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Synergistic Inhibition of HIV-1 Reverse Transcriptase by Combinations of Chain-Terminating Nucleotides

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ABSTRACT: Synergistic inhibition of HIV replication in cell culture has been reported for many combinations of reverse transcriptase inhibitors. However, the biochemical basis underlying this interaction is in most cases unknown. It has been previously shown that combinations of L-697,661 or U-90152s with AZT or ddC synergistically inhibit HIV-1 replication in cell culture. The combination of AZT with ddC is also favorable with respect to the inhibition of viral replication. However, the corresponding combinations showed no synergy in inhibiting enzyme activity when tested on conventional polymerase assays using homo- or heteropolymeric RNA and DNA as template. Data obtained suggest that amplification of the effect of chain terminators, a consequence of the high potential number of termination sites present on the template, override the synergistic effect expected for the combination of two independent nucleotide analogs. When a saturating amount of enzyme over template:primer was used, and a single site on the template was available for each chain terminator, the combination of AZTTP and ddCTP synergistically inhibited enzyme activity, whereas, as expected, the combination of AZTTP and ddTTP behaved as merely additive. Under similar conditions the combination of U-90152s and AZTTP was also synergistic. These results suggest that synergy found in antiviral assays with combinations having nucleosidic inhibitors is not related to the synergistic inhibition of reverse transcriptase and might be due to the presence in the viral population of virus strains with different sensitivity to the inhibitors.

Human immunodeficiency virus type 1 (HIV-1)[1] reverse transcriptase (RT) is responsible for the conversion of viral genomic RNA into double-stranded DNA that is then integrated into the infected host genome. Numerous compounds that inhibit DNA polymerase activity of RT have been described. They can be divided into two broad classes. The first group, that of nucleoside analogs, includes dideoxynucleoside compounds, such as ddC and AZT, that inhibit viral replication by acting as chain terminators of DNA synthesis (Parker, 1991). The second group, that of non-nucleoside reverse transcriptase inhibitors, includes a large number of structurally dissimilar hydrophobic compounds, such as nevirapine (Merluzzi et al., 1990), TIBO (Pauwels et al., 1990), pyridinone derivatives (Goldman et al., 1991) and bisheteroarylpiperazine derivatives (Romero et al., 1991), which bind to a site on the RT palm subdomain adjacent to, but distinct from, the polymerase active site (Kohlstaedt et al., 1992).

Much research has been aimed directly at finding combinations of various HIV inhibitors with therapeutic purposes. Synergistic inhibition of HIV replication in cell cultures has been reported for many combinations of RT inhibitors. These combinations include mixtures of nucleoside analogs (Dornsife et al., 1991; Johnson et al., 1991; Eron et al., 1992; Smith et al., 1993; Merril et al., 1996; Bridges et al., 1996) and combinations of dideoxynucleoside inhibitors with non-nucleoside inhibitors (Goldman et al., 1991; Richman et al., 1991; Buckheit et al., 1994, 1995; Pauwels et al., 1994; Merril et al., 1996). In contrast with these observations, several studies have shown that combinations of dideoxynucleoside triphosphate inhibitors, as well as most combinations of non-nucleosidic inhibitors with nucleosidic inhibitors, showed no synergy in inhibiting RT activity *in vitro* (Tramontano & Cheng, 1992; Balzarini et al., 1992; White et al., 1993; Buckheit et al., 1994; Gu et al., 1995a); it was then concluded that inhibition of HIV RT was not responsible for the synergy found in the inhibition of viral replication (White et al., 1993; Parker et al., 1993; Bridges et al., 1996).

It has been proposed that synergistic inhibition of RT would require simultaneous binding of the inhibitors to the enzyme. Since chain terminators bind to a common catalytic site, it is usually assumed that their combination cannot result in synergistic inhibition of the enzyme activity (Balzarini et al., 1992; White et al., 1993; Gu et al., 1995a). This view, however, is oversimplified. Two inhibitors that bind to independent sites may not produce independent inhibitory effects. For example, two inhibitors can affect indirectly the binding of each other by altering substrate affinity (Palatini, 1983). Conversely, two inhibitors that cannot bind simultaneously to the enzyme may give synergistic inhibition. It has been shown that a combination of two structurally related non-nucleoside inhibitors results in synergistic inhibition of HIV-1 RT (Fletcher et al., 1995). Although both inhibitors bind to the same site on reverse transcriptase, the binding is directed to different mechanistic forms. It should also be expected that the combination of two chain terminators that compete with different natural nucleotides might

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[1] Abbreviations: HIV, human immunodeficiency virus; RT, reverse transcriptase; *I,* interaction index; AZT, 3′-azido-3′-deoxythymidine; AZTTP, AZT triphosphate; ddC, 2′,3′-dideoxycytidine; ddCTP, ddC triphosphate; TIBO, tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepine-2(1*H*)-thione; ddTTP, 2′,3′-dideoxythymidine triphosphate; ddATP, 2′,3′-dideoxyadenosine 5′-triphosphate.

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Chart 1: DNA Duplexes[a]

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3′-AGGGACAAGCCCGCGGTGACGAGCACACAGCAGGTTTT    -5′
5′-TCCCTGTTCGGGCGCCAC                        -3′
38:18-mer

3′-AGGGACAAGCCCGCGGTGAGTCTATCTAAAAGGTGT  -5′
5′-TCCCTGTTCGGGCGCCAC                    -3′
38:16-mer
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[a] The nomenclature used refers to the length of the template:primer. The 38:18-mer was designed to allow the incorporation of dATP only when the synthesis of the primer is finished.

result in synergistic inhibition of the enzyme, since the number of potential termination sites in an heteropolymeric template would be increased. It has been demonstrated that the presence of one of these analogs does not affect the incorporation of a second analog into the DNA chain by the HIV-1 RT (White et al., 1993). In addition, competitive inhibition exerted by chain terminators with respect to the natural deoxynucleoside triphosphates seems to be irrelevant to the mechanism of action of these compounds (Reardon, 1992; Gu et al., 1995b).

It has been previously shown that combinations of L-697,661 or U-90152s with AZT or ddC synergistically inhibit HIV-1 replication in cell culture (Goldman et al., 1991; Chong et al., 1994). Synergistic inhibition of HIV replication has also been reported for combinations of AZT with ddC (Dornsife et al., 1991; Eron et al., 1992). We analyzed the interaction between these inhibitors. Results obtained led to the observation that, when saturating amounts of enzyme with respect to template:primer were used and only a potential site of termination was available for each chain terminator, synergistic inhibition of RT activity took place. On the other hand, if many potential sites of termination are available on the template, the effect of the nucleotide analog is amplified, and the combination of the two chain terminators would have no advantage over the use of an equivalente concentration of a single chain terminator. As far as we know, this is the first report of a synergistic inhibition of enzyme activity by combinations of two nucleoside analogs. The relevance of these findings to the combined chemotherapy is discussed.

