Carboxanilide Derivative Non-Nucleoside Inhibitors of HIV-1 Reverse Transcriptase Interact with Different Mechanistic Forms of the Enzyme†

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ABSTRACT: Researchers at the National Cancer Institute first recognized the anti-HIV potential of the carboxanilide compound oxathiin carboxanilide (UC84) [Bader, J. P., et al. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6740−6744]. We have compared the inhibitory effect of UC84 and a second-generation thiocarboxanilide derivative, UC38, on HIV-1 reverse transcriptase (RT) RNA-dependent DNA polymerase activity. UC38 was a much better inhibitor (IC_{50} = 0.8 μM) than UC84 (IC_{50} = 4.3 μM). Inhibition by UC84 was competitive with respect to primer/template (P/T), whereas that by UC38 was uncompetitive. Both compounds were mixed noncompetitive inhibitors with respect to deoxynucleoside triphosphate (dNTP). Both compounds protected RT from irreversible photoinactivation by an azido derivative of nevirapine, implying that UC84 and UC38 bind to the same region of RT as nevirapine. UC84 photoprotected both free RT and the RT−P/T binary complex, but did not protect the RT−P/T−dNTP ternary complex. In contrast, UC38 completely photoprotected the RT−P/T−dNTP ternary complex, but not free RT or the RT−P/T binary complex. UC84 and UC38 thus appear to bind to different mechanistic forms of RT in the polymerase reaction sequence.

HIV-1 reverse transcriptase (RT)[1] is a virus-specific multifunctional enzyme that catalyzes the conversion of retroviral genomic RNA into double-stranded DNA that can then be incorporated into the infected host cell's genome. Reverse transcription is essential for HIV-1 replication and thus provides a logical target for antiviral intervention. Considerable effort has gone into the development of inhibitors of HIV-1 reverse transcriptase. Generally, these inhibitors can be divided into two classes (Mohan, 1993): (i) dideoxynucleoside analogs such as 3′-azido-3′-deoxythymidine (AZT), 2′,3′-dideoxycytidine (ddC), and 2′,3′-dideoxyinosine (ddI) (Yarchoan et al., 1991) and (ii) non-nucleoside derivatives such as TIBO (Pauwels et al., 1990; Parker & Coburn, 1992), nevirapine (Merluzzi et al., 1990; Kopp et al., 1991), and the pyridinone analogs (Goldman et al., 1991; Carroll et al., 1993). Non-nucleoside RT inhibitors are the most specific and thus potentially the most useful anti-HIV therapeutics. However, despite the large number of non-nucleoside drugs that have been found to inhibit HIV-1 RT, there are relatively few mechanistic and kinetic details as to how these drugs function.

We are studying an interesting group of non-nucleoside RT inhibitors, carboxanilides and related derivatives. One compound of this type, the oxathiin carboxanilide UC84 (**1**, Figure 1) was originally developed by Uniroyal Chemical Company in a search for analogs of the commercial fungicide Vitavax. UC84 was found to be an effective inhibitor of HIV-1 replication in cell culture (Bader et al., 1991). However, little information concerning the mechanism of the anti-HIV action of carboxanilide-type inhibitors was provided in that report. Recently, there has been a renewed interest in the carboxanilide inhibitors, with several groups describing certain antiviral properties of these compounds (Buckheit et al., 1994; Gause & Gonda, 1994; Rubinek et al., 1994). We have been carrying out extensive analyses of the antiviral activities of oxathiin carboxanilide UC84 (**1**, Figure 1) and a second-generation thiocarboxanilide, UC38 (**2**, Figure 1). In this report, we show that UC84 and UC38 are effective inhibitors of HIV-1 reverse transcriptase RNA-dependent DNA polymerase activity *in vitro*. In addition, we show that UC84 and UC38 selectively inhibit different mechanistic forms of RT. Our data may provide new directions for the development and refinement of anti-HIV drug therapies.

MATERIALS AND METHODS

Recombinant HIV-1 p51/p66 heterodimeric reverse transcriptase was purified to a purity of ≥95% from lysates of *Escherichia coli* JM-109 transformed with our expression plasmids pRT66 and pRT51 (Gu et al., 1994), using methods similar to those previously described (Wu et al., 1993). The nevirapine photoaffinity analog 9-azido-5,6-dihydro-11-ethyl-

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[1] Abbreviations: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; P/T, primer/template; dNTP, deoxynucleoside triphosphate; RT−P/T, reverse transcriptase−primer/template binary complex; RT−P/T−dNTP, reverse transcriptase−primer/template−deoxynucleoside triphosphate ternary complex; UC84, 2-chloro-5-[[(5,6-dihydro-2-methyl-1,4-oxathiin-3-yl)carbonyl]amino]benzoic acid, 1-methylethyl ester (oxathiin carboxanilide); UC38, 2-chloro-5-[[(1-methylethoxy)thioxomethyl]amino]benzoic acid, 1-methylethyl ester; TIBO, tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepine-2-(1*H*)-thione; BHAP, bis(heteroaryl)piperazine.

