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Anti-HIV Activity and Mechanism of Action of Macrocyclic Diamide SRR-SB3

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Abstract

The importance of cyclic compounds as anti-cancer and anti-viral agents has been recognized for some time. We have studied a series of macrocyclic amide derivatives for activity against HIV infection of T lymphocytes in-vitro.

Compounds containing aromatic rings and sulphur atoms were generally active, however the selectivity was greatly enhanced when two benzene rings were bridged by a disulphide linkage to produce 7-methyl-6,7,8,9-tetrahydrodibenzo[*c,k*][1,2,6,9]-dithiadiazacyclododecine-5,10-dione (SRR-SB3). This compound was studied in detail with different cell and virus infections including macrophages and chronically infected H9 cells. It was active with an EC50 (the dose affording 50% inhibition of infection) of 0·05–0·1 $\mu g\,mL^{-1}$ and a TC50 (concentration reducing uninfected cell growth by 50%) of 50 $\mu g\,mL^{-1}$. The compound did not inhibit protease, but seemed to act by inhibiting maturation of progeny virus, by interfering with precursor protein processing. It was synergistic with AZT (3′-azido-3′-deoxythymidine; zidovudine) when tested in-vitro.

The unusual mode of action and potent anti-HIV activity in T lymphocytes and macrophages makes this compound a potential candidate for clinical trials.

The anti-viral properties of a series of macrocyclic amides were reported recently (Witvrouw et al 1997). Compound SRR-SB3 (7-methyl-6,7,8,9-tetrahydrodibenzo [*c,k*] [1,2,6,9] - dithiadiazacyclododecine-5,10-dione) was shown to be the most potent. It inhibited infection in different cell lines with an EC50 (the dose affording 50% inhibition of infection) from 1·8–6·5 $\mu g\,mL^{-1}$. Studies of the mode of action of this compound were inconclusive. It was assumed to interact with the zinc fingers of the HIV-1 nucleocapsid protein (NCp7) resulting in ejection of the zinc molecule as reported previously by Tummino et al (1996) for some disulphide benzamides.

Simultaneously with Witvrouw et al (1997), we studied SRR-SB3 for inhibition of HIV infection in-vitro and discovered an unusual mode of action for this compound and good synergy when evaluated in combination with AZT (3′-azido-3′-deoxythymidine; zidovudine).

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Materials and Methods

Antiviral assays

The anti-HIV activity and the toxicity of the compounds were assessed in C8166 T lymphoblastoid cells from man infected with HIV-1MN, HIV-2ROD or SIVMAC and H9 T cell lymphoma cells from man chronically infected with HIV-1IIIB (Mahmood 1995).

Microtiter plate wells were used to mix 4×10^5 cells/well with five-fold dilutions (six dilutions of a 100-$\mu g\,mL^{-1}$ solution in growth medium) of the compounds before addition of 2 CCID50 (50% cell culture infectious dose) of virus. The inhibition of infection was monitored by examining syncytia, by measuring cell viability using the XTT-formazan method (Weislow et al 1989), by estimating viral antigen (gp120 or p24) using ELISA (enzyme-linked immunosorbent assay) or, finally, by titrating progeny virus released after six days incubation at 37°C. The last two assays are most sensitive and were used for calculating EC50 values. The cytotoxicity in drug-treated uninfected cells was measured by the XTT-formazan assay.

Inhibition of viral core protein processing
Chronically infected H9 cells (4×10^5) were centrifuged and resuspended in 1 mL RPMI containing different concentrations of compounds in 24-well plates. After 3 days at 37°C, the cells were centrifuged and the virus in the supernatant was concentrated by PEG precipitation and solubilized in 100 μL SDS (sodium dodecylsulphate) buffer containing 0·1% Triton X-100 for analysis by SDS–PAGE (polyacrylamide gel electrophoresis) and immunoblotting. The separated proteins were visualized by use of a mixture of four anti-p24 antibodies EH12E1, 1E8G2, 3D3 and 4H2B1 (Ferns et al 1987). The relative areas of protein bands p24 and p55 on the autoradiograms were also measured by scanning.

Enzyme assays
In-vitro tests for the inhibitory effects of compound SRR-SB3 on the HIV-1 enzyme reverse transcriptase were performed with concentrated virus and RT-Detect Kit (DuPont Medical Products) with the procedure supplied. The assay for proteinase activity was performed according to the method described by Shearer (1991) using reagents supplied by the MRC AIDS Reagent Project, UK.

