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KINETIC STUDIES WITH THE NON-NUCLEOSIDE HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 REVERSE TRANSCRIPTASE INHIBITOR U-90152E

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Abstract—The bisheteroarylpiperazine U-90152E is a potent inhibitor of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) and possesses excellent anti-HIV activity in HIV-1-infected lymphocytes grown in tissue culture. The compound inhibits both the RNA- and DNA-directed DNA polymerase functions of HIV-1 RT. Kinetic studies were carried out to elucidate the mechanism of RT inhibition by U-90152E. Michaelis–Menten kinetics, which are based on the establishment of a rapid equilibrium between the enzyme and its substrates, proved inadequate for the analysis of the experimental data. The data were thus analyzed using Briggs–Haldane kinetics, assuming that the reaction is ordered in that the template:primer binds to the enzyme first, followed by the addition of dNTP and that the polymerase is a processive enzyme. Based on these assumptions, a velocity equation was derived, which allows the calculation of all the essential forward and backward rate constants for the reactions occurring between the enzyme, its substrates and the inhibitor. The results obtained indicate that U-90152E acts exclusively as a mixed inhibitor with respect to the template:primer and dNTP binding sites for both the RNA- and DNA-directed DNA polymerase domains of the enzyme. The inhibitor shows a significantly higher binding affinity for the enzyme–substrate complexes than for the free enzyme and consequently does not directly impair the functions of the substrate binding sites. Therefore, U-90152E appears to impair an event occurring after the formation of the enzyme–substrate complexes, which involves either inhibition of the phosphoester bond formation or translocation of the enzyme relative to its template:primer following the formation of the ester bond.

Key words: HIV-1 reverse transcriptase; non-nucleoside inhibitors; inhibition kinetics

Certain bisheteroarylpiperazine derivatives are potent inhibitors of HIV-1§ RT (EC 2.7.7.49) [1–7]. Their mechanistic inhibitory activity differs from the one exerted by nucleoside analogs such as AZT, ddI, and ddC, which serve as dNTP substrates for the polymerase. Several classes of non-nucleoside RT inhibitors have been described recently. These include the dipyridodiazepinones [8, 9], the benzodiazepines or TIBO compounds [10, 11], the HEPT or 1-[(2-hydroxyethoxyl)-methyl]-6-(phenylthio)thymine derivatives [12, 13], the pyridinones [14], the quinoline U-78036 [15], polysulfates and polysulfonates [16–25], the α-anilinophenylacetamide derivatives [26], and the bisheteroarylpiperazines or BHAPs [1–7].

The bisheteroarylpiperazine U-90152E {1-(5-methanesulfonamido-1*H*-indol-2-yl-carbonyl)-4-[3-(1-methyl-amino)pyridinyl]piperazine} (Fig. 1) is a member of this last class of compounds [2, 4, 5]. The agent is a potent inhibitor of HIV-1 RT and has excellent antiviral activity at nontoxic doses in experimentally HIV-1-infected lymphocytes grown in tissue culture. In this report, we describe enzymatic kinetic studies, using recombinant HIV-1 RT, to examine the inhibitory effects of U-90152E on both the RNA- and DNA-dependent DNA polymerase functions of HIV-1 RT.

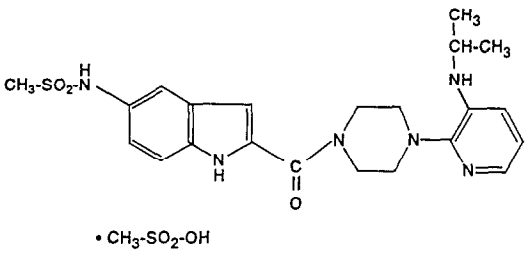

Fig. 1. Chemical structure of U-90152E.

MATERIALS AND METHODS

The expression of HIV-1 RT and its purification have been described [27, 28]. The enzyme was devoid

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§ Abbreviations: HIV-1, human immunodeficiency virus type 1; and RT, reverse transcriptase.

of *Escherichia coli* RNase H activity and consisted of p51/p66 heterodimers as evidenced by gel electrophoresis.

The synthetic template:primers poly(rA), $(dT)_{10}$, poly(rC), $(dG)_{10}$, and poly(dC):$(dG)_{12-18}$ were purchased from Pharmacia (Piscataway, NJ). [α-^{35}S]-Labeled dTTP and dGTP were purchased from Dupont NEN (Wilmington, DE). Nonidet P-40 was purchased from the Sigma Chemical Co., St. Louis, MO.

The standard reaction mixtures for the HIV-1 RT RNA-directed DNA polymerase assay contained 20 mM dithiothreitol, 60 mM NaCl, 0.05% Nonidet P-40, 10 mM $MgCl_2$, 50 mM Tris·HCl, pH 8.3, a 10 μM concentration of the cognate [α-^{35}S]-labeled deoxyribonucleotide-5′-triphosphate (final specific activity 1 Ci/mmol), 10 μg/mL of RNA template [poly(rA) or poly(rC)], 5 μg/mL of the appropriate primer $(dT)_{10}$ or $(dG)_{10}$, and 0.0274 μM HIV RT. The total volume of the reaction mixtures was 50 μL. The samples were incubated at 37° for 15 min. The reactions were terminated by the addition of equal volumes of 10% trichloroacetic acid. Incorporation of radiolabeled precursor was determined by collecting the precipitates on glass fiber filters, drying, and counting the samples.

The DNA-directed DNA polymerase activity of the HIV-1 RT enzyme was assessed as described above for the RNA-directed DNA polymerase assay. The synthetic template:primer used was poly(dC):$(dG)_{12-18}$ (1:1), present at a concentration of 10 μg/mL.

