Inhibition of HIV-1 Reverse Transcriptase by a Quinazolinone and Comparison with Inhibition by Pyridinones

DIFFERENCES IN THE RATES OF INHIBITOR BINDING AND IN SYNERGISTIC INHIBITION WITH NUCLEOSIDE ANALOGS*

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6-Chloro-(4S)-cyclopropyl-3,4-dihydro-4-((2-pyridyl)-ethynyl)quinazolin-2(1H)-one (L-738,372) is representative of a novel structural class of nonnucleoside inhibitors of human immunodeficiency virus, strain 1 (HIV-1), reverse transcriptase (RT), the quinazinolones. L-738,372 is a reversible inhibitor of HIV-1 RT and is noncompetitive against dTTP with a $K_i$ of 140 nM with poly(rA)-oligo(dT) as primer-template. Mixed noncompetitive inhibition by L-738,372 was observed against poly(rC)-oligo(dG) as primer-template. This quinazolinone binds to RT at a site that overlaps the binding site of other nonnucleoside inhibitors as evidenced by the ability of L-738,372 to displace bound radiolabeled L-696,229, a member of the pyridinone class of inhibitors of HIV-1 RT, from complexes of RT and primer-template. Inhibition by L-738,372 shows slow binding characteristics in reactions with all of the primer-templates employed. Synergistic inhibition of RT activity was evident in combinations of L-738,372 and any of the nucleoside analogs, azidothymidine triphosphate, dideoxynosine triphosphate, or dideoxyctosine triphosphate. The azidothymidine-resistant form of RT (D67N, K70R, T215Y, K219Q) is inhibited by L-738,372 with 2-3-fold more potency than is the wild-type RT. Comparison of inhibition by L-738,372 with inhibition by pyridinone inhibitors reveals differences in synergistic inhibition with nucleoside analogs and in the rates of binding of the inhibitors.

Human immunodeficiency virus (HIV), the etiologic agent of acquired immunodeficiency syndrome (AIDS), contains a reverse transcriptase (RT) that is responsible for the conversion of the single-stranded RNA genome of the virus to a double-stranded DNA copy that is integrated into the host cell genome. RT is the presumed target of two kinds of inhibitors, nucleoside analogs and the nonnucleoside inhibitors.

Several different structural classes of nonnucleoside inhibitors of RT have been developed, including TIBO derivatives (Pauwels et al., 1990), nevirapine (Merluzzi et al., 1990), pyridinones (Goldman et al., 1991), and bisheteroaryl piperazines (Romero et al., 1991). All of the nonnucleoside inhibitors are highly specific inhibitors of HIV-1 RT and are noncompetitive against dNTP. The binding site for nevirapine has been determined by x-ray crystallographic studies (Kohlstaedt et al., 1992). The nonnucleoside inhibitors appear to share a common binding site, as evidenced by the displacement of a member of one class of nonnucleoside inhibitor from RT by another class of nonnucleoside inhibitor (Goldman et al., 1991), by the fact that mutations that render the RT resistant to inhibition by one compound can cause resistance to other nonnucleoside inhibitors (Nunberg et al., 1991; Sardana et al., 1992), and by the fact that one class of nonnucleoside inhibitor can protect against labeling by a photoactivatable analog of another class (Wu et al., 1991).

Clinical trials for the efficacy of the pyridinone, L-697,661 (structure shown in Fig. 1A), were hampered by the rapid appearance of resistant strains containing the Y181C mutation in the RT gene (Saag et al., 1993). The ease of development of resistance to inhibition by pyridinones created the need for another class of inhibitors that would be effective against the resistant virus and lead to the investigation of the quinazolinones, of which L-738,372 is a member (Fig. 1B) (Tucker et al., 1994). L-738,372 is a potent inhibitor of the form of RT containing the Y181C mutation, but does not inhibit HIV-2 RT. The development of resistance in strains of HIV-1 to inhibition by L-738,372 is under investigation.

As a basis for comparison with the other structural classes of nonnucleoside inhibitors, this work investigates the mechanism of inhibition of RT activity by L-738,372. L-738,372 shares many mechanistic features with the other classes of nonnucleoside inhibitors, including the same or overlapping binding sites and the same mode of inhibition against dNTP. However, differences in the inhibition of RT activity by members of the group of nonnucleoside inhibitors are apparent in the areas of synergistic inhibition in combination with nucleoside analogs and rates of association of the inhibitors and RT. These differences between structural classes of nonnucleoside inhibitor may impact on the effects of the use of these compounds in clinical trials.