Evaluation of compounds in combination
Combination experiments were performed using six or seven 2-fold dilutions of each of two compounds both alone and in combination. The highest concentration of each compound used was lower than its EC90 (dose affording 90% inhibition of infection). C8166 cells and HIV-1IIIB were used and effects were determined by XTT-formazan assay. For each compound combination isobolograms were plotted for concentrations exerting the same effect (50%). The straight line shown joins the single drug values and represents the line of zero-interaction or additivity. Points above and to the right of the line indicate antagonism, points below and to the left of the line indicate synergy (Berenbaum 1989; Suhnel 1990).

Results and Discussion

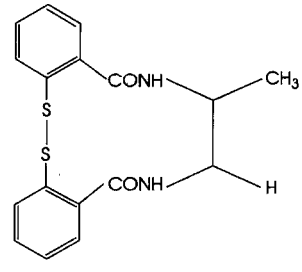
Anti-HIV activity
Compound SRR-SB3 (Table 1) was evaluated in different cell lines including JM cells (thymidine kinase deficient) and macrophages. AZT and other nucleoside analogues are less active in JM cells owing to poor phosphorylation. SRR-SB3 was similarly selective in all types of cell and also against different strains of HIV-1, HIV-2 and SIV and against strains of HIV-1 resistant to AZT and 3TC. The EC50 ranged from 0·05 to 0·1 $\mu g\,mL^{-1}$ by viral infectivity assay.

Compound SRR-SB3 was also tested in chronically infected H9 cells which is a more sensitive test of their effect on maturation of infectious virions. It reduced the infectivity of HIV-1IIIB by more than 99% with an EC50 of 0·05 $\mu g\,mL^{-1}$ and a TC50 (concentration reducing uninfected cell growth by 50%) of 50 $\mu g\,mL^{-1}$ (Table 1). There was no difference between the amount of gp120 or p24 released into the culture supernatant (data not shown), suggesting that there was no inhibition of viral protein synthesis but rather that processing of the precursor proteins or packaging of viral RNA was affected, resulting in the release of non-infectious progeny virus.

Mode of action
The studies of the mode of action of compound SRR-SB3 by Witvrouw et al (1997) were not conclusive. They assumed that viral infectivity was inhibited owing to defective RNA packaging as recently reported for some disulphide benzamides. Rice et al (1993, 1995, 1997) suggested that such compounds interact with the HIV-nucleocapsid protein (NCp7) zinc fingers. Tummino et al (1996) also reported that disulphide benzamides caused ejection of zinc from HIV-1 NCp7 so inhibiting its function and resulting in the production of non-infectious progeny virus.

Here we present evidence that compound SRR-SB3 inhibited processing of precursor proteins as judged by SDS-PAGE and immunoblotting analysis. The ratio of mature capsid protein p24 to the

Table 1. Compound SRR-SB3 and anti-HIV activity.



Compound	EC50 HIV-1MN/C8166	EC50 Chronically infected H9	TC50
SRR-SB3	0·05	0·05	50
AZT	0·004	> 300	> 300
RO31-8959	0·001	0·001	20

EC50 is the concentration of compound ($\mu g\,mL^{-1}$) which inhibited the production of gp120 or virus yield by 50%. TC50 is the concentration that reduces uninfected cell growth by 50% as determined by the XTT-formazan method.

precursor p55 decreased with increasing concentrations of the compound (Figure 1). Some inhibition of processing was evident even at a concentration of 0·03 $\mu g\,mL^{-1}$. The protease inhibitor Ro31-8959 (Roberts et al 1990), supplied by Roche, was 100 times more potent than SRR-SB3. Surprisingly the macrocyclic diamide SRR-SB3 failed to inhibit the viral protease in-vitro, whereas Ro31-8959 inhibited the enzyme with activity similar to that in the Western blot analysis (Mahmood et al 1995).

A number of cyclic compounds with aromatic hydrophobic and heterocyclic rings have previously been shown to inhibit HIV protease (Brinkworth & Fairlie 1992; Vara Prasad et al 1996) and benzodiazepine derivatives have been shown to be highly selective non-nucleoside inhibitors of HIV-1 reverse transcriptase (Pauwels et al 1990; Mertens et al 1993). SRR-SB3 inhibited precursor protein processing and did not inhibit reverse transcriptase, gp120/CD4 interaction or protease. Witvrouw et al (1997) presented some indirect evidence of interaction of SRR-SB3 with Zn^{2+} but they were not able to confirm that it inhibited infection by ejection of Zn^{2+} from Gag proteins of HIV-1.