EXPERIMENTAL PROCEDURES

Chemicals. Non-radiolabeled deoxynucleoside triphosphates, dideoxynucleotides, and the homopolymeric template and primers poly(rA), $(dT)_{10}$, and poly(rC):$(dG)_{12-18}$ were purchased from Pharmacia. AZTTP and [α-^{35}S]dTTP were from Du Pont-New England Nuclear, and [α-^{35}S]dGTP, [α-^{35}S]dCTP, and [α-^{35}S]dATP were obtained from Amersham. Nonidet P-40 was purchased from Boehringer-Mannheim. Non-nucleoside inhibitors U-90152s and L-697,661 were kindly provided from Upjohn Laboratories (Kalamazoo, Michigan). DE-81 paper was from Whatman, glass fiber filters were obtained from Skatron, and Multiscreen glass fiber filter plates were purchased from Millipore.

Oligodeoxynucleotides. FPLC purified oligodeoxynucleotides were obtained from Pharmacia. Oligonucleotide duplexes were formed by mixing equimolar template (36-mer or 38-mer) and primer (18-mer) in 20 mM Tris-HCl, pH 7.5. This mixture was heated to 70 °C for 15 min and slowly cooled to room temperature. Sequences for the 36:18-mer and 38:18-mer are shown in Chart 1.

HIV-1 Reverse Transcriptase. Recombinant p66-p66 RT was expressed in *Escherichia coli* from a clone supplied by Dr. W. G. Tarpley from Upjohn Laboratories. The enzyme was purified by immobilized metal affinity chromatography as described (Chattopadhyay et al., 1992).

Reverse Transcriptase Assay. Enzyme activity was measured in a total volume of 50 μL using a standard reaction mixture containing 50 mM Tris/HCl (pH = 8.3), 100 mM KCl, 0.05% Nonidet P-40, 7 mM $MgCl_2$, 2 mM EGTA, and 2 mM DTT. Reactions containing template:primer, [α-^{35}S]-labeled deoxynucleotides, and inhibitors were preincubated for 2 min and started by adding the enzyme. When homopolymeric RNA was used as template:primer for the combination experiments, reactions were terminated after 10 min by the addition of 50 μL of 10% trichlororacetic acid and incorporation of radiolabeled precursor was determined by collecting the precipitates on glass fiber filters, either on a cell harvester (Skatron instruments) or on a Multiscreen glass fiber plate (Millipore). Filters were washed with 5% trichloroacetic acid, dried, and counted. Both methods gave the same results. For the rest of the assays, reaction was stopped by adding 10 μL of 0.2 M EDTA, pH = 8. Extended primer was bound to DE81 filter paper (Whatman) by adding 10 μL of the reaction mixture on the filter and dried under an infrared lamp. Unincorporated nucleoside triphosphates were removed by washing the filter three times with 0.5 M sodium phosphate (pH = 7) and twice with 95% ethanol. Filters were dried, and radioactivity was quantified in a liquid scintillation counter.

Analysis of Interaction between Inhibitors

Dose−response curves for each inhibitor alone were obtained within a wide range of effects by fitting experimental data to eq 1 by unweighted nonlinear regression:

$$E = \frac{1}{1 + \left(\frac{D_m}{D}\right)^m} \tag{1}$$

where D represents the doses of the inhibitor when tested alone, D_m the doses of the inhibitor giving 50% of inhibition (IC_{50}), and m a parameter giving the sigmoidicity of the dose−response curve (Martinez-Irujo et al., 1996) using the commercial available fitting program Grafit (Erithacus software).

Interaction between inhibitors was evaluated by means of the interaction index (I):

$$I = \frac{d_1}{D_1} + \frac{d_2}{D_2} \tag{2}$$

D_1 and D_2 being the doses of agents 1 and 2 individually producing the same effect as the combination ($d_1 + d_2$) (Berenbaum, 1989). When $I = 1$, agents in the combination do not interact; if $I > 1$ the combination is antagonistic: and if $I < 1$ the combination is synergistic. In preliminary experiments, inhibitors were mixed in a fixed molar ratio. This ratio usually reflects the different potency of the compounds, ensuring in this way that both inhibitors significantly contribute to the total inhibition. In these experiments three different template:primers were used: poly(rA):$oligo(dT)_{10}$ (10 μg/mL and 1.5 μM, respectively) in the presence of 1.5 nM RT and the indicated amounts of

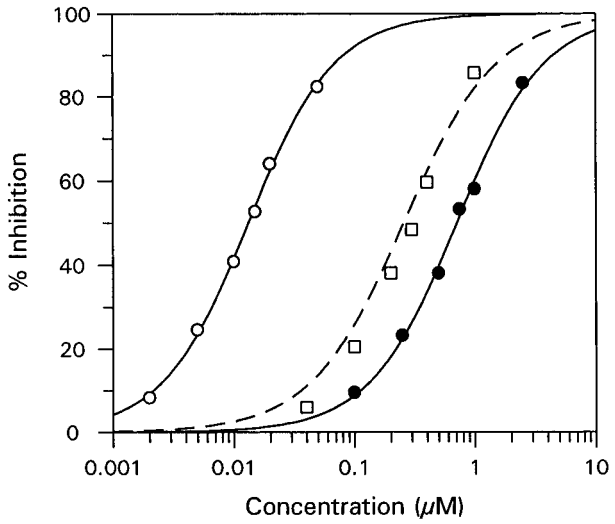

FIGURE 1: Dose−response curves for the inhibition of reverse transcriptase by AZTTP (○), U-90152s (●), and their combination in a proportion 1:29 (□). Reaction assay contained 10 μg/mL and 1.5 μM of poly(rA) and oligo(dT)$_{10}$, respectively, 10 μM [α-^{35}S]-dTTP, and 1.5 nM enzyme. Curves for AZTTP and U-90152s were obtained by fitting experimental data to eq 1 by nonlinear regression as explained in Experimental Procedures. For AZTTP Dm = 0.013 μM, m = 1.22, and for U-90152s Dm = 0.695 μM, m = 1.19. Dashed curve represents the theoretical result expected if no interaction were present and was calculated as described (Martinez-Irujo et al., 1996). This curve properly fits experimental data, showing that the effect of both inhibitors was mutually exclusive.