EXPERIMENTAL PROCEDURES

Materials—Ultrapure dNTPs, dATP, dCTP, dGTP, and dTTP were purchased from Pharmacia Biotech Inc. Deoxynucleoside 5'-triphosphate and 5'-triphosphate were from DuPont NEN. The pyridinone derivatives L-696,229 and L-697,661 (Sarai et al., 1991) were supplied by W. Sarai and J. Hoffmann (Merck Research Laboratories, West Point, PA). L-738,372 was provided by T. Lyle (Merck). [3H]L-696,229 (25 mCi/mmol) was supplied by W.-S. Eng (Merck Research Laboratories, West Point, PA). A plasmid containing the RT gene bearing four mutations known to confer high level

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† The abbreviations and trivial names used are: HIV-1, human immunodeficiency virus, strain 1; AIDS, acquired immunodeficiency syndrome; RT, reverse transcriptase; AZT, azidothymidine; PEG 8000, polyethylene glycol, 8000 molecular weight; AZTTP, azidothymidine triphosphate; DTT, dithiothreitol; L-738,372, 6-chloro-(4S)-cyclopropyl-3,4-dihydro-4-((2-pyridyl)-ethynyl)quinazolin-2(1H)-one; L-697,661, 3-[(4,7-dichloro-1,3-benzoxazol-2-yl)methyl][aminooxy-5-ethyl]-6-methyl-pyridin-2(1H)-one; L-696,229, 3-[3-(1,3-benzoxazol-2-yl)ethyl]-5-ethyl-6-methyl-pyridin-2(1H)-one; TIBO, tetracyydroimidazobenzodiazepine.

‡ V. Sardana, personal communication.
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...resistance to AZT in culture (D6T, R703, T215Y, and K219Q) was provided by J. Condrea (Merck Research Labs, West Point, PA). HIV-1 reverse transcriptase (wild-type and the AZT-resistant form) was purified as described previously (Carroll et al., 1993). RT was purchased from Sierra Biomedical Research (Tucson, AZ).

**Primer Template—** Primers and RNA targets were prepared as described previously (Carroll et al., 1993). Steady-state Kinetic Assays with Poly(rA)-Oligo(dT)—Reactions with poly(rA)-oligo(dT) as primer-template were carried out at 25 °C, except where indicated otherwise, in the presence of poly(rA)-oligo(dT) reaction buffer (50 mM HEPES, pH 7.8, 80 mM KCl, 1 mM MgCl₂, 0.2% FEG 8000, 100 μM dGTP, 50 μg/ml poly(rA)-oligo(dT), 0.5 mM MgCl₂, 2–15 μM [α-³²P]dTMP, and 0–750 nmol L-738,372 to give a final concentration of 4% dimethyl sulfoxide in all reactions. Reactions were initiated by the addition of dTMP after preincubation of the other components for at least 1 h. Aliquots of the reaction mixture were quenched after 15 min into 0.5 ml EDTA solution, pH 8, prior to product analysis by filter binding using DE-52 filters (Whatman) as described previously (Bryant et al., 1983).

**Steady-state Kinetic Assays with Poly(rC)-Oligo(dG)—** Reactions with poly(rC)-oligo(dG) were carried out under the same conditions as for reactions with poly(rA)-oligo(dT) except that MgCl₂ was present at 30 mM, poly(rC)-oligo(dG) was varied from 5 to 75 μg/ml, and [α-³²P]dGTP was present at 3 μM. Aliquots of the reaction were quenched in gel load buffer, and product analysis was carried out with gel electrophoresis and autoradiography, as described previously. **Yonetani-Theorell Plots—** Analysis for synergistic inhibition in combinations of L-738,372 and AZTTP was carried out using reaction conditions as described for poly(rA)-oligo(dT), except that the concentration of dTTP was 5 μM. Analysis for synergistic inhibition in combinations of L-738,372 and AZTTP was carried out using the same buffer conditions as described for assays with poly(rA)-oligo(dT). Reactions included 5–10 nM RT and 500 μM 5'-⁷²P-end labeled primer-template. Reactions containing L-738,372 were initiated by the addition of dNTP to 5 μM after preincubation of the enzyme and inhibitor for 3 h. Aliquots were quenched after various reaction times. Product analysis was carried out, with gel electrophoresis and autoradiography, as described previously (Carroll et al., 1993), or with a PhosphorImager (Molecular Dynamics).