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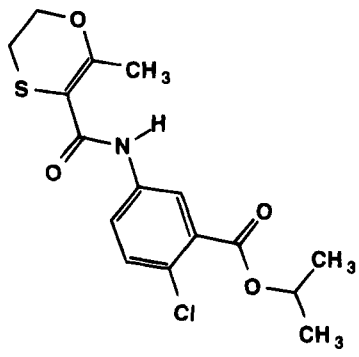

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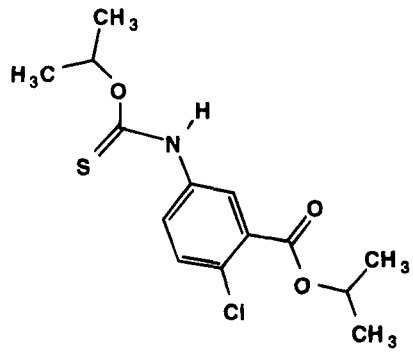

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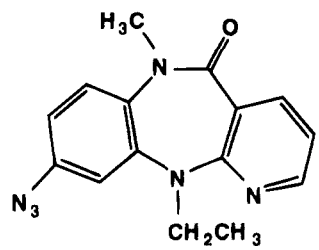

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FIGURE 1: Structures of UC84 (**1**), UC38 (**2**), and nevirapine analog photolabel (**3**) used in the present studies.

6-methyl-11*H*-pyrido[2,3-*b*]][1,5]benzodiazepin-5-enone (**3**, Figure 1) was synthesized according to the procedure of Hargrave and co-workers (Hargrave et al., 1991). NMR spectroscopic characterization of the photolabel was consistent with the structure. Oxathiin carboxanilide UC84 (**1**, Figure 1) and second-generation derivative UC38 (**2**, Figure 1) were kindly provided by Drs. W. A. Harrison and W. Brouwer, Uniroyal Chemical Research Laboratories, Guelph, ON, Canada. Homopolymeric RNA−DNA primer/templates poly(rA)-oligo(dT)$_{12-18}$ and poly(rC)-oligo (dG)$_{12-18}$ were from Pharmacia (Montreal, Quebec, Canada). [^{3}H]dTTP was purchased from NEN-Dupont. 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. Briefly, reaction mixtures (100 μL total volume) contained 50 mM Tris-HCl, pH 7.8 (37 °C), 1 mM dithiothreitol, 60 mM KCl, 8 mM $MgCl_2$, 6 μg/mL poly(rA)-oligo(dT)$_{12-18}$, 10 μM [^{3}H]dTTP, and 2 μL of the inhibitor (dissolved in dimethyl sulfoxide). Reactions were initiated by the addition of p51/p66 RT to a final concentration of 1 nM. The concentration of dimethyl sulfoxide in the reaction mixture was fixed at 2%. Control experiments showed that RT activity was unaffected by concentrations of up to 4% dimethyl sulfoxide. Reaction mixtures were incubated at 37 °C for 10−30 min and then quenched with 500 μL of ice-cold 10% trichloroacetic acid containing 20 mM sodium pyrophosphate. Samples were kept for 30 min on ice and then filtered on Whatman 934-AH glass fiber filters and counted in a liquid scintillation spectrometer.

Inhibition Kinetics of the Carboxanilide Compounds. Kinetic parameters were determined using concentrations of dTTP ranging from 0.5- to 10K_m, with a fixed concentration of primer/template (6 μg/mL), or a similar concentration range for primer/template with a fixed concentration of dTTP (5 μM). The kinetic parameters and inhibition constants were calculated using standard techniques (Segel, 1975) and the programs EZFIT (Perella Scientific, Glenolden, PA) and ENZFITTER (Biosoft, Cambridge, U.K.). All methods gave similar results.

Irreversible Inactivation of RT by the Photolabel 9-Azido-5,6−dihydro-11-ethyl-6-methyl-11H-pyrido[2,3-b]][1,5]-benzodiazepin-5-enone. RT (0.4 μM) and the nevirapine analog pnotolabel (1 μM) in 40 μL of 50 mM Tris-HCl, pH 8.0 (25 °C), were illuminated at 365 nm with a UV lamp (Mineralight UVGL-25, UVP Inc., San Gabriel, CA). The intensity of the UV radiation was kept at 20 μW/cm^2 so that the labeling was gradual enough to be conveniently monitored. The irreversible inactivation of RT by the nevirapine analog was followed by removing 2-μL aliquots of the mixture at different times and diluting the sample 250-fold into 50 mM Tris, pH 7.8 (37 °C) in order to dissociate any reversibly bound photoaffinity probe. A 30-μL aliquot of this diluted sample was then assayed for RNA-dependent DNA polymerase activity as described above. The final dilution of photolabel in the RT assay was 825-fold. This dilution factor produced a final concentration of photolabel of approximately 1 nM, sufficiently low to prevent the photolabel from interfering as a reversible inhibitor of RT in the reaction mixture. Additional control experiments showed no inactivation of RT upon exposure to UV light in the absence of photolabel.

