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Synergistic drug interactions of an HIV-1 protease inhibitor with AZT in different in vitro models of HIV-1 infection

D.M. Lambert[a], H. Bartus[a], A.V. Fernandez[a], C. Bratby-Anders[a], J.J. Leary[a], G.B. Dreyer[b], B.W. Metcalf[b] and S.R. Petteway, Jr.[a]

[a]*Departments of Molecular Virology and Host Defense, and* [b]*Medicinal Chemistry, SmithKline Beecham Research Laboratories, King of Prussia, PA 19406, USA*

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Summary

Synthetic peptide mimetic inhibitors of HIV-1 protease effectively block spread of infectious virus in acutely infected T-cells. These compounds also inhibit production of infectious virions from chronically infected T-cell lines. In order to determine the potential for drug interaction effects on antiviral activity, an HIV-1 protease inhibitor (SK&F 108922) and AZT were studied in three different in vitro models of HIV-1 infection of T-cell lines, specifically, (1) acutely infected cells infected at low multiplicity, (2) HIV-1 chronically-infected cells and (3) co-cultivations of chronically infected with non-infected cells. Upon co-treatment, these compounds demonstrated synergy in Molt4 or H9 cells acutely infected with HIV-1 strain III_B. Either compound alone was a potent inhibitor of HIV-1 in co-cultivations of uninfected and chronically infected cells. In combination treatments of co-cultures, SK&F 108922 demonstrated strong synergy with AZT. Treatment of H9/III_B chronically infected cells demonstrated no inhibitory effect by AZT treatment ($EC_{50} = > 100$ μM) whereas SK&F 108922 was inhibitory ($EC_{50} = 3$ μM). Upon co-treatment of H9/III_B chronically infected cultures with both compounds, the antiviral activity was similar to that of the protease inhibitor alone suggesting no drug interaction. In the co-cultivation experiments, AZT's antiviral effect was most likely due to blocking spread of acute infection to uninfected cells in the culture. No antagonistic effects were observed with AZT and SK&F 108922 co-treatments. These results clearly demonstrate that an

Correspondence to: D.M. Lambert, Department of Molecular Virology and Host Defense, SmithKline Beecham, P.O. Box 1539, L523, King of Prussia, PA 19406, USA.

HIV-1 protease inhibitor can exert a potent antiviral effect on chronically infected T-cells in contrast to AZT and is capable of potent synergy with AZT in acute and co-culture in vitro infection models.

HIV-1; Protease inhibitor; AZT; Drug interaction; Synergy

Introduction

The HIV-1 genome encodes a protease which is required for processing the initial translation products of the viral *gag* and *pol* genes into functional proteins (Dickson et al., 1982). Functional molecules of HIV-1 protease are essential for production of infectious virions. Indeed, mutations within the protease coding region or the cleavage sites in *gag* and *gag-pol* of HIV-1 proviruses result in the formation of non-infectious virions exhibiting immature capsid morphology and reduced reverse transcriptase activity (Kohl et al., 1988; Gottlinger et al., 1989; Peng et al., 1989). Rationally designed synthetic peptide mimetics containing hydroxyethylene dipeptide isosteres are potent inhibitors of the HIV-1 protease (Dreyer et al., 1989, 1992). This class of inhibitors effectively blocks the spread of infectious virus in acute infections of T-cell cultures (Meek et al., 1990a; McQuade et al., 1990; Erickson et al., 1990; Roberts et al., 1990; Ashorn et al., 1990). In addition these compounds block processing of HIV-1 *gag* and *gag-pol* polyproteins in chronically infected T-cells (Meek et al., 1990a) and result in the production of non-infectious virus particles (Lambert et al., 1992).

Combination therapy with two antiviral drugs having different viral targets or mechanisms of action potentially could be additive, synergistic or antagonistic. The rationale for exploring combination treatment regimens is generally accepted as having the potential to increase the activity of the two drugs at lower, less toxic concentrations and such treatments have been suggested to decrease the chances of development of resistance (Rideout and Chou, 1991). Based on previous in vitro work (Poli et al., 1989; Jacobsen et al., 1991), it is possible that infectious virus break-through from infected cells could be a potential problem after in vivo treatment with either drug alone if inhibitory levels of drug cannot be maintained. However, combination treatment with synergistic drugs could potentially enhance the antiviral action of both drugs in vivo.