[α-^{35}S]dTTP; poly(rC):oligo(dG)$_{12-18}$ (20 μg/mL) with 1.5 nM RT and [α-^{35}S]dGTP as substrate; and 38:18-mer (300 nM) with 76 nM RT and 10 μM of each dATP, dGTP, dTTP, and [α-^{35}S]dCTP. If synergy was found when applying this analysis, interaction was corroborated by testing the inhibitors in a checkerboard arrangement as explained in the text. The expected effect of each combination if no interaction was present was calculated by iteration using eqs 1 and 2 as described (Martinez-Irujo et al., 1996). In a different series of combination experiments, we tried to compare the inhibition referred to the number of nucleotides incorporated by the enzyme in the absence of inhibitors with that calculated from the number of completed chains in their presence. In these assays the 38:18-mer was used. This template:primer allows the incorporation of [α-^{35}S]dATP only when the synthesis of the primer is finished, so their incorporation can be taken as a measure of the number of completed chains. Four Ts rather than a single T were present in the template in order to increase the sensitivity of the assay. As control, the inhibition of incorporation of [α-^{35}S]dTTP was measured under the same conditions.

RESULTS

Combination of Reverse Transcriptase Inhibitors Using Different Template:Primers. We analyzed the effect of combining AZTTP and U-90152s using poly(rA)-oligo(dT)$_{10}$ as template primer. Inhibitors were mixed in a constant molar ratio, and the effect of the mixture was measured. The inhibition caused by the combination matched the expected result if the effect of both inhibitors was mutually exclusive (Figure 1). The same result was obtained if poly(rC) or a heteropolymeric DNA oligonucleotide were employed as template for the polymerization reaction (Table 1). Although we have performed several assays varying different conditions, such as proportion of inhibitors, substrate concentration, or the template primer used, in no case synergy was observed. Using an unrelated non-nucleoside compound, such as L-697,661, the same conclusion was reached. Interestingly, the combination of AZTTP and ddCTP that synergistically inhibits virus replication did not interact in the RT assay. These results are in agreement with previous reports showing that combinations of RT inhibitors, that synergistically inhibit virus replication *in vitro*, do not behave similarly in the enzymatic assay.

Factors Affecting the Potency of Chain Terminators in Vitro and in Vivo. It has been proposed that conditions used *in vitro* may not reflect those of the environment where reverse transcription in viral replication takes place. A fundamental difference is that a substantial excess of RT over template:primer exists *in vivo*. In antiviral assays the depletion of viral genome, as a consequence of chain termination, appears to be the crucial event involved in the inhibition of viral replication. However, this factor is not important in conventional steady-state kinetic assays where catalytic amounts of enzyme with respect to template:primers are used. We have examined the effect of increasing enzyme concentration on the inhibition of RT activity by AZTTP. As illustrated in Figure 2, inhibition decreased as concentration of enzyme increased. In addition, the same figure shows that AZTTP significantly inhibited nucleotide incorporation at concentrations that are 2−3 orders of magnitude lower than the concentration of dTTP. It has been demonstrated that the affinity of RT toward AZTTP and dTTP was nearly the same, and that dTTP was as efficient substrate as AZTTP for the enzyme (Reardon, 1992). Accordingly, this graph does not represent the titration of enzyme by the inhibitor, given the large excess of dTTP over AZTTP, nor does it represent the depletion of the substrate (template:primer) by the inhibitor, since the concentration of oligo(dT)$_{10}$ was in the micromolar range. Since the total amount of dTMP incorporated in the absence of AZTTP was held constant in these experiments, the main difference in using a high or a low concentration of RT is the number of times that enzyme must dissociate from the chain terminated primer to go on with DNA synthesis. Under these conditions the probable mode of inhibition is a consequence of the processivity of the enzyme, since the polymerase remains bound to the terminated primer until it dissociates and binds to another template:primer. It should be noted that similar IC$_{50}$'s for AZTTP, about 75 nM, were obtained for all concentrations of enzyme if we allowed the reactions to proceed for the same period of time (1 min). Results presented in Figure 2 indicate that the use of a catalytic amount of enzyme over template:primer increases the inhibition exerted by a chain terminator as a consequence of the dissociation rate of the enzyme from the template:primer. However, this factor seems to be irrelevant to viral replication, where enzyme concentration exceeds that of template:primer.

Another difference between enzymatic and viral assays is that the parameter which is of potential interest in evaluating the potency of a chain terminator inhibitor *in vivo* is the number of chains completed in the presence of the inhibitor. However, what is usually determined in enzymatic assays is the amount of labeled nucleotide incorporated in the presence of a chain terminator. We measured the potency of AZTTP and ddTTP as inhibitors using 350 nM 38:18-mer, 7.6 nM of RT, and 10 μM of the four dNTP as substrate.

Table 1: Interaction Index for Combinations of Reverse Transcriptase Inhibitors against HIV-1 RT[a]

drug combination	ratio	template:primer	[dNTP]	interaction index at % inhibition			
				25%	50%	75%	90%
U-90152s + L-697,661	1:1	poly(rA):oligo(dT)$_{10}$	10 μM	1.10	1.02	0.95	0.88
	1:1	poly(rC):oligo(dG)$_{12-18}$	1 μM	1.20	1.19	1.10	1.08
+ AZTTP	14:1	poly(rA):oligo(dT)$_{10}$	0.25μM	1.02	0.93	0.85	0.78
	29:1	poly(rA):oligo(dT)$_{10}$	10 μM	1.28	1.12	1.02	0.92
	1:1	38:18-mer	10 μM	1.17	1.00	0.93	0.97
+ ddGTP	9:1	poly(rC):oligo(dG)$_{12-18}$	1 μM	1.33	1.01	0.77	0.59
	29:1	poly(rC):oligo(dG)$_{12-18}$	1 μM	1.75	1.24	0.90	0.66
L-697661 + ddGTP	9:1	poly(rC):oligo(dG)$_{12-18}$	1 μM	1.21	1.19	1.16	1.14
	29:1	poly(rC):oligo(dG)$_{12-18}$	1 μM	1.37	1.68	0.85	0.66
AZTTP + ddTTP	2:1	38:18-mer	10 μM	0.80	0.98	1.19	1.48
+ ddCTP	1:1	38:18-mer	10 μM	0.91	0.91	0.92	0.97

[a] Inhibitors were mixed in a constant molar ratio, and interaction index was calculated for each effect level as explained in Experimental Procedures. Interaction index (I) <1, =1, or >1 indicates synergism, no interaction, of antagonism, respectively. This parameter is equivalent to the combination index (CI) proposed by Chou and Talalay for the case of two exclusive inhibitors (Chou & Talalay, 1984; Martinez-Irujo et al., 1996).