Experiments concerning inactivation of the RT primer/template binary complex (RT−P/T) were conducted as descibed above, with the addition of 15 μg/mL of poly(rC)-oligo(dG). Enzyme and primer/template (P/T) were preincubated for several minutes prior to addition of the photolabel and irradiation, in order to ensure formation of the RT−P/T complex. In experiments concerning inactivation of the enzyme−primer/template−deoxynucleoside triphosphate (RT−P/T−dNTP) ternary complex, RT was first preincubated with poly(rC)-oligo(dG), and then deoxynucleoside triphosphate (dGTP or dATP) and photolabel were added sequentially prior to irradiation.

In experiments concerning the ability of UC84 and UC38 to protect RT from photoinactivation, the inhibitors comprised the penultimate addition to the reaction mixture. Photolabel was always added last.

RESULTS

The original report describing the antiviral activity of UC84 had stated that although this compound was highly effective in inhibiting HIV-1 replication in CEM-SS cells, this drug did not inhibit HIV-1 reverse transcriptase in an *in*

Table 1: Some Kinetic Constants for the Inhibition of HIV-1 Reverse Transcriptase by UC84 and UC38[a]

	inhibitor					
	UC84			UC38		
assay condition[b]	IC_{50} (μM)	type of inhibition	K_i (μM)	IC_{50} (μM)	type of inhibition	K_i (μM)
fixed P/T, fixed dNTP	4.3 ± 0.2	n.a.[c]	*d*	0.8 ± 0.1	n.a.	*d*
variable P/T, fixed dNTP	n.a.	C[e]	1.2	n.a.	UC	0.7
fixed P/T, variable dNTP	n.a.	MNC	2.7	n.a.	MNC	0.2

[a] The IC_{50} values are the means ± SD of four or five separate experiments. The K_i data are averages from two separate experiments. [b] Assay conditions are defined in the text. [c] Not applicable. [d] K_i values could not be determined directly from these data, since inhibition by the UC84 compounds was not strictly first-order under these conditions (data not shown). [e] C, competitive; UC, uncompetitive; MNC, mixed noncompetitive.

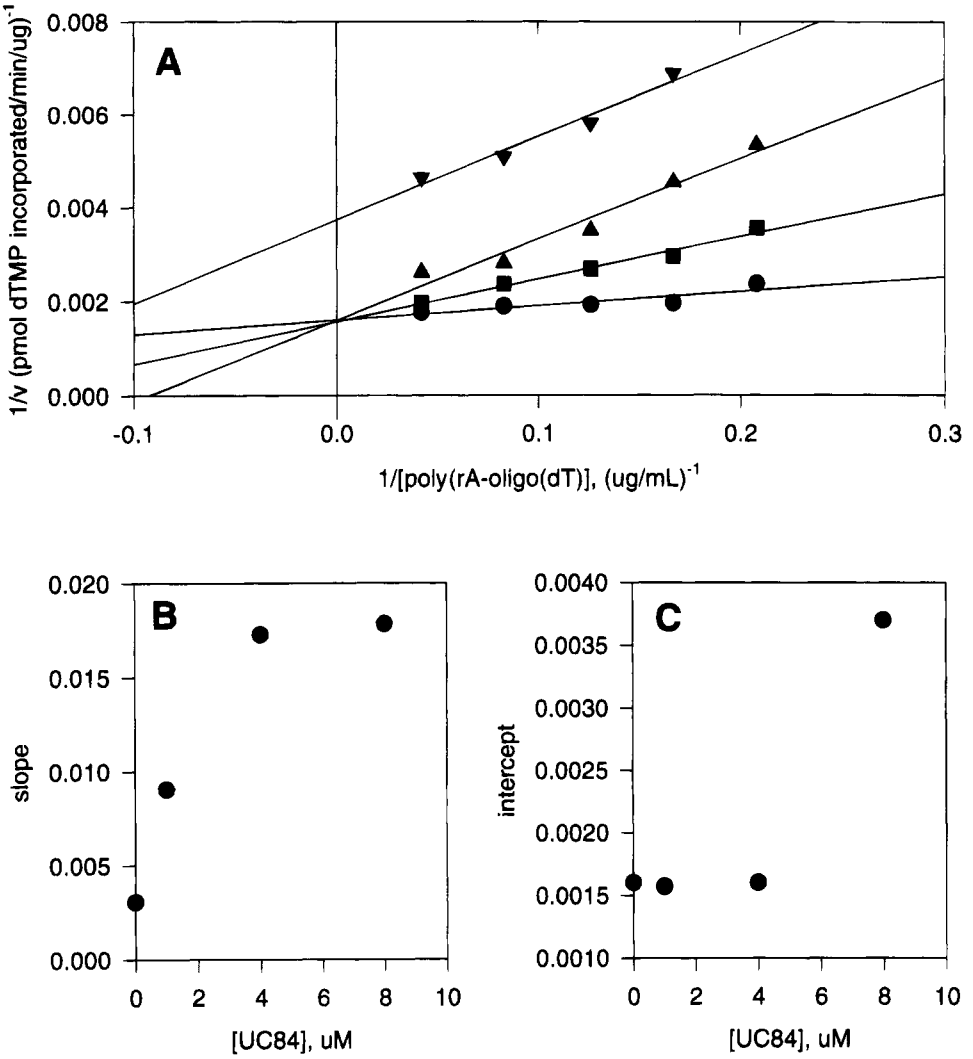

FIGURE 2: Inhibition of reverse transcriptase activity by UC84. (A) Double-reciprocal plot of 1/*v* vs 1/[P/T] in the absence and presence of varying concentrations of UC84. The concentrations of UC84 used were (●) 0, (■) 1, (▲) 4, and (▼) 8 μM. The lines shown are calculated from first-order regression analyses of the data. (B) Replot of the slopes of lines in (A) as a function of UC84 concentration. (C) Replot of the intercepts of lines in (A) as a function of UC84 concentration.