Nucleoside analogs such as 3′-azido-3′-thymidine (AZT) and dideoxyinosine (ddI) have been exploited as inhibitors of HIV-1 replication, both in vitro and in vivo. Nucleoside analogs are currently the only licensed therapeutics for the treatment of HIV infection and AIDS (Fischl et al., 1987; Mitsuya and Broder, 1987). This class of compounds works by inhibiting reverse transcriptase (RT) resulting in a block in cDNA synthesis (Mitsuya and Broder, 1987). However, AZT therapy leads to the development of resistant HIV-1 strains (Larder et al.,

1989, 1991) and demonstrates toxicity in AIDS patients upon long-term therapy (Fischl et al., 1987; Yarchoan et al., 1986). These inhibitors work 'early' in the infectious cycle of HIV-1 and inhibit integration into the T-cell genome. Events after integration, designated as 'late' events, include transcription, protein synthesis and assembly of virions. Protease inhibitors are generally thought to work primarily during or after assembly (i.e., virus budding) to inhibit maturation of virions to a mature infectious state (Meek et al., 1990b; Ashorn et al., 1990; Petteway et al., 1991a, 1991b; Lambert et al., 1992). Thus, not only are the viral targets different, but nucleoside analogs and protease inhibitors differ in the stage of the viral life cycle at which their antiviral activities function.

Two recent reports demonstrate that protease inhibitors can synergistically interact with either AZT, ddI, ddC or rIFN-α in acute HIV-1 infection in vitro (Kageyama et al., 1992; Johnson et al., 1992). However, these studies addressed only acutely infected cultures and did not investigate the role of drug interactions on chronic infection or the spread of infectious virus from chronically infected to uninfected cells in culture.

In the present study, we investigated the interaction of two inhibitors having different mechanisms of action, AZT and an HIV-1 protease inhibitor (SK&F 108922). For these studies, we employed three cell culture infection models, (1) cells acutely infected with HIV-1 at low multiplicity, (2) HIV-1 chronically-infected cells and (3) co-cultivations of chronically HIV-1 infected with uninfected cells. These infection models were used to investigate the effects of inhibitor treatment on the production of virions. Treatment with an HIV-1 protease inhibitor (SK&F 108922) and AZT, both individually and in combination, allowed differentiation of the mode of action of these two antiviral agents based on their activity in the different infection models. Chronically infected cells are important to consider in antiviral testing because such cells have been implicated as an in vivo reservoir of infectious virus, perhaps contributing to the slow progressive nature of HIV-related disease (Poli et al., 1989; Schnittman et al., 1989). It has also been proposed that cell-to-cell spread of HIV-1 from chronically infected cells to uninfected cells may be a sustaining feature of chronic infection in vivo (Sato et al., 1992). We therefore investigated chronic and co-cultivation models of HIV-1 infection to determine whether these compounds, alone or in combination, could block cell-to-cell spread of virus.

In the studies reported here, synergy was observed in both acute and co-cultivation infections but was not observed in cultures of chronically infected cells. Although protease inhibitors have a potent inhibitory effect on infectious HIV-1 production by chronically infected cells (Lambert et al., 1992; Roberts et al., 1990), AZT did not inhibit RT production by these cells. In no experiments did the presence of the protease inhibitor diminish the activity of AZT.

Materials and Methods

Inhibitors

The structure of SKF 108922 is Cbz-Ala-Phe[(S)-$CH(OH)CH_2$]Ala-Val-Valinol, where Phe[(S)-$CH(OH)CH_2$]Ala is the hydroxyethylene isostere of Phe-Ala and Cbz is benzyloxycarbonyl. SKF 108922 was synthesized by methods similar to those previously described (Dreyer et al., 1989, 1992). Briefly, the synthesis proceeded as follows: mixed anhydride coupling of Phe[(S)-$CH(OSiMe_2tBu)CH_2$]Ala to valinol (using iBuOCOCl and *N*-methyl morpholine); selective removal of the Boc group with trifluoroacetic anhydride; mixed anhydride coupling to Cbz-Ala; and finally, removal of the $SiMe_2tBu$ group with trifluoroacetic anhydride. The inhibitor was fully characterized by ^1H NMR, mass spectrometry, and combustion analysis. SKF 108922 exhibited an apparent inhibition constant (K_i) of 2 nM with recombinant HIV-1 protease when assayed as previously described (Dreyer et al., 1992). AZT was purchased from Sigma Chemical Co. (St. Louis, MO). For antiviral assays (see below), 10 mM stocks of inhibitors were prepared in 100% DMSO.

Cells and virus

The H9, CEM, and Molt4 cells have been described (Langlois et al., 1988; Matthews et al., 1987; Minowada et al., 1972). Cell lines were propagated in RPMI1640 medium supplemented with 20% fetal bovine serum, 100 units of penicillin G per ml and 100 μg of streptomycin per ml. HTLV-III_B (Ratner et al., 1985) was used to infect H9 or CEM cells to establish chronically infected cell lines. H9/III_B cells, chronically infected with the HTLV-III_B virus isolate, were established in our laboratories as previously described (Matthews et al., 1987; Meek et al., 1990a; Lambert et al., 1992). Briefly, 7–14 days following acute infection, cells resistant to the cytopathic effect of virus infection were established, yielding a population of chronically infected cells.

