Articles

Synergistic Inhibition of HIV-1 Reverse Transcriptase DNA Polymerase Activity and Virus Replication *in Vitro* by Combinations of Carboxanilide Nonnucleoside Compounds†

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ABSTRACT: The carboxanilides UC84 and UC38 are nonnucleoside inhibitors of both the RNA-dependent and DNA-dependent DNA polymerase activities of HIV-1 reverse transcriptase (RT). We have previously shown that UC84 and UC38 bind to the same site as nevirapine but interact with different RT mechanistic forms, with UC84 preferentially binding to the RT−primer/template complex and UC38 binding only to the RT−primer/template−dNTP ternary complex [Fletcher, R. S., et al. (1995) *Biochemistry 34*, 4346−4353]. Here we demonstrate that combinations of UC84 and UC38 inhibit RT DNA polymerase activity *in vitro* in a synergistic manner. This synergy was noted primarily in reactions containing high concentrations of primer/template and K_m levels of dNTP substrate and was independent of both primer/template identity and the molar ratio of UC84:UC38. Combination indices were in the range of 0.4−0.6, indicating substantial synergy in the inhibition of RT activity. More importantly, combinations of UC84 and UC38 also showed a high degree of synergy in inhibiting HIV-1 replication in both MT-4 and cord blood mononuclear cells. We believe this to be the first example of synergistic inhibition of HIV-1 RT by combinations of structurally related nonnucleoside inhibitors.

The transcription of retroviral RNA into double-stranded DNA is an essential step in the replication cycle of the human immunodeficiency virus type 1 (HIV-1).[1] This process comprises multiple steps, each of which is catalyzed by the multifunctional viral enzyme, reverse transcriptase (RT). This enzyme thus has provided a logical target for the development of antiviral compounds. Numerous compounds that inhibit RT RNA-dependent DNA polymerase (RDDP) activity have been described. These can be divided into two broad classes (Mohan, 1993). The first group includes dideoxynucleoside compounds such as 3′-azido-3′-deoxythymidine (AZT) and 2′,3′-dideoxy-3′-thiacytidine (3TC). The second group, nonnucleoside reverse transcriptase inhibitors (NNRTI), includes a large number of structurally dissimilar hydrophobic compounds, including nevirapine (Merluzzi et al., 1990; Kopp et al., 1991), TIBO (Pauwels et al., 1990), and pyridinone derivatives (Goldman et al., 1991), which bind to a site in the RT palm subdomain that is adjacent to, but distinct from, the polymerase active site (Kohlstaedt et al., 1992; Smerdon et al., 1994; Tantillo et al., 1994; Ren et al., 1995).

We have been studying a new group of NNRTI, the carboxanilides (Bader et al., 1991). We recently noted that two of these compounds, UC84 and UC38, bind to the same nonnucleoside binding site on HIV-1 RT as nevirapine. However, UC84 and UC38 interact with different mechanistic forms of the enzyme (Fletcher et al., 1995). The differential binding of these two structurally related NNRTI suggested to us that combinations of UC84 plus UC38 might show synergy in inhibiting HIV-1 RT. In this report, we show that UC84+UC38 combinations do indeed synergistically inhibit RT DNA polymerase activity *in vitro*. This synergy is dependent on the assay conditions, supporting our premise that different mechanistic forms of RT must be present for combinations of UC84 and UC38 to exert synergy in inhibition. More importantly, combinations of UC84 and

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[1] Abbreviations: AZT, 3′-azido-3′-deoxythymidine; 3TC, 2′,3′-dideoxy-3′-thiacytidine; HIV-1, human immunodeficiency virus type 1; NNI, nonnucleoside inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; DDDP, DNA-dependent DNA polymerase; RDDP, RNA-dependent DNA polymerase; 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; TIBO, tetrahydroimidazo[4,5,1-*jk*][1,4]-benzodiazepine-2(1*H*)-thione; 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); CBMC, cord blood mononuclear cells.

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UC38 show a high degree of synergy in inhibiting virus replication in both MT-4 and cord blood mononuclear cells. To the best of our knowledge, this is the first example of synergistic inhibition of HIV-1 reverse transcriptase by combinations of nonnucleoside inhibitors that interact with the same binding pocket on the enzyme.