**Yonetani-Theorell Plots—** Analysis for synergistic inhibition in combinations of L-738,372 and ddITP was carried out essentially as described for the combination of L-738,372 and AZTTP except that the primer-template used was poly(rC)-oligo(dG). ddGTP was at 5 μM, and the preincubation time was 2 h. Reactions included L-738,372 (0–400 μM) and 0, 0.5, or 1 mM ddGTP. Analysis for synergistic inhibition by combinations of L-738,372 and ddCTP was carried out as described above except that the primer-template used was poly(rC)-oligo(dG), and the reactions included L-738,372 (0–400 μM) and 0, 50, or 100 μM ddCTP.

**Inhibition of RT activity by the combination of L-738,372 and ddITP** was also determined by the method of fractional inhibitory concentrations (Elion et al., 1954). Reactions contained standard reaction buffer except dTTP was omitted, the concentration of ddITP was 4 μM, and 0–20 μM L-738,372 and 0–200 nmol AZTTP were included. The concentration of each inhibitor used individually that was required to give a certain fractional inhibition in the range of 80–100% was determined from a Hill plot. The fractional inhibitory concentration of each inhibitor for each reaction was calculated as the concentration of the inhibitor present in the reaction divided by the concentration required to give the same degree of inhibition when the inhibitor was used alone.

**Reversible Inhibition by L-738,372—** RT (25 μM) was preincubated for 1.5 h at 25 °C with L-738,372 (0 or 1 μM) in the standard poly(rA)-oligo(dT) reaction buffer except that ddTTP was omitted and the concentration of poly(rA)-oligo(dT) was increased to 500 μg/ml. An aliquot of the incubation solution was diluted 50-fold into poly(rA)-oligo(dT) reaction buffer without ddTTP that contained either 0, 20, or 1 μM L-738,372. The enzymatic activity of the diluted solution was monitored over a 9-h period by initiating a reaction with 45 μl of the diluted RT solution and 5 μl of 50 μM (α-³²P)dTTP. After 15 min, the reaction was quenched, and product analysis was carried out by filter binding as described above.

**Displacement of Enzyme-bound [³²P]L-738,372 by L-738,372—** [³²P]L-738,372 (100 nM) was preincubated with 7 nM RT and 30 μg/ml poly(rC)-oligo(dG) in 50 mM Tris, pH 7.8, 80 mM KCl, 6 mM MgCl₂, 1 mM DTT, 40 μM dGTP, and 0.2% FEG 8000 for 20 min. The solution was divided into three aliquots. To one was added L-738,372 to a final concentration of 1 μM. To another was added unlabeled L-738,372 to the same concentration. To the third aliquot was added buffer as a control. Aliquots of each reaction were removed at intervals up to 30 min and centrifuged through a 1-ml Sephadex G-50 column, and the eluate was monitored for radioactivity by scintillation counting.

**Analysis of Slow Binding of L-738,372—** Reactions included the same buffer conditions as described above. Reactions were initiated either by the addition of RT or by the addition of dTTP to the reaction after preincubation of the other buffer components for 3 h. Aliquots were withdrawn after reaction times up to 30 min, quenched, and analyzed for the amount of product formed by filter binding as described. **Steady-state Reactions with Heteromeric Primer-Template—** Reactions were carried out using the same buffer conditions as described for assays with poly(rA)-oligo(dT). Reactions included 5–10 nM RT and 500 μM 5'-⁷²P-end labeled primer-template. Reactions containing L-738,372 were initiated by the addition of dNTP to 5 μM after preincubation of the enzyme and inhibitor for 3 h. Aliquots were quenched after various reaction times. Product analysis was carried out with gel electrophoresis and autoradiography, as described previously (Carroll et al., 1993), or with a PhosphorImager. **Data Analysis—** All nonlinear regression calculations were performed as described previously (Carroll et al., 1993). For determination of IC₅₀ values, inhibitor saturation data were fitted iteratively to Equation 1,

\[
Y = \frac{(1 - a)[I]}{[I] + IC_{50}} + I \quad (Eq. 1)
\]

where Y is the fraction of activity inhibited, a is the residual activity at saturating concentrations of inhibitor, and [I] is the total concentration of inhibitor.

