The Quinoline U-78036 Is a Potent Inhibitor of HIV-1 Reverse Transcriptase*

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The quinoline U-78036 represents a new class of non-nucleoside human immunodeficiency virus (HIV)-reverse transcriptase inhibitors. The quinoline possesses excellent antiviral activity at nontoxic doses in HIV-1-infected lymphocytes grown in tissue culture. Enzymatic kinetic studies of the HIV-1 reverse transcriptase (RT)-catalyzed RNA-directed DNA polymerase function were carried out in order to determine whether the inhibitor interacts with the template-primer or deoxyribonucleotide triphosphate (dTTP) binding sites of the polymerase. The data were analyzed using steady-state or Briggs-Haldane kinetics assuming that the template-primer binds to the enzyme first followed by the dNTP and that the polymerase functions processively. The calculated rate constants are in agreement with this model. The results show that the inhibitor acts as a mixed noncompetitive inhibitor with respect to both the template-primer and the dNTP binding sites of the enzyme. Hence, U-78036 inhibits the RNA-directed DNA polymerase activity of RT by interacting with a site distinct from the template-primer and dNTP binding sites. Moreover, the potency of U-78036 is dependent on the base composition of the template-primer. The equilibrium constants for various enzyme-substrate-inhibitor complexes were at least seven times lower for the poly(rC)·(dG)₉-catalyzed system than the one catalyzed by poly(rA)·(dT)₉. In addition, the inhibitor does not impair the DNA-dependent DNA polymerase activity and the RNase H function of HIV-1 RT nor does it inhibit the RNA-directed DNA polymerase activity of the HIV-2, avian myoblastoma virus, and murine leukemia virus RT enzymes.

The HIV-1 RT enzyme is essential for the replication of the virus and represents a valid target to inhibit the spread of the virus in humans. Thus far, nucleoside analogs targeted to the RT enzyme such as 3'-azido-3'-deoxythymidine (AZT) have shown beneficial effects in AIDS patients in clinical situations. The relative toxicity of these nucleoside analogs and the emergence of resistant viral strains to this class of inhibitors justifies the search for non-nucleoside RT inhibitors. Recently, several classes of non-nucleoside HIV RT inhibitors have been described. These include the tetrahydroimidazo-[4,5,1-jk][1,4]-benzodiazepin-2(1H)-one and -thione or TIBO compounds (1, 2), the dipyrindiazepiones (3, 4), the 1-((2-hydroxyethoxy)-methyl)-6- (phenylthio)thymine or 1-((2-hydroxyethoxy)-methyl)-6-(phenylthio)thymine (HEPT) derivatives (5, 6), polyanilides and sulfonates (7-18), the pyridinone derivatives (19), and the bisbenzazepinones (BHAP compounds) (20-22).

The quinoline U-78036 (1-(4-chlorobenzoyl)-1,2-dihydro-2-quinolincarbonitrile) is a potent inhibitor of HIV-1 RT and differs chemically from the RT inhibitors listed above (Fig. 1). The chemical synthesis of such N-acylhydroquinolines or Reissert compounds is described (23). This communication describes the anti-HIV-1 activity of U-78036 as well as enzymatic kinetic studies dealing with the inhibitory effect of U-78036 on HIV RT.

MATERIALS AND METHODS

The expression of HIV-1 RT and its purification have been described (24). For the polymerase assays, a partially purified RT preparation was used that was judged as 90-95% pure based on SDS polyacrylamide gel electrophoresis. This preparation was devoid of Escherichia coli RNase H activity and consisted of p51/p66 heterodimers of RT, with no evidence of monomeric RT in the form of p66 or p51 alone. For the RNase H assay, a highly purified preparation of the heterodimer p51/p66 RT was used. Its expression and purification have been described (25).

The synthetic template-primers poly(rA), oligo(dT)₁₈, poly(rC), oligo(dG)₁₈, and poly(dC)- (dG)₁₈-₁₈ were purchased from Pharmacia.
was terminated by the addition of equal volumes of culture medium and the total amount of viral RNA synthesized was assayed as described above for the RNA-directed DNA polymerase assay. The synthetic template-primer used was poly(dC)·(dG)₁₂₋₂₀ present at a concentration of 10 µg/ml.