MATERIALS AND METHODS

Recombinant HIV-1 p51/p66 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) by using methods similar to those previously described (Wu et al., 1993). Oxathiin carboxanilide UC84 and the second-generation thiocarbamate derivative UC38 were provided by Drs. W. A. Harrison and W. Brouwer (Uniroyal Chemical Research Laboratories, Guelph, ON, Canada). Homopolymeric primer/templates (P/T) poly(rA)−oligo(dT)$_{12-18}$, poly(dA)−oligo(dT)$_{12-18}$, poly(rC)−oligo(dG)$_{12-18}$, and poly(dC)−oligo(dG)$_{12-18}$ were from Pharmacia (Montreal, Canada). The heteropolymeric P/T for the assay of RT RDDP activity was prepared by using the RNA transcript from plasmid pHIV-PBS (Arts et al., 1994) and a synthetic 18-nt DNA oligonucleotide, as previously described (Gu et al., 1994). Heteropolymeric P/T for RT DDDP activity either was activated calf thymus DNA (Sigma, St. Louis, MO) or was prepared using the synthetic oligonucleotides 5′-GTCCCTGTTCGGGCGCCA-3′ as primer and 5′-TAGCCAAACGGGGCTTTTGTGTAGTAG-TGGCGCCCGAACAGGGAC-3′ as template. Essentially similar results were obtained with both of these DDDP P/T. Synthetic oligonucleotides were obtained from either GSD (Toronto, ON, Canada) or National Biosciences (Plymouth, MN). [^{3}H]TTP and [^{3}H]dGTP were purchased from NEN-Dupont, and [α-^{32}P]dNTP was from Amersham. All other materials were of the highest purity available.

Assay of HIV-1 Reverse Transcriptase DNA Polymerase Activity. HIV-1 reverse transcriptase DNA polymerase activity was determined in a fixed-time assay essentially as previously described (Fletcher et al., 1995). Briefly, reaction mixtures (total volume, 100 μL) contained 50 mM Tris-HCl (pH 7.8, 37 °C), 60 mM KCl, 10 mM $MgCl_2$, 0.5−50 μg/mL primer/template, 0.5−25 μM [^{3}H]- or [α-^{32}P]dNTP substrate, and 2 μL of inhibitor (dissolved in 100% dimethyl sulfoxide). 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. Reactions were initiated by the addition of 10−25 ng of RT p51/p66 heterodimer and then incubated at 37 °C for 10 min. Reaction mixtures were quenched with 500 μL of cold 20 mM sodium pyrophosphate in 10% trichloroacetic acid, placed on ice for 30 min, and then transferred to glass fiber filters (Whatman 934AH). The filters were washed sequentially with 10% trichloroacetic acid and 95% ethanol, and the acid-precipitable radioactivity was determined by liquid scintillation spectrometry.

Cell Culture and Virus Replication. The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID: the CD4$^+$ MT-4 cell line (contributed by Dr. D. D. Richman) and the HTLV-III$_B$ laboratory strain of HIV-1 (contributed by Dr. R. C. Gallo). Cord blood mononuclear cells (CBMC) were isolated by Ficoll-Hypaque centrifugation from cord blood obtained from the Department of Obstetrics, SMBD−Jewish General Hospital. CBMC were prestimulated with phytohemagglutinin prior to use (Rooke et al., 1990). Cells were infected with HIV-1 (multiplicity of infection, 0.2−1.0) for 1 h, and then residual virus was removed by washing and centrifugation. The infected cells were incubated in medium containing the appropriate concentrations of inhibitor. Every 2 days thereafter, one-half of the cell culture medium was replaced with fresh medium either without drug or containing the same concentration of drug used initially. Cytopathic effects of HIV-1 infection in MT-4 cells were scored by microscopic assessment of the extent of syncytium formation (Yao et al., 1992). With CBMC, aliquots of the culture supernatants were harvested and tested for levels of HIV by assay of RT using poly(rA)−oligo(dT) and [^{3}H]TTP as described earlier and by assay of p24 antigen levels (Yao et al., 1992).

Synergy Analysis. Synergistic inhibition of RT was assessed by two methods. In some experiments, the concentration of one inhibitor was varied in the presence of a fixed concentration of the other inhibitor at constant concentrations of substrates. Data were analyzed by Dixon plots (Segel, 1975). In most experiments, synergy of inhibition was assessed by calculation of the combination index (Chou & Talalay, 1984). These calculations were based on the assumption that UC84 and UC38 interact with RT in a mutually exclusive manner. Combination indices of less than 1 indicate synergistic inhibition, whereas values equal to or greater than 1 imply additive and antagonistic inhibition, respectively. Graphical data were fit using the regression analysis subroutine of the program SigmaPlot (Jandel Scientific, San Rafael, CA).

RESULTS

Optimization of the in Vitro Assay of RT RDDP for the Observation of Synergy between UC38 and UC84. We have shown that even though both UC84 and UC38 bind to the same site on RT, these inhibitors bind to different RT mechanistic forms (Fletcher et al., 1995). UC84 exclusively binds to free RT and the enzyme−P/T complex, whereas UC38 almost entirely binds to the RT−P/T−dNTP ternary complex. We speculated that if these multiple mechanistic enzyme forms were present in an *in vitro* RT assay, then combinations of UC38 and UC84 might show synergy in inhibiting the enzyme. Initial experiments aimed at examining this synergy employed RDDP assays in which the P/T and dNTP were present at approximately K_m levels. Dixon plot analysis of these data showed a small slope effect, indicating a slight degree of synergy under these conditions (Figure 1).