**RESULTS**

**Slow Binding Inhibition—** Slow binding was observed in the inhibition of RT activity on poly(rA)-oligo(dT) by L-738,372, as indicated by curvature in a reaction progress curve in the presence of inhibitor. The curvature could be eliminated by preincubation of RT, primer-template, and inhibitor. The rate constant for association of L-738,372 and the complex of RT and poly(rA)-oligo(dT) was determined by initiating reactions by addition of RT in the presence of L-738,372 and analyzing for product formation as a function of reaction time. The observed rate of association was determined by fitting the data from a plot of product versus time, an example of which is shown in Fig. 2, to the integrated form of the rate equation,

\[
p(t) = v_i + [(v_u - v_i)\cdot[1 - e^{-kt}]] \
\]

where p(t) is the concentration of product formed at time t, v_i is the final rate of the inhibited reaction, v_u is the rate of the uninhibited reaction, and k is the observed rate of inhibitor binding (Morrison, 1982). The value of v_i was determined by preincubating inhibitor, RT, and primer-template in reaction buffer for a time sufficient to allow the complete association of the inhibitor and then initiating the reaction by addition of dNTP. The replot, shown in the inset of Fig. 2, of the observed rate of association versus the concentration of L-738,372 was linear over the range of inhibitor concentrations used and was indicative of a one-step mechanism for binding of the inhibitor.

**Fig. 1. Structures of nonnucleoside inhibitors L-697,661, L-698,229, and L-738,372.** A, L-697,661 ([R = Cl, X = NH]); L-698,229 ([R = H, X = CH₃]). B, L-738,372.
The presence of either 0 or 200 μM L-738,372 in poly(rA)-oligo(dT) reaction buffer. Reactions were initiated by the addition of RT (C, O) or by the addition of dTTP after a preincubation time of 2 h (C). The data were fit to a linear equation (C, O) or to the integrated form of the rate equation (Equation 2, O). Inset, the observed rate of inhibitor binding as a function of the concentration of L-738,372. The slope of the line is $2.3 \times 10^{-11}$ M$^{-1}$ s$^{-1}$.

The inhibitory potencies of L-697,661 and L-738,372 are comparable with poly(rA)-oligo(dT) as primer-template, both the rates of association and dissociation of L-738,372 to complexes of RT and poly(rA)-oligo(dT). The rate of association of L-738,372 to complexes of RT and poly(rA)-oligo(dT) increased by a factor of 2.2 with an increase in the reaction temperature from 25 to 37°C.

Slow binding was not observed in the inhibition of RT activity by poly(rA)-oligo(dT) by L-697,661 (data not shown), but it was observed in the inhibition by L-697,661 with poly(rC)-oligo(dG) as primer-template (Goldman et al., 1991). The inhibitory potencies of L-697,661 and L-738,372 are comparable with poly(rA)-oligo(dT) as the primer-template. However, since L-697,661 does not show slow binding characteristics with poly(rA)-oligo(dT) as the primer-template, both the rates of association and dissociation of L-738,372 must be higher than the corresponding rates for binding of L-738,372 to complexes of RT and poly(rA)-oligo(dT).

Reversibility of Inhibition—Reversibility of inhibition by L-738,372 was investigated using two methods. A plot of activity against RT concentration at different fixed concentrations of L-738,372 (Ackermann and Potter, 1949) gave a set of lines that intersected at the origin, indicating reversibility of inhibition (data not shown). The second method involved the preincubation of RT, poly(rA)-oligo(dT), and L-738,372 for 1.5 h to allow the binding of the inhibitor to take place. The complex was then diluted 50-fold, and the level of RT activity in the diluted solution was monitored. The final level of activity in the diluted reaction equaled the activity of a solution with the same final

