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Antiviral properties of Ro 31-8959, an inhibitor of human immunodeficiency virus (HIV) proteinase

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Summary

Ro 31-8959 inhibits the spread of HIV infection and the production of cytopathic effects in cultures of acutely infected cells. IC_{50} values for these effects are in the range 0.5–6.0 nM and IC_{90} values are in the range 6.0–30.0 nM. This inhibitor is effective even when added to cultures at a late stage of infection, after syncytia have started to form. Virus antigen, virus particles and virus cytopathic effects can largely be cleared from cultures treated with compound from 3 days until 6 days post infection. In chronically-infected cells, inhibition of virus maturation can be detected after 24 hours' treatment with 10 nM Ro 31-8959. In addition, a significant reduction of the proteolytic processing of p56 to p24 can be demonstrated in these cells with compound at picomolar concentrations. These properties indicate that Ro 31-8959 is highly effective against HIV with the potential to inhibit acute, established acute and chronic infections.

HIV; Proteinase; Inhibitor

Introduction

Ro 31-8959 is a selective inhibitor of the aspartic proteinase encoded by HIV (Roberts et al., 1990). This proteinase is essential for the maturation of

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infectious virions, as demonstrated in studies of its mutational inactivation (Kramer et al., 1986; Kohl et al., 1988; Peng et al., 1989). Its function is to process two polyprotein products of the gag and pol genes (p56 and p160) into four virion core proteins (p17, p24, p9 and p7) and essential viral enzymes (proteinase, reverse transcriptase and endonuclease) (Ratner et al., 1985; Dunn and Kay, 1990).

Two main forms of HIV infection may be addressed in vitro. In acute infection, newly infected cells enter a replicative cycle in which infectious virus is released into the culture medium and infected cells may fuse with uninfected cells to produce syncytia, thereby causing cell to cell spread of virus. For most cells, acute infection leads to cell death. However some cells may survive initial infection and become chronically infected. These cells contain integrated HIV proviral DNA and can produce infectious virus, but are also capable of their own continued replication.

The ability of peptide analogue inhibitors of HIV proteinase to inhibit both processing of gag and gag-pol polyproteins and HIV production in both acute and chronic infections has been demonstrated previously (McQuade et al., 1990; Meek et al., 1990). However, the analogues employed were of relatively low potency, with 50% inhibitory concentrations (IC_{50}) only in the micromolar region. Here we report antiviral activity of a more selective inhibitor of HIV proteinase with antiviral activities against acute and chronic infections in the picomolar to nanomolar concentration range. In addition, we report the apparent clearance of virus production from cultures of cells which were allowed to establish an infection for 3 days before treatment with compound.

Materials and Methods

Inhibitor Ro 31-8959, systematic name *N-tert*-butyl-decahydro-2-[2(*R*)-hydroxy-4-phenyl-3(*S*)-[[*N*-(2-quinolylcarbonyl)-L-asparaginyl]amino]butyl]-(4aS,8aS)-isoquinoline-3(*S*)-carboxamide, was provided by Dr S. Redshaw, Roche Products Ltd., Welwyn Garden City, U.K., and was produced during a programme of rational design of peptide-based inhibitors of HIV proteinase supervised by Dr J.A. Martin (Roche Products Ltd.). It was originally referred to as compound XVII (Roberts et al., 1990).

Cell lines and viruses

JM cells were obtained from Dr A.S. Tyms, MRC Collaborative Research Centre, Mill Hill, London, U.K. CEM cells chronically infected with HIV-1 (III B) were supplied by Prof. J. Oxford, The London Hospital Medical College, London, U.K. Dr P.J. Greenaway, PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, U.K., provided HIV-1 (GB8). All cell lines were free from mycoplasma contamination, as detected by electron microscopy.