In order to study this possibility further, we performed experiments similar to examples described by Chou and Talalay (1984). Initially, our assays used K_m levels of P/T and dNTP, since these are the conditions normally used in our determinations of inhibitor IC_{50} values. In these experiments, we chose a 4:1 ratio of UC84:UC38 since the inhibition of RT RDDP by UC84 was approximately 4-fold less than that by UC38 (Fletcher et al., 1995). Under these conditions, the combination curve fell in between those curves described by UC84 and UC38 alone (Figure 2A), indicating a small degree of synergy consistent with the

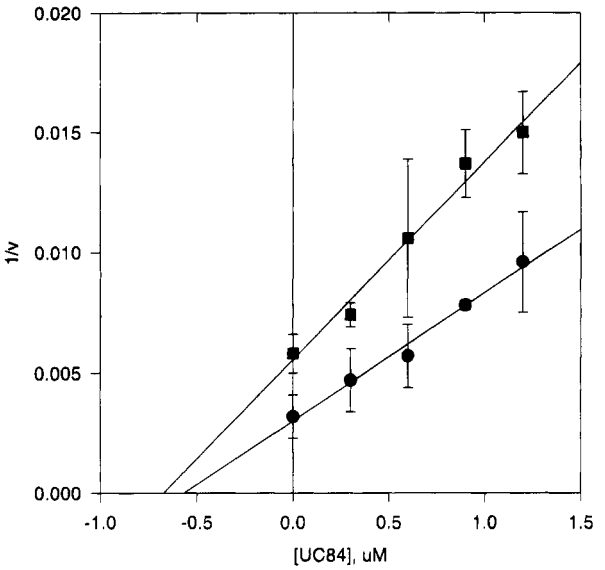

FIGURE 1: Inhibition of HIV-1 RT RDDP activity by various concentrations of UC84 alone (●) and in the presence of 0.3 μM UC38 (■). The reaction assays contained K_m levels of poly(rA)−oligo(dT) (5 μg/mL) and dTTP (5 μM). The data points are the means ± SD of three or four determinations. The lines were calculated by first-order regression analysis of the data.

Dixon analysis described earlier (Figure 1). The combination indices at 70% and 90% inhibition (CI_{70} and CI_{90}) were 0.85 and 0.64, respectively (Table 1), providing additional evidence that UC38 and UC84 in combination inhibit RT in a synergistic manner.

Nonetheless, the degree of synergy noted under these assay conditions was not particularly impressive. We therefore altered the conditions for the *in vitro* assay of RT RDDP in order to determine whether the degree of synergy between UC38 and UC84 could be enhanced. With high concentrations of dTTP (25 μM; approximately 5 times K_m) and K_m levels of the P/T poly(rA)−oligo(dT), inhibition by combinations of UC84 and UC38 (4:1) was clearly additive, with the combination curve coincident with the curve described by UC84 alone (Figure 2B). The CI_{50}, CI_{70}, and CI_{90} values were each approximately 1.0 (Table 1). However, with high concentrations of poly(rA)−oligo(dT) primer/template (25 μg/mL; approximately 5 times K_m) and K_m concentrations of dTTP, significant synergy was noted in the inhibition of RT RDDP by the 4:1 combination of UC84 and UC38. The combination curve was coincident with that described by UC38 alone (Figure 2C), even though the concentration of UC38 in combination with UC84 was only 20% of that in assays containing UC38 alone. Under these conditions, the calculated combination indices were substantially lower than 1.0, with CI_{50}, CI_{70}, and CI_{90} values all approximately 0.40 (Table 1).

The inhibitory potency of UC38 and UC84 was relatively unaffected by alteration of the assay conditions. The IC_{50} for UC38 was invariant, whereas that for UC84 varied by only about 2-fold under the three different assay conditions used (Table 1). Hill plots of the inhibition data, obtained under each of the assay conditions described earlier, were linear with slopes ranging from 0.8 to 1 (Figure 3A−C).