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**TABLE I**

IC$_{50}$ for inhibition by L-738,372 on different primer-templates

| Primer-template | dNTP | IC$_{50}$ | Residual Activity |
|-----------------|------|----------|------------------|
| poly(rA)-oligo(dT) | dTTP | 150 ± 4 | 0.2 ± 0.7 |
| poly(rC)-oligo(dG) | dCTP | 6 ± 2 | |
| p18/AC5U | dTTP | 4 ± 2 | |
| p22/AC5U | dTTP | 1 ± 2 | |
| p22/AC5U | dTTP | 7 ± 2 | |

*The standard deviation is from the fit to a single set of inhibited reactions.*

4 The reaction adds 5 thymidines to the primer.

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**TABLE II**

Association rate constants for inhibition by L-738,372

| Primer-template | dNTP | $k_0$ | $k_a$ | $K_i$ |
|-----------------|------|------|------|------|
| rA-oligo(dT)$^+$ | dTTP | 2.3 x 10$^6$ | 3 x 10$^{-3}$ | 140$^a$ |
| rA-oligo(dT)$^+$ | dTTP | 5.1 x 10$^6$ | 2.1 x 10$^{-3}$ | 410$^b$ |
| rC-oligo(dG) | dGTP | 5.9 x 10$^5$ | 2.8 x 10$^{-4}$ | 4,7$^c$ |
| p22/AC5U | dTTP | 4.1 x 10$^5$ | 3.6 x 10$^{-4}$ | 88$^d$ |

$^a$ $T = 25$ °C.

$^b$ Determined from the measured $k_0$ and $K_i$ with Equation 3.

$^c$ Determined from the intercept of the plot of $k_0$ versus $K_i$.

$^d$ The $K_i$ was determined as the ratio, $k_d/k_{oph}$.

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3 The more complex two-step mechanism of binding where the initial collision complex undergoes a tightening of binding cannot be excluded at inhibitor concentrations higher than those used, although the relevance of a two-step mechanism of binding, if it exists, is dubious since $K_i$ is 140 nM and the one-step mechanism applies up to 600 nM.
concentration of L-738,372. However, maximal activity was regained after ~3 h post-dilution, indicating very slow reequilibration, as shown in Fig. 4. Under these conditions the rate of approach to the new equilibrium that is established after dilution is governed by both the rates of association and dissociation,

$$k_{on} = k_{on}[I] + k_{off}$$  \hspace{1cm} (Eq. 4)

From Equation 4, it can easily be shown that,

$$k_{off} = (1 - f)k_{on}$$  \hspace{1cm} (Eq. 5)

where f is the fraction of inhibition at equilibrium after dilution. The estimate of the rate of dissociation of L-738,372 from the complex of RT and poly(rA)-oligo(dT) in the experiment shown in Fig. 4 is therefore 2.2 x 10^{-4} s^{-1} with f = 0.15, in reasonable agreement with the rate of dissociation of 3 x 10^{-4} s^{-1} calculated from the measured $k_{on}$ and $K_i$, but not in agreement with the rate of dissociation determined from the intercept of the plot in Fig. 2 (1.3 x 10^{-4} s^{-1}). This discrepancy is probably due to the uncertainty in determining the intercept by extrapolation from the data points to the y axis. We therefore favor the value of 3 x 10^{-4} s^{-1} for the dissociation of L-738,372 from complexes of RT and poly(rA)-oligo(dT). In contrast, when the reversibility experiment was carried out using L-697,661, maximal activity was regained within 10 min after the dilution of the complex of RT, primer-template, and L-697,661.

Mode of Inhibition against dNTP—As shown in Fig. 3, L-738,372 was determined to be a linear, noncompetitive inhibitor against dTTP in reactions that employed poly(rA)-oligo(dT) as primer-template and that allowed the equilibration of inhibitor binding by preincubation of L-738,372, RT, and poly(rA)-oligo(dT) for 1 h. The $K_i$ for L-738,372, as determined from the replots of the slopes (Fig. 3, inset), was 140 nM.

Mode of Inhibition against Primer-Template—L-738,372 was determined to be a mixed noncompetitive inhibitor against poly(rC)-oligo(dG) as the variable substrate in reactions that allowed the equilibration of the binding of L-738,372 during a 2-h preincubation (Fig. 5).

