-RG-587, using a heteropolymeric template

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## ABSTRACT

**Steady state kinetics and inhibition by a dipyridodiazepinone of the reverse transcriptase from human immunodeficiency virus type 1 (HIV) were studied using a heteropolymeric RNA template with a sequence from the authentic initiation site on the HIV genome. For addition of the first deoxynucleotide to primer, $k_{cat}/K_M$ is 0.05 $(nM\text{-}min)^{-1}$ and $K_M$ is 10 nM. When all 4 deoxynucleotide triphosphates are present and processive synthesis occurs, catalysis is less efficient; $k_{cat}/K_M = .0077$ $(nM\text{-}min)^{-1}$ and $K_M = 100$ nM for dATP. These results are consistent with a rate determining conformation change involved in translocation of the enzyme along the template. Inhibition by the dipyridodiazepinone BI-RG-587 is noncompetitive with respect to both nucleotide and template-primer; this compound decreases $V_{max}$ but does not affect $K_M$. Thus, this inhibitor binds to a site distinct from the substrate binding sites with $K_I$ of 220 nM. Inhibition by BI-RG-587 results in a uniform decrease in amount of products of all lengths rather than a shift from longer to shorter products, suggesting the inhibitor does not affect processivity of reverse transcriptase.**

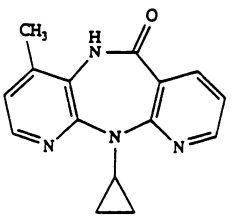
## INTRODUCTION

Human immunodeficiency virus type 1 (HIV)[1] is the major etiological agent for acquired immunodeficiency syndrome. The enzyme responsible for synthesis of proviral DNA from the viral RNA genome, one of the initial steps of HIV replication, is the virally encoded reverse transcriptase (1). Since HIV-RT is absolutely required for replication, it has been the subject of kinetic characterization as well as the object for inhibitor studies (2–8). This enzyme has at least three demonstrated activities: an RNA dependent DNA polymerase, an RNase H, and a DNA directed DNA polymerase (1). In these reactions ascribed to HIV-RT, the viral RNA genome is first transcribed to an RNA:DNA hybrid, the RNA is subsequently cleaved, and a complementary DNA strand is then synthesized to yield a DNA duplex. It is this duplex proviral DNA that will be integrated into the host cell genome to serve as template for all future virus-specific RNAs and proteins.

Detailed kinetic studies of the action of HIV-RT on a mixed-length homopolymeric template-primer, poly(rA)-oligo(dT), have determined that this enzyme has an ordered mechanism. Template-primer binding occurs prior to nucleotide binding (2). Pyrophosphate is the first product released, then as many as several hundred residues are added to the primer before polynucleotide product is released (2,3). Kinetic studies of HIV-RT with a heteropolymeric template of defined length and with a sequence from the HIV genome have not previously been reported.

Several types of HIV-RT inhibitors are known. Many, such as AZT, are nucleoside analogs, which, when incorporated into polynucleotides by HIV-RT, result in chain termination (7,8). Inhibitors that are not nucleoside analogs have also been described. These include our dipyridodiazepinones (4), the TIBO compounds (5), and catechin derivatives (6). Such compounds inhibit by mechanisms other than direct competition for substrate binding sites. Kinetic characterization of HIV-RT inhibition by nucleoside analogs (7,8), by TIBO compounds (9), and by catechin derivatives (6) have been reported.

We report here steady state kinetics of HIV-RT using a defined, 60 base heteropolymeric RNA as template and a 20 base DNA primer. This template-primer combination has a sequence from the authentic initiation site of reverse transcription on the HIV genome. The sequence of this template allows determination of

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$k_{cat}$ and $K_M$ when individual nucleotides are added to the primer. Elongation of the primer by 1, 2, 5 or more residues can be studied by omitting one or more deoxynucleotides from the reaction mixture. This system was used for steady state kinetic studies of inhibition by a representative of a novel and potent series of dipyridodiazepinone HIV-RT inhibitors (4). Preliminary kinetic characterization of this inhibitor using a homopolymer template has appeared (4). A structure-activity relationship for this series of compounds will be reported elsewhere (10). Studies of a photoaffinity analog of the dipyridodiazepinones have also appeared (11).

## MATERIALS AND METHODS

Tritium-labeled nucleotides purchased from New England Nuclear had specific activity of 20−25 Ci/mmole. Unlabeled nucleotides were purchased from Pharmacia. Enzymes and reagents for synthesis of the heteropolymeric template were purchased from Promega. BI-RG-587 was a kind gift from Dr. Karl Grozinger. Recombinant HIV-1 reverse transcriptase was purified to >95% homogeneity from lysates of *E. coli* JM109 containing the plasmid pKRT2 by modifications of a published procedure (11).