Synergistic inhibition of RT RDDP activity was also noted when UC84 and UC38 were combined in a 1:1 molar ratio. With high concentrations of poly(rA)−oligo(dT) (25 μg/mL; approximately 5 times K_m) and low concentrations of dTTP

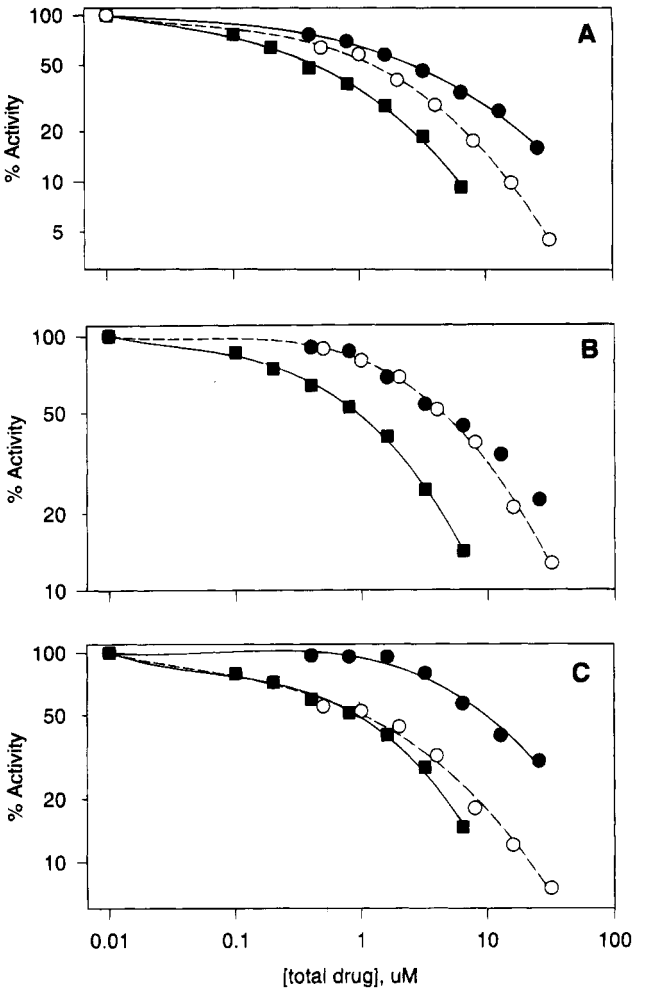

FIGURE 2: Effect of primer/template and dNTP concentrations on the inhibition of HIV-1 RT RDDP activity by various concentrations of UC84 alone (●), UC38 alone (■), and a 4:1 molar combination of UC84:UC38 (○). The assays used poly(rA)−oligo(dT) and dTTP as P/T and substrate, respectively. (A) K_m P/T with K_m dTTP; (B) K_m P/T with $5K_m$ dTTP; (C) $5K_m$ P/T with K_m dTTP. The K_m values were 5 μg/mL for the poly(rA)−oligo(dT) and 5 μM for dTTP. The data shown are averages of duplicate determinations from a representative experiment.

(5 μM; approximately K_m), the combination index was 0.4 (Table 2), which is identical to that obtained with the 4:1 molar ratio combinations of UC84:UC38.

Effect of Primer/Template Identity on the Inhibition of RT by Carboxanilide Compounds. HIV-1 reverse transcriptase can use a variety of homopolymeric and heteropolymeric primer/templates (Cheng et al., 1987). We found that the identity of the P/T significantly influenced the inhibition of RT by UC38 and UC84 (Table 2). With poly(rA)−oligo-$(dT)_{12-18}$ as P/T, the IC_{50} values for UC38 and UC84 were 1.0 μM and 10.2 μM, respectively, depending on the conditions of the assay (Table 1). With poly(rC)−oligo-$(dG)_{12-18}$ as P/T, these inhibitors showed up to 50-fold increases in inhibitory potency, with UC38 inhibiting RT RDDP with an IC_{50} of 0.03 μM and UC84 inhibiting with an IC_{50} of 0.2 μM. The IC_{50} values for the inhibition of RT RDDP activity using a heteropolymeric P/T were 0.3 and 0.15 for UC84 and UC38, respectively. Both UC84 and UC38 inhibited RT DDDP activity (Table 2). As seen for RDDP activity, the inhibitory potency varied with the P/T used, and UC38 was a more effective inhibitor than UC84.

Table 1: Inhibition of RT RDDP Activity by UC84, UC38, and Combinations of UC84+UC38 (4:1 Ratio) with Various Concentrations of Poly(rA)−Oligo(dT) and dTTP

	IC_{50} (μM)					
assay conditions[a]	UC84	UC38	UC84+UC38	CI_{50}[b]	CI_{70}[b]	CI_{90}[b]
K_m P/T + K_m dNTP	4.3 ± 0.4	0.8 ± 0.1	1.2 ± 0.2	1.1 ± 0.06	0.85 ± 0.04	0.64 ± 0.06
K_m P/T + 5K_m dNTP	6.0 ± 0.5	1.0 ± 0.1	4.4 ± 0.6	1.0 ± 0.01	0.94 ± 0.04	0.89 ± 0.14
5K_m P/T + K_m dNTP	10.2 ± 0.7	1.0 ± 0.2	1.0 ± 0.1	0.4 ± 0.04	0.39 ± 0.01	0.45 ± 0.05

[a] The K_m values were 5 μg/mL for the P/T poly(rA)−oligo(dT) and 5 μM for dTTP. [b] Combination indices were calculated according to Chou and Talalay (1984) at 50%, 70%, and 90% inhibition of RT RDDP activity. The values reported are the means ± SD for three separate experiments.

