

## Inhibition of Human Immunodeficiency Virus In Vitro by Combinations of 3'-Azido-3'-Deoxythymidine and Foscarnet

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Received 18 October 1988/Accepted 25 January 1989

**We describe a synergistic effect of combinations of foscarnet and 3'-azido-3'-deoxythymidine against human immunodeficiency virus type 1 multiplication in cell culture, an additive effect of foscarnet and 3'-azido-3'-deoxythymidine triphosphate against human immunodeficiency virus type 1 reverse transcriptase, and a low toxicity in cell culture of combinations of the two drugs.**

The mechanisms of inhibition of human immunodeficiency virus (HIV) replication by 3'-azido-3'-deoxythymidine (AZT) and foscarnet in vitro have been well studied. AZT, which is a thymidine analog, is phosphorylated by cellular enzymes and, as a 5'-triphosphate, inhibits HIV type 1 (HIV-1) reverse transcriptase (RT) and terminates viral DNA chain elongation (8, 15, 22, 24). Foscarnet interacts with HIV-1 RT at a site where pyrophosphate is split off during polymerization of nucleoside triphosphates (18, 19, 25). The activity of AZT TP is competitive with dTTP (24), whereas foscarnet is a noncompetitive inhibitor (25).

The possibility of combining foscarnet and AZT against HIV infections and the possibility of using foscarnet against cytomegalovirus retinitis (20; S. L. Walmsley, E. Chew, S. E. Read, H. Vellend, I. Salib, A. Rachlis, and M. M. Fanning, *J. Infect. Dis.*, in press) in patients with acquired immunodeficiency syndrome treated with AZT (6, 7) called for an evaluation of the effects of combining these two compounds. The combinations of AZT or foscarnet plus alpha interferon (12, 13) have shown synergistic effects. AZT plus ribavirin, on the other hand, has shown antagonistic effects (1, 23).

The HIV-1 RT activity was assayed from lysed virus particles isolated from human T-lymphotropic virus type III<sub>B</sub>-infected U937 clone 2 cells (25). Foscarnet (Astra Alab AB, Södertälje, Sweden), AZT (99% pure; Sigma Chemical Co., St. Louis, Mo.), and AZT TP (J. Chattopadhyaya, Biomedical Center, Uppsala, Sweden) were used. The viability of H9 cells (a human CD4<sup>+</sup> lymphoid cell line) in cytotoxicity studies with antiviral compounds was assayed by counting the total number of cells in a hemacytometer and by trypan blue exclusion, both after 6 days of incubation. The mean of two experiments differing less than 15% is given. Cell inhibitory concentrations inhibiting culture growth to 50% of the control cell viability (CIC<sub>50</sub>) were calculated. The 50% inhibitory concentrations (IC<sub>50</sub>) of compounds were determined on HIV-1 replication. Uninfected H9 cells (10<sup>5</sup> cells per 0.5 ml) were seeded in 24-well microplates (Costar, Cambridge, Mass.) with 0.5 ml of medium with AZT or foscarnet or both in all possible combinations. Immediately after the cells and drugs were mixed, 1 ml of HIV-1 (supernatant fluid of persistently human T-lymphotropic virus type III<sub>B</sub>-infected H9 cells) (10, 16) was added in concentrations giving 50 to 60% and 30 to

40% infected cells. After a 6-day incubation, HIV-1 antigen content was measured in the cells by immunofluorescence (9) and in the supernatants by enzyme-linked immunosorbent assay (V.-A. Sundqvist, J. Albert, J. Hinkula, E. Ohlsson, and B. Wahren, *J. Med. Virol.*, in press).

