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THE INHIBITORY ACTIVITY OF A PEPTIDE DERIVATIVE AGAINST THE GROWTH OF SIMIAN IMMUNODEFICIENCY VIRUS IN C8166 CELLS

*Joseph A. Martin, +Margaret A. Mobberley, *Sally Redshaw, ++Andy Burke, ++A. Stanley Tyms and +Timothy A. Ryder

*Research Centre, Roche Products Ltd., P.O. Box 8, Welwyn Garden City, Herts AL7 3AY, UK

+Electron Microscopy Unit, Queen Charlotte's and Chelsea Hospital, Goldhawk Road, London W6 OXG, UK

++MRC Collaborative Centre, 1-3 Burtonhole Lane, Mill Hill, London NW7 1AD, UK

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SUMMARY: The peptide derivative Ro 31-8959 is a potent and selective inhibitor of the aspartic proteinases encoded by HIV-1 and HIV-2 and it arrests the growth of both viruses in cell culture. We have demonstrated similar effects against the simian immunodeficiency virus SIV_{mac251} in the human T-cell line, C8166 (ED_{50} = 6nM) with a therapeutic index of 4,500. The antiviral activity of Ro 31-8959 was 250 and 22 times greater than that of ddI and ddC, respectively. The mode of action was confirmed by accumulation of the polyprotein p55 with concomitant reduction of the cleavage product, p27, and by the production of immature virions.

The simian immunodeficiency virus (SIV) is similar to its human counterpart (HIV) in virion morphology, gene structure and biological properties (1,2,3). These agents were originally isolated from immunosuppressed rhesus macaques (SIV_{mac}) (4) but similar viruses have been recovered from other macaques (5,6) and African green monkeys (7). SIV is similar to HIV in its requirement for the CD4 receptor and has the ability to induce disease in macaques (8) which resembles the acquired immunodeficiency syndrome (AIDS) of man. These similarities provide encouragement that SIV infection and disease in macaques or rhesus monkeys may provide a realistic model for the evaluation of putative drugs and vaccines directed against HIV infection.

Recently there has been a marked interest in compounds which inhibit the HIV proteinase as potential antiviral agents

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(9,10,11). The target enzyme which has been classified as an aspartic proteinase is essential for the processing of the gag and gag-pol polyproteins into the various structural proteins (p17, p24, p9 and p7) and virion-associated enzymes (proteinase, reverse transcriptase, integrase and RNase-H). In an earlier publication (9) we described a series of peptide derivatives which inhibit the proteinases of HIV-1 and HIV-2 at concentrations in the nanomolar range and show antiviral effects at similar concentrations in cell culture models. These compounds are highly selective (>10,000) for the HIV enzyme with little or no affinity for other human aspartic proteinases such as renin, pepsin, cathepsin D, cathepsin E and gastricin.

Because of the potency and selectivity of these peptide derivatives we decided to investigate their effect on the growth of SIV_{mac} with a view to evaluating this type of compound against simian immunodeficiency disease in monkeys. The results with the lead compound Ro 31-8959 (Fig.1) against the growth of SIV_{mac251} in the human T-cell line C8166 suggests the SIV virion proteinase has similar physical and biological properties to the enzymes of HIV-1 and HIV-2. An evaluation of this compound for therapeutic effect in SIV infected monkeys would appear to be warranted.

MATERIALS AND METHODS

Virus and Cells: The SIV_{mac251} strain, an isolate from the New England Primate Centre, was provided by the MRC AIDS Directed Programme. Virus (SIV_{mac251}) was adapted to growth in the human T-cell line C8166 and titrated by end-point dilution. C8166 cells were grown in RPMI 1640 with 10% FCS and infected with SIV_{mac251} at a multiplicity of approximately 0.01 infectious unit per cell. Virus was absorbed for one hour at room temperature before infected cells were washed three times in fresh growth medium prior to use.

Drugs: Ro 31-8959 was made-up as a stock solution in dimethyl sulphoxide at a concentration of 10mg/ml. The nucleoside analogue dideoxyinosine (ddI) was purchased from Sigma.