Assays to quantify HIV-1 replication

Cell-free medium of treated and control cultures was harvested and assayed for reverse transcriptase (RT) activity (Micro RT Assay) at 5 and 7 days post-infection as previously described (Goff et al., 1981; Willey et al., 1988). Assessment of anti-HIV-1 activity was based on reverse transcription levels and/or p24 levels using an ELISA assay (NEN/DuPont). EC_{50} values were calculated as the concentration at which virus replication was reduced by 50% compared to untreated controls.

Cytotoxicity testing

Cytotoxicity was assessed by both direct microscopic examination of trypan blue stained cells and by the treated culture's ability to metabolize the tetrazolium salt, XTT, to its formazan dye (Weislow et al., 1989). The XTT assay allows determination of the 50% toxic concentration (TC_{50}) of compounds for the cell/virus system used.

SDS-PAGE and Western blots

HIV-1 *gag* proteins in H9/III_B: Molt4 co-cultures treated with SK&F 108922, AZT or a combination of the two inhibitors were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970). Proteins were separated in 12.5% polyacrylamide gels at 40 mA/gel for 4 h and electroblotted onto nitrocellulose filters (Towbin et al., 1979). HIV-1 *gag* proteins were detected using monoclonal antibodies to p24 and p17 (Beckman), as previously described (Meek et al., 1990a; Lambert et al., 1992; Dreyer et al., 1992). Anti-mouse IgG antibodies conjugated with horseradish peroxidase (Bio-Rad, Richmond, CA) were detected using an enhanced chemiluminescence (ECL) kit (Amersham).

Antiviral assays for drug interaction studies

Three types of virus-cell culture systems (acute, co-cultivation, and chronic) were used to assess the effects of protease inhibitor and AZT interaction on viral infectivity. All assays were carried out in triplicate in 24-well plates (Nunc). 5-Fold serial dilutions of inhibitor were made in 100% DMSO to yield 200× final concentrations. Addition of 1/200 vol. of dilutions to culture wells resulted in a final concentration of 0.5% DMSO and the desired final concentration of inhibitor. Experiments were carried out either with dilutions of a fixed ratio of the two inhibitors (i.e., 1:10 or 1:40, AZT:SK&F 108922) or in a 'checkerboard' motif in which the concentrations of the two inhibitors were varied independently.

For low multiplicity acute infectivity assays, 3×10^4 uninfected Molt4 cells per well were infected with 50 TCIDs of HIV-1 (strain III_B). Stocks of inhibitors (10 mM) were prepared in 100% DMSO. Inhibitors were added on day 0, immediately after the 1.5 h virus adsorption period. Cultures were re-fed on days 1 and 4 with growth medium containing inhibitors at their original concentrations. Samples were harvested on day 7, the peak of virus production.

For chronic infection assays, chronically infected H9/III_B cells were washed three times in growth medium and plated at a density of 6×10^4 cells per well. Inhibitors were added on day 0. Cultures were re-fed on days 1 and 3 with growth medium containing inhibitor and harvested on day 5.

For co-cultivation infectivity assays 3×10^4 uninfected Molt4 cells were co-cultivated with 3×10^3 H9/III_B or CEM/III_B chronically infected cells per well in 24-well plates. Inhibitors were added at time 0, and the assay plates were re-fed on days 1 and 3 with growth medium containing inhibitors. The assay was harvested on day 5. Antiviral activity was evaluated by one of several parameters: Western blot analysis on pelleted cells from treated cultures, reverse transcriptase (RT) levels, and p24 antigen levels in the supernatant.

Two-drug interaction calculations

The interaction between SK&F 108922 and AZT in 'checkerboard' experiments was analyzed using the method of Pritchard and Shipman (1990), and their 'MacSynergy' computer program (C. Shipman, University

of Michigan). For experiments using fixed ratios of the two inhibitors, Chou and Talalay's combination method (1984) and 'Dose-Effect Analysis with Microcomputers' software (Chou and Chou, 1987) were used.

