Volume 247, number 2, 349-352 FEB 07059 April 1989

Inhibition of HIV replication in cell culture by the specific aspartic protease inhibitor pepstatin A

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Received 17 February 1989

After incubation of H9 cells infected with human immunodeficiency virus (HIV) with pepstatin A at 10^{-4} M for 2, 4, or 11 days, the culture medium contained significantly less HIV core antigen (p24) than controls without pepstatin A and no or only borderline activity of reverse transcriptase was detected. In addition, after pepstatin A treatment no infectious HIV at 2 or 4 days and only minimal amounts at 11 days were detectable in the culture medium.

HIV protease; Pepstatin A; Virus inhibition

1. INTRODUCTION

Virus-encoded enzymes are suitable targets for antiviral therapy because most of these enzymes have a more restricted range of substrate specificity than their cellular or extracellular counterparts. The functions of the viral enzymes are limited to the replication cycle of the virus, and agents specifically inhibiting viral-encoded enzymes should not impair cellular functions.

Retroviral proteases, first described in avian and murine retroviruses [1,2], process their viral gag (and gag-pol) protein precursors but not other proteins (unless they are denatured). Processing of the viral (gag-pol) protein precursors is necessary for the production of infectious virus.

Before searching for suitable inhibitors of retrovirus-encoded proteases, the active site of the enzyme had to be identified. A conserved Asp-Thr-Gly sequence present in the amino acid sequences of retroviral proteases suggested that such proteases are aspartic-type proteases [3-5] (and a structural dimer model for viral aspartic proteases was recently proposed [4]). Further, it was reported that the viral-encoded proteases of bovine leukemia virus (BLV), murine leukemia virus (MLV) and human T cell leukemia virus (HTLV I) could be inhibited in vitro by pepstatin A [6], an aspartic protease inhibitor [7]. Recent site-specific mutagenesis of Asp-25 proved that the HIV protease has an aspartic proteolytic site [8-10], and it was demonstrated that pepstatin A but not some other substances known to block aspartic proteases inhibited in vitro the HIV protease [10-12]. In checking whether pepstatin A also inhibited in vivo the HIV gag processing in cell cultures, we found that intracellular gag processing was partially inhibited [10], and we have now extended these experiments in HIV-infected cultured cells, studying the inhibitory effect of pepstatin A on viral antigen production, on the formation of active reverse transcriptase RT and on the production of infectious HIV.

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2. MATERIALS AND METHODS

2.1. *Cells and virus*

H9 cells were grown in RPMI 1640 supplemented with 20% fetal calf serum (FCS) at 37°C in air plus 5% CO_2. Human immunodeficiency virus (HIV strain III B) was propagated in H9 cells; the supernatant of infected cultures with an infectivity titer

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

of $10^{4.5}$ $TCID_{50}$/ml was used as virus stock (H9 cells and HIV were kindly supplied by Dr R. Gallo).

2.2. *HIV infectivity assay*

Undiluted and serially diluted test materials (0.1 ml for stock virus titration or 0.5 ml for evaluation of experimental cultures) were added to 10^6 H9 cells in 1 or 0.6 ml culture medium, respectively, in microtiter plates and incubated with a medium renewal after 5 days. Supernatants were checked for virus replication by determining HIV core antigen (CA(p24)) and reverse transcriptase (RT) activity on the days indicated in table 1. HIV CA(p24) was determined after detergent lysis using a commercially available antigen capture assay (Abbott Laboratories, North Chicago). RT was measured as described [13].

2.3. *Pepstatin A*

Pepstatin A (Boehringer, Mannheim or Bachem, Switzerland) was dissolved in DMSO (Merck, Darmstadt) at a concentration of 10^{-2} M. This solution was added very slowly to cell culture medium, with constant stirring to prevent precipitation, to a final concentration of 10^{-4} M.

2.4. *Protocol for determining inhibition of HIV replication by pepstatin A*

Aliquots of 10^6 H9 cells were suspended in 1 ml medium containing 10^{-4} M pepstatin A in 24-well microtiter plates and were infected with $10^{0.5}$, $10^{1.5}$ or $10^{2.5}$ $TCID_{50}$ of HIV in 0.1 ml culture medium: duplicate cultures per virus concentration were used. The cultures were incubated at 37°C and 80% of the culture medium was renewed daily until day 4 and every second day thereafter with 50% conditioned medium (by non-infected H9 cells) and 50% fresh medium both containing 10^{-4} M pepstatin A to counteract a possible decay of the inhibitor, although the exact half-life of the activity of pepstatin A at 37°C is unknown. HIV CA(p24), RT activity, and infectious virus present in the supernatants were determined after various incubation periods (see table 1).

3. RESULTS AND DISCUSSION

The experiments were designed to test the inhibitory effect of pepstatin A (i) on the production of viral antigen, (ii) on the formation of active RT, and (iii) on the production of infectious HIV (table 1). As pepstatin A is relatively water insoluble, it was dissolved in DMSO and then diluted very carefully into the medium to a maximal concentration of 10^{-4} M. The concentration of 1% DMSO in the culture medium (resulting from the dilution of the stock solution) did not affect virus propagation (not shown), and 10^{-4} M pepstatin A did not appear to be toxic for HIV-infected H9 cells, as had been previously discussed [10].