Experimental Protocols: Infected cells treated with varying drug concentrations or left untreated were scored for syncytia between three and six days post-infection. Samples of culture fluid were taken during this period for measurement of extracelluar levels of p27 core antigen (Coulter SIV Kit).

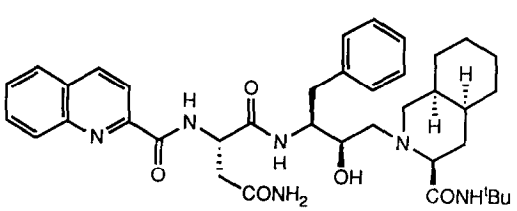
SDS-PAGE Analysis: SIV_{mac251} infected cells were incubated (in drug-free condition) for 2 days post-infection and then treated with either Ro 31-8959 or left untreated. After 24 hours incubation, cells were washed accordingly with either medium containing drug or medium alone and maintained for a further 2 days in medium containing Ro 31-8959 or drug-free. At the end of this time cells were pelleted and viral proteins analyzed by

SDS-PAGE under reducing conditions. Western blot analysis was carried out with monkey immune or non-immune sera or monoclonal antibody to SIV core proteins (Dr J. Stott, National Institute for Biological Standards & Control, South Mimms, Herts) along with an anti-species alkaline phosphatase-labelled detection system. Results were recorded photographically and quantified by scanning densitometry (LKB Ultrascan) using an integration programme.

Electron Microscopy: Infected cells treated with Ro 31-8959 or left untreated (as prepared above) were fixed in glutaraldehyde and processed for transmission electron microscopy as described previously (12). Sections were examined using an Hitachi Hu12A transmission electron microscope.

RESULTS

The effect of Ro 31-8959 (Figure 1) on the growth of SIV_{mac251} was evaluated in the human T-cell line C8166. Concentrations of the proteinase inhibitor greater than 100nM, completely protected cells from cytopathic effect, assessed by lack of syncytium formation and cell deterioration. Inhibition of infectious progeny virus production was demonstrated after titration of the supernatant fluids in C8166 cells (results not shown). The dose response characteristics of Ro 31-8959 in C8166 cells were determined by measuring the extracellular p27 core antigen by ELISA (Table 1). The 50% effective dose (ED50) measured 4 or 5 days post-infection (p.i.) was 10.5nM or less. The activity of the proteinase inhibitor Ro 31-8959 was compared with two drugs currently under clinical investigation for the treatment of AIDS: it was 255 times more active than ddI and 22 times more active than ddC when assayed by the production of p27 core antigen (Fig.2). When Ro 31-8959 was used in combination with the nucleoside analogue ddC, a moderate increase in antiviral activity was observed suggestive of an additive effect (Table 2).



Ro 31-8959

Figure 1
Structure of the proteinase inhibitor.

Table 1. THE EFFECTS OF RO-8959 AGAINST THE GROWTH OF SIV_{mac251} IN C8166 CELLS

Compound	nM	4 Days+	5 Days+
Drug free	-	107 (100%)	543 (100%)
RO-8959	300	N.D. (0%)	N.D. (0%)
	150	N.D. (0%)	N.D. (0%)
	75	N.D. (0%)	N.D. (0%)
	38	N.D. (0%)	8.4 (1.6%)
	19	2.9 (2.7%)	30.1 (5.5%)
	10	5.9 (5.5%)	243.0 (45%)
	5	32.6 (30%)	633.0 (>100%)
	2.5	113.0 >(100%)	670.0 (>100%)
*ED_{50}		4.1nM	10.5nM

N.D. = None detected.
*ED_{50} = 50% Effective Dose.
+P27 antigen at times post-infection.

The proteinase inhibitor Ro 31-8959 was well tolerated by a number of human T-cell lines at concentrations which far exceeded those necessary to prevent the growth of HIV-1 and HIV-2 (9). In the present study, the cytotoxicity of this compound was evaluated in the host-cell line C8166, by exposing cells to varying concentrations of inhibitor for up to 5 days (Fig.3). After this time the viability of each cell-population was determined by a tetrazolium reduction assay (13). The therapeutic index (4,500) was calculated for Ro 31-8959 by comparing the 50% cytotoxic dose (CD50) in C8166 cells (27.5μM) to the mean ED50 for antiviral effect (0.006μM n=4).