Results

Effects of SK&F 108922 and AZT on HIV-1 acute infections

Treatment of acutely infected Molt4 cells showed that both compounds were effective inhibitors of spread of HIV-1 infection in T-cell lines. Under the conditions of these experiments, the EC_{50} values of AZT and SK&F 108922 were 5 nM and 54 nM, respectively. In comparison, a fixed ratio of AZT:SK&F 108922 (1:10) appeared to potentiate virus inhibition, yielding EC_{50} values of 3.7 and 37 nM, respectively. Analysis of these results with the computer program of Chou and Chou (1987) confirmed that synergistic drug interaction occurred. A more comprehensive 'checkerboard' experiment (see Materials and Methods) with H9 cells acutely infected with HIV-1, also showed that enhanced inhibition was obtained by combination treatments (Table 1). Data shown in Table 1 were analyzed using the MacSynergy computer program (Prichard and Shipman, 1990) and are represented in a three dimensional bar graph showing that over a range of concentrations of the two inhibitors, synergystic interactions were obtained (Fig. 1). The surface area plot of this data (not shown) yielded a volume of synergy of 107.8% μM^2. No indication of either antagonism or enhancement of AZT cytotoxicity was observed in these experiments. Thus, combination treatment of two different acutely infected cell lines (Molt4 and H9) with SK&F 108922 and AZT resulted in synergistic drug interaction.

TABLE 1

Checkerboard analysis of AZT/SK&F 108922 drug interactions in an HIV-1 acute infectivity assay in Molt4 cells measured by RT activity[a]

SKF108922 μM							
31	1.00	1.00	1.00	1.00	1.00	1.00	1.00
6.25	1.00	1.00	1.00	1.00	1.00	1.00	1.00
1.25	0.16	0.20	0.26	0.56	0.76	1.00	1.00
0.25	0	0	0	0	0.28	1.00	1.00
0.05	0	0	0	0	0.20	1.00	1.00
0.01	0	0	0	0	0.28	1.00	1.00
0	0	0.10	0	0	0.20	1.00	1.00
	0	0.001	0.005	0.025	0.125	0.625	3.125
				AZT μM			

[a]Values represent fractional inhibition compared to untreated control and are averages of triplicate samples. RT activity in untreated HIV-1 infected cultures was an average of 21 800 CPM per 10 μl of supernatant. Background counts in uninfected untreated Molt4 samples were an average of 150 CPM per 10 μl of supernatant.

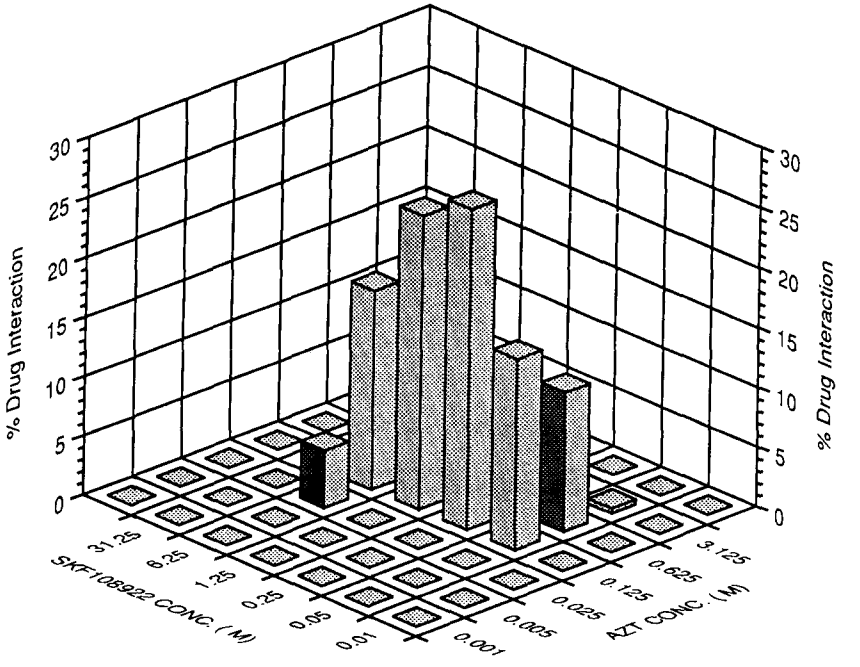

Fig. 1. Analysis of SK&F 108922 and AZT interactions in HIV-1 strain III_B acutely infected Molt4 cells. The amount of synergy (i.e., potentiation of inhibition above expected additivity) observed with combinations of the two compounds (data shown in Table 1) was calculated using the MacSynergy computer program and is represented by the height of bars in the graph (Pritchard and Shipman, 1990). The % of drug interaction is plotted vs. drug concentrations. Additive levels were subtracted out by the program and are represented by the floor of the 3-dimensional graph. If antagonism had occurred, negative values would be plotted below the floor of the graph. Analysis of a surface area plot (not shown) of the same data allowed quantification of the volume of synergy which was calculated to be 107.8% μM^2.