### Heteropolymeric RNA Template

This was generated by *in vitro* transcription of the plasmid pRT2. A 60 base sequence corresponding to the HIV-1 primer binding region was cloned under control of the SP6 promoter in pGEM3Zf(+) to yield pRT2 (Figure 1). *In vitro* transcription was performed using the following protocol: 3.5 μg of pRT2 previously linearized with EcoRI was incubated for 2 h at 40°C with 72 to 80 units of SP6 RNA polymerase in a total volume of 50 μL containing 40 mM Tris, 10 mM DTT, 6 mM $MgCl_2$, 2 mM spermidine, 100 mM NaCl, 90 to 100 units of RNasin, and 0.75 mM each of ATP, CTP, GTP, and UTP at pH 7.5. Following incubation, the reaction mixture was extracted once with an equal volume of phenol:chloroform (1:1); followed by extraction with chloroform. Nucleic acids were precipitated by addition of one volume of 7.5 M ammonium acetate and 2.5 volumes of absolute ethanol and stored overnight at −20°C. The precipitated nucleic acids were collected by centrifugation, washed with 70% ethanol, dried, and redissolved in doubly distilled water. RNA was isolated by fractionation through a 10% (w/v) polyacrylamide gel containing 8.3 M urea. The transcript was visualized with ethidium bromide, cut out of the gel and the RNA recovered by electroelution followed by ethanol precipitation as before. The RNA was dissolved in buffer consisting of 10 mM Tris, 100 mM NaCl and 1 mM EDTA at pH 8 and further purified by passage through an RNase-free Sephadex G-25 Quick-Spin column (Boehringer Mannheim Biochemicals) followed by ethanol precipitation. Pellets were dissolved in water at 1.5 μg/ml and stored at −70°C. A 20 base DNA primer was synthesized using an Applied Biosystems Inc. DNA synthesizer. The oligodeoxynucleotide primer used in these experiments corresponds to the 3′ end of the natural primer $tRNA^{Lys}$, and has the following sequence: 5′-AAT TCC CTG TTC GGG CGC CA-3′. Template and primer were annealed by heating a solution 400 nM in each polynucleotide at 65°C for 5 min in 5 mM Tris and 1 mM $MgCl_2$ at pH 7.8. The solution was allowed to cool slowly to room temperature and was stored at −70°C. The structure of the resulting template:primer hybrid is shown in Figure 1.

```
5′                                                                  3′
GAAUACUCAAGCUUAGUCAGUGUGGAAAAUCUCUAGCAGUGGCGCCCGAACAGGGAAUU
                                                ACCGCGGGCTTGTCCCTTAA
                                              3′                    5′
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**Figure 1.** The sequence of the 60 base RNA template annealed to the 20 base DNA primer.

### Kinetics Varying Deoxynucleoside Triphosphate

These experiments were performed with an assay mixture consisting of 50 mM Tris, 50 mM glutamate, 1 mM DTT, 2 mM MgCl2, 0.04% CHAPS, 40 nM template-primer and 2 nM HIV-RT. Nucleotides were varied in the following combinations: a) 20 nM to 300 nM [$^3$H]-dCTP; b) 300 nM dCTP with 20−300 nM [$^3$H]-dTTP; c) 300 nM each of dCTP and dTTP with [$^3$H]-dGTP varied from 20−300 nM; d) 300 nM each of dCTP, dTTP, and dGTP and 20−300 nM [$^3$H]-dATP. For inhibition studies, combination d was used and BI-RG-587 was present at 0, 60, 125 or 330 nM. Reactions were started by addition of the nucleotide mix. At the desired intervals, a 50 μL aliquot was removed from each reaction mixture and added to 10 μL of 0.5 M EDTA. 50 ul of this mixture was spotted on Whatman DE81 paper, washed 3 times (8 minutes each) with 0.3 M ammonium formate (pH 8.0), and then rinsed with 95% ethanol (12). The dried filters were then counted in 5 ml of ReadySafe scintillation cocktail. Initial rates were calculated from linear plots of cpm incorporated versus time with <10% of substrate converted to products. Counting efficiency for nucleotide was determined in the same system to convert cpm to molar amounts. Kinetic parameters were determined by direct fitting of the initial rates to the Michaelis-Menton equation. Linear initial rates indicate that RNaseH catalyzed degredation of substrate was negligible.