Recently, Turpin et al (1996) presented convincing evidence that disulphide-substituted benzamides inhibited precursor processing without inhibiting protease. The compounds induced the formation of cross-linked Gag proteins by disulphide bond formation. As SRR-SB3 did not directly inhibit protease, it is apparent that it is binding to the precursor proteins and interfering with proteolytic processing.

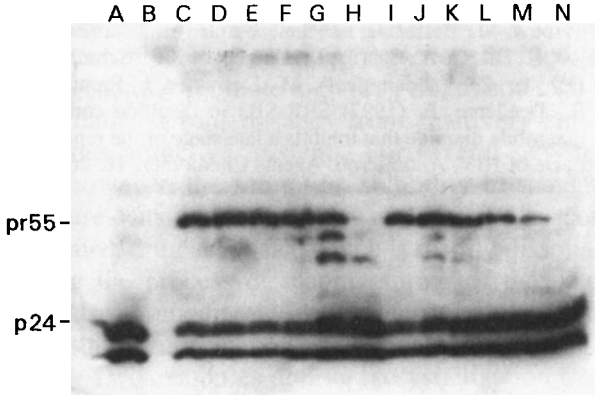


Figure 1. Inhibition of viral precursor protein processing. Chronically infected H9 cells (4×10^5) were incubated with different concentrations of the compounds in 1 mL medium at 37°C for 3 days. Cell culture supernatant was concentrated by precipitation with PEG for Western blot analysis using a mixture of three anti-p24 monoclonal antibodies and ECL system. Lanes A and N, untreated infected culture supernatant; lane B, untreated and uninfected; lanes C–H, treated with protease inhibitor RO31-8959, 5–0·001 $\mu g\,mL^{-1}$ (five-fold dilutions); lanes I–M, treated with amide SRR-SB3, 20–0·032 $\mu g\,mL^{-1}$ (five-fold dilutions). Molecular weight standards were included as controls.

Inhibition of infection by combinations of compounds

Because the mechanism of action of SRR-SB3 is different from that of AZT (they target different stages in the HIV replicative cycle), they were studied for combined inhibitory effects. Isobolograms are shown in Figure 2; the results (average from three separate experiments with s.d. 20%) indicate that the effect of SRR-SB3 seems to be synergistic with that of AZT.

The macrocyclic amides are a different and new class of compound; they are easy to synthesize and quite stable, maintain inhibitory activity for 5–6 days at 37°C and are resistant to freezing and thawing. Further studies are in progress to

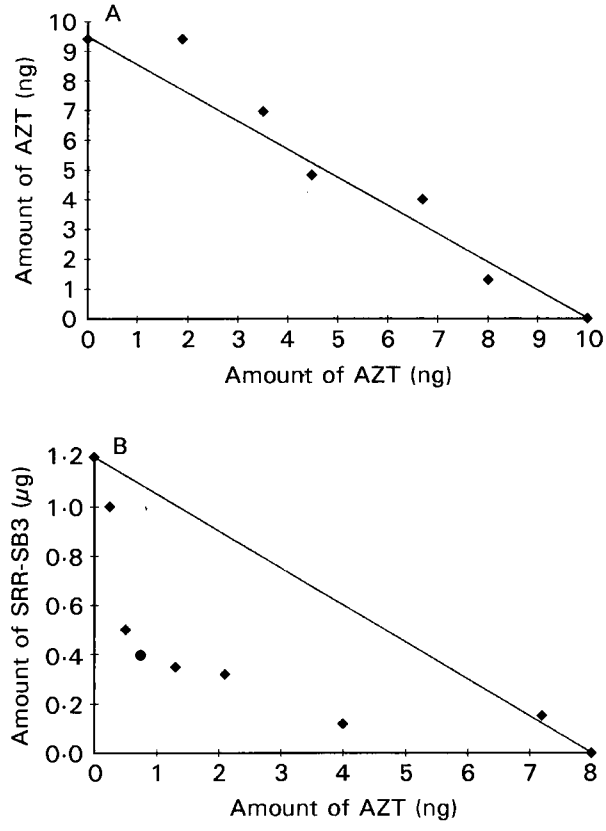


Figure 2. Isobolograms for combinations of compounds SRR-SB3 and AZT. Each point on the graphs is the concentration of compound affording 50% inhibition of infection as measured by cell viability assay (XTT-formazan method). Because this is a less sensitive method for compounds inhibiting viral infectivity, the EC50 value for SRR-SB3 is much higher than that determined by the infectivity assay, as shown in Table 1. A. AZT mixed with AZT to show additive effect; B. AZT mixed with SRR-SB3 showing additive to synergistic effect.

synthesize compounds with higher selectivity and to study bioavailability.

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