To determine whether the inhibitory effect of pepstatin A changed with the length of treatment, we evaluated the effect after incubation of the cell cultures for 2, 4 or 11 days with 10^{-4} M pepstatin A. The dependence of the inhibitory effect on the titer of the HIV inoculum was tested by using three different concentrations of virus inoculum.

This study confirms our previous observation [10] that pepstatin A inhibits the proteolytic processing of the HIV gag precursor in cultured cells; in addition, the studies are extended by the demonstration that pepstatin A inhibits the production of infectious HIV in cell cultures.

No RT activity was detected throughout the entire experimental period in the pepstatin A-containing cultures, not even with the highest titered HIV inoculum. The production of CA(p24) was substantially reduced or undetectable, depending on the HIV inoculum and the length of incubation.

The supernatants of infected cultures were also titrated for infectious virus after 2, 4 and 11 days of incubation. No infectious virus was detected in the culture medium of 10^{-4} M pepstatin A-containing cultures after 2 or 4 days of incubation, and only low titers of HIV ($10^{1.5}$ $TCID_{50}$/ml) were observed after 11 days of incubation in media of cultures originally infected with the highest titered virus inoculum ($10^{2.5}$ $TCID_{50}$). Cultures originally infected with $10^{1.5}$ $TCID_{50}$ showed only borderline values at this time.

The results reported in this paper are interesting because pepstatin A is hydrophobic and it was uncertain whether it would penetrate cells [14], although it has been reported to enter cells and accumulate in organelles [15]. Three possibilities can be envisioned for the site of inhibition; first, pepstatin A may permeate into HIV-infected cells, and thus inhibit the HIV protease intracellularly. Second, it may act at the cytoplasmic membrane, blocking maturation of the gag and gag-pol precursor, thereby preventing virus particle assembly and release, and possibly also activation of RT. The second possibility is more likely, because studies of the topology of gag (-pol) processing of Rauscher and Moloney MLV [16] suggested the membrane as a maturation site. Third, pepstatin A may act extracellularly on released but immature virus particles. In this case, the maturation of immature virus particles released during the first cycle of virus replication would be prevented by pepstatin A. Such extracellular maturation would occur through viral protease within the released particles.

Table 1

Infection of H9 cells with different concentrations of HIV, with (A) and without pepstatin A (B)

HIV inoculum $TCID_{50}$	CA(p24) antigen[a], days post-infection			Infectious HIV[b], days post-infection			RT activity, 10 days post-infection	
	2	4	11	2	4	11	ratio $\frac{rA_c}{dA}$	cpm[d]
A								
$10^{2.5}$	0.339[e]	0.191	1.974	<0.3	<0.3	1.5	1.7	1881
	0.367	0.119	>2.0	<0.3	<0.3	1.5	0.95	1634
$10^{1.5}$	0.095	0.068	1.396	<0.3	<0.3	≤0.3	4.1	3792
	0.081	0.061	0.068	[f]	<0.3	<0.3	1.6	1681
$10^{0.5}$	0.051	0.054	0.059	<0.3	<0.3	<0.3	1.8	1625
	0.051	0.054	0.001	<0.3	<0.3	<0.3	1.5	1649
no HIV	0.053	0.053	0.051	<0.3	<0.3	<0.3	1.4	1573
	0.054	0.050	0.051	<0.3	<0.3	<0.3		
		cut off	0.098					
B								
$10^{2.5}$	0.712	>2.0	>2.0	>2.5	>2.5	>2.5	15.7	17859
	0.681	>2.0	>2.0	>2.5	>2.5	>2.5	9.0	10151
$10^{1.5}$	0.130	1.936	>2.0	1.5	>2.5	>2.5	9.3	12685
	0.104	>2.0	>2.0	1.5	>2.5	>2.5	16.3	17445
$10^{0.5}$	0.056	0.341	>2.0	<0.3	2.0	>2.5	33.5	35905
	0.055	0.329	>2.0	<0.3	2.0	>2.5	24.0	31424
no HIV	0.050	0.054	0.051				1.5	1791
	0.050	0.059	0.124					
		cut off	0.098					

[a] *A* of the antigen capture assay; mean values of duplicate assays
[b] Infectious HIV log $TCID_{50}$/ml
[c] Ratio of incorporated [^{3}H]TTP with poly dA:dT over poly dA:dT
[d] cpm of incorporated [^{3}H]TTP with poly rA:dT template
[e] Results of duplicate cultures
[f] Could not be evaluated for technical reasons

Further virus replication cycles would then not be initiated, even if the immature virus particles could adsorb to and penetrate new cells. The third possibility would require a release of an amount of antigen comparable to the non-inhibited control. However, we found a significantly reduced amount of CA(p24) in the presence of the inhibitor. It is conceivable that a combination of membrane and extracellular inhibitor action could take place, reducing the amount of released CA(p24) considerably; this would not have been detected under our experimental conditions. In these circumstances, however, because antibodies to CA(p24) recognize gag protein precursors, immature particles would have been detected had they been present.

It is also possible that pepstatin A not only blocks the virus protease but also affects other steps in HIV replication: MuLV containing a protease defect [17,18] or HIV which contains an Asp-25 mutant protease [9] produce a significant amount of viral antigen.

Studies continue, in particular to elucidate the topology of the protease action and the exact mechanism of the inhibition of virus replication in this system by pepstatin A.

Acknowledgements: We thank Hans Fritz for valuable discussions, E. Oker and G. Pleyl-Wisgickl for excellent technical assistance and the Bundesministerium für Forschung und Technologie for financial support.

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