Syncytium reduction assay

JM cells (2×10^5 cells/ml) were exposed to HIV-1 (GB8) at a multiplicity of infection (MOI) of 0.01 syncytium-forming units/cell. Aliquots (0.2 ml) of cells were placed in microtitre wells with appropriate dilutions of Ro 31-8959 added to 5 replicate cultures. After incubation for 3 days at 37°C in a humidified atmosphere of 5% CO_2 in air, syncytia were counted and the percentage inhibition was calculated:

$$\% \text{ Inhibition} = \left[1 - \frac{\text{syncytia formed in presence of compound}}{\text{syncytia formed in absence of compound}} \right] \times 100$$

IC_{50} and IC_{90} were estimated from graphs of % inhibition plotted against concentration of Ro 31-8959.

Measurement of antiviral activity with compound added 3 to 6 days post infection

JM cells were infected with HIV-1 (GB8) as described above and maintained in culture in 75-cm^2 flasks for 3 days. The cells were counted, passaged at 2×10^5 cells/ml into 25-cm^2 flasks and Ro 31-8959 was added to each culture at different concentrations. After a further 3 days in culture, the cells were counted, and the viability of each culture was measured by means of trypan blue exclusion. Proliferation values were assigned according to the multiplicity of increase in cell number between 3 and 6 days post infection. Cells were harvested and prepared for immunofluorescent staining, immunoblotting and electron microscopy.

Immunofluorescent staining

Cells were harvested, washed twice with 0.01 M phosphate-buffered saline, (PBS) pH 7.4, and resuspended at 1.0×10^7 cells/ml in PBS. Smears were prepared by drying the cells on microscope slides and fixing with methanol for 10 min at 18°C. The cells were covered with mouse anti-p56/p24 monoclonal antibody (Roche Diagnostica, Basle), diluted in 0.01 M PBS, pH 7.4. After incubation in a humidified atmosphere at 37°C for 1 h, the cells were washed for 15 min in PBS and covered with goat anti-mouse IgG antiserum conjugated to fluorescein isothiocyanate (Dako Ltd.). They were then maintained at 37°C and washed as before, mounted in 90% glycerol in PBS (pH 8.0) with 0.1% (w/v) p-phenylenediamine (Johnson and Nogueira Araujo, 1981) and examined by means of fluorescence microscopy.

Electrophoretic and immunological analysis

Experimental samples were collected by centrifugation and resuspended in SDS-PAGE sample buffer (Laemmli, 1970) and heated for 10 min at 100°C.

Aliquots (10 μl) were fractionated through a 15% SDS-polyacrylamide gel via a 3.3% stacking gel (Laemmli, 1970). Fractionated proteins were transferred to nitrocellulose as described by Towbin et al. (1979). Immunological detection was accomplished using an HIV-1 gag p24 monoclonal antibody. Determination of immunoreactive polypeptide was via a horseradish peroxidase-coupled second antibody and enhanced chemoluminescence (Amersham). For reference a prestained marker mix, containing proteins in the relative molecular mass (M_r) range 14–200 kDa (Gibco-BRL), was run in parallel.

Electron microscopy

Cell pellets containing approximately 1.0×10^6 cells were formed by centrifugation of 5 ml cell culture at $300 \times g$ for 3 min. These were fixed in 2.5% glutaraldehyde in isotonic (300 mosM) sodium cacodylate buffer (pH 7.4) for 30 min at 18°C and then for at least 24 h at 4°C. The treated pellets were then post-fixed in 1% osmium tetroxide in isotonic buffer and treated with 0.5% aqueous uranyl acetate. Specimens were dehydrated in ethanol and embedded via epoxypropane in Araldite resin. Sections were cut with a diamond knife, stained with uranyl acetate and lead citrate and examined with a Philips CM12 electron microscope. Electron micrographs of infected cells were prepared and used to count virions with electron dense central cores (mature) and those with an electron dense periphery (immature). The percentages of mature and immature virions were calculated. Some virus particles with uniform electron density were classified as indeterminate and were eliminated from the calculation.