vitro assay (Bader et al., 1991). We have confirmed that UC84 is an effective inhibitor of HIV-1 replication in infected cells, with an IC_{50} of about 2 μM against viral replication in both MT-4 and cord blood mononuclear cells (unpublished data). However, in contrast to the previously reported data, we found that UC84 was an effective inhibitor of HIV-1 RT RNA-dependent DNA polymerase activity *in vitro*, with an IC_{50} of 4.3 μM (Table 1). UC38 was found to be a much more effective inhibitor than UC84 of HIV-1 RT polymerase *in vitro*, with an IC_{50} value of 0.8 μM (Table 1).

Kinetics of Inhibition of RT by UC84 and UC38 in the Presence of Variable Primer/Template Concentrations. The *in vitro* inhibition of RT polymerase activity by UC84 and UC38 was examined by varying the concentration of the homopolymeric P/T poly(rA)-oligo(dT) while maintaining [^{3}H]dTTP at a fixed concentration of 5.0 μM, approximately K_m for this dNTP. Essentially similar results were obtained when the dTTP concentration was increased to 10 μM (data not shown); thus for most experiments the lower concentration of dTTP was employed. The K_m for poly(rA)-oligo(dT) under our reaction conditions was 6 μg/mL. The inhibition pattern for UC84 was rather complex. As illustrated in Figure 2A, inhibition of RT at low concentrations of UC84 (<4 μM) was apparently competitive with respect to P/T. At higher concentrations of UC84, the inhibition

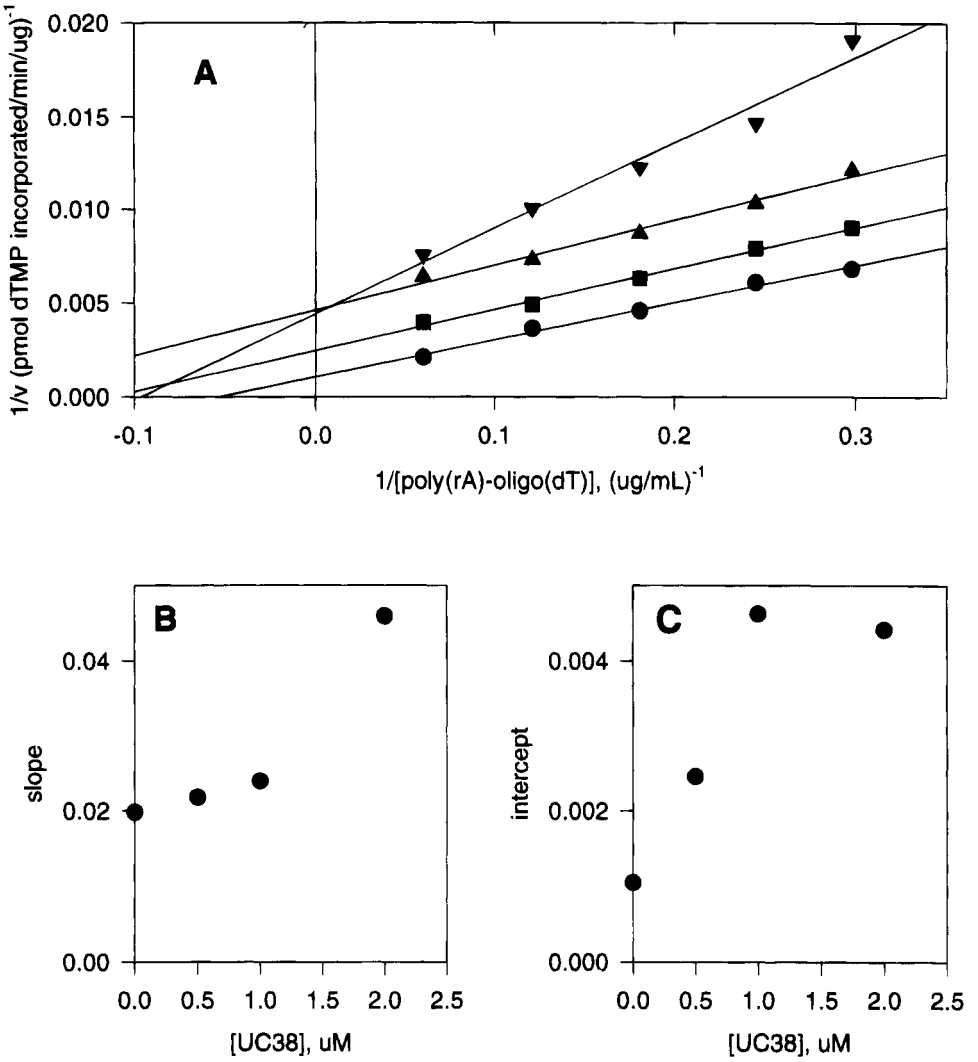

FIGURE 3: Inhibition of reverse transcriptase activity by UC38. (A) Double reciprocal plot of 1/v vs. 1/[P/T] in the absence and presence of varying concentrations of UC38. The concentrations of UC38 used were (●) 0, (■) 0.5, (▲) 1, and (▼) 2 μM. The lines shown are calculated from first-order regression analyses of the data. (B) Replot of the slopes of the lines in (A) as a function of UC38 concentration. (C) Replot of the intercepts of lines in (A) as a function of UC38 concentration.