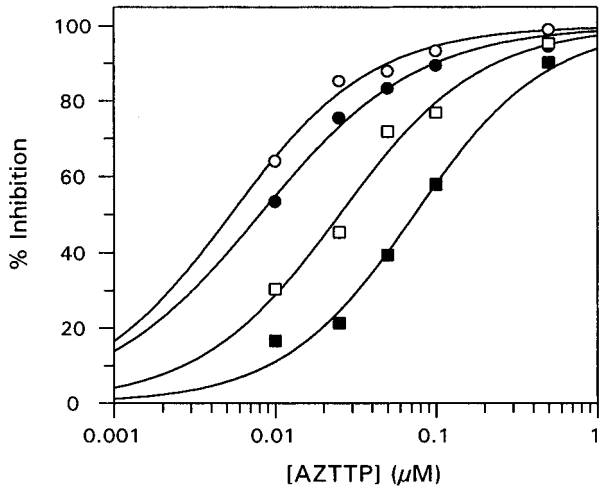

FIGURE 2: Effect of the enzyme concentration on AZTTP inhibition. Inhibition of reverse transcriptase activity by AZTTP was measured in the presence of poly(rA):oligo(dT)$_{10}$, 10 μg/mL and 1.5 μM, respectively, and 10 μM [α-^{35}S]dTTP using four enzyme concentrations: (○) 1.9 nM, (●) 7.6 nM, (□) 30.4 nM, and (■) 76 nM. Each reaction was incubated for 40, 10, 2.5, and 1 min, respectively, ensuring in this way that, in the absence of the inhibitor, the same amount of substrate was incorporated in all reactions (103, 107, 104, and 108 pmol of dTMP, respectively). IC$_{50}$ values for each curve were 0.0055, 0.0079, 0.025, and 0.074 μM.

This template:primer allows the incorporation of [α-^{35}S]-dATP only when the synthesis of the primer is completed (Chart 1). When the labeled substrate was [α-^{35}S]dTTP, 50% of inhibition was reached with 1.45 μM AZTTP or 1.87 μM ddTTP, whereas, if [α-^{35}S]dATP was used instead, these values lowered to 0.71 and 0.93 μM, respectively. If inhibition was referred to the number of chains completed, it was greater, as expected, than if referred to the amount of nucleotides incorporated.

The importance of these factors on the interaction between AZTTP and ddCTP was evaluated using 100 nM 38:18-mer as template:primer, 10 μM of each dTTP, dGTP, dCTP, and [α-^{35}S]dATP and a saturating amount of enzyme (170 nM). Even in this case, more closely resembling *in vivo* conditions, the combination behaved as merely additive, suggesting mutually exclusive inhibition (data not shown). When, under the same conditions, the concentration of dNTP was lowered to 1 μM, the same result was obtained.

Effect of the Number of Potential Sites of Termination on the Inhibition of Chain Terminators. In order to complete DNA synthesis, RT must add in a sequential manner the correct nucleotide at each position. When a chain terminator is incorporated into the growing chain, not only the incorporation of the correct nucleotide is excluded, but the addition of subsequent nucleotides is also blocked. Since the total amount of nucleotides incorporated by RT in each assay depends on the experimental conditions used, the inhibition exerted by a chain terminator might depend on the amount of nucleotides sequentially added by reverse transcriptase in the absence of the inhibitor. To test this hypothesis we have measured the effect of AZTTP on the incorporation of dTTP on poly(rA) depending on the total amount of dTMP incorporated by RT in the absence of the chain terminator. In these experiments a saturating amount of RT over the primer was used, ensuring in this way that enzyme dissociation rate was not contributing to the measured inhibition. Under these conditions, the amount of substrate incorporated is related to the number of nucleotides sequentially added by the enzyme. As shown in Figure 3 there was a rise in inhibition as the number of available sites of termination on the template increased. We measured the interaction between AZTTP and ddCTP under conditions where a single site of termination was available for each inhibitor and a saturating amount of enzyme was used (Figure 4A). Under these conditions, the combination synergistically inhibited RT activity. Interaction indexes calculated from the four combinations resulting from the mixing of 1, 2, 4, and 10 μM AZTTP with 0.4, 0.8, 1.6, and 4 μM of ddCTP, respectively, the diagonal where a constant proportion (2.5:1) between both inhibitors was maintained, were equal to 0.71, 0.69, 0.55, and 0.26 respectively. As reference control, we measured the interaction between AZTTP and ddTTP, two nucleoside analogs competing for the same site of incorporation on the template, using concentrations of ddTTP producing the same effect as those of ddCTP. In sharp contrast with the former case, the effect of combining AZTTP with ddTTP resulted in no interaction (Figure 4B). Interaction indexes calculated for combinations situated on the diagonal of the graph, where a constant proportion of AZTTP to ddTTP (4:1) was maintained, are 1.01, 1.10, 1.04, and 1.02, respectively. The interaction between AZTTP and U-90152s was also analyzed using the same template:primer with [α-^{35}S]dTTP 1 μM as the only substrate. Concentrations of U-90152s giving the same effects as those of ddCTP in Figure 4A were selected. Under these conditions, synergy was also found with interaction indexes ranging from 1.03,

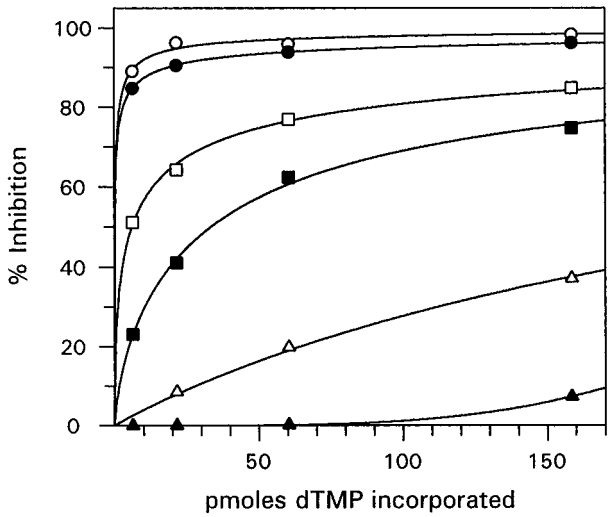

FIGURE 3: Influence of the length of the chain synthesized on the effect of AZTTP. The inhibition of reverse transcriptase activity by six concentrations of AZTTP (○) 1 μM, (●) 0.5 μM, (□) 0.1 μM, (■) 0.05 μM, (△) 0.01 μM, and (▲) 0.001 μM on the poly(rA):oligo(dT)$_{10}$-directed poly(dT) synthesis was measured at four different times of incubation (0.5, 1, 2, and 5 min) in the presence of a saturating amount of enzyme with respect to the primer. Reaction mixture contained in a total volume of 50 μL of 10 μg/mL poly(rA), 30 nM oligo(dT)$_{10}$, 10 μM [α-^{35}S]dTTP, and 100 nM of reverse transcriptase. Inhibition was calculated with respect to the amount of dTMP incorporated by the enzyme in the absence of AZTTP.

at low inhibitor concentrations, to 0.20, when the concentrations were increased.