Results

When JM cells were infected with HIV and incubated in the presence of Ro 31-8959, reduced numbers of syncytia were formed 3 days post infection compared with cultures not treated with compound (Fig. 1). Results from 8 experiments were averaged and the mean IC_{50} was found to be 2.7 nM, with a range of 0.7–5.3 nM; a mean IC_{90} of 16 nM, with a range from 6.2–30 nM, was calculated. (Results from 2 experiments carried out in Dr A.S. Tyms' laboratory, MRC Collaborative Centre, Mill Hill are included with permission). In addition, at concentrations of compound > 10 nM, inhibition of syncytium formation and of virus antigen production was maintained for at least 6 days post infection without further addition of compound. By comparison, ddC in the same system produced 50% inhibition at a concentration of 46 nM and 90% inhibition at 849 nM. (AZT is inactive in JM cells due to a lack of thymidine kinase activity. P. Wong-Kai-In, unpublished observation).

If the addition of Ro 31-8959 was delayed until 3 days post infection, when syncytia were formed with a density of approximately 2×10^3/ml (Fig. 2A), a

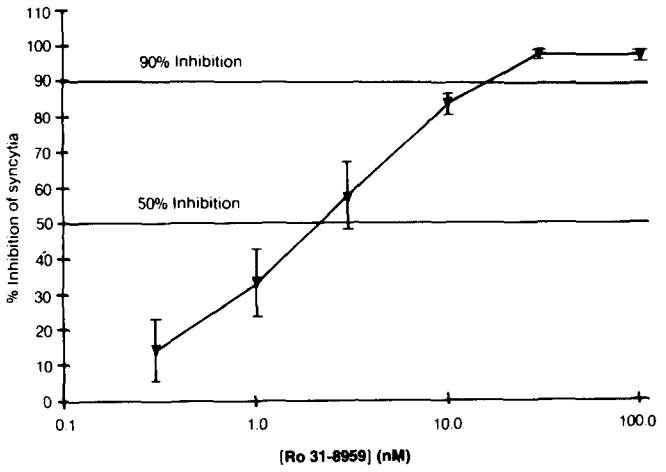

Fig. 1. Percent inhibition of syncytium formation in cultures of JM cells infected with HIV-1 (GB8) treated with Ro 31-8959. 50% and 90% inhibition levels are indicated.

potent antiviral effect on cultures could still be demonstrated 6 days post infection. Widespread cytopathic effects can be seen in untreated cultures (Fig. 2B) and in cultures treated with 1 nM compound (Fig. 2C), while these are rarely found in cultures with 10 nM Ro 31-8959 (Fig. 2D). This inhibition is also seen in terms of virus antigen depletion, demonstrated by means of immunofluorescence (Fig. 3A–E) and immunoblot (Fig. 4). Cultures treated with 10 nM Ro 31-8959 contained virtually undetectable levels of virus antigen. Examination of sections of glutaraldehyde-fixed cells from these cultures indicated that mature virions were produced in the presence of up to 1 nM compound, while they were rarely detected in cultures treated with $>$ 10 nM Ro 31-8959. In the cultures with 1 nM Ro 31-8959 or less, inhibition of the breakdown of p56 to p24 and inhibition of viral maturation could not be demonstrated by immunoblot or electron microscopy, respectively. Finally, infected cultures treated with 10–10 000 nM Ro 31-8959 showed a similar level of proliferation and cell viability to control cultures of uninfected cells (proliferation $\times$ 6–9; 95% viable), while those treated with $<$ 1 nM or without compound contained fewer cells and showed reduced viability (proliferation $<$ 1; viability $<$80%).

CEM cells infected chronically with HIV-1 strain IIIB continued to produce virions and virus antigen after treatment with Ro 31-8959 for 24 h. However, a significant reduction of proportions of mature virions was found in cultures treated with $>$10 nM Ro 31-8959 (mature virions in untreated cultures: 35%; in cultures treated with 10 nM Ro 31-8959: 10%; 100 nM Ro 31-8959: 0.4%; 1000 nM Ro 31-8959: 6.0%; Fig. 5). As expected, treatment of these cells with 1000 nM AZT did not prevent the maturation of virions. In addition, analysis of virus antigen by means of immunoblots showed reduced levels of p24 core protein in cells from cultures treated with 300 pM Ro 31-8959 compared with untreated cultures (Fig. 6).