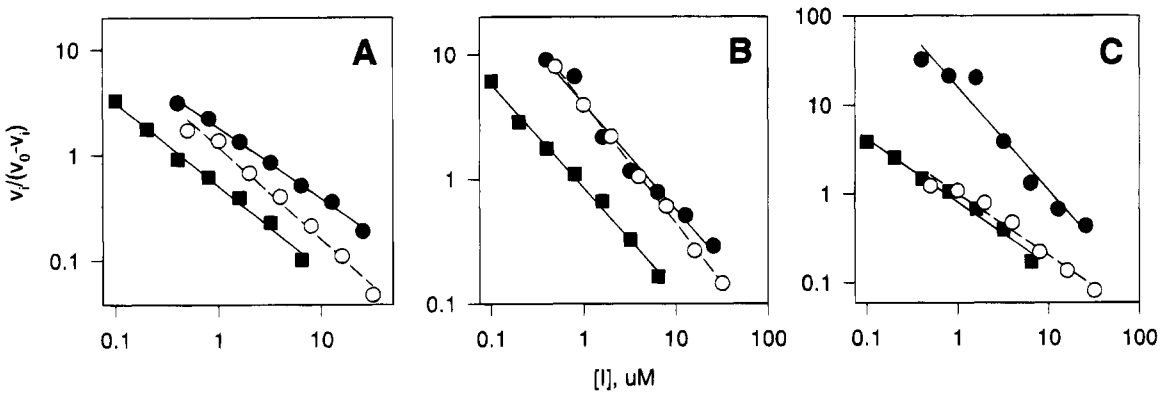

FIGURE 3: Hill plot analysis of the inhibition of HIV-1 RT RDDP activity by various concentrations of UC84 alone (●), UC38 alone (■), and a 4:1 molar contration of UC84:UC38 (○). The assays used poly(rA)−oligo(dT) and dTTP as P/T and substrate, respectively. (A) K_m P/T with K_m dTTP; (B) K_m P/T with 5K_m dTTP; (C) 5K_m P/T with K_m dTTP. The K_m values were 5 μg/mL for the poly(rA)−oligo(dT) and 5 μM for dTTP. The data shown are averages of duplicate determinations from a representative experiment.

Table 2: Inhibition of HIV-1 RT RDDP and DDDP Activities by UC84, UC38, and 1:1 Combinations of UC84+UC38 under Conditions Promoting Synergy[a]

	IC_{50} (μM)			
primer/template	UC84	UC38	UC84+UC38 (1:1)	CI_{50}[b]
		RT RDDP activity		
poly(rA)−oligo(dT)	10.2 ± 1.4	1.0 ± 0.1	1.0 ± 0.2	0.40 ± 0.10
poly(rC)−oligo(dG)	0.2 ± 0.03	0.03 ± 0.01	0.03 ± 0.01	0.53 ± 0.10
heteropolymeric	0.3 ± 0.05	0.15 ± 0.06	0.16 ± 0.02	0.60 ± 0.15
		RT DDDP activity		
poly(dA)−oligo(dT)	*c*	*c*	*c*	not applicable
poly(dC)−oligo(dG)	1.6 ± 0.2	0.6 ± 0.05	0.5 ± 0.1	0.39 ± 0.05
heteropolymeric	6.8 ± 1.2	1.9 ± 0.3	2.0 ± 0.2	0.63 ± 0.07

[a] The values reported are the means ± SD for three or four separate experiments. Assays were performed with 5K_m P/T and K_m levels of dNTP substrate. [b] Combination index was calculated according to Chou and Talalay (1984) at 50% inhibition of RT activity. [c] RT was inactive with this primer/template in either the presence or absence of UC84 or UC38.

Effect of Primer/Template Identity on the Observation of Synergy with Combinations of UC84 and UC38. The detailed synergy experiments described earlier (Figures 1 and 2) were obtained in assays using poly(rA)−oligo(dT) as P/T. Since the apparent affinity of RT for the UC inhibitors is strongly dependent on the identity of the P/T used in the assay of RT DNA polymerase activity, we carried out experiments to determine whether P/T identity affected synergistic inhibition of RDDP by combinations of UC84 and UC38. Kinetic parameters for all P/T−dNTP combinations were determined in order to allow the alteration of assay conditions similar to those used with poly(rA)−oligo$(dT)_{12-18}$ and dTTP (data not shown). In all cases, significant synergy in the inhibition of RT polymerase activity was noted with combinations of UC84 and UC38 in assays employing 5K_m P/T and K_m levels of dNTP (Table 2). Synergistic inhibition of RT RDDP and DDDP activities by combinations of the carboxanilides UC84 and UC38 thus appears to be a general phenomenon.