Mutually Exclusive Inhibition by L-738,372 and Pyridinones—Two experimental methods were used to determine whether L-738,372 and the pyridinones, L-697,661 and L-696,229, bind to overlapping sites on RT. Inhibition of RT activity on poly(rA)-oligo(dT) by mixtures of L-697,661 and L-738,372 was analyzed by the method of Yonetani and Theorell (Yonetani and Theorell, 1964), which showed parallel lines indicating mutually exclusive inhibition by the two compounds (data not shown). Both L-738,372 and unlabeled L-696,229 displaced radiolabeled L-696,229 from RT-primer-template complexes in experiments implementing a spin-column technique (Goldman et al., 1991) for resolving enzyme-bound L-696,229 from free compound (data not shown). In the absence of added L-738,372, the complex of enzyme and L-696,229 was stable for the time course of the experiment. Both methods are consistent with binding of L-696,229 and L-738,372 to the same or overlapping sites on the enzyme.

Synergistic Inhibition with Nucleoside Analogues—Synergistic inhibition of RT activity by combinations of AZTTP and L-738,372 was examined by the method of Yonetani and Theorell (1964). The combination exhibited synergistic inhibition as evidenced by nonparallel lines in the Yonetani-Theorell plot.
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**Fig. 5.** Steady-state inhibition by L-738,372 of HIV-1 reverse transcriptase-catalyzed synthesis on poly(rC)-oligo(dG). Reactions included 0.4 nm RT, 3 μM (α-32P)GTP, from 5 to 75 μg/ml poly(rC)-oligo(dG), and 0 (○), 4 (▲), 10 (△), or 20 (×) nM L-738,372. Reactions were initiated by the addition of dGTP after a 2-h preincubation, and time points from 10 to 100 min were quenched by the addition of gel load buffer prior to electrophoresis on 20% acrylamide, 8% gels. Product was quantified with a PhosphorImager.

(Fig. 6A). Mutually exclusive inhibition by the combination of AZTTP and L-697,661 in the range of inhibition tested (activity <92% inhibited) was indicated in a Yonani-Theorell plot (Fig. 6B). Similar results were obtained with the combinations of each nonnucleoside inhibitor and either dITTP or dCTP.

The possibility of synergistic inhibition by combinations of L-697,661 and AZTTP at higher concentrations of both inhibitors was investigated using the method of fractional inhibitory concentrations (Elion et al., 1954), and the results are shown in Fig. 7. Synergistic inhibition, when the enzyme was inhibited by more than 92%, was evident because the data points fall below and to the left of the line of additivity. In reactions in which RT activity is inhibited by less than 92%, the data points fall close to the line of additivity.

**Inhibition of AZT-resistant RT**—The AZT-resistant RT was inhibited by L-738,372 with an IC50 on poly(rA)-oligo(dT) of 60 nM, under conditions that allowed for the equilibration of inhibitor binding by preincubating RT, poly(rA)-oligo(dT), and L-738,372. Under the same conditions, the wild-type RT was inhibited with an IC50 of 150 nM. L-697,661 showed the same ratio of inhibitory potencies against the wild-type and AZT-resistant Rts.

**Inhibition by L-738,372 on Heteromeric Primer-Templates**—The inhibitory potency of L-738,372 was determined using the heteromeric primer-template systems shown in Table I. The sequence of the synthetic 32-mer RNA template is taken from the HIV-1 genome (Alizon et al., 1990). Reactions were limited to the incorporation of a defined number of nucleotides by incubating only one dNTP that allowed the evaluation of inhibitory potency at a specific region of the template. To allow for the complete association of L-738,372 and complexes of RT and primer-template, the inhibitor was preincubated with the other reaction components in the absence of dNTP. Preincubation times of over 1 h were required for complete binding of L-738,372, whereas L-697,661 was completely bound in less than 10 min.

As previously reported for inhibition of HIV-1 RT activity by L-696,229 (Carroll et al., 1993), the potency of inhibition by L-738,372 is dependent on the position of the template that is being transcribed. For example, the IC50 for inhibition on substrate p22/tC5U is 6-fold greater than the IC50 on p15/tC5U. L-738,372 inhibits the DNA-dependent DNA polymerase activity of RT on substrate p22/tD32 with a potency similar to that with which it inhibits the RNA-dependent DNA polymerase activity of RT on substrate p22/tCSU, which has the same sequence as p22/tD32 with uracil substituting for thymidine. At saturating concentrations, L-738,372 inhibits completely all of the reactions on the heteromeric RNA sequences catalyzed by RT shown in Table I.