Effects on an HIV-1 chronic infection in H9 cells

It was of interest to investigate the relative antiviral effectiveness of both SK&F 108922 and AZT in the $H9/III_B$ chronically infected cell model and also to determine whether any synergistic interaction could be detected. $H9/III_B$ cells, which constitutively produce infectious virions, were treated with dilutions of either AZT or SK&F 108922 alone or in combination over the same dose range of these compounds. The RT released into the medium was measured after 5 days of exposure to the compounds (data not shown). As shown previously by others (Poli et al., 1989; Roberts et al., 1990) AZT treatment alone had little or no effect on RT activity released into the medium of this cell line, compared to its potent antiviral effect in acutely infected H9 or Molt4 cells. In contrast, SK&F 108922 demonstrated effective inhibition of infectious HIV-1 virus indicated by reduction of active RT in the medium. Combination of AZT with SK&F 108922 did not significantly reduce the production of RT-containing particles by this cell line beyond that obtained with the protease inhibitor alone, suggesting that in this infection model, there

was no potential for synergistic interaction.

Effect of SK&F 108922 and AZT on HIV-1 replication in co-cult infections

The effects of these inhibitors on the spread of infectious virus from chronically infected cells to uninfected cells in culture were evaluated. In separate experiments, chronically infected H9/III_B or CEM/III_B cells were mixed with a 10-fold excess of uninfected Molt4 cells. Compared to untreated cultures, treatment with each individual compound effectively blocked HIV-1 replication as evidenced by reductions in both RT activity and p24 antigen

TABLE 2

Effects of the HIV-1 protease inhibitor, SK&F 108922, and AZT alone and in fixed ratio combination (AZT:SKF 108922 = 1:40) on HIV-1 replication in co-cultures of H9/III_B or CEM/III_B and Molt4 cells measured by RT activity and p24 activity. Combination Index (CI) values at F_a values of 50, 75, 90 and 95% are given at the bottom of the graph for each co-culture system. CI values < 1 indicate synergy

Drug Treatment Conc. (nM)		Fraction Inhibited[a]			
		H9/IIIB:Molt4		CEM/IIIB:Molt4	
SKF108922	AZT	RT	p24	RT	p24
50 000		0.985	0.999	0.981	0.997
10 000		0.977	0.998	0.98	0.995
2000		0.91	0.995	0.97	0.973
400		0.836	0.8	0.21	0
80		0.176	0.35	0	0
16		0	0	0	0
3.2		0	0	0	0
	1250	0.916	0.97	0.89	0.64
	250	0.856	0.86	0.73	0.15
	50	0.768	0.64	0.47	0.05
	10	0.628	0.62	0.33	0
	2	0.38	0.38	0.06	0
	0.4	0	0.13	0	0
	0.08	0	0	0	0
50 000	1250	0.996	0.999	0.990	0.999
10 000	250	0.992	0.999	0.989	0.998
2000	50	0.992	0.998	0.980	0.990
400	10	0.986	0.996	0.860	0.818
80	2	0.959	0.991	0.170	0
16	0.4	0.968	0.958	0	0
3.2	0.08	0.94	0.94	0	0
CI[b] at Inhibitions of		H9/IIIB:Molt4		CEM/IIIB:Molt4	
50%		0		0.3575	
75%		0		0.3691	
90%		0.0002		0.3881	
95%		0.0012		0.4045	

[a]Values represent fractional inhibition compared to untreated infected control and are averages duplicate RT assays of triplicate samples. EC_{50} values for this data shown in Table 4.
[b]Combination Index Values (CI) calculated from Chou and Chou computer program (Biosoft).

levels present in the medium of these cultures (Table 2). When combination treatments (1:40 fixed ratio of AZT:SK&F 108922) were carried out, potentiation of the inhibitory effect of either drug alone was obtained. A larger synergistic effect was observed in H9/III_B:Molt4 co-cultures than in CEM/III_B:Molt4 co-cultures. Combination index (CI) values <1 indicate synergy. Stronger synergy effects are reflected by lower numbers. For H9/III_B:Molt4 and CEM/III_B:Molt4 co-cultures the CI values at 50% inhibitory concentrations were 0 and 0.357.

This conclusion was further supported by the Western blot analysis of treated H9/III_B:Molt4 co-cultures (Fig. 2). This experiment showed that greatly enhanced inhibition of both synthesis and processing of viral *gag* polyprotein was obtained by combination treatment compared with either compound alone. At the highest concentrations of these inhibitors in the co-treatment samples (1.25 μM AZT and 50 μM SK&F 108922), no $Pr55^{gag}$ was detected (Fig. 2). The dramatic reduction in *gag* proteins being synthesized in the cells after co-treatment provides an explanation for the reduction of RT and p24 released into the medium of these cultures (Table 2).