Michaelis–Menten kinetics, which are based on the establishment of a rapid equilibrium between the enzyme, its substrates and the inhibitor and the various enzyme–substrate complexes, proved inadequate for the analysis of the kinetic data (see, for instance, Refs. 25 and 29). The reason for this inadequacy resides in the fact that the enzyme needs to form an initiation complex with its respective substrates before elongation of the primer can commence. Therefore, no immediate equilibrium is established between the reactants involved. The experimental data were thus analyzed using steady-state Briggs–Haldane kinetics. These kinetics assume that the enzyme–substrate complex is not necessarily in equilibrium with the enzyme and its substrate. However, shortly after initiation of the reaction, enzyme–substrate complex is formed at the same rate as it dissociates. A steady-state scheme that includes all the forward and backward reaction rate constants to be considered here yields complex velocity equations that are impractical to solve. However, the general steady-state kinetic system used in this study was simplified significantly, as detailed in Fig. 2. The specific rate constants used in the following text are defined in this figure. The steady-state kinetics were limited to the reactions occurring between the enzyme and its substrates, and rapid equilibrium kinetics were applied to the reactions between the inhibitor on the one hand and the free enzyme and the various enzyme–substrate complexes on the other. This treatment is admissible since the low molecular weight inhibitor will react instantly with the respective components of the reaction. Moreover, an ordered mechanism was assumed, whereby the template:primer complex binds first to the enzyme, followed by the addition of dNTP [30, 31]. The polymerase is a processive enzyme and, after the addition of the first nucleotide, translocation occurs along the template, resulting in the incorporation of further nucleotides into the growing chain [30]. Under these conditions the formation of the phosphoester bond can be considered as irreversible as the reverse reaction occurs at an extremely low rate and the dissociation of the enzyme–product complex into its components is also negligible during the initial phase of the reaction. Thus, the enzyme–product does not differ from the initial enzyme–template:primer complex in that the former shuttles back to the enzyme–template:primer state, and this reaction rate constant (k_{2p}) is equal to k_{cat}, representing the turnover number. The quaternary enzyme–inhibitor–template:primer–dNTP complex should be non-productive as no translocation to the enzyme–inhibitor–template:primer state should occur and k'_{2p} should be 0. These simplifications reduce the number of parameters to be considered in the system to twelve or thirteen if we include k'_{2p}.

The HIV RT catalyzed system considered here consists of two substrates, S_1, (representing the template:primer) and S_2 (representing the dNTP), and I, an inhibitor. The reactions between the enzyme (E) and the low molecular weight inhibitor can be deemed as diffusion-controlled reactions [32–34]. Consequently, the conversions between E and EI, ES_1 and EIS_1, and ES_1S_2 and EIS_1S_2 occur at a much faster rate than the interconversions between the enzyme and its substrates. Thus, although the whole system is a steady-state one, there is an equilibrium between E and EI, ES_1 and EIS_1, and ES_1S_2 and EIS_1S_2. Accordingly, these three pairs of enzyme species can be treated as three combined species, i.e. E + EI, ES_1 + EIS_1, and ES_1S_2 + EIS_1S_2, where the fractions of E, EI, ES_1, EIS_1, ES_1S_2 and EIS_1S_2 are:

$$\begin{cases} f_E = \dfrac{[E]}{[E]+[EI]} = \dfrac{K_0}{K_0+[I]} \\ f_{EI} = \dfrac{[EI]}{[E]+[EI]} = \dfrac{[I]}{K_0+[I]} \\ f_{ES_1} = \dfrac{[ES_1]}{[ES_1]+[EIS_1]} = \dfrac{K_1}{K_1+[I]} \\ f_{EIS_1} = \dfrac{[EIS_1]}{[ES_1]+[EIS_1]} = \dfrac{[I]}{K_1+[I]} \\ f_{ES_1S_2} = \dfrac{[ES_1S_2]}{[ES_1S_2]+[EIS_1S_2]} = \dfrac{K_2}{K_2+[I]} \\ f_{EIS_1S_2} = \dfrac{[EIS_1S_2]}{[ES_1S_2]+[EIS_1S_2]} = \dfrac{[I]}{K_2+[I]} \end{cases} \quad (1)$$

and the equilibrium constants K_0, K_1 and K_2 are defined as follows:

$$K_0 = \frac{[E][I]}{[EI]}, \quad K_1 = \frac{[ES_1][I]}{[EIS_1]}, \quad K_2 = \frac{[ES_1S_2][I]}{[EIS_1S_2]}. \quad (2)$$

Therefore, instead of the mechanism depicted in Fig. 2 which contains six enzyme species, we can consider a simplified mechanism in which there are only three independent enzyme species, as shown in Fig. 3. In doing so, however, the corresponding rate

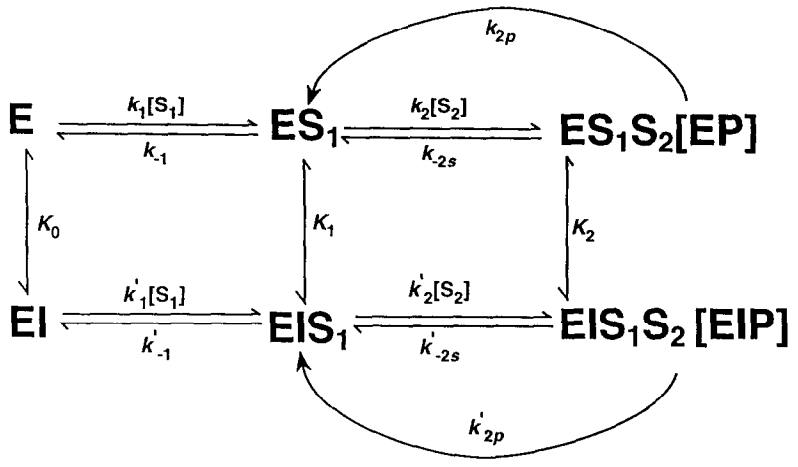


Fig. 2. Steady-state reaction scheme for HIV RT. E = enzyme; S_1 = template:primer; S_2 = dNTP; K_0, K_1, K_2 = equilibrium constants between the inhibitor (I), the enzyme and its substrates; EP = enzyme–product complex; EIP = enzyme–inhibitor–product complex.

