The Thiocarboxanilide Nonnucleoside UC781 Is a Tight-Binding Inhibitor of HIV-1 Reverse Transcriptase†

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Received January 22, 1997; Revised Manuscript Received April 17, 1997⊗

ABSTRACT: The thiocarboxanilide nonnucleoside inhibitor (NNI) UC781 inhibited HIV-1 reverse transcriptase (RT) DNA polymerase activity at a 1:1 molar ratio of inhibitor to enzyme. Inhibition was linear uncompetitive with respect to template/primer (T/P) and mixed noncompetitive with respect to deoxynucleoside triphosphate (dNTP), typical of NNI. When the RT−T/P binary complex was incubated with UC781 and then separated from unbound inhibitor, recovery of enzyme activity was slow, with only about 60% activity recovered after 25 min. The inactivation of the RT−T/P complex was prevented by the presence of a large excess of UC84, another carboxanilide NNI that interacts with this RT mechanistic form. UC781 protected the RT−T/P−dNTP ternary complex from irreversible inactivation by a photoactivatable azido analog of nevirapine, implying that UC781 binds to the NNI pocket of this RT mechanistic form. UC781 did not photoprotect either the free enzyme or the RT−T/P binary complex; however, protein fluorescence quenching studies indicated that UC781 interacted with all RT mechanistic forms, with the order of affinity being RT−T/P−dNTP ternary complex > RT−T/P binary complex > free RT. Reaction progress curve analysis showed that the binding of UC781 to RT is rapid ($k_{on} \sim 1.7 \times 10^6$ M^{-1} s^{-1}), but that dissociation is slow ($k_{off} \sim 1.6 \times 10^{-3}$ s^{-1}). UC781 is therefore a rapid tight-binding inhibitor of HIV-1 RT, the first NNI to demonstrate this property.

The reverse transcriptase (RT; EC 2.7.7.49)[1] of human immunodeficiency virus type 1 (HIV-1) is multifunctional, with DNA polymerase [RNA-dependent DNA polymerase (RDDP) and DNA-dependent DNA polymerase (DDDP)] and ribonuclease H (RNase H) activities. These retroviral enzyme functions are crucial for replication, providing a double-strand proviral DNA copy of the retroviral genomic RNA which can then be integrated into the infected host cell genome. HIV-1 RT has therefore provided a logical target for the development of antiviral agents. Current anti-RT chemotherapeutics can be grouped into two classes, dideoxynucleoside (ddN) analogs such as 3′-azido-3′-deoxythymidine (AZT), which require "activation" by host cell kinases (Furman *et al.*, 1986), and nonnucleoside inhibitors (NNI). The NNI are hydrophobic compounds with diverse structural features, and do not require cellular modification for antiviral activity. Examples include nevirapine (Grob *et al.*, 1992; Merluzzi *et al.*, 1990), the pyridinones (Carroll *et al.*, 1993; Goldman *et al.*, 1991), and the carboxanilides (Bader *et al.*, 1991; Balzarini *et al.*, 1995, 1996a).

The latter series of NNI, developed by Uniroyal Chemical Ltd. Research Laboratories (hence the UC designation), are particularly interesting. Our laboratory previously showed that different carboxanilide NNI, while structurally similar, interact with different mechanistic forms of HIV-1 RT *in vitro* (Fletcher *et al.*, 1995a). Appropriate combinations of these carboxanilide NNI show synergy in inhibiting HIV-1 replication (Fletcher *et al.*, 1995b), and prolong the time required for the development of resistant viral strains.[2] In addition, carboxanilide NNI have excellent activity against HIV-1 resistant to other NNI, such as nevirapine (Balzarini *et al.*, 1995, 1996a,b). Continued development of the UC series of carboxanilide NNI has resulted in *N*-[4-chloro-3-(3-methyl-2-butenyloxy)phenyl]-2-methyl-3-furanocarbothiamide (UC781) (Figure 1), which we now show acts as a tight-binding inhibitor of HIV-1 reverse transcriptase. This property is unique among the NNI so far described. The tight-binding inhibition of HIV-1 RT by UC781 has important implications for the chemotherapeutic use of this compound, and suggests that UC781 may be of use in

† The research of M.A.P. was supported in part by an International Research Scholar's grant from the Howard Hughes Medical Institute, and in part by Grants UI-13039 and GR-13918 from the Medical Research Council of Canada. M.A.P. is a Chercheur-boursier senior (Career Research Scholar) of the Fonds de la recherche en santé du Québec. G.B. is recipient of a postdoctoral fellowship from the Medical Research Council of Canada.

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⊗ Abstract published in *Advance ACS Abstracts,* June 1, 1997.