RT inhibition values for foscarnet, AZT TP, and a mixture of the two in a ratio of 10:1 are shown in Fig. 1. The data were analyzed according to the median effect plot (5). Linear regression analysis resulted in parallel lines. This suggests that the two inhibitors are mutually exclusive. The slopes of the lines are similar and close to 1: 0.961 for foscarnet, 0.910 for AZT TP, and 0.956 for the foscarnet-AZT TP mixture, which shows that the system follows Michaelis-Menten kinetics. The dose-effect curves in Fig. 1 also show hyperbolic relations. The drug combination index (5) was 0.83. Since the drug combination index value is below 1, the assumption can be made that foscarnet and AZT TP had a weakly synergistic effect on inhibition of HIV-1 RT from human T-lymphotropic virus type III<sub>B</sub>. In three other experiments, the drug combination index values were 0.84, 0.86, and 0.90.

AZT combined with foscarnet was evaluated in cell culture by enzyme-linked immunosorbent assay of HIV-1 antigens in supernatants (Fig. 2). The median effect analysis showed that the compounds were mutually nonexclusive drugs in cell culture. By the fractional-product method (3, 4, 21), the observed values were found to be less than the expected values.

Immunofluorescence of infected cells showed that the IC<sub>50</sub>s varied in the range of 0.01 to 0.05 μM for AZT and 10 to 25 μM for foscarnet, depending on the virus dose. Combinations resulted in better antiviral activity against HIV-1, with drug combination index values of 0.49 (60% infected cells in the control) and 0.18 (30% infected cells in the control). These results indicate a synergistic anti-HIV-1 effect of the combination of AZT and foscarnet at concentrations at which the effects were strongly dose dependent and a stronger synergy in tissue culture than with RT alone.

In assessing combined cytotoxicity, it was noted that counting the total number of cells gave lower CIC<sub>50</sub>s than estimation of the percent viable cells (Table 1). In no case was the observed value similar to or lower than the expected values obtained by the fractional-product method. The two compounds thus did not exhibit any synergistic cytotoxic effect in noninfected H9 cells.

This study demonstrates an additive and mutually exclu-

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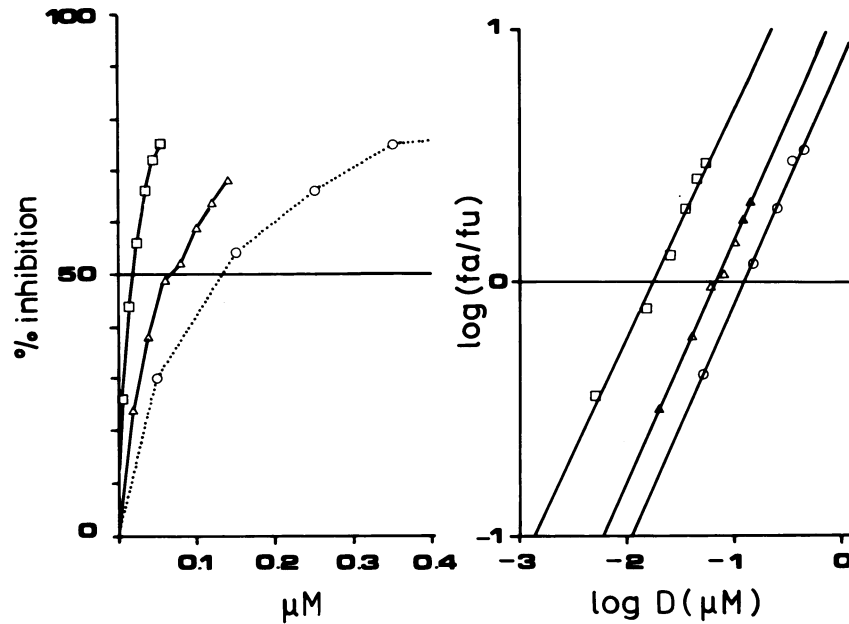


FIG. 1. Dose-effect plot (left) and median effect plot by linear regression analysis (right) of HIV-1 RT inhibition by foscarnet (O), AZT TP (□), and a 10:1 mixture of the two (Δ). The experiment was done in triplicate with coefficients of variation of less than 15% for each point. fa, Fraction of system affected by drug; fu, fraction of system unaffected by drug (5).

sive effect against HIV-1 of the combination of AZT TP and foscarnet at the enzyme level. The binding sites of HIV-1 RT for AZT TP and foscarnet seem to overlap (14), and mutants with resistance to foscarnet can, but need not, also have

resistance to AZT TP (L. Vrang and B. Öberg, manuscript in preparation). The mechanism for the moderate synergy in tissue culture is unknown. One explanation may be that AZT has additional antiviral effects when metabolized in the cell.