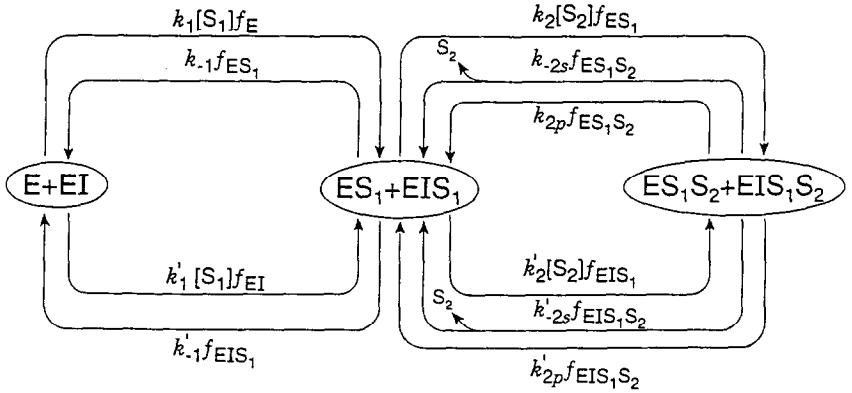


Fig. 3. The enzyme-catalyzed mechanism derived from Fig. 2 by means of the apparent rate constant method [35].

constants should be multiplied with a fractional factor [35]. For the case considered here, the rate constant associated with E should be multiplied by f_E, that associated with EI multiplied by f_{EI}, that associated with ES_1 multiplied by f_{ES1}, and so forth, as shown in Fig. 3. According to the graphic rules of Chou [36, 37], the mechanism in Fig. 3 can be expressed by a directed graph G (Fig. 4a) and the transformed graph $G\dagger$ (Fig. 4b) in which E_1, E_2, and E_3 represent $E + EI$, $ES_1 + EIS_1$, and $ES_1S_2 + EIS_1S_2$, respectively, and

$$\begin{cases} k_{12} = k_1[S_1]f_E + k'_1[S_1]f_{EI} \\ k_{21} = k_{-1}f_{ES_1} + k'_{-1}f_{EIS_1} \\ k_{23} = k_2[S_2]f_{ES_1} + k'_2[S_2]f_{EIS_1} \\ k_{32} = k_{-2s}f_{ES_1S_2} + k_{2p}f_{ES_1S_2} + k'_{-2s}f_{EIS_1S_2} + k'_{+2p}f_{EIS_1S_2} \end{cases} \quad (3)$$

Substitution of the fractional factors of equation 1 into equation 3 yields

$$\begin{cases} k_{12} = \frac{(k_1K_0+k'_1[I])[S_1]}{K_0+[I]} \\ k_{21} = \frac{k_{-1}K_1+k'_{-1}[I]}{K_1+[I]} \\ k_{23} = \frac{(k_2K_1+k'_2[I])[S_2]}{K_1+[I]} \\ k_{32} = \frac{(k_{-2s}+k_{2p})K_2+(k'_{-2s}+k'_{+2p})[I]}{K_2+[I]} \end{cases} \quad (4)$$

The concentration of the mth enzyme species is then given by

$$[E_m] = \frac{N_m}{\sum_{i=1}^{n} N_i}e_0, \quad (m = 1, 2, \cdots, n) \quad (5)$$

where e_0 is the total concentration of all enzyme species and N_m can be immediately derived using Chou's graphic rule 2 [36, 37]. When applying this graphic rule to calculate N_m, E_m should be assigned as the terminative point, while the initial reference point may be any of the points shown in Fig. 4b. For example, in deriving N_1, the terminative point

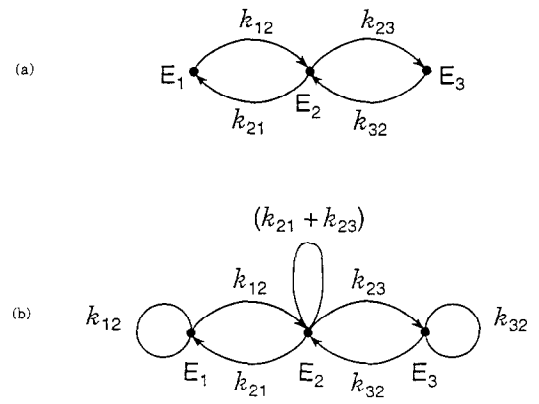
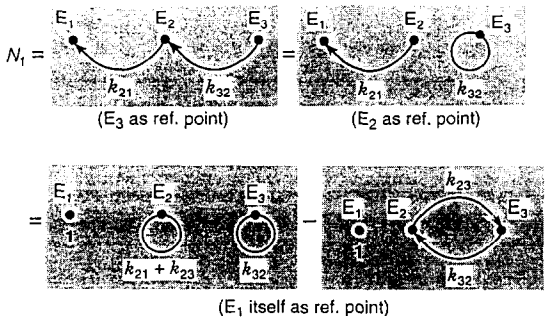


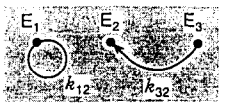
Fig. 4. Graphical expression of the mechanism shown in Fig. 3(a) The directed graph G, and (b) its transformed graph $G\dagger$, where $E_1 = E + EI$, $E_2 = ES_1 + EIS_1$, $E_3 = ES_1S_2 + EIS_1S_2$ and k_{12}, k_{21}, k_{23} and k_{32} are given by equation 4.

should be E_1, while the reference point can be either E_3, E_2, or E_1. The final results obtained should be the same, i.e.