The DNA-directed DNA polymerase activity of the RT enzyme was assessed as described above for the RNA-directed DNA polymerase assay. The RNase H assay was conducted as described (26). In general, the assay follows the loss of trichloroacetic acid-precipitable radiolabeled RNA-DNA hybrid as a function of time. The specific assay mixtures contained 2.5 µg and 2 µCi/ml [³H]poly(rG)·poly(dC) (1:1), 50 mM Tris·HCl, pH 8.5, 5 mM MgCl₂, 0.02% Nonidet P-40, and 5% glycerol. Incubation was for 10 min at 25 °C, and the reactions were terminated by the addition of equal volumes of 10% trichloroacetic acid. The loss of substrate was determined by collecting the precipitates on glass fiber filters, drying, and counting the samples.

The infectivity assays in HIV-infected lymphocytes grown in culture were carried out by the syncytia reduction method (27) or by measuring the total amount of core p24 protein released and the IC₅₀ in terms of cytotoxicity to the host cells (28).

The avian myeloblastosis virus and murine leukemia virus RT preparations were purchased from Life Technologies, Inc. These enzymes were assayed in the same standard reaction mixture as described above for the HIV RT. Sufficient amounts of the latter RT species were added per reaction mixture to incorporate approximately 0.04 nmol of dNTP in 15 min at 37 °C.

RESULTS

Antiviral Activity—The quinoline U-78036 is a potent inhibitor of HIV-1 RT and has shown excellent antiviral activity at nontoxic doses in HIV-1-infected lymphocytes grown in tissue culture. In the syncytia reduction assay, using MT-2 cells infected with HIV-1 (IIIb isolate), the IC₅₀ was <5 pM U-78036 and the IC₅₀ in terms of cytotoxicity to the host cells was >10 µM inhibitor (Table I). In the p24 release and total viral RNA synthesis assays carried out in HIV-1 (D34 isolate)-infected peripheral blood mononuclear cells, the IC₅₀ values in terms of p24 core protein released and total viral RNA synthesized were <0.1 µM U-78036 at day 3 and <1 µM at day 4 post-infection of the cells (Table II). No apparent toxic effects on the host cells were observed at a concentration of 10 µM inhibitor. The IC₅₀ values for inhibition of the cellular DNA polymerases α and δ were 1300 and >3400 µM, respectively. These concentrations are significantly above those required to inhibit HIV-1 RT to the same extent as shown below.

Enzyme Kinetics—The kinetic data were first analyzed using Michaelis-Menten kinetics, which are based on a rapid equilibrium system wherein the enzyme, the substrate, and rapid equilibrium kinetics were applied to the interactions between the inhibitor and the enzyme or the various enzyme-substrate complexes. Moreover, an ordered mechanism was assumed, whereby the template-primer complex binds first to the enzyme, followed by the addition of dNTP (29, 30). 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 (29). Under these condi-

**Table I**

| Sample | Number of syncytia/plate |
|--------|--------------------------|
| Control | 29                       |
| U-78036 | 33.9 µM                  |
|         | 0                        |
|         | 3.4 µM                   |
|         | 10                       |
|         | 0.33 µM                  |
| Control | 22                       |
| U-78036 | 17.8 µM                  |
|         | 0                        |
|         | 1.78 µM                  |
|         | 7                        |
|         | 0.178 µM                 |
|         | 20                       |

**Table II**

| Sample | ng HIV p24/ml | pg HIV RNA/ml | Cell viability % of control |
|--------|---------------|---------------|----------------------------|
| Day 3  |               |               |                           |
| Control | 25            | 90.8          | ND                        |
| U-78036 |               |               |                           |
| 10 µM  | 0             | 0             | ND                        |
| 1 µM   | 1             | 2.3           | ND                        |
| 0.1 µM | 7             | 55.9          | ND                        |
| Day 4  |               |               |                           |
| Control | 58.4          | 278           | 100                       |
| U-78036 |               |               |                           |
| 10 µM  | 0             | 0             | 100                       |
| 1 µM   | 5.5           | 0             | 100                       |
| 0.1 µM | 53            | 196           | 100                       |

* ND, no data.