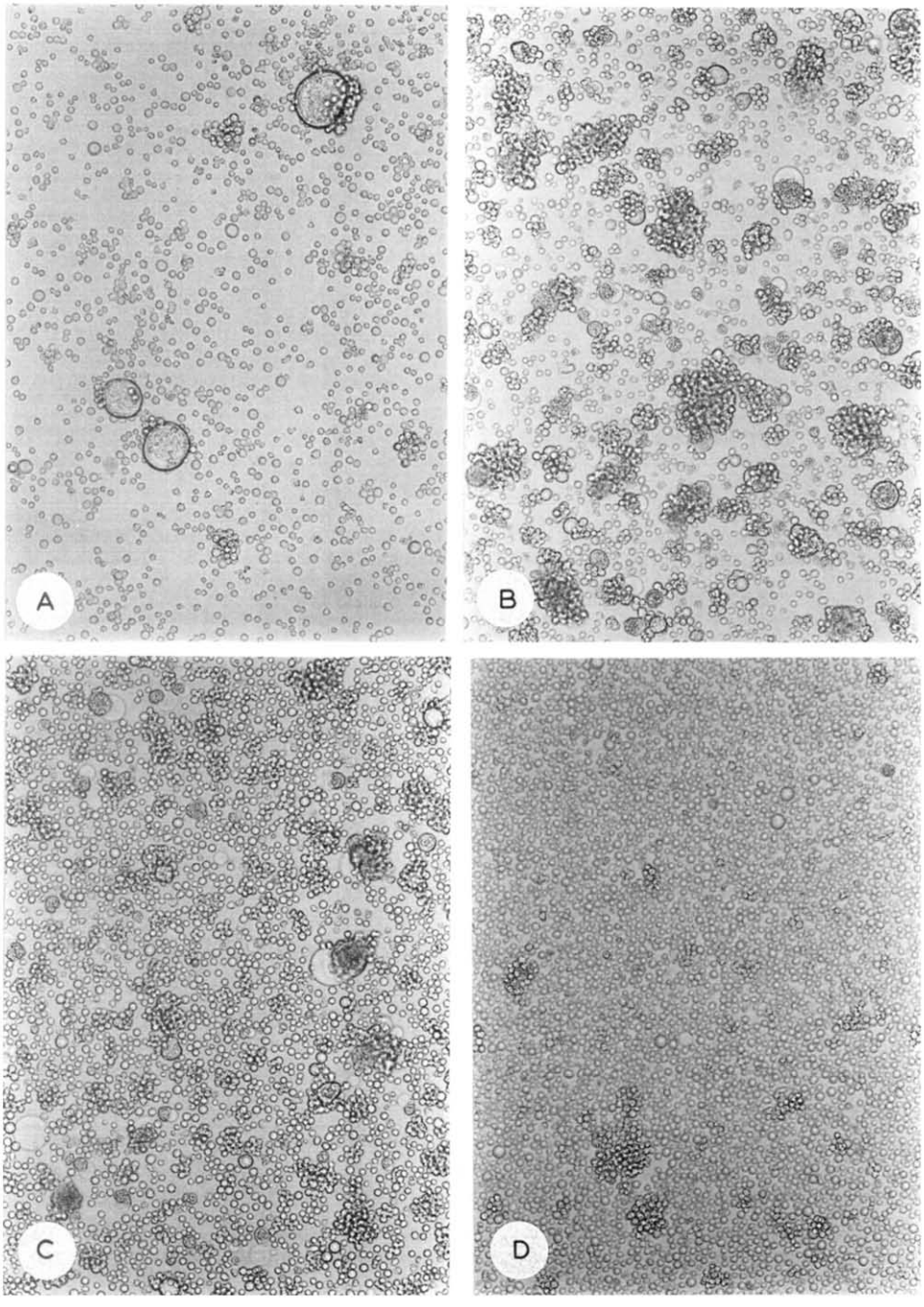

Fig. 2. Cultures of JM cells infected with HIV-1 (GB8): (**A**) 3 days post infection; (**B**) 6 days post infection; treated from day 3 with Ro 31-8959 at concentrations of (**C**) 1 nM; (**D**) 10 nM (× 130).