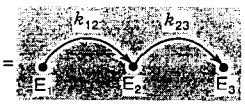


$$= k_{32}k_{21} \tag{6}$$

As we can see from the above, the more distant the reference point is located from the terminative point, the simpler the operation. Therefore, it is advisable to select a reference point as far removed as possible from the terminative point, as recommended by Chou [36, 37]. Thus, for N_2 and N_3 we have



$$= k_{12}k_{32} \tag{7}$$



$$= k_{12}k_{23} \tag{8}$$

Substituting equations 6–8 into equation 5, we immediately obtain equation 9.

$$[E_3] = \frac{k_{12}k_{23}e_0}{k_{32}k_{21} + k_{12}k_{32} + k_{12}k_{23}} \tag{9}$$

The rate of product formation is (cf. Figs. 2 and 3)

$$\frac{d[P]}{dt} = (k_{2p}f_{ES_1S_2} + k'_{2p}f_{ES_1S_2})\,[E_3] \tag{10}$$

According to equations 1 and 9, equation 10 can be written as

$$\frac{d[P]}{dt} = \frac{k_{12}k_{23}(k_{2p}K_2 + k'_{+2p}[I])/(K_2 + [I])}{k_{32}k_{21} + k_{12}k_{32} + k_{12}k_{23}}e_0 \tag{11}$$

The merit of using Chou's graphic rules resides in the fact that the more complicated the system is, the more efficient will be its derivation. Some graphic rules, e.g. Rule 4 in Chou [36, 37], can also be used to analyze none steady-state enzyme-catalyzed systems [38].

RESULTS

RNA-dependent DNA polymerase. This function was studied in the presence of various concentrations of poly(rA):$(dT)_{10}$ and a fixed concentration of dTTP and vice versa in the presence of different concentrations of dTTP and a fixed concentration of poly(rA):$(dT)_{10}$. Three inhibitor concentrations were used in addition to controls containing no U-90152E. As each sample was run in duplicate, each experiment generated a total of sixty-four data points for analysis. The analysis of the kinetic data was carried out with a computer using the steady-state kinetic scheme described in Materials and Methods. The rate constants were derived by fitting the experimental data to equation 11, and the thirteen kinetic parameters defined in Fig. 2 were calculated from this equation. The experimental results are shown in Fig. 5, and the calculated essential forward and backward reaction rates and equilibrium constants of the system are presented in Fig. 6. For k_1, the association constant and k_{-1}, the dissociation rate constant of the enzyme–poly(rA):$(dT)_{10}$ complex, values of 4.4×10^4 M^{-1} sec^{-1} and 0.3 sec^{-1} were calculated for the control reactions in the absence of the inhibitor. The ratio k_{-1}/k_1 amounts, thus, to 6.8 μM of template:primer. Published estimates for this ratio range from 0.6 nM to 3 μM of template:primer, although these latter values were obtained under the assumption that the system is at equilibrium [39–41]. In the presence of U-90152E, the corresponding values were 3.8×10^4 M^{-1} sec^{-1} for k'_1 and 0.2 sec^{-1} for k'_{-1}. The forward rate constant k_2, for the association of dTTP to the enzyme–poly(rA):$(dT)_{10}$ complex was 3.7×10^4 M^{-1} sec^{-1}. The backward rate constant k_{-2s}, representing the dissociation rate of the ternary enzyme–poly(rA):$(dT)_{10}$–dTTP complex was 0.85 sec^{-1}. The corresponding values in the presence of U-90152E were $k'_2 = 1.0 \times 10^4 M^{-1} sec^{-1}$, and $k'_{-2s} = 0.3$ sec^{-1}. The turnover number k_{2p}, representing k_{cat} was 0.4 sec^{-1} in the control reaction and the corresponding value k'_{2p} in the presence of the inhibitor was essentially 0. The value for k_{2p} is somewhat lower than the one reported by Reardon [31] (14 sec^{-1}), but reasonably close to the one reported by Anderson and Coleman [42] (1.2 sec^{-1}), although both of the latter values were calculated with different mathematical schemes than the one applied here. Moreover, the respective inhibition constants or K_i values calculated for the enzyme (K_0), the enzyme–poly(rA):$(dT)_{10}$ (K_1), and the enzyme–poly(rA):$(dT)_{10}$–dTTP (K_2) complexes with the inhibitor were 5.2, 1.1, and 1.6 μM U-90152E. The