**Fig. 2. Steady-state reaction scheme for HIV RT.** E, enzyme; S₁, template-primer; S₂, dNTP. Kₑ, Kₛ, and Kₑₛ represent equilibrium constants between the inhibitor (I), the enzyme, and its substrate. EP, enzyme-product complex.
tions, the formation of the phosphoester bond can be considered as irreversible, since the reverse reaction occurs at an extremely slow rate and the dissociation of the enzyme-product complex into its components is also negligible during the initial reaction phase. Thus, the enzyme-product complex does not differ from the initial enzyme-template-primer complex in that the former shuttles back to the enzyme-template-primer state where another nucleotide is added and this rate constant, designated as \( k_{\text{tp}} \), is equal to \( k_{\text{cat}} \) representing the turnover number. The constant \( k_{-2} \) represents the backward rate constant for the enzyme-template-primer-dNTP complex. The quaternary enzyme-inhibitor-template-primer-dNTP complex should be nonproductive, as no translocation of the enzyme-inhibitor-template-primer state should occur (thus, \( k_{\text{ip}}' \to 0 \)).

Hence, the HIV RT-catalyzed system considered here consists of two substrates, \( S_1, S_2 \), and one inhibitor, \( I \). Therefore, the system contains eight enzyme species, i.e., \( E, ES_1, ES_2, EI, EIS_1, EIS_2, \) and \( EIS_1S_2 \). If conversion between any two of these enzyme species is possible, then the directed graph \( G \) representing the enzyme-catalyzed system is shown in Fig. 3 (34, 35). This system is very complex, and its kinetic equations involve an excessive number of rate constants. To illustrate this, one can use Chou's graphic rule 1 of enzyme kinetics (34, 35) to estimate how many terms need to be considered in deriving the concentration for each of the enzyme species. The conversion between any two enzyme species can be expressed by a "zero-one" matrix \( A \) in which the element at the \( i \)th row and \( j \)th column is one if the enzyme species \( i \) can be converted to the enzyme species \( j \); otherwise, the element is zero. Such a zero-one matrix for the enzyme-catalyzed system shown in Fig. 3 is given by Equation 1.

\[
A = \begin{bmatrix}
0 & 1 & 1 & 0 & 0 & 0 & 1 & 0 \\
1 & 0 & 0 & 1 & 0 & 0 & 0 & 1 \\
1 & 0 & 0 & 1 & 1 & 0 & 0 & 0 \\
0 & 1 & 0 & 0 & 1 & 0 & 0 & 1 \\
6 & 0 & 1 & 0 & 0 & 1 & 1 & 0 \\
0 & 0 & 0 & 1 & 1 & 0 & 0 & 1 \\
1 & 0 & 0 & 1 & 0 & 0 & 0 & 1 \\
0 & 1 & 0 & 0 & 0 & 1 & 1 & 0 
\end{bmatrix}
\]  

(Eq. 1)

According to Chou's graphic rule 1 of enzyme kinetics (34, 35), the number of terms in the numerator for the concentration of the \( i \)th enzyme species, say \( i = 1 \), at the steady-state will be

\[
P_1 = \det B_{11} = 304
\]  

(Eq. 3)

where \( B_{11} \) denotes the submatrix obtained by removing the first row and first column from the matrix \( B \). Since \( G \) of Fig. 3 is a symmetric graph, i.e., a graph in which whenever there is an arc from the enzyme species \( i \) to \( j \), there must be an arc from enzymes species \( j \) to \( i \). Then the corresponding number of terms for each of the other seven enzyme species must be the same and also equal to 304 (34, 35). The number of terms for the denominator is even larger and equal to 304 \( \times 8 = 2432 \) rendering the system impractical to manageable solutions.