pattern changed toward mixed noncompetitive. The slope replot of the data was hyperbolic (Figure 2B). The K_i value calculated for UC84 under these reaction conditions was 1.2 μM (Table 1).

UC38 also displayed a complex inhibition pattern with respect to P/T. At low concentrations of UC38 (<1.0 μM), the pattern was essentially uncompetitive, changing to mixed noncompetitive at concentrations above 1 μM (Figure 3A). The slope replot for UC38 was parabolic (Figure 3B). The K_i value calculated for UC38 was 0.7 μM (Table 1).

Kinetics of Inhibition of RT by UC84 and UC38 in the Presence of Variable Deoxynucleoside Triphosphate Concentrations. Inhibition by UC84 and UC38 was also examined with varying concentrations of [^{3}H]dTTP at a fixed concentration of poly(rA)-oligo(dT) P/T (6 μg/mL). Inhibition by both UC84 and UC38 under these conditions was mixed noncompetitive with respect to the dNTP substrate (data not shown). Slope replots in both cases were linear. The K_i values calculated for UC84 and UC38 were 2.7 and 0.2 μM, respectively (Table 1).

Irreversible Inhibition of HIV-1 RT by Photoaffinity Label 9-Azido-5,6-dihydro-11-ethyl-6-methyl-11H-pyrido[2,3-b]][1,5]benzodiazepin-5-enone. In order to confirm that inhibition by our preparation of the photoaffinity label **3** was consistent with results reported in the literature, various concentrations of the photolabel were assayed with HIV-1 RT in the dark. The IC_{50} value derived from our data was 140 nM, essentially identical to the previously reported values of 140 and 160 nM (Hargrave et al., 1991; Wu et al., 1991).

HIV-1 RT was irreversibly inactivated upon UV irradiation in the presence of the nevirapine analog photolabel. This compound was able to irreversibly inactivate the various mechanistic forms of RT, under conditions described in Materials and Methods. The rate constant for photoinactivation of the RT−poly(rC)-oligo(dG) primer/template complex was increased approximately 30% relative to that of the free enzyme (Table 2). However, the ability of the photolabel to inactivate the RT−P/T complex was highly dependent on the identity of the primer/template used, since no inactivation was noted in the presence of poly(rA)-oligo(dT). The rate of inactivation of the RT−P/T−dNTP ternary complex was decreased relative to that of the free enzyme (Table 2).

Photoprotection of Free Enzyme by the Carboxanilide Compounds. Various concentrations of either UC84 or UC38 were added to fixed concentrations of reverse transcriptase and photolabel. UC84 was quite effective in

Table 2: Rates of Photoinactivation of Various Forms of HIV-1 Reverse Transciptase

enzyme form[a]	k_{inact} (min^{-1})[b] no inhibitor	+UC84	+UC38
(1) free RT	0.035 ± 0.001	0.012 ± 0.001 (0.3 μM)[c] 0 (0.9 μM)	0.035 ± 0.001 (1.5 μM)
(2) RT−P/T complex			
poly(rA)-oligo(dT)	0	n.d.[d]	n.d.
poly(rC)-oligo(dG)	0.044 ± 0.002	0 (0.3 μM)	0.043 ± 0.002 (1.5 μM)
(3) RT−P/T−dNTP complex			
poly(rC)-oligo(dG)			
+ dGTP (2.5 μM)	0.027 ± 0.001	0.010 ± 0.001 (0.9 μM)	0.009 ± 0.001 (0.6 μM)
+ dGTP (10 μM)	0.022 ± 0.002	0.021 ± 0.002 (0.9 μM)	0.002 ± 0.001 (0.6 μM)
+ dATP (10 μM)	0.036 ± 0.002	0.022 ± 0.002 (0.9 μM)	0.013 ± 0.003 (0.6 μM)

[a] Enzyme forms were "prepared" as described in Materials and Methods. [b] Data are means ± SD calculated from three or four separate experiments, each comprising four to eight individual time points. [c] Concentrations of UC84 and UC38 are indicated in parentheses. [d] Not determined.