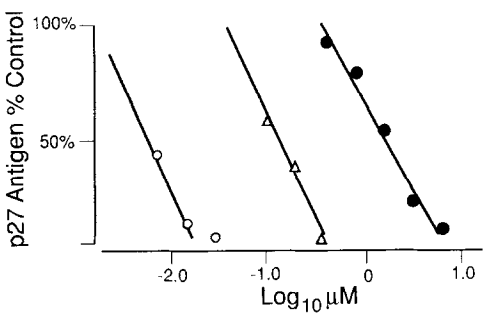


Figure 2

Dose response curves for the anti-SIV activity of ddI (● - ●), ddC (▲ - △) and Ro 31-8959 (○ - ○) determined by the level of p27 antigen in the supernatant fluids of infected C8166 cells.

Table 2. EFFECT OF RO 31-8959 AND ddC IN COMBINATION AGAINST THE GROWTH OF SIV_{mac251}

		ddC (nM)		
		300	60	0
Ro 31-8959 (nM)	30	0%	0%	0%
	15	0%	30%	30%
	7.5	24%	53%	49%
	3.8	14%	67%	64%
	1.9	37%	75%	83%
	0	58%	91%	100%
	ED_{50}	<1.5nM	7.1nM	7.0nM

Values represent percentage levels of p27 antigen in drug-free control. ED_{50}: 50% effective dose for Ro 31-8959.

To confirm the mode of action of Ro 31-8959, C8166 cells were exposed to SIV at a multiplicity of infection of 0.01 and incubated for 48 hours p.i. Infected cells were thoroughly washed in growth medium and a proportion of cells then received Ro 31-8959 at relatively high concentration (5μM) with the remainder of cells maintained in drug-free medium. After 24 hours, cells were washed and fed fresh medium with or without drug. After a further 48 hours incubation, cells were recovered and the constituent proteins separated by SDS-PAGE under reducing conditions. The SIV core proteins were identified by Western-blot analysis using monkey immune sera (Fig.4) or monoclonal

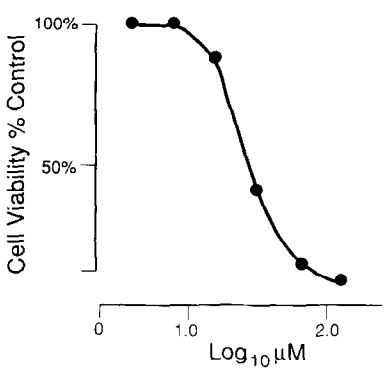
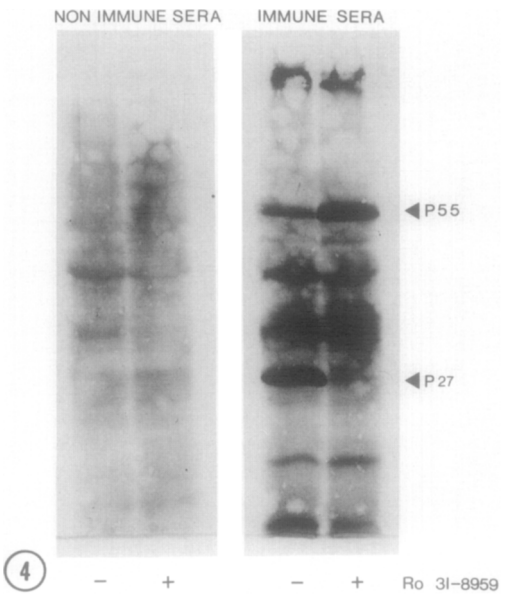
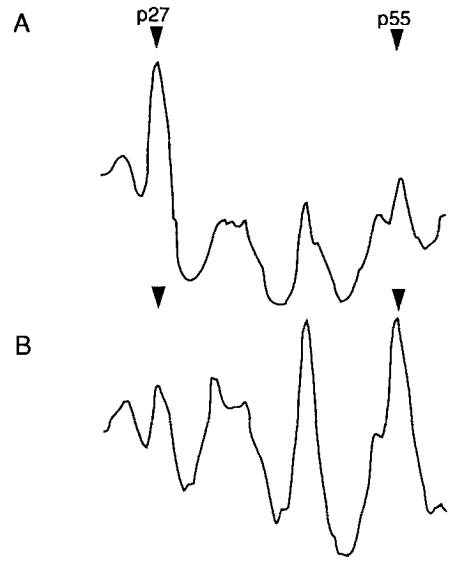