DISCUSSION

In spite of the growing attention to combined chemotherapy in the treatment of AIDS, the biochemical mechanism underlying the antiviral synergy of combinations of reverse transcriptase inhibitors is in most cases unknown. Most combinations of RT inhibitors, that synergistically inhibit viral replication in cell culture, showed mutually exclusive effects in the inhibition of RT activity *in vitro* (Tramontano & Cheng, 1992; Balzarini et al., 1992; White et al., 1993; Buckheit et al., 1994; Gu et al., 1995a). In agreement with these results we found that, irrespective of the nature of the primer, combinations of two nucleosidic inhibitors, or that of a nucleosidic inhibitor with another non-nucleosidic, resulted in no interaction under conditions usually employed in these assays. However, convincing evidence has been reported showing that the binding of non-nucleoside compounds does not exclude the binding of dNTP to the catalytic site of the enzyme (Spence et al., 1995).

The mechanism of action of chain-terminating nucleotides has been studied in detail (Parker, 1991; Reardon, 1992), but the implications of these findings in combined chemotherapy has not yet been analyzed. The potent inhibition of DNA polymerase activity in enzymatic assays by a chain terminator is related to the slow dissociation rate of the enzyme from the chain-terminated template:primer due to the processivity of the enzyme. This factor, however, seems to be irrelevant *in vivo* where a substantial excess of enzyme over genomic RNA exists (Huang et al., 1994). As shown by several authors, chain terminators are potent inhibitors of viral replication not because they compete very efficiently with their natural deoxynucleotides, but because of the high

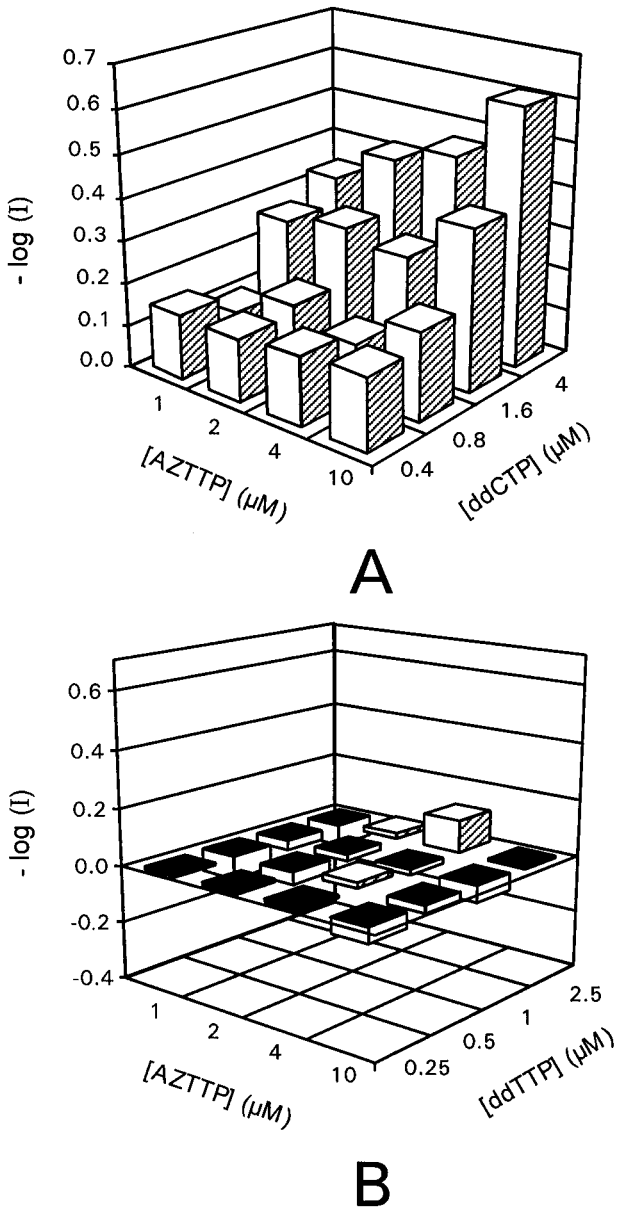

FIGURE 4: Interaction between nucleotide analogs on the inhibition of HIV-1 RT. Reaction mixture contained 50 nM 38:16-mer, 1 μM dTTP, 1 μM [α-^{35}S]dCTP, and a saturating amount of enzyme (100 nM). Under these conditions only two nucleotides, dTTP and dCTP (or their respective analogs), can be sequentially added to the primer. (A) Interaction between AZTTP and ddCTP (for AZTTP, D_m = 0.78 μM, m = 0.93; for ddCTP, D_m = 2.0 μM, m = 0.99). (B) Interaction between AZTTP and ddTTP (for AZTTP, D_m = 0.86 μM, m = 0.98; for ddTTP, D_m = 1.08 μM, m = 0.93). In both cases interaction was assessed by plotting the logarithm of the interaction index for each combination with its sign changed (Martinez-Irujo et al., 1996). Positive values indicate synergy, zero or near-zero values indicate no interaction, and negative values (columns having black bases) indicate antagonism.

number of potential sites of chain termination in the viral genome (Goody et al., 1991; Reardon, 1992). To illustrate this point let us consider a homopolymeric template:primer in the presence of a saturating amount of RT. If we added a mixture of complementary dNTP and a competitive chain terminator, the probability (p) that substrate, and not chain terminator, be incorporated in each step would depend on their relative concentrations and on the efficiency of incorporation of both substrates. We calculated the expected inhibition caused by a chain terminator depending on the number of residues that can be sequentially added by the

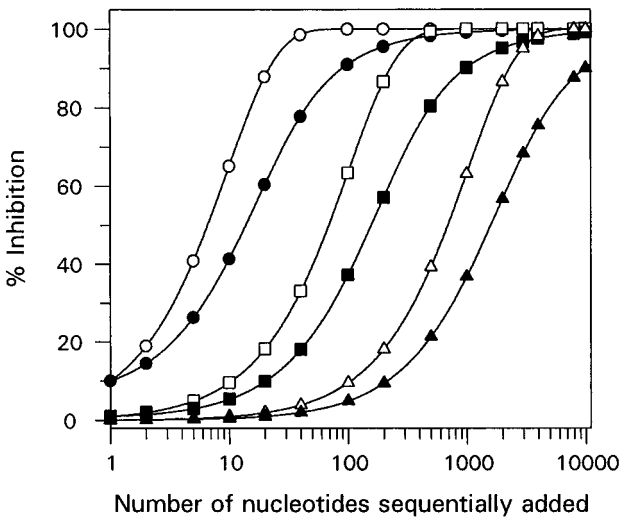

FIGURE 5: Effect of the number of nucleotides sequentially added by the polymerase on the inhibition caused by a chain terminator. Percent of inhibition refers to the number of chains completed in the absence of the inhibitor (open symbols) or to the amount of dNTP incorporated by the enzyme (closed symbols). The inhibition was calculated assuming that the probability of incorporation of the nucleotide analog in each position was equal to 0.1 (○, ●), 0.01 (□, ■), or 0.001 (△, ▲).