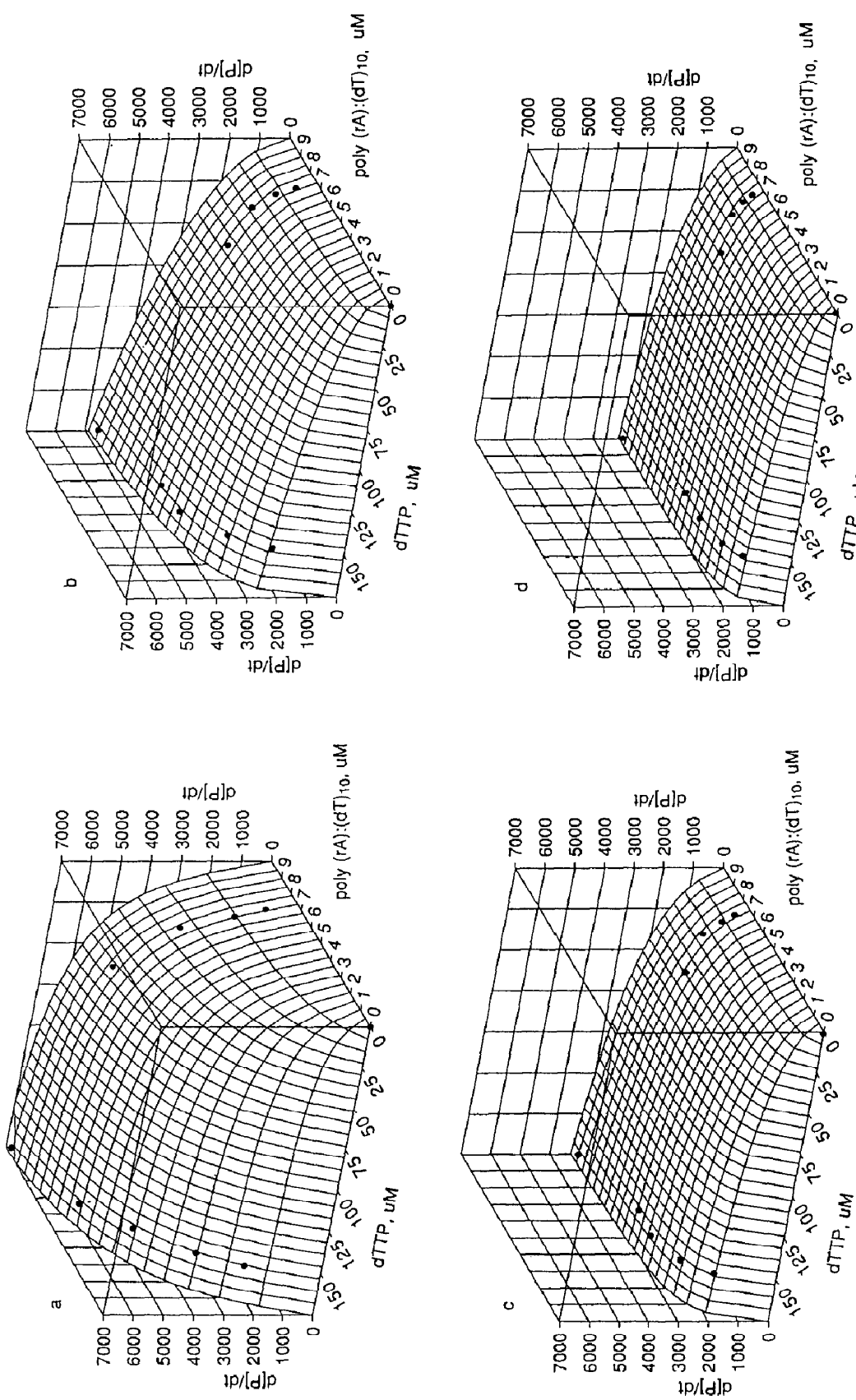


Fig. 5. Inhibition of HIV-1 RT poly(rA):$(dT)_{10}$-directed poly(dT) synthesis by U-90152E. Enzyme = 0.0274 μM, and $d[P]/dt = \mu M \times 10^{-6}$ of dTMP incorporated per sec. (a) No inhibitor, and (b) 1 μM, (c) 2 μM, and (d) 4 μM U-90152E.

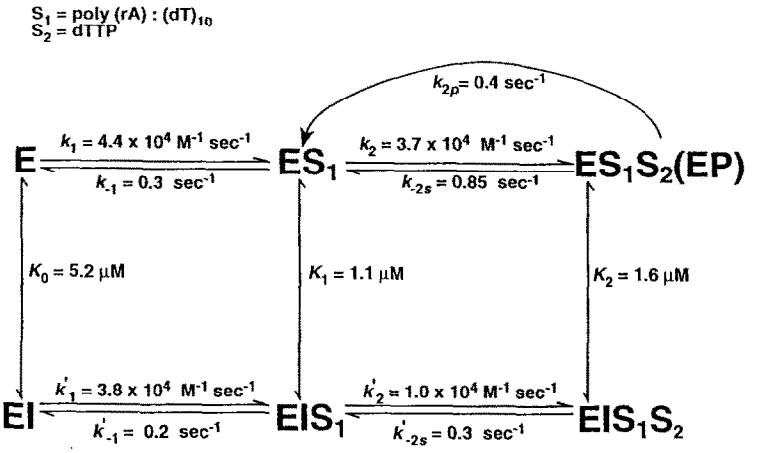


Fig. 6. Inhibition of poly(rA):$(dT)_{10}$-directed poly(dT) synthesis by U-90152E; steady-state kinetic parameters.

overall fitting error for the analysis was 1×10^{-5}. The low value for k_{-1}, amounting to $0.3\,sec^{-1}$, is indicative of a processive enzyme, and the results are consistent with an ordered mechanism whereby the template:primer binds to the enzyme first, followed by the addition of dNTP. The equilibrium constants K_0, K_1, and K_2 assess the reactions between the inhibitor and the enzyme or the enzyme–substrate complexes. The value for K_0 is at least 3–4 times larger than the ones for K_1 and K_2, which means that U-90152E possesses a higher binding affinity for the enzyme–mono and bisubstrate complexes than for the free enzyme.

Similar experiments were carried out with the homopolymeric template:primer poly(rC):$(dG)_{10}$ and the cognate nucleotide dGTP. The experimental results are given in Fig. 7, and the calculated values for the rate and equilibrium constants are shown in Fig. 8. The forward rate constant k_1 for the formation of the enzyme–poly(rC):$(dG)_{10}$ complex was $5.3 \times 10^4\,M^{-1}\,sec^{-1}$ and the corresponding backward rate k_{-1} was $0.3\,sec^{-1}$. In the presence of the inhibitor, k'_1, assessing the formation of the ternary enzyme–U-90152E-poly(rC):$(dG)_{10}$ complex, was $2.6 \times 10^4\,M^{-1}\,sec^{-1}$, and the value for k'_{-1}, the reverse reaction, was $0.1\,sec^{-1}$. Furthermore, the forward reaction rate for the formation of the ternary enzyme–poly(rC):$(dG)_{10}$–dGTP complex k_2 was $3.3 \times 10^4\,M^{-1}\,sec^{-1}$ and the backward rate k_{-2s} was $0.7\,sec^{-1}$. In the presence of the inhibitor, k'_2, the rate constant for the formation of the quaternary enzyme–U-90152E-poly(rC):$(dG)_{10}$–dGTP complex was $0.8 \times 10^4\,M^{-1}\,sec^{-1}$, and the corresponding reverse rate constant k'_{-2s} for this complex was $0.2\,sec^{-1}$. The rate of translocation (k_{2p}) or turnover number in the reaction without inhibitor was $0.7\,sec^{-1}$, and the corresponding value k'_{2p} for the quaternary enzyme-U-90152E-poly(rC):$(dG)_{10}$–dGTP complex was essentially zero. The equilibrium constant K_0 was 5.5 μM U-90152E for the enzyme–inhibitor complex but decreased to 0.25 μM for K_1, the enzyme–inhibitor–template:primer complex, as well as for K_2, the enzyme–inhibitor–template:primer–dGTP complex. The fitting error was 3.3×10^{-5} for this system. The value for K_0 exceeds the ones for K_1 and K_2 by a factor of at least twenty, although both K_1 and K_2 are equal. This demonstrates that U-90152E binds more tightly to the enzyme–substrate complexes than the free enzyme if the template:primer is poly(rC):$(dG)_{10}$ and the cognate nucleotide is dGTP. Moreover, the inhibitor binds equally well to the enzyme–template:primer and the enzyme–template:primer–dGTP complexes. The values for K_1 and K_2 are smaller than the corresponding ones obtained with the poly(rA):$(dT)_{10}$-catalyzed systems where K_1 was 1.1 μM and K_2 was 1.6 μM, and manifests that the potency of U-90152E depends on the base composition of the template:primer.