[1] Abbreviations: 9-AN, 9-azido-5,6-dihydro-11-ethyl-6-methyl-11*H*-pyrido[2,3-*b*][1,5]benzodiazepin-5-one; AZT, 3′-azido-3′-deoxythymidine; DDDP, DNA-dependent DNA polymerase; DMSO, dimethyl sulfoxide; HIV-1, human immunodeficiency virus type 1; NNI, nonnucleoside inhibitor; dNTP, deoxynucleoside triphosphate; RDDP, RNA-dependent DNA polymerase; RT, reverse transcriptase; RT−T/P, reverse transcriptase−template/primer binary complex; RT−T/P−dNTP, reverse transcriptase−template/primer−deoxynucleoside triphosphate ternary complex; TCA, trichloroacetic acid; TIBO, tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazapine-2(1*H*)-thione; T/P, template/primer; UC38, 2-chloro-5-[[[(1-methylethoxy)thioxo]methyl]amino]-benzoic acid 1-methylethyl ester; UC84, 2-chloro-5-[[5,6-dihydro-2-methyl-(1,4-oxathiin-3-yl)carbonyl]amino]benzoic acid 1-methylethyl ester (oxathiin carboxanilide); UC781, *N*-[4-chloro-3-(3-methyl-2-butenyloxy)phenyl]-2-methyl-3-furanocarbothiamide.

[2] G. Borkow, N. Kaushik, and M. A. Parniak, manuscript in preparation.

S0006-2960(97)00140-2 CCC: $14.00

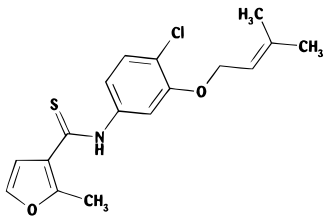

FIGURE 1: Structure of UC781 {*N*-[4-chloro-3-(3-methyl-2-butenyloxy)phenyl]-2-methyl-3-furancarbothioamide}.

retrovirucidal/microbicidal formulations for the prevention of sexual transmission of HIV from infected individuals to noninfected individuals.

MATERIALS AND METHODS

Recombinant HIV-1 p51/p66 RT was expressed and purified by the procedure of Fletcher *et al.* (1996). The nevirapine analog photolabel 9-azido-5,6-dihydro-11-ethyl-6-methyl-11*H*-pyrido[2,3-*b*][1,5]benzodiazepin-5-one (9-AN) was a generous gift from Dr. G. I. Dmitrienko (University of Waterloo, Waterloo, ON, Canada). The carboxanilides UC38, UC781, and UC84 were kindly provided by Drs. A. W. Harrison and W. G. Brouwer, Uniroyal Chemical Research Laboratories (Guelph, ON, Canada). The homopolymeric T/P poly(rC)−oligo(dG)$_{12-18}$ was from Pharmacia Biotech (Montreal, Quebec, Canada). [^{3}H]dGTP was purchased from Amersham (Mississauga, ON, Canada) or NEN−Dupont (Mandel Scientific, Guelph, ON, Canada). Bio-Spin 30 centrifugal chromatography columns were obtained from Bio-Rad (Mississauga, ON) and used according to the suppliers directions. All other materials were of the highest purity available.

HIV-1 Reverse Transcriptase Assay. Inhibition studies used a fixed-time assay for HIV-1 reverse transcriptase RNA-dependent DNA polymerase activity. Reaction mixtures (50−100 μL total volume) contained 50 mM Tris-HCl (pH 7.8, 37 °C), 60 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 5 μg/mL poly(rC)−oligo(dG)$_{12-18}$, variable concentrations (0.5−20 μM) of [^{3}H]dGTP, and variable concentrations of inhibitor dissolved in dimethyl sulfoxide (DMSO). Reactions were initiated by the addition of 5−25 ng of RT. The maximum concentration of DMSO in the reaction assays was fixed at 2%, and control experiments determined that neither RT RDDP nor DDDP activity was affected by this concentration of DMSO. Reaction mixtures were incubated at 37 °C for 10−30 min, and then quenched with 500 μL of ice-cold 10% trichloroacetic acid (TCA) containing 20 mM sodium pyrophosphate. Quenched samples were left on ice for 30 min, then filtered on Whatman 934-AH glass fiber filters, and washed sequentially with 10% TCA containing 20 mM sodium pyrophosphate and ethanol, and the extent of radionucleotide incorporation was determined by liquid scintillation spectrometry.

Inhibition kinetic parameters were determined using concentrations of [^{3}H]dGTP ranging from 0.5 to 10 × K_m with a fixed concentration of T/P (5 μg/mL) or a similar concentration range for T/P with a fixed concentration of dGTP (2.5 μM).

Spin-Column Experiments. Bio-Spin 30 columns (Bio-Rad) were prepared for use according to the manufacturer's instructions, and then equilibrated with 50 mM Tris (pH 7.8, 37 °C) containing 60 mM KCl. The RT−T/P binary complex [formed by preincubating 0.3 μM p51/p66 RT with 15 μg/mL poly(rC)−oligo(dG)$_{12-18}$] was incubated for 5 min at 4 °C with 1 μM UC781, 20 μM UC84, or a mixture of 1 μM UC781 + 20 μM UC84 in a total volume of 50 μL; then sample was applied to a Bio-Spin 30 column, and the RT−T/P complex was rapidly separated from unbound inhibitor by centrifugation at 2300*g* for 4 min at 4 °C. Control experiments showed that both RT and the T/P were found in the spin-column eluate, whereas the free inhibitors were retained within the included volume. The eluate containing the RT−T/P complex was diluted 12.5-fold with 50 mM Tris (pH 7.8, 37 °C) containing 60 mM KCl and incubated at 37 °C. At various times thereafter, 10 μL aliquots were withdrawn and assayed for RT RDDP activity as described above. The RT−T/P complex was used in these experiments in order to improve the stability of RT during incubation after spin-column chromatography, and because this is the RT mechanistic form to which UC84 preferentially binds (Fletcher *et al.*, 1995a).