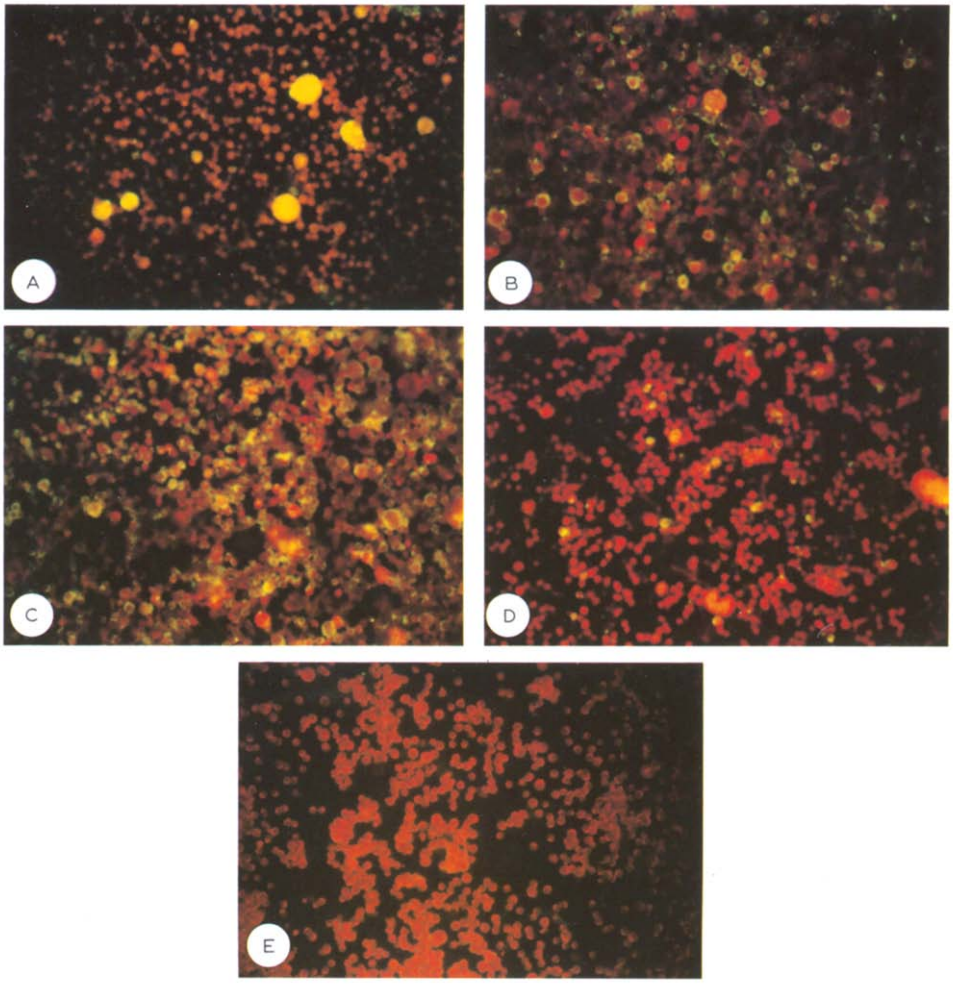

Fig. 3. Immunofluorescent staining of cells harvested from cultures (**A** to **D**) as described in the legend to Fig. 2 (×91). (**E**) Immunofluorescent staining of uninfected cells treated with 100 nM Ro 31-8959 for 3 days. Yellow/green immunofluorescent stain of virus antigen on cells counterstained red is shown.