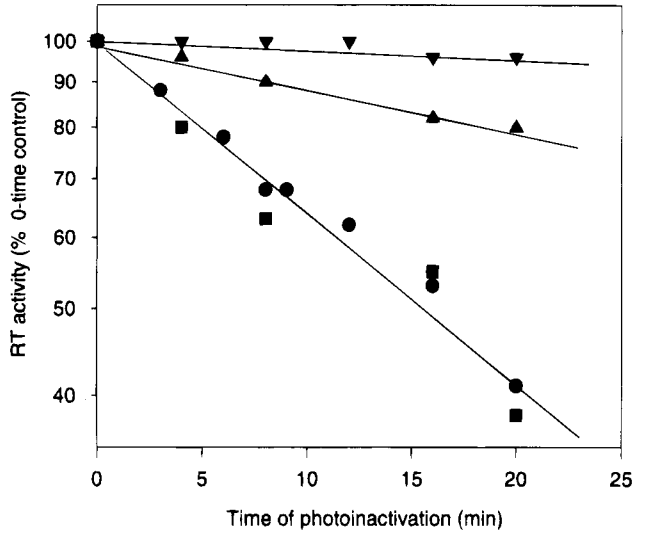

FIGURE 4: Protection of *free* RT from photoinactivation. A mixture of RT (0.4 μM heterodimer) and nevirapine analog **3** (1.0 μM) was photoirradiated as described in Materials and Methods in the absence (●) or the presence of 0.3 μM UC84 (▲), 0.9 μM UC84 (▼), or 1.5 μM UC38 (■). Aliquots were withdrawn at the times indicated and assayed for RT RNA-dependent DNA polymerase activity.

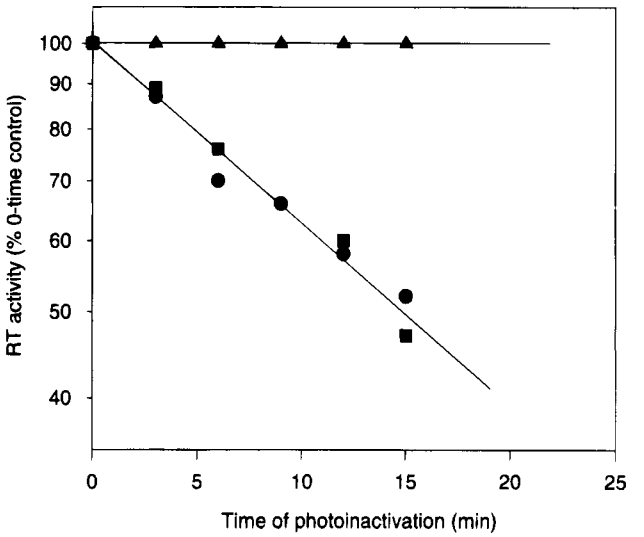

FIGURE 5: Protection of RT−P/T *binary complex* from photoinactivation. A mixture of RT (0.4 μM heterodimer), poly(rC)-oligo(dG) (15 μg/mL), and nevirapine analog **3** (1.0 μM) was photoirradiated in the absence (●) or the presence of 0.3 μM UC84 (▲) or 1.5 μM UC38 (■). Aliquots were withdrawn at the times indicated and assayed for RT RNA-dependent DNA polymerase activity.

protecting RT from irreversible photoinactivation at concentrations well below its apparent IC_{50} (Figure 4). In contrast, UC38 was unable to protect the free enzyme from photoinactivation, even at concentrations well above the apparent IC_{50} for this compound. We explored the possibility that UC38 was decomposing or undergoing a possible conformational change as a result of UV irradiation. Therefore, UC38 was subjected to 30 min of photoirradiation at the same wavelength and intensity of UV light used in the photoinactivation experiments, and then compared with untreated UC38 for inhibition of RT RNA-dependent DNA polymerase activity. No differences in inhibitory potency were found. Thus, UC38 appears to be unable to protect free enzyme from irreversible photoinactivation.

Photoprotection of the Enzyme−Primer/Template Complex by UC84 and UC38. It was previously shown that the nevirapine analog photolabel irreversibly inactivated HIV-1 RT in the presence of the primer/template poly(rC)-oligo(dG) (Wu et al., 1991). When similar experiments were carried out in the presence of UC84 at a concentration well below its IC_{50} value, complete photoprotection of the enzyme−primer/template complex was observed (Figure 5). In contrast, UC38 was unable to protect the RT−primer/template complex from photoinactivation.

Photoprotection of the RT−Primer/Template−dNTP Ternary Complex by the Carboxanilide Compounds. Irreversible photoinactivation of HIV-1 RT was also observed in the presence of poly(rC)-oligo(dG) and the complementary deoxynucleoside triphosphate, dGTP, conditions which would allow formation of the ternary complex. In the presence of 15 μg/mL poly(rC)-oligo(dG) (approximately 2.5 times the apparent K_m) and 2.5 μM dGTP (approximately equal to the K_m value), the rate of irreversible inactivation in the absence of the carboxanilide compounds was 80% of that observed with the free enzyme, and 67% of that for the enzyme−primer/template complex (Table 2). Under these conditions, both UC84 and UC38 showed similar photoprotection profiles (Figure 6A). However, the protection by UC84 was not nearly as complete as for the enzyme−primer/template complex. When the concentration of dGTP was increased to 10 μM, conditions which would be expected to promote formation of the E−P/T−dNTP ternary complex, UC38 was very effective in preventing photoinactivation (Figure 6B). In contrast, the protection afforded by UC84 was significantly decreased under these conditions (Figure 6B; Table 2).