Effect of UC781 on Photoinactivation of RT by 9-Azido-5,6-dihydro-11-ethyl-6-methyl-11H-pyrido[2,3-b][1,5]-benzodiazepin-5-one (9-AN). These studies were carried out as previously described (Fletcher *et al.*, 1995a), except that samples were irradiated at 254 nm. We have found that use of this wavelength provides more reproducible results than previously obtained with irradiation at 365 nm. Wild-type RT (0.4 μM) and 9-AN photolabel (2 mM) in 40 μL of 50 mM Tris-HCl (pH 8.0, 25 °C) were illuminated at 254 nm (20 mW/cm^2; Mineralight UVGL-25, UVP Inc., San Gabriel, CA) in the absence and the presence of various concentrations of UC781. The irreversible inactivation of RT was followed by the removal of 2 μL aliquots of the mixture at various time intervals and diluting the sample 250-fold into 50 mM Tris-HCl (pH 7.8, 37 °C) in order to dissociate reversibly-bound photolabel. A 20 μL aliquot of the diluted sample was assayed for residual RDDP activity as described above. Control experiments showed that no loss of RT RDDP activity was noted upon illumination of the enzyme at 254 nm in the absence of photoaffinity label. Similarly, irradiation of RT in the presence of UC781 only did not result in any additional inhibition of RT above that noted for nonirradiated mixtures of RT and UC781. Finally, irradiation of UC781 did not diminish its ability to inhibit RT, implying that the inhibitor was stable under the photoinactivation experimental conditions.

Photoinactivation experiments concerning the RT−T/P binary complex were conducted as described above, with the addition of 15 μg/mL poly(rC)−oligo(dG)$_{12-18}$. RT and T/P were preincubated for 5 min at 25 °C prior to addition of UC781 and/or photolabel to ensure formation of the RT-P/T complex. In experiments involving the RT−T/P−dNTP ternary complex, RT was first preincubated with 15 μg/mL poly(rC)−oligo(dT)$_{12-18}$ in the presence of 6 mM $MgCl_2$ for 5 min prior to addition of dGTP. UC781 and 9-AN were added after the addition of dGTP. In all experiments with UC781, this inhibitor was the penultimate addition to the reaction mixtures. The 9-AN photolabel was always added last.

Fluorescence Measurements. Studies of the intrinsic protein fluorescence of the different RT mechanistic forms were performed using an SLM-Aminco SPF-500C spectrofluorometer, at ambient temperature (20 °C). An excitation wavelength of 280 nm was used and fluorescence emission

was monitored between 300 nm to 400 nm. Assay samples comprised 85.5 nM p51/p66 RT heterodimer and where appropriate 5 μg/mL poly(rC)−oligo(dG)$_{12-18}$ and/or 2.5 μM dGTP in 50 μL of 50 mM Tris-HCl, pH 7.8, 37 °C, in the absence or the presence of variable concentrations of UC781 or UC38. All data were corrected for background fluorescence using samples containing all experimental components except RT.

Reaction Progress Curve Analysis. Progress curves for the interaction between UC781 and RT were analyzed using the equation (Morrison & Walsh, 1988):

$$P = v_s t + (v_o - v_s)[1 - \exp(-k_{app} t)]/k_{app} \quad (1)$$

where P is the product concentration, v_o is the reaction velocity at $t = 0$, v_s is the steady-state reaction velocity, and k_{app} is the observed rate constant. In this particular study, progress curves were generated at 2.5, 5, and 10 nM UC781. The resultant data were fit to eq 1 by nonlinear regression analysis using the program Sigma Plot (Jandel Scientific, San Rafael, CA) to calculate values for v_o, v_s, and k_{app}.

Equation 1 is applicable to both a single-step and a two-step process (Morrison & Walsh, 1988). The single-step process can be described by

$$k_{app} = k_{off} + [k_{on}[I]/(1 + S/K_m)] \quad (2)$$

whereas the two-step process involves an isomerization step of the EI complex to an EI* complex and is described by

$$k_{app} = k_{isom,r} + k_{isom,f}[(I/K_i)/(1 + S/K_m + I/K_i)] \quad (3)$$

where $k_{isom,r}$ is the rate constant for the disappearance of the EI* complex and $k_{isom,f}$ is the rate constant for EI* formation. The two mechanisms may be distinguished experimentally by the dependence on inhibitor concentration, since a plot of k_{app} vs [I] will be linear for the single-step process.