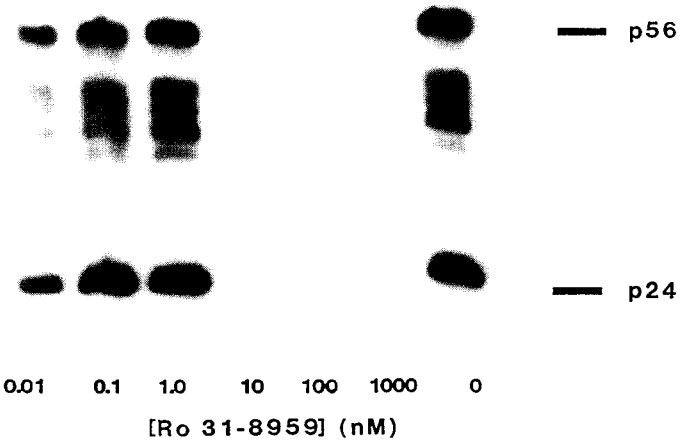

Fig. 4. Immunoblot obtained with cells harvested from cultures of JM cells 6 days post infection with HIV-1 (GB8), treated from day 3 with Ro 31-8959 at concentrations as shown.

Discussion

Results from this study demonstrate a potent antiviral effect of Ro 31-8959 against HIV-1 if it is added to cultures early or late in acute infection or to cultures of chronically infected cells. In all instances, a concentration of 10 nM was sufficient to effect more than 50% reduction of normal virus production, while analysis by immunoblotting demonstrated a significant reduction of gag processing at a concentration of 0.3 nM. The retrieval from cultures with cytopathic effects 3 days post infection of apparently healthy cultures of cells within 3 days of addition of compound demonstrates the ability of Ro 31-8959 to inhibit virus-induced cytopathology and reduce the amount of virus antigen expressed in JM cells under these in vitro conditions. The level of activity reported here in acute infection is of the same order as that reported previously in studies of inhibition of p24 production in cultures of C8166 cells infected with HIV-1 strain RF (Roberts et al., 1990).

Since Ro 31-8959 was designed as a specific inhibitor of HIV aspartic proteinase (Roberts et al., 1990), it is likely that the effects found in cultures of acutely infected cells can be attributed to the inhibition of virus maturation, rendering the released particles non-infectious. Preliminary experiments (unpublished) have confirmed this. Ro 31-8959 is unlikely to inhibit the production of gp120 and gp41 by direct action since these are thought to be the products of cleavage by host cell proteinases. Factors which may contribute to the clearance of virus from cultures by 6 days post infection include the proliferation of uninfected cells and the disintegration of pre-formed syncytia. The possibility that products of HIV aspartic proteinase processing might be involved indirectly in the regulation of syncytium formation cannot be excluded.