DNA-directed DNA polymerase of HIV-1 RT. U-90152E was also tested for its effect on the DNA-directed DNA polymerase activity of RT. This reaction was studied with poly(dC):$(dG)_{12-18}$ as the template:primer and dGTP as the nucleotide being incorporated. The experimental results are given in Fig. 9 and the calculated rate and equilibrium constants in Fig. 10. In the absence of any inhibitor the calculated value for k_1 was $5.1 \times 10^4\,M^{-1}\,sec^{-1}$ and the one for k_{-1} was $0.3\,sec^{-1}$, representing the respective forward and backward reaction rate constants for the formation and dissociation of the enzyme–poly(dC):$(dG)_{12-18}$ complex. In the presence of U-90152E the corresponding forward and backward reaction rate constants were: $k'_1 = 3.1 \times 10^4\,M^{-1}\,sec^{-1}$ and $k'_{-1} = 0.3\,sec^{-1}$. The forward rate constant for the formation of the ternary enzyme–poly(dC):$(dG)_{12-18}$–dGTP complex k_2 was $7.4 \times 10^4\,M^{-1}\,sec^{-1}$ and the backward rate constant k_{-2s} was $0.7\,sec^{-1}$. The corresponding values in the presence of the inhibitor were $k'_2 = 4.9 \times 10^4\,M^{-1}\,sec^{-1}$ and $k'_{-2s} = 0.1\,sec^{-1}$. The turnover number k_{2p} was $1.3\,sec^{-1}$ for the control reaction and near 0 for k'_{2p} in this system. The dissociation constant for the enzyme–U-90152E complex, K_0, was 5.2 μM of inhibitor and the ones for both K_1 and K_2 were equal and amounted to 1.1 μM U-90152E. The fitting error

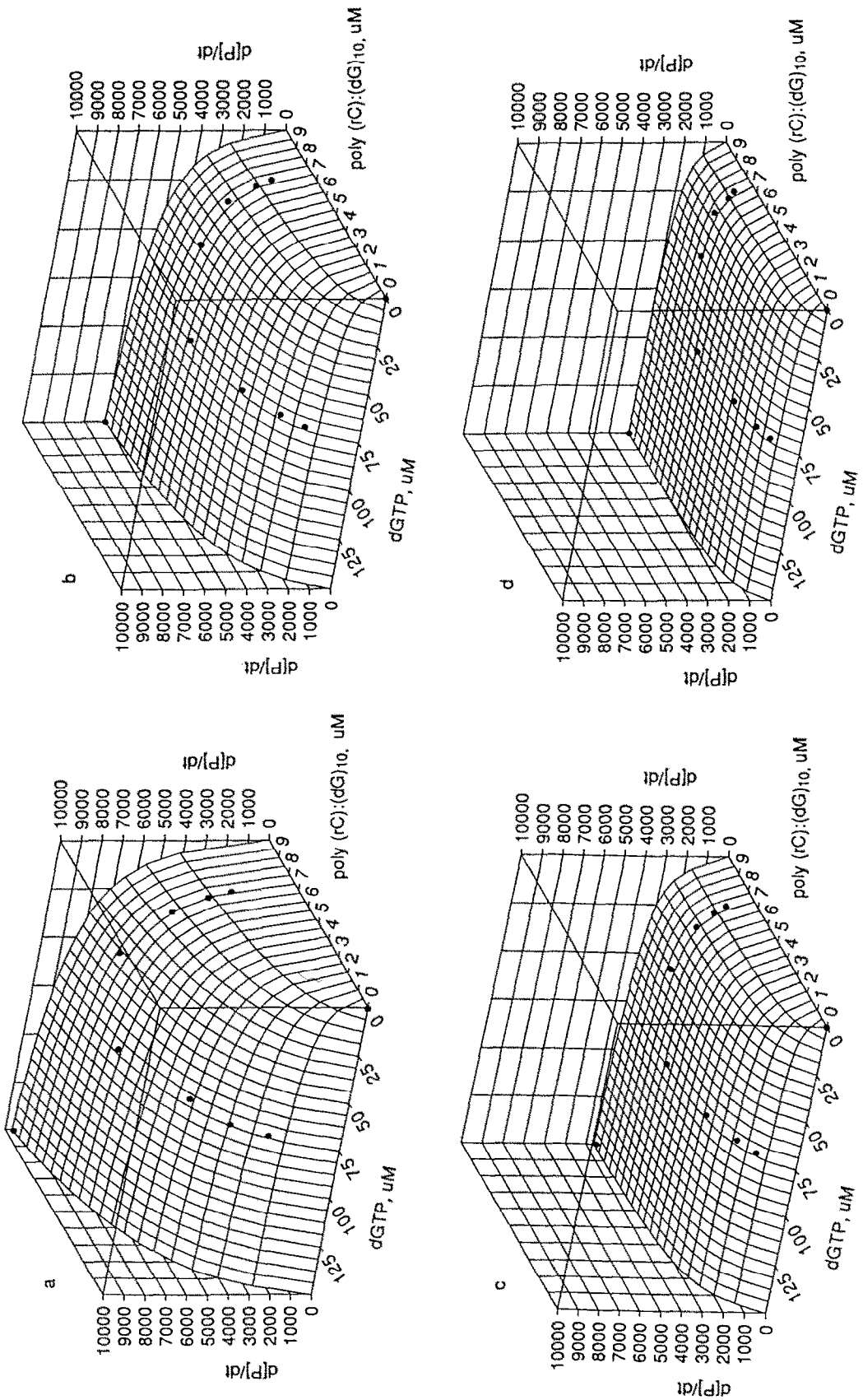


Fig. 7. Inhibition of HIV-1 RT poly(rC):$(dG)_{10}$-directed poly(dG) synthesis by U-90152E. Enzyme = 0.0274 μM, and $d[P]/dt = \mu M \times 10^{-6}$ of dGMP incorporated per sec. (a) No inhibitor, and (b) 0.25 μM, (c) 0.5 μM, and (d) 1 μM U-90152E.