RESULTS

During our initial characterizations of the *in vitro* inhibitory activity of UC781, we noted that the inhibitor showed exceptional potency against RT RDDP activity using poly(rC)−oligo(dG)$_{12-18}$ as T/P (Borkow *et al.*, 1997). The observed IC$_{50}$ (0.2 nM) was similar to the concentration of p51/p66 RT heterodimer employed in the assays (*ca.* 0.5 nM). Inhibition at 1:1 molar ratios of inhibitor−target is characteristic of "tight-binding" inhibitors (Williams & Morrison, 1979; Morrison & Walsh, 1988; Szedlacsek & Duggleby, 1995). We tested this possibility more directly by first incubating the RT−T/P complex with UC781 or another less potent NNI, UC84, for a brief period of time, and then rapidly separating the RT from excess unbound NNI using "spin-column" gel chromatography. As illustrated in Figure 2, complete and rapid recovery of RT activity was noted with enzyme treated with UC84. In contrast, recovery of RT activity in samples treated with UC781 was slow, with only about 60% RT RDDP activity recovered 25 min following removal of unbound inhibitor. When the RT−T/P complex was incubated with a stoichiometric amount of UC781 in the presence of a large excess of UC84, complete recovery of RT activity was noted immediately following the chromatographic separation step (Figure 2), implying that the excess of UC84 prevented UC781 from binding to the enzyme. The RT−T/P complex was used in these experiments in order to improve the stability of RT during incubation after spin-column chromatography, and because this is the RT mechanistic form to which UC84 preferentially binds (Fletcher *et al.*, 1995a).

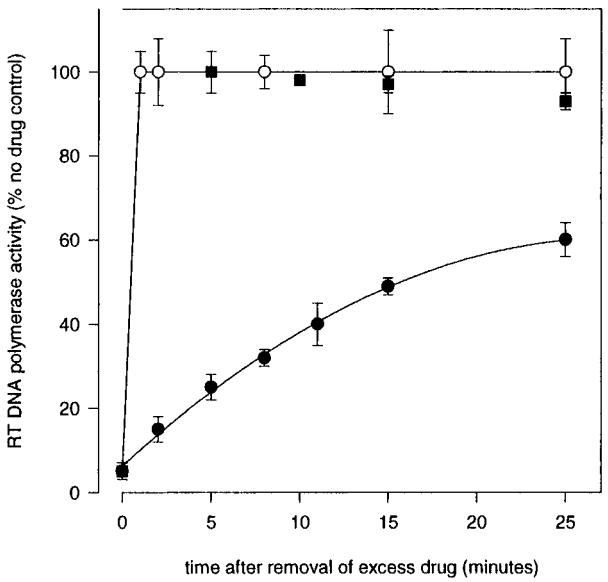

FIGURE 2: Time dependence for recovery of RT DNA polymerase activity following removal of unbound inhibitor by "spin-column" chromatography. HIV-1 p51/p66 RT−T/P binary complex (0.3 μM) was incubated with 1 μM UC781 (●), 20 μM UC84 (○), or a mixture of 1 μM UC781 + 20 μM UC84 (■) for 5 min at 4 °C, and then rapidly separated from unbound inhibitor by spin-column chromatography. The eluate containing the RT was diluted 12.5-fold and incubated at 37 °C. At various times, aliquots were withdrawn and assayed for RT RDDP activity as described under Materials and Methods.

Kinetics of Inhibition of RT by UC781. We examined UC781 inhibition of RT RDDP activity using poly(rC)-oligo(dG)$_{12-18}$ as T/P and [^{3}H]dGTP as dNTP substrate. Inhibition by UC781 was uncompetitive with respect to T/P (Figure 3A). Both slope and intercept replots were linear (Figure 3B,C), and yielded a K_i of 2.9 ± 0.2 nM. In contrast, inhibition by UC781 with respect to dGTP as variable substrate was essentially mixed, but with a significant competitive component (Figure 4A). Slope replots were linear, whereas intercept replots appeared to be curvilinear (Figure 4B,C). The K_i and K_i' were calculated to be 1.9 ± 0.25 nM and 5.2 ± 0.8 nM, respectively.

Ability of UC781 to Protect HIV-1 RT from Irreversible Photoinactivation by 9-Azido-5,6-dihydro-11-ethyl-6-methyl-11H-pyrido[2,3-b][1,5]benzodiazepin-5-one (9-AN). We have previously used this technique to probe whether a given NNI can bind to the NNI-binding pocket of HIV-1 RT, and to determine which of the RT mechanistic forms interacts with the NNI inhibitor (Fletcher *et al.*, 1995a; Arion *et al.*, 1996a). UC781 (at a 1:1 molar ratio with RT) was unable to protect either free RT or the RT−T/P binary complex from photoinactivation by 9-AN. Interestingly, the presence of UC781 appeared to slightly enhance the rate of photoinactivation of these RT mechanistic forms, compared to the rate of photoinactivation in the presence of 9-AN alone (Table 1). In contrast, UC781 protected the RT−T/P−dNTP ternary complex from photoinactivation by 9-AN (Table 1). The protective effect was dependent on the concentration of UC781.