Synergistic Inhibition of HIV-1 Replication by Combinations of UC84 and UC38. The synergistic inhibition of RT DNA polymerase activity *in vitro* by combinations of UC84 and UC38 suggested that combinations of these two structurally similar nonnucleoside compounds might also exhibit synergy in inhibiting HIV-1 replication in infected cells. We therefore examined the ability of UC84, UC38, and a 1:1 molar combination of UC84+UC38 to inhibit HIV-1 replication in the MT-4 lymphoid cell line and in cord blood mononuclear cells (CBMC). Infection of MT-4 cells, in the absence of antiviral compounds, resulted in extensive cytopathic effects, as determined from the levels of syncytium formation and cell death within 3−4 days after infection (Figure 4). Both UC84 and UC38 alone (at 0.2 μM) delayed viral replication, with significant syncytium formation evident only after 7 days postinfection. However, 0.2 μM of a 1:1 molar combination of UC84+UC38 effectively delayed syncytium formation for up to 10 days postinfection (Figure 4).

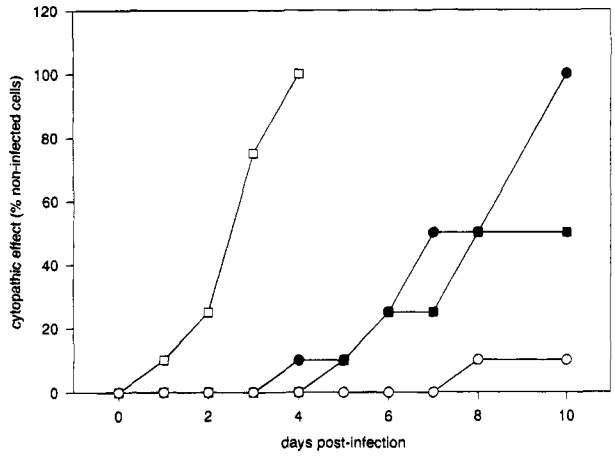

FIGURE 4: Inhibition of HIV-1-mediated cytopathicity in MT-4 cells by UC84 and UC38. MT-4 cells (3×10^5 cells/mL) were infected with HIV-1 (multiplicity of infection, 0.2) for 1 h. Residual virus was washed out, and the cells were resuspended in medium containing no drug, UC84, UC38, or a 1:1 molar combination of UC84+UC38. Cells were examined daily by microscopy. Every 2 days, one-half of the culture medium was removed and replaced with fresh medium containing the appropriate concentration of drug: (□) MT-4 cells cultured in the absence of drug; (●) cells cultured in the presence of 0.2 μM UC84; (■) cells cultured in the presence of 0.2 μM UC38; (○) cells cultured in the presence of 0.2 μM total concentration of a 1:1 molar combination of UC84+UC38. Cytopathicity was assessed by microscopic determination of syncytium formation, as described in Materials and Methods.

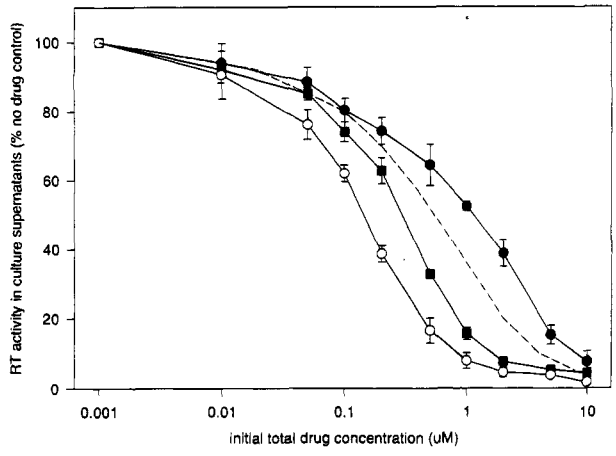

FIGURE 5: Synergistic inhibition of HIV-1 replication in cord blood mononuclear cells by combinations of UC84 and UC38. CBMC were isolated and preactivated as described in Materials and Methods. Activated CBMC (5×10^5 cells/mL) were infected with HIV-1 (multiplicity of infection, 1.0) for 1 h, and then residual virus was washed off and the infected cells were cultured in fresh medium containing the indicated concentrations of UC84 (●), UC38 (■), or a 1:1 molar combination of UC84+UC38 (○). At day 2, one-half of the medium was replaced by fresh medium without drug. This process was repeated on day 4. Aliquots of the culture supernatants were harvested on day 6 and assessed for the presence of HIV-1. The dashed line is calculated (Chou & Talalay, 1984) for *additive* inhibition by combinations of UC84+UC38, using the experimentally determined inhibition constants for each drug alone. The data shown are the means ± SD of triplicate determinations from a representative experiment.