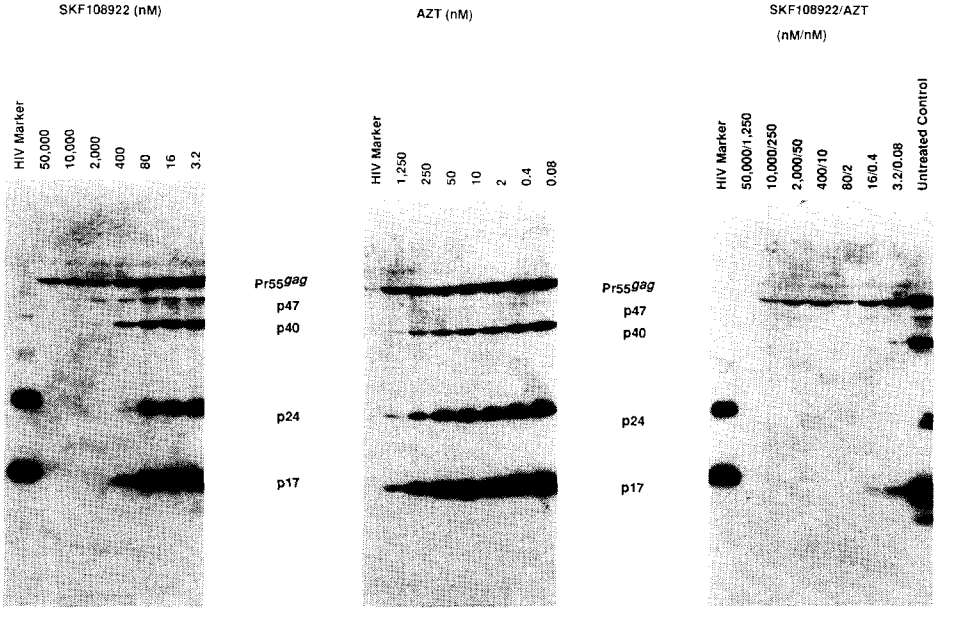

Fig. 2. Effects of HIV-1 protease inhibitor treatment on viral proteins. Co-cultures of H9/III_B chronically infected cells with Molt4 cells were treated with SK&F 108922, AZT or a combination of both drugs at a fixed ratio of 40:1 (SK&F 108922:AZT) over the same concentration range. Cell pellets were disrupted in sample buffer and analyzed by SDS-PAGE and Western blots. Detection of HIV-1 *gag* proteins ($Pr55^{gag}$, p47, p40, p24 and p17) on the electroblots utilized p24 and p17 mouse monoclonal antibodies and detected by an HRP-labeled second antibody and ECL. Samples include an HTLV-III_B Virion Control (HIV Marker), extracts of H9/III_B cells treated with the indicated concentrations of compounds, and an untreated control sample.

TABLE 3

Checkerboard assay of AZT and SK&F 108922 drug interactions in an HIV-1 co-cultivation infectivity assay as measured by RT activity[a]

SKF108922 (μM)									
50	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
10	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
2	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
0.4	0.21	0.79	1.00	1.00	1.00	1.00	1.00	1.00	1.00
0.08	0	0	0.75	0.92	0.79	0.88	1.00	1.00	1.00
0.016	0	0	0.50	0.54	0.75	0.83	0.92	1.00	1.00
0.0032	0	0	0	0.33	0.67	0.75	0.92	0.92	0.92
0	0	0	0	0.33	0.54	0.63	0.75	0.92	0.92
	0	0.0004	0.002	0.01	0.05	0.25	1.25	6.25	31.25
				AZT (μM)					

[a]Values represent fraction inhibited compared to untreated control and are the means of triplicate samples. RT activity in untreated co-cultures was an average of 24000 CPM per 10 μl of supernatant. Background counts in uninfected untreated Molt4 samples were an average of 200 CPM per 10 μl of supernatant.