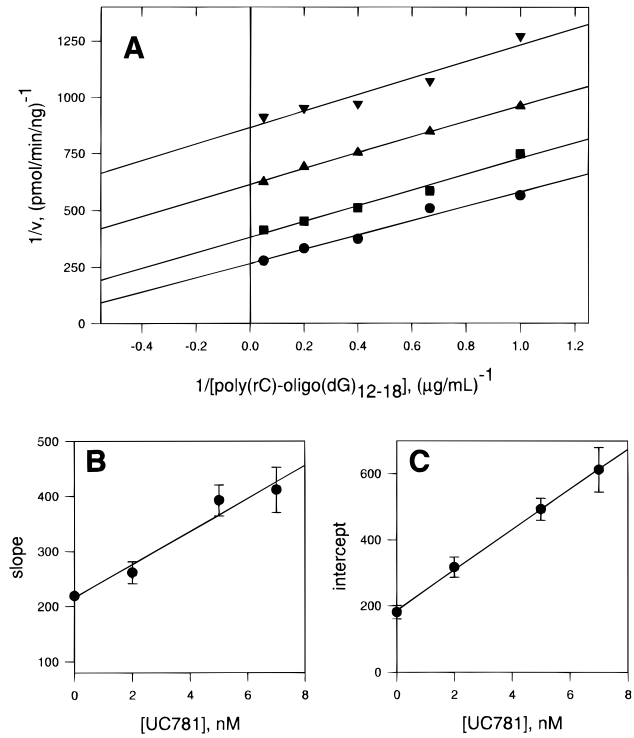

FIGURE 3: Inhibition of HIV-1 RT by UC781: Kinetics with respect to template/primer. (A) Double-reciprocal plot ($1/v$ vs 1/[poly(rC)−oligo(dG)$_{12-18}$]) in the presence of (●) 0, (■) 2, (▲) 5, and (▼) 7 nM UC781. The data points are the averages of duplicate determinations from a typical experiment. The lines shown are calculated from first-order regression analyses of the data. (B) Slope replots and (C) intercept replots as a function of UC781 concentration. The data shown are means ± SD of three separate experiments, each carried out in duplicate. The lines are calculated by first-order regression analysis (r^2 values were >0.98 for both replots).

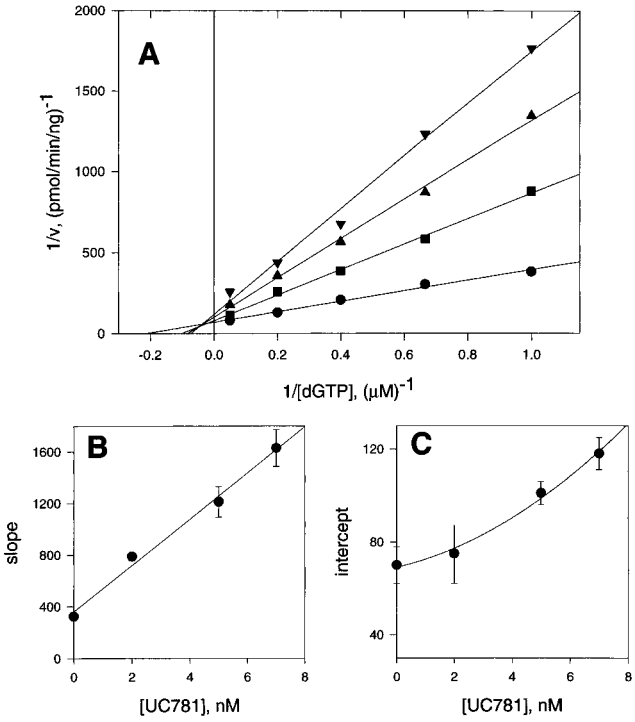

FIGURE 4: Inhibition of HIV-1 RT by UC781: Kinetics with respect to dGTP substrate. (A) Double-reciprocal plot ($1/v$ vs 1/[dGTP]) in the presence of (●) 0, (■) 2, (▲) 5, and (▼) 7 nM UC781. The data points are the averages of duplicate determinations from a typical experiment. The lines shown are calculated from first-order regression analyses of the data. (B) Slope replots and (C) intercept replots as a function of UC781 concentration. The data shown are means ± SD of three separate experiments, each carried out in duplicate. The lines are calculated by first-order (slope) or second-order (intercept) analysis (r^2 values were >0.98 for both replots).

Control experiments showed that no loss of RT RDDP activity was noted upon illumination of the enzyme at 254 nm in the absence of photoaffinity label. Similarly, irradiation of RT in the presence of UC781 only did not result in any additional inhibition of RT above that noted for nonirradiated mixtures of RT and UC781. Finally, irradiation of UC781 did not diminish its ability to inhibit RT, implying that the inhibitor was stable under the photoinactivation experimental conditions.