### Kinetics Varying Template:Primer

These were studied using assay conditions as described above with the following exceptions: HIV-RT was at 0.2 nM, template-primer was varied from 1 nM to 40 nM. Reactions were started by addition of [$^3$H]-dGTP, [$^3$H]-dCTP, dATP and dTTP each to 1 υM. At 15 min intervals, a 50 μL aliquot was removed from the reaction mixture and added to 10 μL of 0.5 M EDTA. When the reaction was complete, 60 μL of ice-cold 10% (w/v) trichloroacetic acid and 2% (w/v) sodium pyrophosphate was added. Samples were cooled at 4°C for 15 min before the contents were harvested onto #30 glass fiber filters (Schleicher & Schuell). Dried filters were counted in an LKB 1205 Betaplate Liquid Scintillation Counter. Initial rates and kinetic constants were calculated as above.

### Studies of Product Length

These were accomplished using reactions run as above but with primer that had been 5′-end labeled with $^{32}$P. Labelling reactions were performed as described (13) using gamma$^{32}$P-ATP (New England Nuclear) and T4 polynucleotide kinase (New England Biolabs). Products were separated by electrophoresis through 20% (w/v) polyacrylamide gels containing 8.3 M urea and visualized by autoradiography. Observations of full length products suggests our template-primer is stable in the presence of the RNaseH activity of HIV-RT.

## RESULTS

Steady state kinetic constants for each of the substrates in HIV-RT catalyzed transcription of the heteropolymeric template are shown in the Table. The sequence of the template adjacent to the primer is 3'-GACGAU..-5'; therefore, the order of the first 6 additions to the primer is C, T, G, C, T, then A. Extension of the primer by 1, 2, 5 and more nucleotides is illustrated in Fig. 2A. When the HIV-RT reaction was run with dCTP; the primer was only extended by one residue. When dCTP and dTTP were present, two residues were added to the primer. When only dATP was lacking, the primer extension was 5 nucleotides. In the presence of all four nucleotides, full length product was obtained. For kinetic analysis, the concentration of the last nucleotide in the sequence was varied with the others fixed at saturating concentrations. All nucleotides were saturating when template-primer was varied. Lineweaver-Burke plots of data obtained varying dATP and template-primer are shown in Figure 3. These plots are typical of the data obtained when varying other nucleotides.

The effect of BI-RG-587 on the steady state kinetics of HIV-RT catalyzed transcription of the heteropolymeric RNA template was studied and the results are shown in Fig. 3. For this reaction all four deoxynucleotides were included in order to observe the effect of BI-RG-587 on the complete polymerization reaction. With dATP as the variable substrate (Fig. 3A), inhibition by BI-RG-587 is noncompetitive; the compound decreases $V_{max}$ but does not affect $K_M$ for dATP. When the concentration of heteropolymeric template-primer is varied, the same pattern of noncompetitive inhibition is observed (Fig. 3B). The $K_i$ for BI-RG-587 was determined from secondary plots (not shown) of the intercept of the double reciprocal plot against the concentration of inhibitor; the x-intercept is equal to $-K_i$ (14). These plots were linear and yielded values of $K_i$ of 206 nM and 244 nM for the data from Figs. 3A and 3B, respectively. These numbers are equal within experimental error.

The effect of BI-RG-587 on the length distribution of HIV-RT products was determined (Figure 2B). Inhibition of HIV-RT by BI-RG-587 results in a uniform decrease in amount of products of all lengths rather than a shift from longer to shorter products.