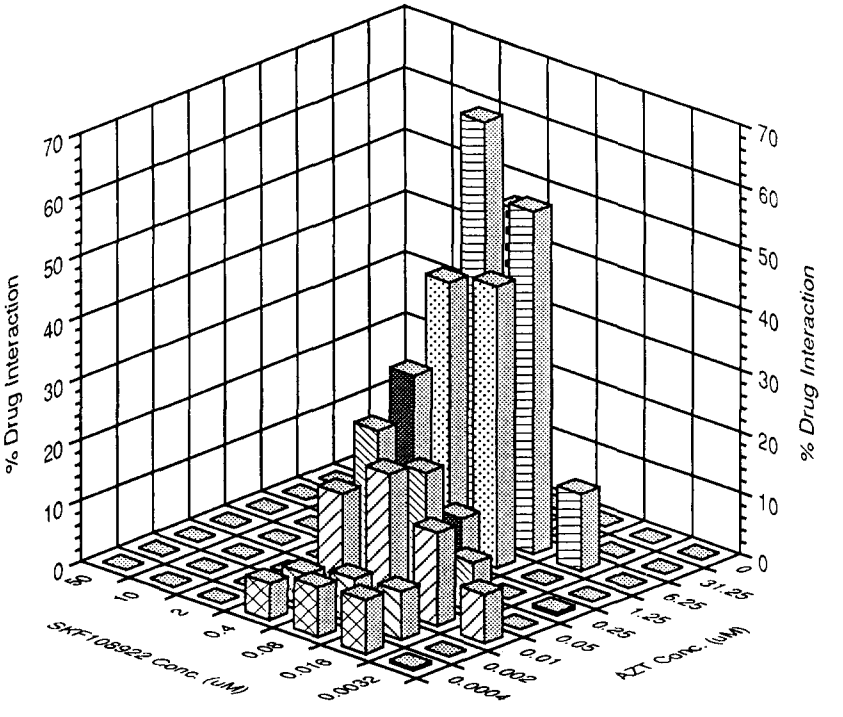

Fig. 3. Analysis of SK&F 108922 and AZT interactions in H9/III$_B$:Molt4 Co-cultures. The % of drug interaction is plotted vs. drug concentrations. The MacSynergy generated graph demonstrates the amount of synergy (i.e., potentiation of inhibition above expected additivity) observed with combinations of the two compounds (data shown in Table 3). Additive levels were subtracted out by the computer program and are represented by the floor of the three-dimensional graph. Surface area plot analysis (not shown) of the same data revealed that the calculated volume of synergy in the three dimensional peak was 477.1% μM^2.

A more detailed 'checkerboard' study of the effects of these compounds on the H9/III_B:Molt4 co-cultures was carried out. The results shown in Table 3 demonstrate potentiation of inhibition during co-treatment with these two drugs. Analysis of this data using the MacSynergy program further demonstrated potent synergy over a range of AZT to SK&F 108922 concentrations (Fig. 3A). The volume of synergy determined from the surface area plot (not shown) was calculated by the MacSynergy program to be 477% μM^2.

Table 4 summarizes results obtained by comparing the EC_{50} values obtained with the individual inhibitors as well as fixed ratios of SK&F 108922 and AZT in the three in vitro models of HIV-1 infection used in these studies. AZT alone was 2–10-fold less effective (EC_{50} = 10–40 nM) at inhibiting HIV-1 replication in the co-cultivation model, compared to the acute infection model (EC_{50} = 5 nM), and was not active in the chronic model (up to 100 μM) in reducing RT activity released into the culture medium. No potentiation of drug interaction was observed in the chronically infected H9/III_B cell line after co-treatment as predicted since AZT is not active in chronically infected T-cells (Poli et al., 1989). Protease inhibitor treatment was effective in all three infection models although the inhibitory effect of SKF 108922 based on relative EC_{50} values was 4–14-fold lower in the H9/III_B:Molt4 and CEM/III_B:Molt4 co-cultivation assays (EC_{50} = 200–710 nM, respectively) than in the acute infection assay in Molt4 cells (EC_{50} 54 nM) and 66-fold less effective in the chronically infected H9/III_B cells (EC_{50} = 3600 nM). Co-treatment resulted in reductions in the EC_{50} values compared with either compound alone in both the acute and co-cultivation infection models. Furthermore, analysis by the Chou computer program indicated synergistic interaction between these compounds in these

TABLE 4

Comparison of the antiviral activities of SK&F 108922 and AZT treatments on HIV-1 replication in acutely infected H9 cells, co-cultures of uninfected Molt4 cells with H9/III_B or CEM/III_B and chronically infected H9/III_B cells. Combination treatments were carried out at the indicated fixed ratios of drug. Results were determined from duplicate RT activity assays on culture supernatants and are presented as the EC_{50} value

EC_{50} Drug concentrations (nM)				
Drug treatment	Acute infection	Molt4 Co-Cult H9/III_B[a]	CEM/III_B[a]	Chronic H9/III_B
SK&F108922	54	200	710	3600
AZT	5	10	50	>100 000
SK&F108922 / AZT	37[b] / 3.7	<3.2[c] / <0.08	180[c] / 4.5	3000[c] / >100 000
Chou analysis	Synergy	Synergy	Synergy	No interaction

[a]EC_{50} values calculated from RT data in Table 2.
[b]10:1 ratio of SK&F108922:AZT.
[c]40:1 ratio of SK&F108922:AZT.

two models (Table 4). In contrast, no drug interaction was evident in the H9/III_B chronic infection model.