Quenching of RT Protein Fluorescence by UC781. The 9-AN photolabel probe experiments implied that UC781 interacted primarily with the RT NNI pocket in the RT−T/P−dNTP ternary complex. However, the experiments illustrated in Figure 2 indicated that UC781 must bind to the RT-T/P binary complex as well. Studies of protein fluorescence quenching have proven useful in analysis of the binding of substrates and inhibitors to HIV-1 RT (Rittinger *et al.*, 1995; Thrall *et al.*, 1996; Arion *et al.*, 1996b). We therefore used this technique to investigate the interaction of UC781 with the different RT mechanistic forms, in comparison with UC38, an NNI previously shown to bind almost exclusively to the RT−T/P−dNTP ternary complex (Fletcher *et al.*, 1995a). As seen in Figure 5A, UC38 altered the fluorescence of the RT ternary complex to a far greater extent than that of either the free enzyme or the RT−T/P binary complex, consistent with the conclusions of our previously described photoprotection experiments with this compound. In contrast, UC781 provided significant changes in the fluorescence emission of free RT, the RT−T/P binary complex, and also the RT−T/P−dNTP ternary complex (Figure 5B), even though UC781 was able to protect only the latter mechanistic species from inactivation by the 9-AN photolabel. The UC781-induced quenching of RT fluorescence was saturable, and indicates an end point of 0.92 mol of UC781 bound per mole of p51/p66 RT heterodimer in the ternary complex (Figure 5C). Interestingly, the binding curves for the interaction of UC781 with the RT−T/P binary complex and particularly with the free enzyme demonstrated apparent cooperativity.

RT DNA Polymerase Reaction Progress Curve Analysis. RT RDDP progress curves were generated in the absence and in the presence of various concentrations of UC781, and analyzed according to the procedure of Morrison and Walsh (1988) as described under Materials and Methods. Good agreement between observed and calculated curves was noted (Figure 6A), implying that this method provides a satisfactory description of the inhibition process. The plot of k_{app} vs [UC781] was linear (Figure 6B), indicating that the interaction between UC781 and RT proceeds *via* a one-step mechanism, without an isomerization process. In this particular instance, formation of the RT−UC781 complex is rapid, with a calculated second-order rate constant of 1.7×10^6 M^{-1} s^{-1}. The first-order rate constant for disappearance of the RT−UC781 complex is about 1.6×10^{-3} s^{-1}. The dissociation constant ($K_d = k_{off}/k_{on}$) for UC781 under our assay conditions is approximately 0.9 nM.

Table 1: Effect of UC781 on Rates of 9-AN-Induced Photoinactivation of Different RT Mechanistic Forms

RT mechanistic form[a]	k_{inact} (min^{-1})[b] −UC781	k_{inact} (min^{-1})[b] +UC781	UC781:RT[c] ratio
(1) free RT	0.028 ± 0.002	0.034 ± 0.002	1.0
(2) RT−T/P binary complex	0.027 ± 0.001	0.036 ± 0.002	1.0
(3) RT−T/P−dNTP ternary complex	0.043 ± 0.003	0.038 ± 0.005	0.5
		0.033 ± 0.004	1.0
		0.027 ± 0.002	2.0

[a] RT mechanistic forms were generated as described under Materials and Methods. [b] The data are the means ± SD calculated from three separate experiments, with four time points/experiment. [c] Each experiment used 400 nM p51/p66 RT heterodimer.