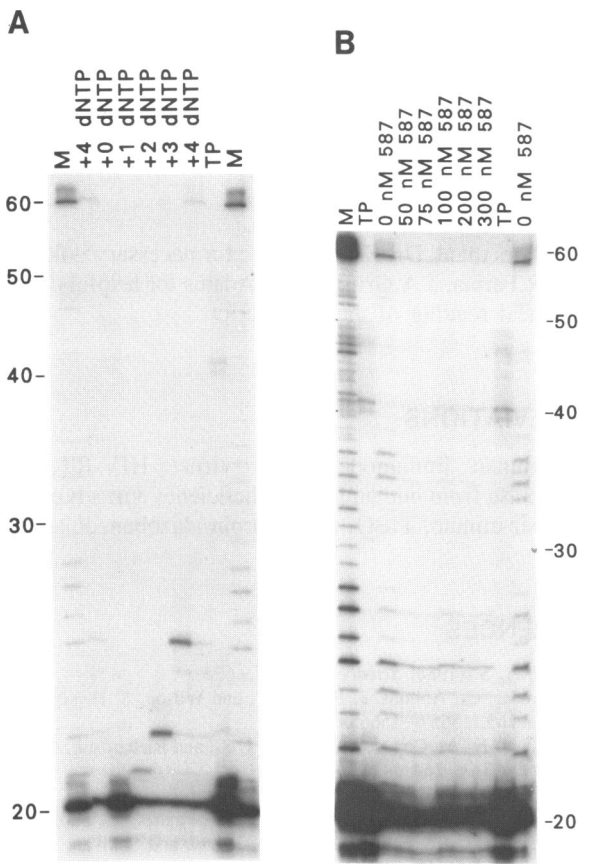


**Figure 2.** Product analysis of HIV-RT reactions with 60 base heteropolymeric template, after separation through a denaturing 20% polyacrylamide gel. *Panel A:* HIV-RT products produced in the presence of: lane 4, only dCTP; lane 5, dCTP and dTTP; lane 6, dCTP, dTTP and dGTP; lane 7, all 4 dNPTs. Lanes marked M are $^{32}P$ labelled product ladders; each band differing from adjacent ones by a single base. Lanes labelled TP are the template-primer used as substrate for the reactions. *Panel B*: The products produced by HIV-RT in the presence of varying concetrations of BI-RG-587. Lanes 3 trough 8 have 0 nM, 50 nM, 75 nM, 100 nM, 200 nM, and 300 nM BI-RG-587 respectively included in the reaction. Lanes TP and M are as in panel A.

**Table 1.** Steady state kinetic constants for each substrate in reverse transcription of heteropolymer template. The first six nucleotides are added to the primer in the order C,T,G,T,G, then A. Addition of 1,2,5, or >6 residues to the primer results from omission of nucleotides from the reaction. This allows determination of kinetic constants for addition of each nucleotide.

| Variable Substrate | $k_{cat}$ $min^{-1}$ | $K_M$ nM | $k_{cat}/K_M$ $(nM\text{-}min)^{-1}$ |
|---|---|---|---|
| dCTP | 0.500 | 10 | 0.050 |
| dTTP[a] | 0.430 | 40 | 0.0110 |
| dGTP[b] | 0.120 | 20 | 0.0062 |
| dATP[c] | 0.770 | 100 | 0.0077 |
| Template-primer[d] | 0.960 | 6.3 | 0.1520 |

[a]dCTP was at 300 nM
[b]dCTP and dTTP were present at 300 nM
[c]Concentrations of dCTP, dTTP and dGTP were 300 nM
[d]dCTP, dTTP, dGTP and dATP were at 1 $\mu$M

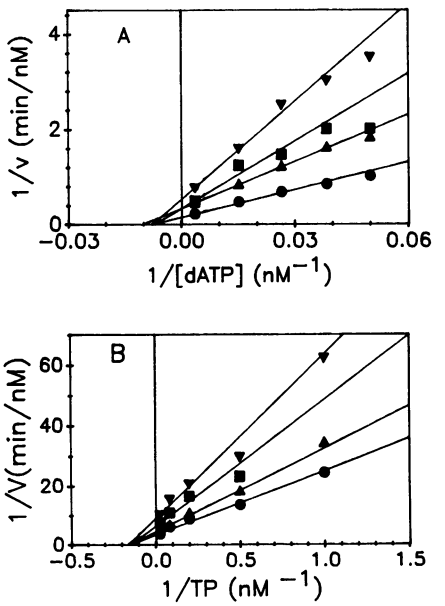


**Figure 3.** A)A double reciprocal plot of inhibition of RT by BI-RG-587 using a heteropolymeric template-primer with dATP as the variable substrate. Concentrations of BI-RG-587 were as follows: ● 0 nM, ▲ 60 nM, ■ 125 nM, ▼ 330 nM. B) Inhibition of HIV-1 RT by BI-RG-587 varying the heteropolymeric template-primer. BI-RG-587 was: ● 0 nM, ▲ 30 nM, ■ 150 nM, ▼ 350 nM.

## DISCUSSION

Characterization of steady state kinetics of HIV-RT on a heteropolymeric template was undertaken prior to studies of inhibitors of this reaction. The first six residues are added to the primer in the order C, T, G, T, G, then A. Thus, extension of the primer by 1, 2, 5 or more nucleotides can be studied by omission of the appropriate deoxynucleotide(s). Comparison of kinetic constants for the first addition to those for subsequent additions shows an interesting trend. Although $k_{cat}$ is approximately constant for addition of each of the nucleotides, $k_{cat}/K_M$ varies 7-fold. Addition of the first nucleotide has a larger $k_{cat}/K_M$ than processive synthesis. This suggests that the rate determining step in processive synthesis follows addition of the first nucleotide. Such a step might be a slow conformation change that causes HIV-RT to move along the template. It has previously been observed that HIV-RT dissociates from template-primer much more readily after the first addition to the primer than after subsequent additions (3). Our results are consistent with this. A slow step after addition of the first nucleotide would give the enzyme time to dissociate from the template. When all 4 nucleotides are present and HIV-RT can catalyze processive synthesis, variation of [dATP] yields $K_M$ for addition of this nucleotide that is 10-fold larger than $K_M$ for the first nucleotide added to the primer. Not only does HIV-RT slow down after the first addition to primer, but it apparently interacts more weakly with nucleotides as well. Studies of pre-steady state kinetics of HIV-RT also show a trend toward increasing $K_m$ with elongation of the primer (15).