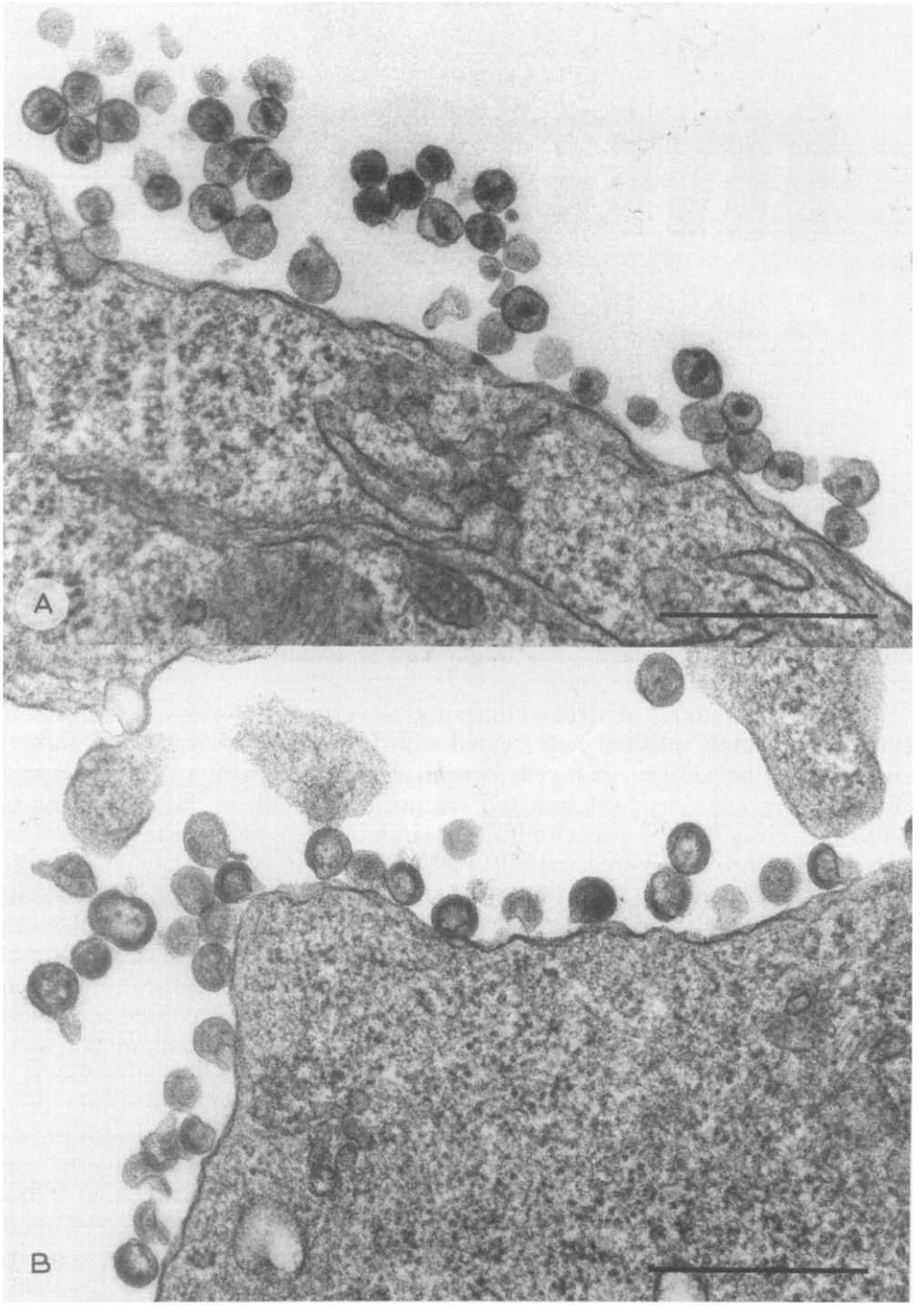

Fig. 5. (**A**) Untreated chronically-infected CEM cell; HIV-1 virus particles have a central condensed core which is typical of mature virus. (**B**) Chronically-infected CEM cells treated with 100 nM Ro 31-8959 for 24 h; HIV-1 virus particles have an electron-dense outer layer which is typical of immature virus. Magnification × 66 000; scale bar represents 0.5 μm.

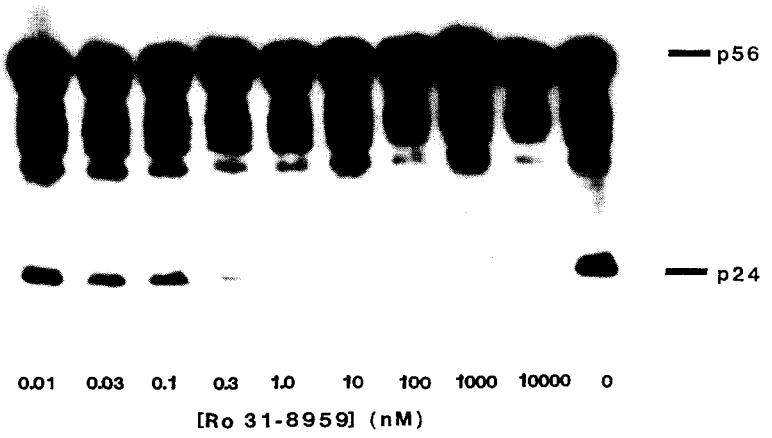

Fig. 6. Immunoblot obtained with CEM cells chronically infected with HIV-1 (IIIB) and treated for 24 h with Ro 31-8959 at concentrations as shown.

In addition, the increased intensity of immunofluorescent stain in cells treated with 1 nM Ro 31-8959 compared with that in untreated cells suggests that impairment of proteinase activity might lead to accumulation of virus antigen under these conditions.