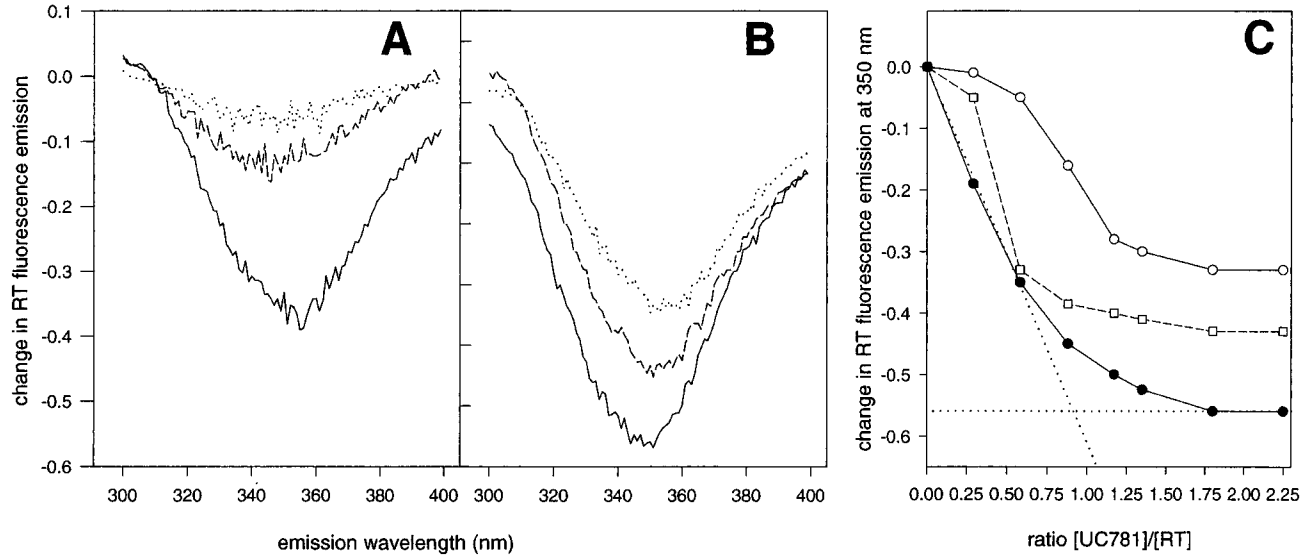

FIGURE 5: Quenching of the protein fluorescence of various RT mechanistic forms by (A) 10 μM UC38 and (B) 100 nM UC781. (···) Free RT; (- - -) RT−T/P binary complex; (—) RT−T/P−dNTP ternary complex. (C) Titration of RT fluorescence emission at 350 nm as a function of UC781 concentration. (○) Free RT; (□) RT−T/P binary complex; (●) RT−T/P−dNTP ternary complex. The concentration of RT in all experiments was 85.5 nM of p51/p66 heterodimer.

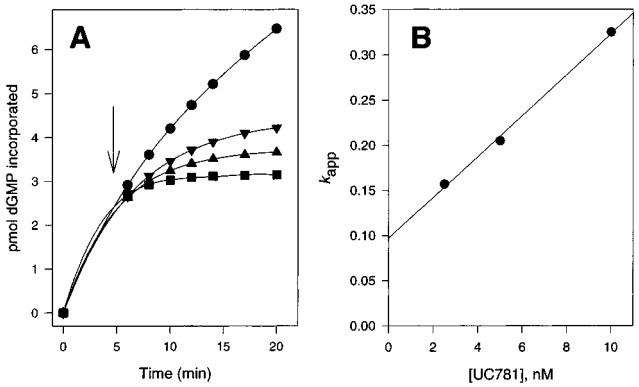

FIGURE 6: (A) Progress curves for RT RDDP activity in the absence (●) or presence of (▼) 2.5, (▲) 5, and (■) 10 nM UC781. Reaction assays employed 5 ng of p51/p66 RT, 5 μM [^{3}H]dGTP, 5 mg/mL poly(rC)−oligo$(dG)_{12-18}$, and other components as described under Materials and Methods. The arrow indicates the time of addition of UC781 to the reaction assays. (B) Dependence of k_{app} on UC781 concentration. The line was calculated from the best fit of the parameters of eq 2 to the data. Details are provided under Materials and Methods.

DISCUSSION

The data presented in this report show that the thiocarboxanilide UC781 is a rapid tight-binding inhibitor of HIV-1 RT, a property that is so far unique among the large number of RT inhibitors described to date. UC781 must therefore interact with RT in a manner somewhat distinct from that of other inhibitors of the enzyme. Nonetheless, UC781 inhibition has many characteristics of the nonnucleoside class of RT inhibitors. The inhibition profiles are mixed and/or uncompetitive with respect to the normal RT substrates, similar to those noted for other NNI such as nevirapine (Kopp *et al.*, 1991), TIBO (Balzarini *et al.*, 1992), and the pyridinones (Carroll *et al.*, 1993). UC781 competes with a nevirapine analog for binding to RT. Nevirapine and other NNI have been shown to interact with a reasonably well-defined region of HIV-1 RT, termed the nonnucleoside-binding pocket that includes residues Y181 and Y188 (Kohlstaedt *et al.*, 1992; Smerdon *et al.*, 1994; Ding *et al.*, 1995; Ren *et al.*, 1995). Other carboxanilide NNI, namely, UC38 and UC84, have been shown to interact with the NNI-binding pocket (Fletcher *et al.*, 1995a). UC84 prevents UC781 from binding to RT (Figure 2), competing with UC781 for interaction with the RT−T/P binary complex. We therefore think it likely that inhibition by UC781 results from interaction of the compound with the HIV-1 RT NNI-binding pocket. Nonetheless, this binding must involve some unique contacts with the residues of RT that comprise the NNI pocket, in order to provide the exceptionally high binding forces that result in the observed "tight" binding.

Inhibition by UC781 is uncompetitive with respect to T/P, implying that this inhibitor does not preferentially bind to free RT. This is consistent with our observation that UC781 was unable to protect free RT from photoinactivation by 9-AN. In fact, the presence of UC781 seemed to enhance the rate of 9-AN photoinactivation of free RT, which would imply that UC781 must be able to interact in some manner

with this RT mechanistic form. Indeed, our fluorescence quenching studies demonstrated that UC781 interacts with all three RT mechanistic forms, including free RT. However, the greatest extent of UC781-dependent quenching of RT protein fluorescence occurred with the RT-P/T-dNTP ternary complex, the only mechanistic form of RT to which UC781 afforded any degree of protection from photoinactivation by 9-AN. We have previously shown that another thiocarboxanilide NNI, UC38, also specifically protected only the RT−T/P−dNTP ternary complex from photoinactivation (Fletcher *et al.*, 1995a). The 9-AN photolabel has been shown to form a covalent link with RT residue Y181 (Wu *et al.*, 1991). Thus, while we believe that UC781 binds to the NNI pocket of RT, this binding in the free RT and RT−T/P binary complex may involve primary contacts with residues other than those critical for the binding of nevirapine. UC781 may have significant contacts with Y181 only in the RT−T/P−dNTP ternary complex, thereby interfering with the 9-AN labeling process. It is also possible that UC781 interacts with RT at a site other than the NNI-binding pocket, as we recently suggested for the interaction of the RT inhibitor TSAO (Arion *et al.*, 1996a). Although we feel this is unlikely, for the reasons discussed above, unequivocal identification of the enzyme site to which UC781 binds must await crystallographic analysis.