Inhibition of HIV-RT by BI-RG-587 was studied with respect to two substrates for this enzyme. When tested with respect to either template-primer or dATP, BI-RG-587 affects the rate at which the enzyme catalyzes product formation but not $K_M$ for the substrates; this is noncompetitive inhibition. The absence of an effect on $K_M$ for either substrate indicates that BI-RG-587 interacts with HIV-RT at a site distinct from the template-primer or nucleotide binding sites. This same conclusion was drawn from preliminary kinetic work (4) and studies of a photoaffinity analog of BI-RG-587 (11). Inhibition of HIV-RT by catechin derivatives is noncompetitive with respect to deoxynucleotide and mixed with respect to template-primer (6). The TIBO compounds are noncompetitive against nucleotides and uncompetitive with respect to template-primer (9). Like BI-RG-587, these compounds bind to a domain distinct from the substrate sites (6). In fact, TIBO and BI-RG-587 compete (11). Apparently, there are one or more sites on HIV-RT that are distinct from the substrate sites; that bind compounds that are not nucleoside analogs; and that when occupied, cause inhibition of the enzyme.

No effect of BI-RG-587 on the processivity of HIV-RT was observed. Product analysis reveals that the length of polynucleotide products remains unchanged, only the quantity decreases. If the inhibitor, BI-RG-587, decreased the processivity of HIV-RT or was a chain terminator (7,8), shorter products would predominate.

The steady state kinetic constants we report for catalysis using a heteropolymeric template-primer are typically lower than those reported with homopolymeric templates (2–4). The values of $K_M$ (Table) that we observe for the deoxynucleoside triphosphates are a factor of 10 to 100 lower than those observed by us for dGTP with a poly(rC) template, 570 nM (4), and by others for dTTP using a template of poly(rA), 3 $\mu$M (2). Other workers (15,16) also observe similar decreases in $K_m$ on going from homopolymer to heteropolymeric template. Some characteristic of a heteropolymeric template, such as its defined shorter length or perhaps just the varied sequence, might allow more optimal binding interaction with the deoxynucleotide triphosphate resulting in lower $K_M$. This binding does not show up in catalysis, however, as turnover is slower with our heteropolymeric template. With poly(rC) template $k_{cat}$, based on primer sites, is 15 $min^{-1}$ (4), some 20-fold higher than observed for elongation of the heteropolymeric template. Our $k_{cat}$ for elongation of the heteropolymer template-primer, 0.9 $min^{-1}$, is comparable to the value of 1.5 $min^{-1}$ obtained by Reardon and Miller (15). They and another group (16) observe a lower $k_{cat}$ using heteropolymeric templates than with homopolymer templates. An interesting contrast is found in the changes in the overall efficiency of catalysis, $k_{cat}/K_M$, which differs little from homo- to heteropolymeric templates. For heteropolymeric it is about 0.01 $(nM\text{-}min)^{-1}$, for poly(rC) about 0.03 $(nM\text{-}min)^{-1}$ (4). Reardon and Miller also observe template independent values of $k_{cat}/k_m$ (15). The difference in values of $k_{cat}/K_M$ is smaller than the difference in either constant alone because both $K_M$ and $k_{cat}$ are larger for the homopolymeric template-primer system. More detailed kinetic studies are required to determine the reason for these trends. There is little in the literature on affinity of HIV-RT for template-primer. However, a binding constant for poly(rA):oligo(dT) of 3 nM has been reported (3). This is similar to the $K_M$ of 6.3 nM that we obtained for our heteropolymeric template-primer.

## ACKNOWLEDGEMENT

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## ABBREVIATIONS

HIV, human immunodeficiency virus; HIV-RT, reverse transcriptase from human immunodeficiency virus type 1; cpm, counts per minute, TIBO, tetrahydroimidazobenzodiazepinone.

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