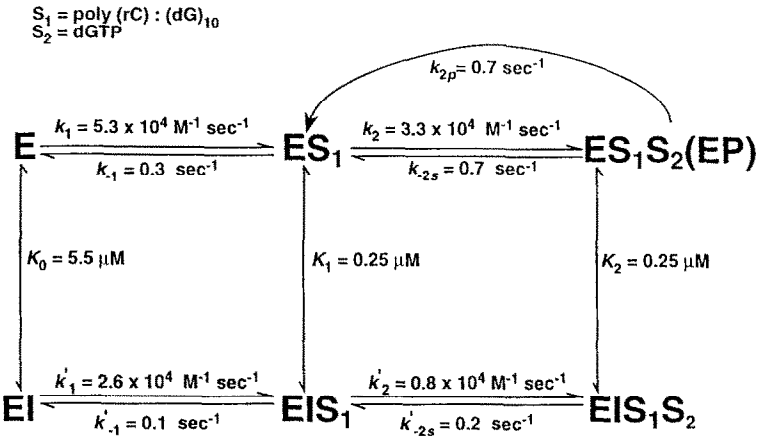


Fig. 8. Inhibition of poly(rC):$(dG)_{10}$-directed poly(dG) synthesis by U-90152E; steady-state kinetic parameters.

was 2.5×10^{-4} for this system. It should be noted that K_0 exceeds the values for K_1 and K_2 approximately 5-fold, which indicates that U-90152E binds much tighter to the enzyme–substrate complexes than the free enzyme.

DISCUSSION

The inhibition kinetics of U-90152E on the RNA- and DNA-directed DNA polymerase domains of HIV-1 RT were studied with respect to the nucleic acid and dNTP binding sites, using homopolymeric template: primers. The analysis of the experimental results was carried out using a modified Briggs–Haldane kinetic scheme. These kinetics take into consideration that (a) the reaction is ordered in that the template:primer binds first to the enzyme and is followed by the binding of dNTP, (b) the enzyme–product complex elongated by one base does not dissociate into free enzyme and product but is recycled back to the enzyme–template:primer state by the process of translocation and the polymerase is thus processive, (c) the formation of the phosphoester bond and the concomitant release of pyrophosphate is irreversible as the reverse reaction rate is extremely slow, and (d) the binding of the low molecular weight inhibitor to the free enzyme or the various enzyme–substrate complexes follows rapid equilibrium kinetics. The rate equation 11 derived for this mechanism contains thirteen rate and equilibrium constants, as defined in Fig. 2. Equation 11 allows the calculation of these constants as it relates the rate of product formed by the RT enzyme to the two substrates, the template: primer and the dNTP. The calculated dissociation constants for the enzyme–template: primer (k_{-1}) complexes in the absence of inhibitor were 0.3 sec^{-1} in both the RNA- and DNA-catalyzed DNA polymerase reactions studied. These values are very small and characteristic for processive polymerases. The turnover numbers k_{2p} or k_{cat} for the RNA- and DNA-directed polymerase functions ranged from 0.4 to 1.3 sec^{-1} and are somewhat smaller than a previously published value of 14 sec^{-1} for HIV-1 RT by Reardon [31] but in good agreement with a value of 1.2 sec^{-1} published by Anderson and Coleman [42]. In all the systems tested, the equilibrium constants K_0 for the enzyme–inhibitor (EI) complexes exceeded those of the enzyme–inhibitor–substrate complexes (EIS_1 and EIS_1S_2) by several-fold, but the values for K_1 and K_2 were equal within each system studied. This indicates that, although U-90152E binds to the free enzyme, it binds much tighter to the enzyme–substrate–complexes, and the inhibitor does, therefore, not directly impair the template: primer nor the dNTP binding sites of the RT enzyme. Since the values for K_1 and K_2 are equal, it follows that the binding of the first substrate, the template: primer to the enzyme, suffices to amplify the binding affinity and no further enhancement results following the binding of the second substrate, the dNTP. Moreover, as the functions of the substrate binding sites appear largely unaffected by U-90152E, the inhibitor must impair an event occurring after the formation of the enzyme–substrate complexes, which includes either the formation of the phosphoester bond or the translocation of the enzyme relative to the template: primer following the generation of the ester bond.

We had studied previously the inhibition kinetics of U-87201E and U-88204E, two bisheteroaryl-piperazines structurally related to U-90152E [6, 7]. They differ from each other structurally in that U-87201E possesses a 5-methoxy group, U-88204E is unsubstituted, and U-90152E possesses a 5-(methyl)sulfonylamino moiety on the indole portion of the molecule. They all contain a piperazine moiety in the central portion of the molecule and a 3-substituted alkylaminopyridine. U-87201E differs from U-88204E and U-90152E in that it contains a 3-ethylamino substituent on the pyridine, while U-88204E and U-90152E contain a 3-isopropylamino substituent group. All three compounds share the common property in that they inhibit both the RNA- and DNA-directed DNA polymerase functions of RT but not the RNase H function. U-90152E, the