With "classical" inhibitors, the attainment of equilibrium between enzyme, inhibitor, and the enzyme−inhibitor complexes is rapid and requires a large excess of inhibitor to enzyme. In contrast, with tight-binding inhibitors, the attainment of equilibrium may be rapid, but the total concentration of inhibitor needed to inhibit enzyme is similar to the total concentration of enzyme (Morrison & Walsh, 1988). Two types of tight-binding inhibitor have been identified, and may be differentiated by determination of the relative rates of binding and of dissociation of the inhibitor. The bimolecular association rate constant, k_{on}, for rapid tight-binding inhibitors (10^6-10^8 M^{-1} s^{-1}) is rapid compared to slow tight-binding inhibitors ($<10^6$ M^{-1} s^{-1}). Typical dissociation rate constants, k_{off}, for tight-binding inhibitors are $10^{-3}-10^{-4}$ s^{-1} (Yiotakis *et al.*, 1994; Furfine *et al.*, 1994). The bimolecular association rate constant for UC781 was calculated as 1.7×10^6 M^{-1} s^{-1}, and the dissociation rate constant was determined to be 1.6×10^{-3} s^{-1}. UC781 is therefore a rapid tight-binding inhibitor. Based upon these numbers, the K_d value was calculated to be approximately 0.9 nM, in reasonable agreement with our experimentally-determined K_i values (Figures 3 and 4).

It is interesting to comment on our data in light of the extensive kinetic analyses of NNI action carried out by Spence *et al*. (1995, 1996). Resistance to NNI has been proposed to result not from any decrease in the k_{on} for NNI binding to mutant RT, but rather from a large increase in k_{off} compared to wt RT. Thus, in this model, the NNI binds normally, but cannot remain bound to RT, and thus is unable to exert a significant inhibitory effect. UC781 binds normally to RT, but once bound does not readily dissociate from the enzyme. It is important to note that UC781 shows excellent inhibitory activity against several mutant HIV-1 strains resistant to other NNI, including those with the L100I, V106A, E138K, and Y181C mutations (Balzarini *et al*., 1996a,b). Each of these residues forms part of the NNI-binding pocket of HIV-1 RT (Kohlstaedt *et al.*, 1992; Smerdon *et al.*, 1994; Ding *et al.*, 1995; Ren *et al.*, 1995). The RT residues contacted by UC781 must therefore differ to some degree from those involved in binding of other NNI such as nevirapine, and may account for the inability of UC781 to protect the free RT and RT−T/P binary complexes from inactivation by the 9-azidonevirapine photolabel. Such possibilities can only be verified by extensive structural analysis. Identification of the primary residue contacts for UC781 is important in view of the fact that UC781 binds very tightly to RT, unlike other NNI so far described. Identification of the important contacts between the enzyme and the inhibitor might facilitate development of other tight-binding inhibitors.

Tight-binding inhibitors of HIV-1 RT have important implications in the development of new approaches to the treatment of the HIV pandemic. Current clinical treatments for HIV infection involve antiviral agents such as 3′-azido-2′,3′-dideoxythymidine (AZT), a reverse transcriptase inhibitor, as well as inhibitors of the viral protease, and are intended for use in patients already infected with HIV. However, there is a crucial need to identify prophylactic strategies to minimize the spread of HIV from already-infected individuals to noninfected individuals. Heterosexual contact is the primary mode of transmission of HIV infection worldwide (Merson, 1993). Thus, retrovirucidal spermicides/vaginal microbicides could minimize the spread of HIV from infected to noninfected individuals. Accordingly, the World Health Organization has established a research priority for the development of effective anti-HIV vaginal microbicides (Potts, 1994). The tight-binding nature of UC781 inhibition suggests that this compound may be useful in retrovirucidal formulations, since UC781, once bound to RT, does not readily dissociate from the enzyme. We have recently found that UC781 readily penetrates isolated HIV-1 particles, and that treatment of isolated HIV-1 virions with UC781 results in rapid inactivation (loss of infectivity) of the virus (Borkow *et al.*, 1997). Perhaps more importantly, pretreatment of uninfected cells with UC781 renders these cells refractory to subsequent infection by HIV, even in the absence of the continued extracellular presence of the drug. UC781 therefore appears to have considerable promise for use in both systemic and retrovirucidal therapies.

ACKNOWLEDGMENT

We thank Drs. A. W. Harrison and W. G. Brouwer, Uniroyal Chemical Research Laboratories (Guelph, ON), for providing us with the UC series of NNI. We are grateful to Dr. G. I. Dmitrienko for synthesis of the 9-azidonevirapine photolabel.

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BI970140U