Since the system considered here is ordered in that the binding of \( S_1 \) to \( E \) precedes the binding of \( S_2 \), the system can be simplified significantly. Furthermore, the reactions between the inhibitor and the enzyme and various enzyme-substrate complexes are assumed to be diffusion-controlled (32, 33), and the interconversion rates between \( E \) and \( EI, ES_1, ES_2, EIS_1, EIS_2, \) and \( EIS_1S_2 \), respectively, occur much faster than those between the enzyme and its substrates. Thus, although the whole system is a steady-state one, there is an equilibrium between the low molecular weight inhibitor and the enzyme and the enzyme-substrate complexes (31). The whole system can be expressed as shown in Fig. 2, where the equilibrium constants or \( K \) values defined as \( K_0, K_1, \) and \( K_2 \), respectively, are shown in Equation 4.

\[
K_0 = \frac{[E][I][S_1]}{[EI]}, \quad K_1 = \frac{[EIS_1][I]}{[EIS_1]}, \quad K_2 = \frac{[EIS_1S_2][I]}{[EIS_1S_2]}
\]  

(Eq. 4)

For such a simplified system, the rate of product formation is given by the velocity Equation 5 (see Ref. 22)

\[
\frac{d[P]}{dt} = \frac{k_{iz}k_{iz}k_{iz}(h_{iz}K_0 + h_{iz}'[I])/[K_0 + [I])}{k_{iz}k_{iz} + k_{iz}k_{iz} + k_{iz}k_{iz}} e_0
\]  

(Eq. 5)

where

\[
k_{iz} = \frac{(k_{iz}K_0 + h_{iz}'[I])[S_1]}{K_0 + [I]} \]  

(Eq. 6)

RNA-directed DNA Polymerase—Enzymatic kinetic studies were performed with U-78036 and synthetic template-primers to determine the type of inhibition pattern on the
U-78036, an Inhibitor of HIV-1 Reverse Transcriptase

RNA-directed DNA polymerase function of HIV-1 RT with respect to the dNTP and template-primer binding sites.

In one set of experiments using the template-primer combination poly(rA)- (dT)$_{10}$ and dTTP as the two polymerase substrates, alternatively, one of the substrates was varied while the other one was kept constant. Three concentrations of inhibitor (25, 50, and 100 µM) were studied in addition to appropriate controls containing no drug. The data were analyzed via computer using the steady-state kinetic model described above. The program simultaneously calculates the essential forward and backward rate constants as well as the inhibition constants of the reaction. The experimental results are listed in Fig. 4, and the calculated reaction rates are shown in Fig. 5. The rate constants derived by fitting the experimental data to Equation 5 for the association and dissociation rate constants $k_1$ and $k_1^\prime$, respectively, representing the association and dissociation rate constants for the enzyme-template-primer-U-78036 complex, were $4.7 \times 10^4$ M$^{-1}$ s$^{-1}$ and 0.26 s$^{-1}$. The corresponding association and dissociation rate constants for the enzyme-template-primer-dTTP complex were $3.2 \times 10^4$ M$^{-1}$ s$^{-1}$ for $k_1^\prime$ and 0.7 s$^{-1}$ for $k_1$. The forward rate constant $k_2$ for the formation of the enzyme-template-primer-dTTP complex was $3.9 \times 10^4$ M$^{-1}$ s$^{-1}$, and the reverse rate constant $k_{2^\prime}$ for its dissociation was 0.8 s$^{-1}$. The corresponding values in the presence of the inhibitor were $0.9 \times 10^4$ M$^{-1}$ s$^{-1}$ for $k_1^\prime$ and 0.23 s$^{-1}$ for $k_{2^\prime}$. The translocation rate $k_{2p}$ was 1 s$^{-1}$ for the control reaction and essentially zero for $k_{2p}$. The equilibrium constants or $K_i$ values for the enzyme-U-78036 $(K_0)$, enzyme-template-primer-U-78036 $(K_1)$, and enzyme-template-primer-dTTP-U-78036 $(K_2)$ complexes were 4.8, 57, and 51 µM inhibitor, respectively. The much smaller value for $K_0$ as compared with $K_1$ and $K_2$ suggests that U-78036 acts as a mixed inhibitor with respect to the nucleic acid and dNTP binding sites of the enzyme if the substrates are poly(rA)- (dT)$_{10}$ and dTTP.