More quantitative observations were obtained in studies of the ability of the carboxanilides alone and in combination to inhibit HIV-1 replication in CBMC (Figure 5). In these experiments, UC84 and UC38, alone or in a 1:1 molar combination, were present at the concentrations shown in Figure 5 only for the first 2 days postinfection. After 6 days postinfection, aliquots of culture supernatants were assessed for the presence of HIV-1 by assay of RT RDDP activity. IC_{50} values of about 0.4 μM for UC38 and 1 μM for UC84 were noted. However, the IC_{50} value for the 1:1 molar combination of the two drugs was approximately 0.1 μM, which is significantly lower than the values obtained for each drug alone. The combination index at 50% inhibition for the 1:1 combination of UC84+UC38 was calculated to be 0.40, indicating an exceptionally high degree of synergy in the inhibition of HIV-1 replication.

DISCUSSION

Although a number of drugs targeted against HIV RT have been approved for clinical use, the toxicities of these compounds generally limit their clinical utility. Moreover, therapeutic use of these drugs individually is also complicated by the rapid development of viral drug resistance (Richman, 1993). Thus, there is a critical need to develop new approaches in anti-HIV chemotherapy. One possibility is the use of combination chemotherapy, an approach that has been successful in the treatment of other diseases. Ideally, combinations of antiviral agents should include inhibitors that bind to different sites on the viral protein target. This would allow simultaneous binding of the inhibitors to the enzyme and possibly result in additivity or synergy in the inhibition of enzyme activity. In addition, appropriate combinations of antiviral agents may slow the development of viral strains resistant to the drugs.

Combinations of dideoxynucleoside triphosphate inhibitors or combinations of nonnucleoside inhibitors showed no synergy in inhibiting RT RDDP activity *in vitro* (Balzarini et al., 1991; White et al., 1993; Yuasa et al., 1993). However, synergistic inhibition of HIV-1 replication has been reported for combinations of dideoxynucleoside inhibitors with phosphonoformate (Kong et al., 1991) and for combinations of dideoxynucleoside inhibitors with nonnucleoside RT inhibitors (Yuasa et al., 1993; Buckheit et al., 1993), although others have failed to observe synergy with the latter combinations (Balzarini et al., 1992). Interestingly, combinations of AZT and ddC appeared to synergistically inhibit viral replication in cell culture (Dornsife et al., 1991; Eron et al., 1992). However, this may be related to the possibility that AZT, as the monophosphorylated derivative, may also inhibit another viral target in addition to RT polymerase activity (Tan et al., 1991; Zhan et al., 1994).

The rationale for the present work stems from our observation that certain carboxanilide nonnucleoside inhibitors interact with mechanistically distinct forms of HIV RT (Fletcher et al., 1995). The RT polymerase reaction pathway follows an ordered mechanism (Majumdar et al., 1988; Kedar et al., 1990; Kati et al., 1992; Reardon, 1993), with RT first binding P/T to form the RT−P/T complex. This complex then binds dNTP to form the RT−P/T−dNTP ternary complex. The carboxanilide derivative nonnucleoside inhibitors UC84 and UC38 bind to the same NNI binding site on RT and are thus mutually exclusive inhibitors (Chou & Talalay, 1984). UC84 exclusively binds to free RT and the RT−P/T complex, whereas UC38 almost entirely binds to the RT−P/T−dNTP ternary complex (Fletcher et al., 1995). We reasoned that if the different enzyme mechanistic forms were present in the *in vitro* RT DNA polymerase assay, then

combinations of UC84 and UC38 might demonstrate synergy in the inhibition of this activity. Indeed, the combination of UC84 and UC38 showed clearly synergistic inhibition that was highly dependent on the relative concentrations of P/T and dNTP employed in the *in vitro* RDDP assay.