Because the amount of virus antigen and the number of virions produced in cultures of acutely infected cells treated with Ro 31-8959 were limited, further studies were carried out with cells chronically infected with virus. These cells contain integrated virus genome and are not dependent on the production of infectious virus for the perpetuation of virus antigen and virion production. Most of the chronically-infected cells ($>80\%$) contained virus gag antigen and continued to express it after treatment with proteinase inhibitor (J.C. Craig, unpublished observation). The results of the study by electron microscopy show a variability of the reduced proportion of mature virions in those cultures treated with >10 nM Ro 31-8959 compared with untreated cultures. This might be due to virus produced before the addition of compound being retained in association with some cells. In addition, high levels of p56 were found by immunoblot of untreated chronically-infected cell-associated antigens. This finding was consistent with the observation of only 35% mature virions present in these preparations. However, inhibition of both the maturation of virus and the processing of gag protein p56 to produce virus core protein p24 in cultures treated with Ro 31-8959 is clearly demonstrated. These findings are consistent with the inhibition of HIV proteinase by this compound.

The effects of Ro 31-8959 described here in the picomolar to nanomolar range, combined with other evidence of low toxicity (Roberts et al., 1990), indicate that this compound is a promising candidate for further development as a selective antiviral agent in AIDS chemotherapy.

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References

Dunn, B.M. and Kay, J. (1990) Targets for antiviral chemotherapy: HIV-proteinase. Antiviral Chem. Chemother. 1, 3–8.

Johnson, G.D. and Nogueira Araujo, G.M. de C. (1981) A simple method of reducing the fading of immunofluorescence during microscopy. J. Immunol. Methods 43, 349–350.

Kohl, N.E., Emini, E.A., Schlief, W.A., Davis, L.J., Heimbach, J.C., Dixon, R.A.F., Scolnick, E.M. and Sigal, I.S. (1988) Active human immunodeficiency virus protease is required for viral infectivity. Proc. Natl. Acad. Sci. USA 85, 4686–4690.

Kramer, R.A., Schaber, M.D., Skalka, A.M., Ganguly, K., Wong-Staal, F. and Reddy, E.P. (1986) HTLV-III *gag* protein is processed in yeast cells by the virus *pol*-protease. Science 231, 1580–1584.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

McQuade, T.J., Tomasselli, A.G., Liu, L., Karacostas, V., Moss, B., Sawyer, T.K., Heinrikson, R.L. and Tarpley, W.G. (1990) A synthetic HIV-1 protease inhibitor with antiviral activity arrests HIV-like particle maturation. Science 247, 454–456.

Meek, T.D., Lambert, D.M., Dreyer, G.B., Carr, T.J., Tomaszek, T.A. Jr., Moore, M.L., Strickler, J.E., Debouck, C., Hyland, L.J., Matthews, T.J., Metcalf, B.W. and Petteway, S.R. (1990) Inhibition of HIV-1 protease in infected T-lymphocytes by synthetic peptide analogues. Nature 343, 90–92.

Peng C., Ho, B.K., Chang, T.W. and Chang, N.T. (1989) Role of human immunodeficiency virus type 1-specific protease in core protein maturation and viral infectivity. J. Virol. 63, 2550–2556.

Ratner, L., Haseltine, W., Patarca, R., Livak, K.J., Starchich, B., Josephs, S.F., Doran, E.R., Rafalski, J.A., Whitehorn, E.A., Baumeister, K., Ivanoff, L., Petteway, S.F. Jr., Pearson, M.L., Lautenberger, J.A., Papas, T.S., Ghrayeb, J., Chang, N.T., Gallo, R.C. and Wong-Staal, F. (1985) Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature 316, 277–284.

Roberts, N.A., Martin, J.A., Kinchington, D., Broadhurst, A.V., Craig, J.C., Duncan, I.B., Galpin, S.A., Handa, B.K., Kay, J., Kröhn, A., Lambert, R.W., Merrett, J.H., Mills, J.S., Parkes, K.E.B., Redshaw, S., Ritchie, A.J., Taylor, D.L., Thomas, G.J. and Machin, P.J. (1990) Rational Design of peptide-based HIV proteinase inhibitors. Science 248, 358–361.

Towbin, H., Staehelin, T. and Gordon, J., (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350–4354.