polymerase (n). When fractional inhibition (f) was referred to the proportion of chains completed in the absence of inhibitor, the equation employed was

$$f = 1 - p^n \tag{3}$$

However, if the fractional inhibition refers to substrate incorporation, as is usually done when reverse transcriptase activity is measured, the following equation was used (Goody et al., 1991):

$$f = 1 - \frac{p(p^n - 1)}{n(p - 1)} \tag{4}$$

Figure 5 shows that, as expected, inhibition was greater when referred to the first parameter. It should be stressed that even when the probability of incorporation of the chain terminator is very low, complete inhibition of DNA replication could be achieved, provided the template is long enough. This seems to be the most relevant factor *in vivo* where the incorporation of a single chain inhibitor during the polymerization would prevent the completion of DNA synthesis. Although the model used is oversimplified, since it assumes that the probability of incorporation of a chain terminator at all positions is the same, something which, in fact, probably depends on the sequence context (Gu et al., 1995b), it may be useful to understand the mechanism of action of chain-terminating nucleotides. The sensitivity of enzymes carrying mutations conferring substantial viral resistance to chain-terminating nucleotides has been analyzed by single nucleotide incorporation assay (Lacey et al., 1992; Martin et al., 1993; Gu et al., 1994). Some of these enzymes showed reduced sensitivity to these compounds in enzymatic assays. For enzymes carrying mutations conferring virus resistance to AZT, the slight changes seen in the sensitivity to AZTTP of RT account only partly for the significantly reduced sensitivity of the virus carrying such mutations (Lacey et al., 1992; Martin et al., 1993). It is interesting to point out that resistance to a chain terminator can arise from even a very low reduction in enzyme affinity, since this effect will be amplified *in vivo* as a consequence of the existence of thousands of potential termination sites in the viral genome. Substantial differences in the sensitivity to a chain-terminating nucleotide between wild type and mutant reverse transcriptases should be expected if the number of chains completed in the presence of AZTTP is measured using long templates and a saturating amount of enzyme over template: primer, ensuring in this way that enzyme dissociation rate was not contributing to the inhibition being measured.

When the interaction between AZTTP and ddCTP was measured under conditions where only a potential site of termination for each inhibitor exists, synergy between AZTTP and ddCTP was clearly seen, whereas, as expected, the effect of combining AZTTP and ddTTP, two analogs competing with the same nucleotide, was mutually exclusive (Figure 4). It should be stressed that it is the depletion of DNA substrate and not their binding to the enzyme what is responsible for their antiviral activity. As Figure 5 shows, chain terminators are so effective in inhibiting viral replication not because they are potent competitive inhibitors, but for the fact that incorporation of a single chain terminator would preclude the synthesis of genomic DNA. This is an unrecognized point in other combination studies. Papers published to date dealing with combinations of nucleoside analogs assume that two chain terminators cannot be synergistic because they bind to the same site on the enzyme. What is relevant, however, in the effect of this combination is not the simultaneous binding of the chain terminators to the RT, but the fact that their combination could give a more effective depletion of template:primer by increasing the number of sites where the primer can be terminated. Results obtained suggest that amplification of the effect of chain terminators override the synergistic effect expected for the combination of two independent inhibitors. For a combination to be synergistic, it is necessary that the mixture be more effective than the addition of a equivalent dose of a single inhibitor. If the effect of an inhibitor is amplified, as occurs with chain terminators, the combination of two independent inhibitors would be of no advantage over their individual use.

In fact, that could be predicted if the aforementioned factors are taken into account. Let us suppose that we have two chain terminators that compete with different dNTP, such as AZTTP and ddCTP, and that we are measuring their combined effect using a template:primer with the same number of potential sites of termination for each inhibitor; for the sake of clarity we will consider that the relative efficiency of incorporation of each natural deoxynucleotide and their competitive analog is the same. The probability that in the presence of a chain terminator DNA synthesis be completed can be calculated as

$$1 - f = \frac{1}{(1 + D/s)^n} \tag{5}$$

where f is the fractional inhibition, D the concentration (dose) of chain terminator when tested alone, s the concentration of the competing substrate (dNTP), and n the number of potential sites of termination for the chain terminator on the template:primer. Let us now prepare combinations containing both inhibitors holding a 1:1 molar ratio between them and measure their combined effect using an equimolecular

Table 2: Effect of the Potential Number of Sites of Termination for Each Inhibitor (n) on the Interaction between Two Independent Chain Terminators

	interaction index (I)[a]			
% inhibition	$n = 1$	$n = 5$	$n = 10$	$n = 100$
1	1.00	1.00	1.00	1.00
5	0.99	1.00	1.00	1.00
10	0.97	0.99	1.00	1.00
25	0.93	0.99	0.99	1.00
50	0.83	0.97	0.98	1.00
75	0.67	0.93	0.97	1.00
90	0.48	0.89	0.94	0.99
95	0.37	0.85	0.93	0.99
99	0.18	0.77	0.89	0.99
99.9	0.06	0.67	0.83	0.98

[a] Interaction index (I) <1, $=1$, or >1 indicates synergism, no interaction, or antagonism, respectively. This parameter is equivalent to the combination index (CI) proposed by Chou and Talalay (1984) for the case of two exclusive inhibitors.

mixture of the four natural dNTPs. The probability of termination in the presence of both inhibitors ($d_1 + d_2$) will be the product of their respective probabilities of termination, i.e.,

$$1 - f = \frac{1}{(1 + d_1/s_1)^n} \times \frac{1}{(1 + d_2/s_2)^n} = \frac{1}{(1 + d/s)^{2n}} \quad (6)$$

where d_1 and d_2 are the concentration of each inhibitor in the combination ($d_1 = d_2 = d$). From eqs 5 and 6 we can calculate the interaction index (I) for each combination.

$$I = \frac{d_1}{D_1} + \frac{d_2}{D_2} = 2 \times \frac{d}{D} = 2 \times \frac{d/s}{D/s} = 2 \times \frac{\frac{1}{(1-f)^{1/2n}} - 1}{\frac{1}{(1-f)^{1/n}} - 1} \quad (7)$$