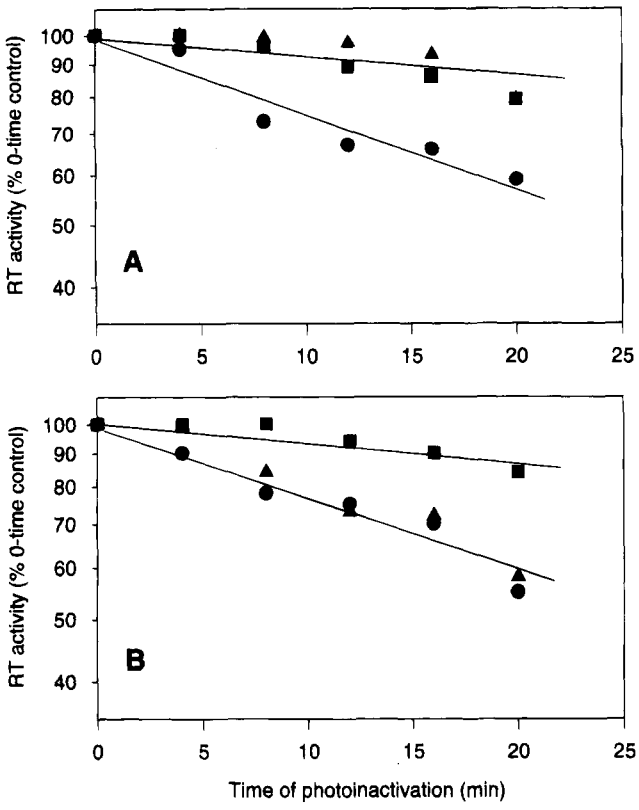

FIGURE 6: Protection of RT−P/T−dGTP *ternary complex* from photoinactivation. A mixture of RT (0.4 μM heterodimer), nevirapine analog **3** (1.0 μM), poly(rC)-oligo(dG) (15 μg/mL), and either 2.5 μM (A) or 10 μM dGTP (B) was photoirradiated in the absence (●) or the presence of 0.9 μM UC84 (▲) or 0.6 μM UC38 (■). Aliquots were withdrawn at the times indicated and assayed for RT RNA-dependent DNA polymerase activity.

Interestingly, UC38 also afforded photoprotection to the ternary complex containing a noncomplementary deoxynucleoside triphosphate, dATP (Table 2). However, the degree of photoprotection was less than that observed in the presence of the complementary dGTP.

DISCUSSION

The mechanism of HIV-1 reverse transcriptase RNA-dependent DNA polymerase is rather complex (Majumdar et al., 1988; Kedar et al., 1990; Kati et al., 1992; Reardon, 1992) and comprises at least three mechanistic forms of RT prior to catalysis—free enzyme (RT), enzyme−primer/template binary complex (RT−P/T), and enzyme−primer/template−deoxynucleoside triphosphate ternary complex (RT−P/T−dNTP). The enzyme−dNTP complex is believed not to play a significant role in enzyme activity. At least two different ternary complexes are possible, one comprising RT−P/T and a dNTP noncomplementary to the template base and the other comprising RT−P/T and the complementary dNTP. Both complementary and noncomplementary dNTPs have been shown to bind with apparently equal affinity to the RT−P/T complex (Painter et al., 1991).

The carboxanilide derivatives UC84 and UC38 were found to be mixed noncompetitive inhibitors of HIV-1 RT with respect to dNTP. Such inhibition is typical of other non-nucleoside inhibitors, such as nevirapine (Kopp et al., 1991), TIBO (Balzarini et al., 1992), BHAP (Althaus et al., 1993), and pyridinones (Carroll et al., 1993), and is consistent with the idea that these compounds interact through a nonsubstrate binding site (Cohen et al., 1991; Wu et al., 1991). It has generally been accepted that most non-nucleoside antivirals are also mixed noncompetitive inhibitors with respect to P/T. However, evidence for the latter is inconsistent. For example, inhibition by the TIBO derivative R82150 has been observed to be mixed noncompetitive (Frank et al., 1991; Balzarini et al., 1992) or uncompetitive (Debyser et al., 1993) with respect to P/T. We found that the inhibition kinetics exhibited by the carboxanilide non-nucleosides were rather complex, with UC84 acting as a partial competitive inhibitor, and UC38 acting as a partial uncompetitive inhibitor, with respect to P/T. However, the type of inhibition appeared to change to mixed noncompetitive at high concentrations of each inhibitor, i.e., at concentrations considerably above their respective IC_{50} values. We considered that this change might reflect the presence of a second site for inhibition. However, Hill plots of inhibition data obtained under a variety of conditions were linear, with slopes approximately equal to 1 (data not shown). It is therefore likely that UC84 and UC38 inhibit by binding at a single site on RT. Instead, we feel that the differences in inhibition patterns given by UC84 and UC38 imply that these two structurally similar compounds interact with different RT mechanistic forms in the polymerase reaction sequence. However, although enzyme inhibition kinetic studies are very useful mechanistic probes (Segel, 1975), complex inhibition patterns such as those shown by UC84 and UC38 (Figures 2A and 3A) can be difficult to interpret.