Complete mechanistic details of the inhibition of RT by carboxanilide compounds are not yet known. Nonetheless, by assuming that the binding of UC84 and UC38 is mutually exclusive, the observed synergy may be considered in terms of the RT forms that would be present under various concentrations of P/T and dNTP. When P/T and dNTP are at their respective K_m levels, the predominant enzyme forms would be free RT and RT−P/T, with a low level of ternary complex. This would favor the binding of UC84 and minimize binding of UC38. It is interesting to note that UC84 had its greatest inhibitory potency under these conditions (Table 1). Inhibition of RDDP by combinations of UC84 and UC38 under these conditions showed only a small degree of synergy. With K_m levels of P/T and high concentrations of dNTP, the RT−P/T−dNTP ternary complex would predominate, favoring binding of UC38. Indeed, under similar conditions, only UC38 afforded significant photoprotection of RT against inactivation by a nevirapine photolabel (Fletcher et al., 1995). Although the inhibition curve for the UC38+UC84 combination was coincident with that described by UC84 alone (Figure 2B), it must be emphasized that UC38 provided only a small proportion of the total inhibitor concentration in the UC38+UC84 combination experiments. If the RT activity data for the inhibitor combination in Figure 2B are plotted against the concentration of UC38 in the combination, the resulting curve is coincident with that described by the data for UC38 alone (results not shown). This implies that only UC38 provides significant inhibition of RT RDDP activity under conditions of low P/T and high dNTP concentrations.

Significant synergy of inhibition by combinations of UC84 and UC38 was noted only in assays employing high concentrations of P/T and K_m levels of dNTP. Under these conditions, both the RT−P/T binary complex and the RT−P/T−dNTP ternary complex would be present. These are the enzyme mechanistic forms that bind UC84 and UC38, respectively. Only under these conditions could both UC84 and UC38 protect RT from photoinactivation by the nevirapine photolabel (Fletcher et al., 1995). Binding of UC38 to the RT−P/T−dNTP ternary complex would "back up" the RDDP reaction sequence, allowing the accumulation of the RT−P/T binary complex to which UC84 would bind. Thus, under these conditions, dissociation of both UC84 and UC38 from their respective complexes would be necessary for observable catalysis.

Combinations of UC38 and UC84 gave synergistic inhibition of both RT RDDP and DDDP activities *in vitro* when assayed with heteropolymeric P/T. Such a P/T likely reflects more accurately the situation in virus-infected cells. This suggested that combinations of the carboxanilide NNRTI might exhibit synergy in inhibiting HIV-1 replication in infected cells. As seen in Figures 4 and 5, UC38+UC84 combinations provided excellent synergy in inhibition of HIV-1 replication in both MT-4 cells and CBMC. Synergistic inhibition of RT DNA polymerase activity *in vitro* was noted under relatively restricted conditions of P/T and dNTP. The actual levels of viral genomic RNA P/T and RT in HIV-infected cells are not well characterized. However, it is interesting to note that retrovirus particles, and thus virus-infected cells, contain a substantial excess of RT relative to viral genomic RNA (Panet et al., 1975; Huang et al., 1994). The role of this excess RT, if any, is unknown. It is also likely that the level of dNTP will exceed that of the viral P/T. Thus, the predominant mechanistic forms of RT in virus-infected cells might be free RT and the RT−P/T−dNTP ternary complex, which selectively bind UC84 and UC38, respectively (Fletcher et al., 1995). The observed synergy in inhibition of viral replication by UC84+UC38 combinations might imply that the excess free RT in infected cells may in fact play an important role in viral replication.

We are certain that the illustrated inhibition curves with CBMC (Figure 5) reflect a reduction in viral replication, and not merely the inhibition of RT by residual drug in the culture supernatants, for several reasons. First, the IC_{50} values for UC84 and UC38 determined in this experiment are significantly different from those determined by *in vitro* assay of purified RT RDDP and DDDP (see Table 2). This is even more obvious when one considers that, under the conditions of the experiment, drug concentrations at the time of assay would be at a maximum of 25% of the starting levels indicated for Figure 2. Second, we found no inhibition of the RDDP activity of purified recombinant p51/p66 RT added to these culture supernatants. Finally, viral p24 antigen levels in selected culture supernatants effectively mirrored the inhibition profiles obtained by assay of HIV-1 RT activity (data not shown).

We believe that our studies provide the first demonstration of synergistic inhibition of HIV-1 replication by structurally similar nonnucleoside inhibitors. The synergy between UC84 and UC38 may be due to interaction of these inhibitors with different mechanistic forms of RT in the infected cell. However, complete mechanistic details underlying the synergy between UC84 and UC38 must await more extensive information concerning reverse transcription in HIV-infected cells. Nonetheless, the identification of additional combinations of structurally similar nonnucleoside inhibitors that show synergy in inhibition of HIV-1 replication may provide a most useful direction for the development of new therapeutic anti-HIV strategies. For example, combinations of synergistic NNRTI pairs such as UC84 and UC38 with dideoxynucleoside inhibitors such as AZT or 3TC may provide a significant degree of inhibition of HIV-1 replication compared to those drug combinations used to date. In addition, such combinations may also result in a significant delay in the appearance of drug-resistant viral strains, compared to that seen with the use of single drugs. Studies to assess this possibility *in vitro* are currently underway in our laboratory.

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