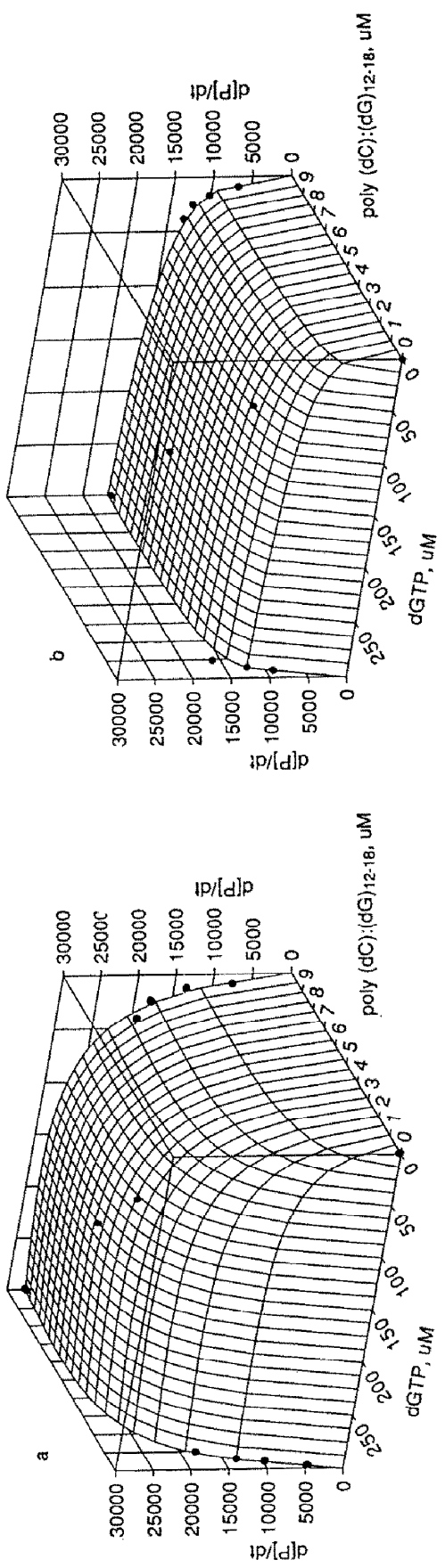


Fig. 9. Inhibition of HIV-1 RT poly(dC):$(dG)_{12-18}$-directed poly(dG) synthesis by U-90152E. Enzyme = 0.0274 μM, and $d[P]/dt = \mu M \times 10^{-6}$ of dGMP incorporated per sec. (a) No inhibitor, and (b) 0.5 μM, (c) 1 μM, and (d) 2 μM U-90152E.

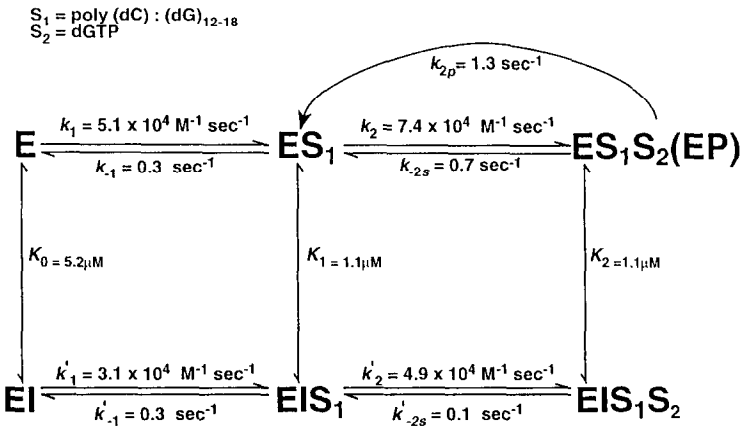


Fig. 10. Inhibition of poly(dC):$(dG)_{12-18}$-directed poly(dG) synthesis by U-90152E; steady-state kinetic parameters.

compound discussed here, is the most potent of the three. Their specific kinetic inhibition patterns differ in some significant ways. U-87201E acts predominantly as a noncompetitive inhibitor with respect to both substrate binding sites of the enzyme regardless of the template:primer used. U-88204E acts as a noncompetitive inhibitor if the template: primers are either poly(rA):$(dT)_{10}$ (RNA-directed DNA polymerase) or poly(dC):$(dG)_{12-18}$ (DNA-directed DNA polymerase), but it acts as a mixed inhibitor if the template:primer is poly(rC):$(dG)_{10}$. U-90152E, on the other hand, acts as a mixed inhibitor in all the systems tested in that it possesses a higher binding affinity for the enzyme–substrate complexes than for the free enzyme. Moreover, U-90152E is a much more potent inhibitor of the DNA-catalyzed DNA polymerase function of RT than either U-88204E or U-87201E, with U-87201E being the least potent one.

Kinetic studies with other non-nucleoside HIV-1 RT inhibitors have been described. The benzodiazepine or TIBO compound R82150 appears to be a specific inhibitor of the HIV-1 RT catalyzed RNA-directed DNA polymerase function [11]. The inhibitor acts uncompetitively with respect to the nucleic acid binding site and noncompetitively with respect to the dNTP site. The IC_{50} for the DNA-directed DNA polymerase was 40 times higher than the one required to inhibit the RNA-directed DNA polymerase, and the RNase H activity was not impaired by the inhibitor. The dipyridodiazepinone nevirapine acts as a mixed inhibitor with respect to the poly(rA):$(dT)_{10}$ and poly(rC):$(dG)_{10}$ binding sites and noncompetitively with respect to the dNTP binding sites during RNA-directed DNA synthesis by HIV-1 RT [43]. The pyridinone derivative L-697,639 indicated noncompetitive inhibition with respect to dGTP and poly(rC):$(dG)_{12-18}$ [14]. The quinoline U-78036 acts as a mixed to noncompetitive inhibitor with respect to both the substrate binding sites of the enzyme [15]. Moreover, this compound is a specific inhibitor of the RNA-catalyzed DNA polymerase function of RT as it has no inhibitory effect on the DNA-catalyzed one. Compared with these other classes of RT inhibitors, U-90152E differs from all of them in that it acts exclusively as a mixed inhibitor of both the RNA- and DNA-directed DNA polymerase domains of RT and has a much higher binding affinity for the enzyme–substrate complexes than for the free enzyme. It should be emphasized, however, that all of these data, except for the ones obtained with the quinoline U-78036 and the arylpiperazines U-90152E, U-87201E, and U-88204E, were derived from rapid equilibrium kinetics and not Briggs–Haldane kinetics.

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