Table 2 shows the interaction index (I) obtained for each inhibition level displayed by the combination depending on the number of potential sites of termination for each inhibitor on the template. Two main conclusions can be drawn from the table. First, interaction index decreased as the effect of the combination increased, as expected if the effect of two inhibitors were non-exclusive (Martinez-Irujo et al., 1996). This has a physical meaning. At low concentration of the inhibitor the effect obtained is usually proportional to the amount of inhibitor present. As the concentration of the inhibitor is raised, saturation is more manifest. If we mix two non-exclusive inhibitors at low concentrations, their combined effect is the same as if we were using an equivalent concentration of only one of them. Only when saturation approaches, the combination would be advantageous over the individual use of an inhibitor. For this reason we selected the concentrations of ddCTP and ddTTP in Figures 4A and 4B giving the same inhibition. This allows a direct comparation between their respective interaction index. On the other hand, if interaction is assessed as the difference between actual and expected effects, as it is usually done in antiviral assays (Prichard et al., 1993; Chong et al., 1994), maximum synergy would be observed when inhibitors are combined at rather moderate concentrations. That could induce to think that there is an "optimum" for the combination, when, in fact, it is simply an artifact generated by the way synergy is measured. The reason for this discrepancy is that when several inhibitors are mixed at high concentrations, the effect of each inhibitor alone is usually high, so that the combination of these agents cannot result in an inhibition noticeably higher than that obtained with agents assayed alone (Martinez-Irujo et al., 1996). However, the concentration needed to reach a specific effect can be substantially decreased as a consequence of the interaction. This should be especially taken into account when interactions are studied in order to guide clinical assays, as usually happens in AIDS research where an adequate animal model is not available.

The second conclusion is that synergy for the combination of two independent chain terminators is only seen when the potential number of sites of termination in the template for each inhibitor is very low. If that is not the case, amplification of the effect of the chain terminator will make the interaction index close to 1; the combination will behave as if the effect of both inhibitors was mutually exclusive, something which could be mistakenly taken as if the binding of both inhibitors were mutually exclusive. This result can be interpreted as follows. If only a site, or very few sites, of termination are available on the template for each chain terminator, it would be advantageous to combine them to block DNA elongation. This would reduce the concentration needed to obtain some specific effect as a consequence of the increased number of potential termination sites. On the other hand, if many potential sites of termination are present for a nucleotide analog, its effect is amplified and the combination has no advantage over the use of an equivalent concentration of a single chain terminator. In addition, and depending on the experimental design used, this amplification can be increased by factors that being irrelevant *in vivo* have a considerable effect *in vitro*, such as the slow dissociation rate of the enzyme from the template:primer. In fact, any factor that raises the processivity of the enzyme will increase the effect of a chain terminator. It is unlikely, however, that the synergistic effect observed in Figure 4 could be relevant to the synergy obtained in antiviral assays, given the large number of potential termination sites, possibly thousands, for each chain terminator present in the viral genome. This would not imply that combinations of reverse transcriptase inhibitors cannot synergistically inhibit enzyme activity in a standard reverse transcriptase assay. If the binding of a non-nucleoside RT inhibitor decreases the dissociation rate of the enzyme to the template:primer, their combination with a nucleosidic inhibitor would result in synergy. In fact, although exclusive effects have been found in many combinations of nucleosidic with non-nucleosidic inhibitors (Balzarini et al., 1992; Tramontano & Cheng, 1992; White et al., 1993; Gu et al., 1995a; Carroll et al., 1994), it has been reported that synergistic inhibition of RT activity can occur for some combinations (Romero et al., 1991; Yuasa et al., 1993; Carroll et al., 1994). Many teams are searching for positive interactions between these inhibitors on enzyme activity on the assumption that this synergistic inhibition might be of relevance to the inhibition of the viral replication. We feel that those results must be critically reviewed because the molecular mechanism of these interactions is, in most cases, unknown. Mechanistic studies are needed to discern whether the synergy found is related to parameters, which affect the inhibition measured in enzymatic assays, that are irrelevant to viral replication, such as the rate of dissociation of the enzyme from the template:primer.

The results obtained in this work suggest that synergistic inhibition of RT by combinations of RT inhibitors is not responsible for the synergy found in antiviral assays. Attempts to determine the mechanism of antiviral synergy of combinations of nucleoside analogs have been unsuccessful to date. Combination of another nucleoside with AZT does not appear to affect the metabolism of either nucleoside analog or to significantly affect the levels of the natural triphosphate pools, even when these studies are carried out in HIV-infected cells of the type in which antiviral synergy was measured (Parker et al., 1993; White et al., 1993; Veal et al., 1994; Bridges, 1996). It has also been suggested that AZTTP, or its metabolites, might have a second target in addition to that of inhibiting the RT that would account for their synergistic interaction when combined with other RT inhibitors (White et al., 1993). Increasing evidence is showing, however, that positive interactions are not restricted to combinations containing AZTTP (Bridges, 1996; Merril et al., 1996; Palmer et al., 1996).

We think that the inhibition of the RT must be related to the synergy found by combinations or reverse transcriptase inhibitors on HIV-1 replication. If it were not so, it would be difficult to understand why so many combinations of very different inhibitors of this enzyme synergistically inhibit HIV replication. In fact, it would not be necessary for two inhibitors to synergistically inhibit enzyme activity so that their combination were more effective than the use of each inhibitor alone. If in the viral population there are virus strains with different susceptibility to reverse transcriptase inhibitors, the combination of several inhibitors will be more effective than if used independently. It has been estimated that in each replication cycle, HIV incorporates at least a mutation in their genome. The presence of a single mutation on the HIV-RT can lead to reduced susceptibility, or even total resistance, to an inhibitor. It should be kept in mind that it would not be necessary that a marked resistance to an inhibitor be present in the global population to explain the synergy found in antiviral assays. In particular, synergistic interaction will be diagnosed if both inhibitors do not show cross-resistance, in such a way that each inhibitor is capable of efficiently inhibiting the fraction of the viral population with reduced sensitivity to the other inhibitor. It is then possible that the favorable interaction found in *in vitro* antiviral assays using combinations of inhibitors could in fact be due to the presence in the viral population of virus with different susceptibility to them. This would explain why the type and extent of the interaction depend on the virus isolate tested (Palmer et al., 1996). In any case, it seems likely that this will be an essential factor affecting the interaction between inhibitors *in vivo*, where viral population is by far much more heterogeneous (Coffin, 1995).

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REFERENCES

Balzarini, J., Pérez-Pérez, M. J., San-Félix, A., Camarasa, M. J., Bathurst, I. C., Barr, P. J., & De Clerq, E. (1992) *J. Biol. Chem. 267*, 11831−11838.

Berenbaum, M. C. (1989) *Pharmacol. Rev. 41*, 93−141.

Bridges, E. G., Dutschman, G. E., Gullen, E. A., & Cheng, Y. C. (1996) *Biochem. Pharmacol. 51*, 731−736.

Buckheit, R. W., Jr., White, E. L., Germany-Decker, J., Allen, L. B., Ross, L. J., Shannon, W. M., Janssen, P. A. J., & Chirigos, M. A. (1994) *Antiviral Chem. Chemother. 5*, 35−42.

Buckheit, R. W., Jr., Fliakas-Boltz, V., Decker, W. D., Roberson, J. L., Stup, T. L., Pyle, C. A., White, E. L., McMahon, J. B., Currens, M. J., Boyd, M. R., & Bader, J. P. (1995*) Antiviral Res. 26*, 117−132.