Figure 3

Dose response curve for the growth of C8166 cells in the presence of Ro 31-8959 for five days by the tetrazolium reduction assay.





A: Drug-free B:Ro 31-8959

Viral protein	Peak Area Au/mm	%	Peak Area Au/mm	%	Relative Change (B:A)
p55	0.567	33	0.795	60	Increase (40%)
p27	1.141	67	0.541	40	Decrease (53%)
Total :	1.708	100	1.336	100	

5

Figure 4

SIV infected cells were treated with Ro 31-8959 at 2 days post-infection or left untreated and the cells recovered at 4 days post-infection. After SDS PAGE analysis, SIV proteins were identified by Western-blot analysis using monkey immune serum and the p55 and p27 core antigens identified by comparison with molecular weight standards. Similar Western-blots were carried out using non-immune monkey sera.

Figure 5

The levels of synthesis of SIV core proteins as analysed in figure 4 were quantified by scanning densitometry and the relative amounts of each protein in Ro 31-8959 treated or untreated cells determined by integration. Details of the estimated amounts of each protein as computed by the densitometer is included.

antibody to p27/p55 core antigen and precursor (results not shown). It was evident from these data that exposure of infected cells to the proteinease inhibitor Ro 31-8959 caused an accumulation of the precursor protein p55 with a concomitant depletion of the major core antigen, p27. The relative change in the proportion of core protein was determined by scanning densitometry: the quantified results are summarised in Fig.5.

Evidence that virus production continued after the addition of Ro 31-8959 was obtained by electron microscopy. Mature particles in the untreated control cells showed the classical lentivirus morphology with an oblique core structure (Fig.6b). In contrast,