Discussion

We investigated the antiviral activity of an HIV-1 protease inhibitor (SK&F 108922) in combination with AZT using three separate assays which may be relevant in vitro models for the study of antiviral compounds active at different stages of HIV-1 infection. First, the acute infection assay may model the rapid replication and cytopathic effects contributing to the loss of $T4^+$-lymphocytes in vivo (Schnittman et al., 1989). Second, chronically infected cells, containing integrated provirus and exhibiting moderate to low levels of continuous virus expression, are likely to represent in vivo reservoirs of infectious virions, which ultimately contribute to disease progression (Fauci, 1988; Poli et al., 1989; Schnittman et al., 1989). Third, the co-cultivation assay used in these studies is perhaps a more relevant model of in vivo infection since it involves 'cell-to-cell' as well as 'cell-free' spread of HIV-1 within the culture (Sato et al., 1992).

Acute infection of T-cell lines and treatment with SK&F 108922 or AZT effectively blocked HIV replication and virus spread whereas combination treatments potentiated viral inhibition. Others have recently demonstrated that HIV-1 protease inhibitiors can synergistically interact with either AZT, ddI, ddC or rIFN-α in acute HIV-1 infections in vitro (Kageyama et al., 1992; Johnson et al., 1992).

In agreement with Roberts et al. (1990), we found that AZT was unable to inhibit production of RT in the medium of treated chronically infected T-cells whereas an HIV-1 protease inhibitor was able to block production of active RT in the medium. Consequently, the lack of synergy observed in chronically infected H9/III_B cells can be attributed to the fact that AZT had no antiviral effect on production of infectious virions by these cultures and thus no drug synergy with SK&F 108922 was possible. Although immature virions are produced under these conditions, their ability to subsequently infect a T cell line is reduced to non-detectable levels by protease inhibitor treatment (Lambert et al., 1992). AZT is not an effective inhibitor of HIV-1 production in chronically infected cells because viral cDNA synthesis and integration do not appear to play a role in the production of infectious virions from these cultures (Poli et al., 1989). Pincus and Wehrly (1990) reported that AZT inhibited HIV-1 in chronically-infected H9 cells. This conclusion resulted from studies which measured the ability of AZT-treated chronically infected H9 cells to form infectious centers when co-cultivated on $CD4^+$ HeLa cell monolayers in the presence of AZT. These results are not unexpected since AZT also completely blocks cell to cell fusion of a chronically infected T-cell line with uninfected T-cells (Buckheit et al., 1992).

Treatment of H9/III_B:Molt4 co-cultures with either AZT or SK&F 108922, effectively inhibited the amplification of HIV-1 replication observed in control

untreated co-cultures (Fig. 2). However, the substantial enhancement of synergy compared to that seen in acute infections was initially somewhat surprising. Surface area plot analysis using MacSynergy demonstrated approximately 4-fold greater synergy (477% μM^2) compared to acute infection (108% μM^2) in spite of the fact that both AZT and SKF 108922 were less effective at inhibiting HIV-1 in co-cultures (see EC_{50} values, Table 4). Indeed, Western blots of cell extracts from these cultures showed that co-treatment with AZT and SK&F 108922 enhanced inhibition of both synthesis and processing of viral *gag* polyproteins. The differences in synergistic interaction of these compounds in the acute and co-culture infection models may be partially due to the antiviral action of AZT and SK&F 108922 at different phases of the virus replication cycle.

Interesting differences between EC_{50} values were evident when comparing the three in vitro models of infection (Table 4). In contrast to their individual effects in the acute infection model, both SK&F 108922 and AZT were less effective in the co-cultivation infection model. This result may have been due to either (i) greater virus load contributed by the chronic cells present in the co-culture compared to the lower multiplicity acute infection model, or (ii) an increased efficiency of infection through cell to cell spread (Sato et al., 1992). However, combination treatment of acutely infected cells and co-cultures resulted in enhanced inhibition of HIV-1 replication, evident in markedly lowered EC_{50} values and shown to be synergistic by computer analysis (Chou and Chou, 1987).

Studies reported here demonstrate the potentiation of antiviral effects of SK&F 108922, an HIV-1 protease inhibitor, and AZT, an RT inhibitor, on the replication of HIV-1 in different in vitro models of infection. It is hoped that the synergy exhibited in vitro will translate to in vivo synergy with existing RT inhibitor antiviral therapies when protease inhibitors are investigated in the clinic. Importantly, based on the in vitro studies presented here, it is likely that neither of these antiviral agents will interfere with the action of the other.

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