Carroll, S. S., Stalhut, M., Geib, J., & Olsen, D. B. (1994) *J. Biol. Chem. 269*, 32351−32357.

Chattopadhyay, D., Evans, D. B. Deibel, M. R., Vosters, A. F., Eckenrode, F. M., Einspahr, H. M., Hui, J. O., Tomasselli, A. G., Zurcher-Neely, H. A., Heinrikson, R. L., & Sharma, S. K. (1992) *J. Biol. Chem. 267*, 14227−14232.

Chong, K. T., Pagano, P. J., & Hinshaw, R. R. (1994) *Antimicrob. Agents Chemother. 38*, 288−293.

Coffin, J. M. (1995) *Science 267*, 483−489.

Dornsife, R. E., St Clair, M. H., Huang, A. T., Panella, T. J., Koszalka, G. W., Burns, C. L., & Averett, D. R. (1991) *Antimicrob. Agents Chemother. 35*, 322−328.

Eron, J. J., Jr., Johnson, V. A., Merrill, D. P., Chou, T. C., & Hirsch, M. S. (1992*) Antimicrob. Agents Chemother. 36*, 1559−1562.

Fletcher, R. S., Arion, D., Borkow, G., Wainberg, M. A., Dmitrenko, G. I., & Parniak, M. A. (1995) *Biochemistry 34*, 10106−10112.

Goldman, M. E., Nunberg, J. H., O'Brien, J. A., Quintero, J. C., Shleif, W. A., Freund, K. F., Gaul, S. L., Saari, W. S., Wai, J. S., Hoffman, J. M., Anderson, P. S., Hupe, D. J., Emini, E. A., & Stern, A. M. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6863−6867.

Goody, R. S., Müller, B., & Restle, T. (1991) *FEBS Lett. 291*, 1−5.

Gu, Z., Quan, Y., Li, Z., Arts, E. J., & Wainberg, M. A. (1995a) *J. Biol. Chem. 270*, 31046−31050.

Gu, Z., Arts, E. J., Parniak, M. A., & Wainberg, M. A. (1995b) *Proc. Natl. Acad. Sci. U.S.A. 92*, 2760−2764.

Huang, Y., Mak, J., Cao, Q., Li, Z., Wainberg, M. A., & Kleiman, L. (1994) *J. Virol. 68*, 7676−7883.

Johnson, V. A., Merril, D. P., Videler, J. A., Chou, T. C., Byington, R. E., Eron, J. J., D'Aquila, R., & Hirsch, M. S. (1991) *J. Infect. Dis. 164*, 646−655.

Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A., & Steitz, T. A. (1992) *Science 256*, 1783−1790.

Lacey, S. F., Reardon, J. E., Furfine, E. S., Kundel, T. A., Bebenek, K., Ecker, K. A., Kemp, S. D., & Larder, B. A. (1992) *J. Biol. Chem. 267*, 15789−15794.

Martin, J. L., Wilson, J. E., Haynes, R. L., & Furman, P. A. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6135−6139.

Martinez-Irujo J. J., Villahermosa, M. L., Alberdi, E., & Santiago, E. (1996) *Biochem. Pharmacol. 51*, 635−644.

Merluzzi, V. J., Hargrave, K. D., Labadia, M., Grozinger, K., Skoog, M., Wu, J. C., Shih, C. K., Eckner, K., Hattox, S., Adams, J., Rosenthal, A. S., Faanes, R., Eckner, R. J., Koup, R. A., & Sullivan, J. L. (1990) *Science 250*, 1411−1413.

Merril, D. P., Moounis, M., Chou, T. C., & Hirsch, M. S. (1996) *J. Infect. Dis. 173*, 355−364.

Palatini, P. (1983) *Mol. Pharmacol. 24*, 30−41.

Palmer, S., Harmenberg, J., & Cox, S. (1996) *Antimicrob. Agents Chemother. 40*, 1285−1288.

Parker, W. B., White, E. L., Shaddix, S. C., Ross, L. J., Buckheit, R. W., Germany, J. M., Secrist, J. A., III, Vince, R., & Shannon, W. M. (1991) *J. Biol. Chem. 266*, 1754−1762.

Parker, W. B., Shaddix, S. C., Bowdon, B. J., Rose, L. M., Vince, R., Shannon, W. M., & Bennet, L. L., Jr. (1993) *Antimicrob. Agents Chemother. 37*, 1004−1009.

Pauwels, R., Andries, K., Desmyter, J., Schols, D., Kukla, M. J., Breslin, H. J., Raeymaeckers, A., Van Gelder, J., Woestenborghs,

R., Heykants, J., Schellekens, K., Janssen, M. A. C., De Clercq, E., & Janssen, P. A. J. (1990) *Nature 343*, 470−474.

Pauwels, R., Andries, K., Debyser, Z., Kukla, M. J., Schols, D., Breslin, H., Woestenborghs, R., Desmyter, J., Janssen, M. A. C., De Clercq, E., & Janssen, P. A. J. (1994) *Antimicrob. Agents Chemother. 38*, 2863−2870.

Prichard, M. N., Prichard, L. E., & Shipman, C., Jr. (1993*) Antimicrob. Agents Chemother. 37*, 540−545.

Reardon, J. E. (1992) *Biochemistry 31*, 4473−4479.

Richman, D., Rosenthal, A. S., Skoog, M., Eckner, R. J., Chou, T. C., Sabo, J. P., & Merluzzi, V. J. (1991) *Antimicrob. Agents Chemother. 35*, 305−308.

Romero, D. L., Busso, M., Tan, C. K., Reusser, F., Palmer, J. R., Poppe, S. M., Poppe, S. M., Aristoff, P. A., Downey, K. M., So, A. G., Resnick, L., & Tarpley, W. G. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8806−8810.

Smith, M. S., Kessler, J. A., Rankin, C. D., Pagano, J. S., Kurtzberg, J., & Carter, S. G. (1993) *Antimicrob. Agents Chemother. 37*, 144−147.

Spence, R. A., Kati, W. M., Anderson, K. S., & Johnson, K. A. (1995) *Science 267*, 988−993.

Tramontano, E., & Cheng, Y. C. (1992) *Biochem. Pharmacol. 43*, 1371−1376.

Veal, G. I., Wild, M. J., Barry, M. G., & Back, D. J. (1994) *Br. J. Clin. Pharmacol. 38*, 323−328.

White, E. L., Parker, W. B., Ross, L. J., & Shannon, W. M. (1993) *Antiviral Res. 22*, 295−308.

Yuasa , S., Sadakata, Y., Takashima, K., Sekiya, K., Inouye, N., Ubasawa, M., & Baba, M. (1993) *Mol. Pharmacol. 44*, 895−900.

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