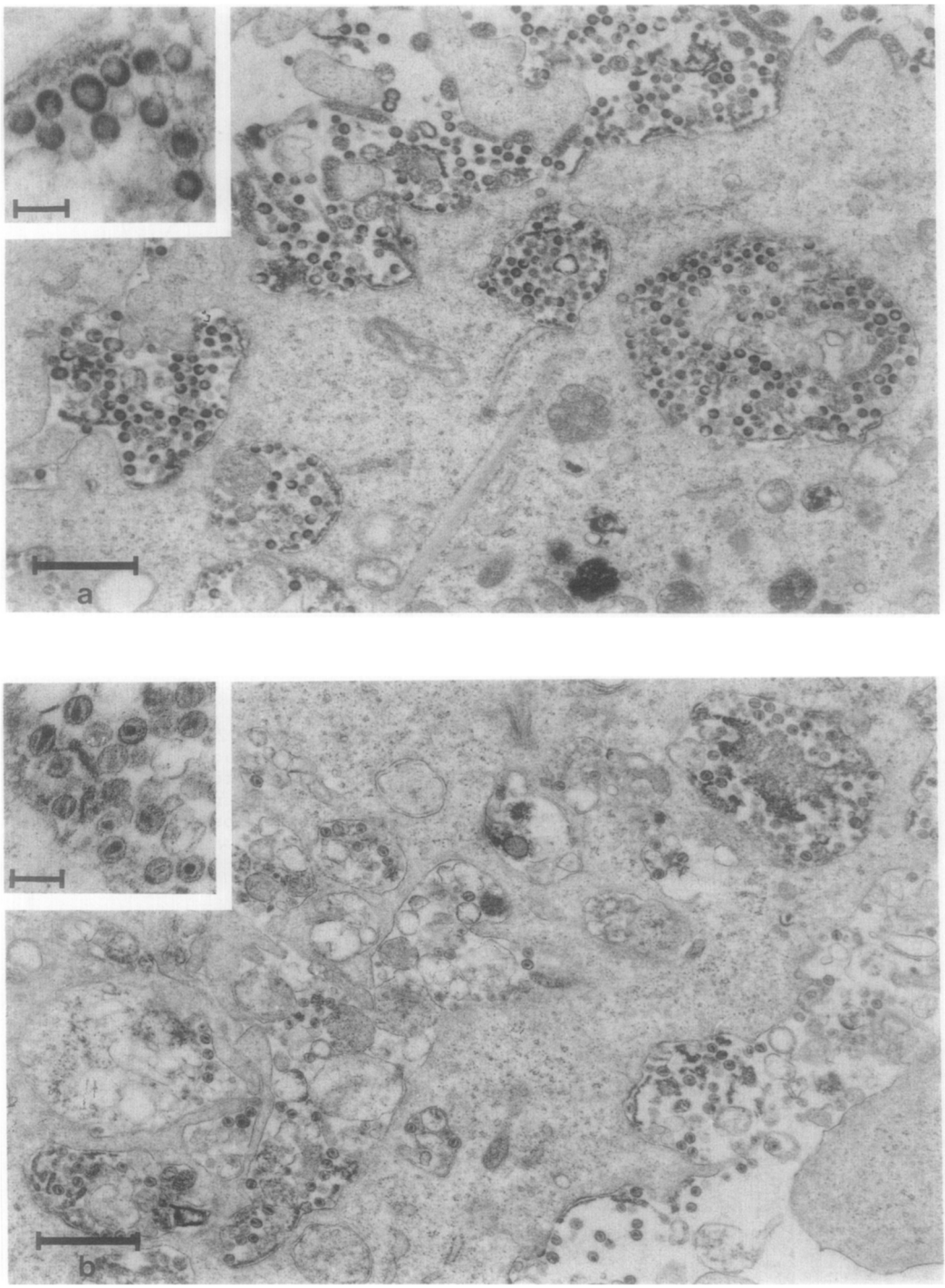


Figure 6

SIV infected cells treated with Ro 31-8959 (a) or left untreated (b), as indicated in the legend to figure 4, were processed for ultra-thin sectioning and compared by electron microscopy. After treatment with the proteinase inhibitor the characteristic retroviral particles (b) were predominantly replaced by abberant immature particles (a). Bar markers represent 1μm (main figure) and 200nm (inset).

Ro 31-8959 treated cultures were characterised by a predominance of apparently immature virions which lacked the core and retained a distinct electron opaque edge (Fig.6a). Both sets of cell cultures were typified by the accumulation of virions in vesicles as well as at the cell surface.

DISCUSSION

Ro 31-8959 is a potent and highly selective inhibitor of the HIV-1 and HIV-2 encoded proteinases and has been shown to inhibit the growth of both viruses in tissue culture (9). Here we report data which demonstrates a similar antiviral against SIV_{mac251}. This compound has remarkable selectivity for the proteinases encoded by primate retroviruses with little or no effect on related mammalian aspartic proteinases. These data suggest that Ro 31-8959 could be well tolerated in vivo. This compound, unlike the nucleoside reverse transcriptase inhibitors, does not require metabolic activation to exert an antiviral effect. It is possible that Ro 31-8959 could be useful in the treatment of HIV infections including those which fail to respond to inhibitors of reverse transcriptase, by bringing about a reduction in both virion and antigen load. A critical factor in the clinical application of traditional peptides is their in vivo stability. However, peptide mimetics such as Ro 31-8959, which does not contain normal peptide bonds, should be more stable towards metabolic degradation by proteolytic enzymes. Although the behaviour of peptide derivatives may be different in monkey when compared to man, the in vitro potency of Ro 31-8959 and mode of action justifies an in vivo evaluation against SIV.

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