Similar experiments were carried out with the template-primer poly(rC)- (dG)$_{10}$ and dGTP. The inhibitor was tested at concentrations of 2.5, 5, and 19 µM. The experimental data are shown in Fig. 6, and the calculated values for the rate constants for the system are presented in Fig. 7. The forward rate constant $k_1$ for the formation of the enzyme-template-primer(dG)$_{10}$ complex was $5.1 \times 10^4$ M$^{-1}$ s$^{-1}$, and its backward rate $k_{1^\prime}$ was 0.3 s$^{-1}$. The forward reaction rate $k_2$ for the formation of the enzyme-template-primer-dGTP complex was $2.8 \times 10^4$ M$^{-1}$ s$^{-1}$, and the backward rate $k_{2^\prime}$ was 0.8 s$^{-1}$. The translocation rate $k_{2p}$ was 0.6 s$^{-1}$ for the control and close to zero for
\(k_0\) in these experiments. Hence, in the case of the substrates poly(rA)·(dT)\(\_\_\) and dTTP, the translocation rate in the absence of the inhibitor was somewhat higher (1 s\(^{-1}\)) than for the poly(rC)·(dG)\(\_\_\) system. This difference shows that the incorporation of dTMP into the nascent DNA chain occurs at a slightly more efficient rate than the incorporation of dGMP by HIV-1 RT. The association and dissociation rate constants for the corresponding association and dissociation rate constants  
\[ k^+ \quad k^- \]

were higher for the poly(rA)·(dT)\(\_\_\) system. These values are much smaller than the ones containing poly(rC)·(dG)\(\_\_\) containing complexes than to the ones containing poly(rA)·(dT)\(\_\_\). Recently, other non-nucleoside classes of HIV-1 RT inhibitors have been described. These include the tetrahydromidazolo-[4,5,1-k-j][1,4]-benzo-diazepin-2(1H)-one and -thione or TIBO compounds (1, 2), the dipyridiazepinones (3, 4), the 1-[2-hydroxyethoxy]-methyl]-6-(phenylthio)thymine or HEPT derivatives (5, 6), the pyridinone derivatives (19), and the bisheptolopylpirazines or BHP compounds (20–22). The TIBO compound R82150 appears to be a specific inhibitor of HIV-1 RT-catalyzed RNA-directed DNA synthesis (3). Kinetic studies suggest that the inhibitor acts noncompetitively with respect to the nucleic acid binding site and noncompetitively with respect to the dNTP site. The 
\[ IC_{50} \]

for DNA-directed DNA synthesis was 40 times higher than the one required for effective inhibition of RNA-directed DNA synthesis. Moreover, R82150 did not inhibit RNase H. The dipyrdo-diazepinone nevirapine acts as a mixed inhibitor with respect to the poly(rA)·(dT)\(\_\_\) and poly(rC)·(dG)\(\_\_\) binding sites and noncompetitively with respect to the dNTP binding sites during RNA-directed DNA synthesis by HIV-1 RT (36). Nevirapine also inhibits the DNA-catalyzed DNA polymerase function of HIV-1 RT. The pyridinone derivatives, like the TIBO compounds mentioned above, also seem to act as competitive inhibitors with respect to the nucleic acid binding site and as noncompetitive inhibitors with respect to the dNTP binding site of the enzyme if the RT functions in the RNA-directed DNA mode (19). Moreover, the pyridinone inhibits the DNA-directed DNA polymerase of HIV-1 RT and showed a noncompetitive inhibition pattern with respect to the nucleic acid and dNTP binding sites in this case. The bisheptolopylpirazine U-87201E is a noncompetitive inhibitor of both the nucleic acid and dNTP binding sites (22). It should be noted that all of these assessments, except for the arylpirazine U-87201E, are based on Michaelis-Menten kinetics and not steady-state kinetics. Compared with these other classes of non-nucleoside RT inhibitors mentioned, the quinoline U-78036 appears to possess a somewhat unique inhibition pattern in that it specifically inhibits the DNA-directed DNA polymerase of HIV-1 RT without affecting the DNA-directed DNA polymerase or RNase H activities.

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