Perhaps a more important clinical effect of combining compounds than obtaining synergy is that the side effects of each compound may be reduced considerably. On a molar level, AZT is more active than foscarnet against HIV-1 in H9 cells, but it is also more toxic. Since the antiviral effects related to the anticellular effects may be measured during different growth conditions, both activities should always be determined. Our experiments to assay the cellular toxicity of each compound alone and in combination did not show any

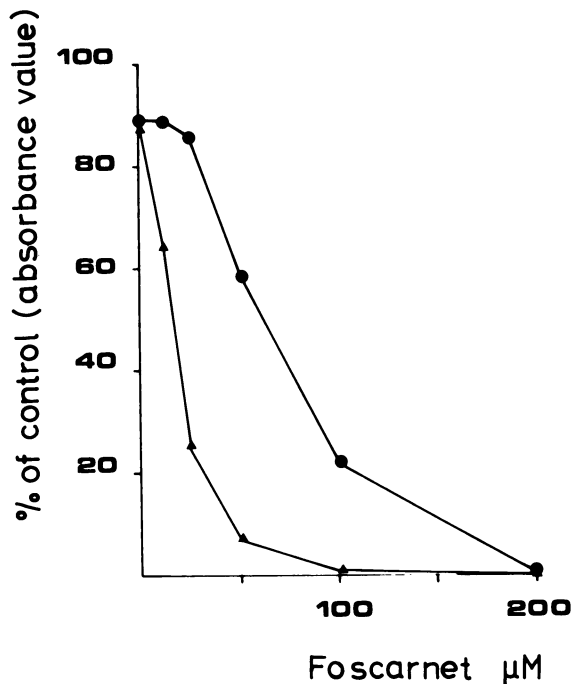


FIG. 2. Synergistic effect of foscarnet in combination with 0.01 μM AZT. Cells were infected with HIV-1, giving 60% infected cells. Observed values (▲) achieved experimentally when AZT and foscarnet were used together at the indicated concentrations and expected values (●) calculated by the fractional-product method are shown. The method for virus antigen determinations was enzyme-linked immunosorbent assay of supernatants.

TABLE 1. Cellular cytotoxicity of antiviral compounds individually and in combinations in noninfected H9 cells

Drug concn (μM)	% Viable cells		% of total cells	
	Observed	Expected <sup>a</sup>	Observed	Expected <sup>a</sup>
Foscarnet alone				
2,400	50			
800				
AZT alone				
22	50			
15				
Both (foscarnet/AZT)				
200/20	83	47	17	14
400/40	61	52	13	8
800/80	30	25	55	25
1,600/10	64	42	38	12
3,200/40	28	19	11	0.4

<sup>a</sup> The expected percent viable and total cells were obtained by the fractional-product method. The drug effect on viable cells is measured at a similar time point as in virus inhibition, i.e., after 6 days in culture. The total number of noninfected cells was 1.59 to 1.86 × 10<sup>6</sup> per well at that time, with a viability of 96 to 100%.

synergistic effects and not even convincing additive effects in dose ranges which cause very strong antiviral effects.

Clinical experience with AZT in patients with acquired immunodeficiency syndrome has shown an increased survival (7), but the treatment was also associated with severe hematological problems (17). The limited clinical experience with foscarnet has shown a reversible reduction in HIV-1 p24 antigen during intravenous treatment (2, 6, 11). The synergistic effects on HIV-1 and the low toxicity indicate that treatment with combinations might be a method of achieving clinical effects against HIV-1 at dose levels likely to cause less toxicity in patients.

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