In order to obtain additional information concerning the RT forms with which the carboxanilide compounds interact, we carried out a series of experiments involving the ability of the compounds to protect RT from irreversible photoinactivation by a nevirapine analog photolabel (**3**, Figure 1). This compound has been shown to be a very specific inactivator of HIV-1 reverse transcriptase (Wu et al., 1991), and it provides an effective tool for comparing the binding sites of nevirapine and other classes of non-nucleoside drugs. Previous work had shown that this photolabel was able to interact both with free RT and with the RT−P/T complex (Wu et al., 1991). In the present study, we have extended these observations and shown that the photolabel **3** can bind to three mechanistic forms of RT—the free enzyme, the enzyme−P/T binary complex, and the enzyme−P/T−dNTP ternary complex. From the inactivation rate constants (Table 2), the nevirapine photolabel appears to bind preferentially in the order RT−P/T > RT > RT−P/T−dNTP. The ability of this photolabel to inactivate all three mechanistic forms of RT provides an extraordinarily useful tool for the study of the interaction of RT with other non-nucleoside inhibitors, such as UC84 and UC38. The data obtained from these studies, coupled with our kinetic analyses, have allowed us to make certain conclusions in this respect.

The noncompetitive nature of inhibition by UC84 and UC38 with respect to dNTP indicates that the carboxanilide inhibitors do not bind at the dNTP binding site and must be interacting with a separate site on the enzyme. We (Wu et al., 1993) and others (Cheng et al., 1993) have shown that the dNTP binding site may involve residues 65−73 in the "fingers" subdomain of the enzyme. In contrast, non-nucleoside compounds are believed to bind in a region defined by residues in the "palm" subdomain, including Y181 and Y188 (Cohen et al., 1991). The ability of both UC84 and UC38 to protect RT from photoinactivation, under appropriate conditions, implies that these compounds bind to a region at or near that for the binding of nevirapine, and

distinct from that for the binding of dNTP. Interestingly, the mutation L100I has recently been identified with resistance to UC84 (Buckheit et al., 1994). This mutation has also been correlated with resistance to the non-nucleoside inhibitor TIBO (Mellors et al., 1993). Residue L100 of HIV-1 RT has been implicated in the binding of nevirapine (Smerdon et al., 1994).

The competitive inhibition by UC84 with respect to P/T implies that this compound interacts with the same enzyme form as the primer/template, i.e., free RT. This is consistent with the observation that UC84 protects free RT from photoinactivation. The increasing complexity of the inhibition pattern at higher concentrations of UC84 suggests two possibilities, either that the free enzyme−inhibitor complex is catalytically inactive or that UC84 can interact with additional enzyme forms. The latter is supported by our observation that UC84 photoprotects both free RT and the RT−P/T complex. In contrast, UC84 was unable to protect the RT−P/T−dNTP ternary complex from photoinactivation. Although UC84 showed photoprotection at low concentrations of dGTP (Figure 6A), the conditions used in this experiment (K_m values of dGTP, saturating levels of P/T) were such that both RT−P/T and RT−P/T−dGTP complexes would be present. When conditions were adjusted so that the RT−P/T−dGTP ternary complex would predominate, UC84 was unable to photoprotect (Figure 6B).

The uncompetitive inhibition by UC38 with respect to P/T suggests that this inhibitor does not interact significantly with the free enzyme (Segel, 1975). Indeed, UC38 was completely ineffective in protecting free RT from photoinactivation, even at concentrations exceeding its IC_{50} and significantly in excess of the photolabel concentration. More interesting is the observation that UC38 also failed to photoprotect the RT−P/T complex. Only when all substrates were present to form the RT−P/T−dNTP ternary complex did UC38 show a high degree of photoprotection. Therefore, UC38 appears to bind only to a single mechanistic species, the RT−P/T−dNTP ternary complex.

Photoinactivation of the RT−P/T complex was noted only when poly(rC)-oligo(dG) was used as P/T. No inactivation was found when poly(rA)-oligo(dT) was used. These data imply that the nevirapine analog **3** binds only poorly to the latter RT−P/T complex. This observation was somewhat surprising, given that nevirapine can inhibit RT RNA-dependent DNA polymerase activity when poly(rA)-oligo-(dT) is used as P/T (Merluzzi et al., 1990). The structure of the P/T can have a significant effect on RT activity (Cheng et al., 1987), and could alter the mechanism of the enzyme. However, as discussed above, the results of our kinetic analyses, which used poly(rA)-oligo(dT) as P/T, are highly consistent with those obtained from our photoprotection experiments, which used poly(rC)-oligo(dG). We are therefore confident of the validity of our conclusions concerning the interaction of UC84 and UC38 with different mechanistic species of RT.

To the best of our knowledge, the present studies provide the first evidence of structurally similar non-nucleoside inhibitors interacting with different mechanistic forms of HIV-1 reverse transcriptase. Our discovery of the differential binding of two structurally related non-nucleoside inhibitors to different mechanistic forms of RT suggests the possibility that such drugs may be able to inhibit HIV-1 RT in a synergistic manner. Preliminary experiments indicate that UC84 and UC38 do in fact show synergy in inhibiting RT under appropriate conditions *in vitro* (R. S. Fletcher, G. I. Dmitrienko, and M. A. Parniak, unpublished data).

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