**DISCUSSION**

Several different structural classes of nonnucleoside inhibitors of HIV-1 RT have been discovered in screening programs (for review, see De Clercq (1993)). Clinical studies of pyridinone compounds have revealed the rapid emergence of viral strains resistant to these inhibitors and have pointed out the need for compounds that are more effective against the broad range of mutants of RT. The appearance of virus resistant to L-697,661 lead to the development of L-738,372, the lead compound of another structural class of inhibitors of HIV-1 RT, the quinazolines.

The inhibition of RT activity by L-738,372 has several features in common with inhibition by other nonnucleoside inhibitors of HIV-1 RT. L-738,372 is a linear, noncompetitive inhibitor against dNTP. Other nonnucleoside inhibitors have also been reported to be noncompetitive against dNTP (Pauweis et al., 1990; Merluzzi et al., 1990; Goldman et al., 1991; Romero et al., 1991; Carroll et al., 1993). L-738,372 is a mixed noncompetitive inhibitor against primer-template. Other nonnucleoside inhibitors have been reported to be either noncompetitive, mixed, or uncompetitive against primer-template (Debyser et al., 1991; Tramontano and Cheng, 1992; Goldman et al., 1991; Althaus et al., 1993). Nonnucleoside inhibitors of HIV-1 RT, including L-738,372 (Tucker et al., 1994) have been reported to be highly specific, with no detectable activity against cellular polymerases or HIV-2 RT.

An unusual feature of L-738,372 compared with most other nonnucleoside inhibitors is the slow binding nature of inhibition by L-738,372. The inhibition of RT activity on poly(rC)-oligo(dG) by L-738,372 exhibited slow binding characteristics with an association rate constant of $5 \times 10^8$ M$^{-1}$ s$^{-1}$ at 37°C. The pyridinone class of nonnucleoside inhibitor has also been reported to have slow binding characteristics with poly(rC)-oligo(dG) as primer-template but not with
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poly(rA)-oligo(dT) as primer-template (Goldman et al., 1991). Although the mechanistic basis for the slow binding inhibition of L-738,372 has not been established, the structural basis for the difference in association rate constants with poly(rA)-oligo(dT) as the primer-template for the two non-nucleoside inhibitors may be related to the greater conformational rigidity of L-738,372. The observation that inhibition by TIBO of the presteady-state burst amplitude of synthesis by HIV-1 RT required preincubation of inhibitor and enzyme was suggested to be due to a requirement of TIBO to bind to the free enzyme (Gopalakrishnan and Benkovic, 1994). Preincubation of L-738,372 and RT for 10 s prior to initiation of the reaction with primer-template and dNTP did not eliminate the slow binding, indicating that binding to the free enzyme is also slow, although that may be the preferred mode of binding.

Another important difference between inhibition of RT activity by L-738,372 and by L-697,661 is their synergistic inhibition in combination with nucleoside analogs. Synergistic inhibition of RT activity by the combination of L-738,372 and AZTTP was evident in the nonparallel lines in a Yonetani-Theorell plot. It is important to note that synergistic inhibition does not require synergy in the binding of the two inhibitors but only that it be possible operationally for both inhibitors to bind to the enzyme simultaneously (Segel, 1975). Therefore it is possible for both L-738,372 and whatever form of AZT that causes the inhibition, which may be the AZTTP-terminated primer (Heidenreich et al., 1990; Reardon and Miller, 1990); to bind simultaneously to RT. Similar results were found with the combinations of L-738,372 and either ddITP or ddCTP.

In contrast, inhibition by the combination of L-697,661 and AZTTP appeared to be additive at less than 92% inhibition of activity, but synergistic inhibition became apparent in plots of fractional inhibitory concentrations at greater than 92% inhibition. The most likely explanation for the requirement of a high fraction of inhibition in order to observe synergistic inhibition is that binding of the first inhibitor decreases the affinity of the second inhibitor for the complex of enzyme and first inhibitor. Therefore higher concentrations of both inhibitors are required to populate the doubly inhibited enzyme complex required for synergistic inhibition. The observation of synergis-
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