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HIV-1 protease does not play a critical role in the early stages of HIV-1 infection

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Abstract

We asked whether human immunodeficiency virus type 1 (HIV-1) protease plays a major role in the early stages of infection (i.e. from viral entry to reverse transcription) by using various protease inhibitors (saquinavir, ritonavir, and KNI-272). When assessed in the two-day multinuclear activation of a galactosidase indicator (MAGI) assay, involving a single cycle of HIV-1 replication, all protease inhibitors failed to block infection of HeLa-CD4-LTR-β-gal cells by HIV-1, while reverse transcriptase (RT) inhibitors (AZT and ddI) completely blocked the infection. Moreover, when HIV-1 proviral DNA synthesis was examined by polymerase chain reaction in HeLa-CD4-LTR-β-gal cells exposed to HIV-1 and cultured in the presence of protease inhibitors, a significant amount of proviral DNA was detected, while no proviral DNA synthesis was detected when the cells were cultured in the presence of RT inhibitors. Protease inhibitors also failed to block chloramphenicol acetyltransferase (CAT) expression in HLCD4-CAT cells exposed to HIV-1, while RT inhibitors completely suppressed CAT expression. These results strongly suggest, contrary to a previous report by Nagy et al. (1994), that HIV-1 protease does not play a major role in the early stages of infection. © 1997 Elsevier Science B.V.

Keywords: Human immunodeficiency virus type 1; Protease inhibitors; Proviral DNA

1. Introduction

The final stages in HIV-1 replication involve crucial virus-specific secondary processing of viral precursor proteins mediated by a viral protease (Kramer et al., 1986; Katoh et al., 1987). Indeed, the design of HIV-1 protease inhibitors based on the transition state mimetic concept led to the

Abbreviations: AIDS, acquired immunodeficiency syndrome; AZT, 3′-azido-2′,3′-dideoxythymidine or 3′-azido-3′-deoxythymidine; CAT, chloramphenicol acetyltransferase; ddI, 2′,3′-dideoxyinosine; FCS, fetal calf serum; HIV-1, human immunodeficiency virus type 1; MAGI, multinuclear activation of a galactosidase indicator; PCR, polymerase chain reaction; RT, reverse transcriptase; $TCID_{50}$, 50% tissue culture infectious dose.

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generation of a variety of peptide derivatives highly active against viral replication (Erickson et al., 1990; Roberts et al., 1990) and several different classes of HIV protease inhibitors are presently clinically available. A number of HIV protease inhibitors are now in various stages of preclinical and clinical trials. However, whether HIV-1 protease plays a major role in the early stages of infection (i.e. from viral entry to reverse transcription) as yet remains to be defined.

The possible involvement of protease in the early stages of the retroviral replication was suggested on the basis of results of experiments with equine infectious anemia virus (EIAV) (Roberts and Oroszlan, 1989; Roberts et al., 1991) and HIV-1 (Baboonian et al., 1991; Venaud et al., 1992; Nagy et al., 1994). However, there have been contradictory reports that the viral protease had no role in the early stages in HIV-1 replication (Jacobsen et al., 1992; Kaplan et al., 1996).

In this study, we employed a battery of protease inhibitors (two substrate-based inhibitors and one symmetry-based inhibitor) and asked whether they blocked HIV-1 infection and proviral DNA synthesis in HeLa-CD4-LTR-β-gal cells, and chloramphenicol acetyltransferase (CAT) expression in HLCD4-CAT cells exposed to HIV-1.

2. Materials and methods

2.1. Reagents

3′-Azido-2′,3′-dideoxythymidine (AZT or zidovudine) and 2′,3′-dideoxyinosine (ddI or didanosine) were purchased from Sigma (St. Louis, MO) and CalBioChem (La Jolla, CA), respectively. HIV-1 protease inhibitors, saquinavir (Overton et al., 1990; Roberts et al., 1990) and ritonavir (Kempf et al., 1995), were kindly provided by Roche (Welwyn Garden City, UK) and Abbott (Abbott Park, Ill.), respectively. KNI-272 was synthesized as previously described (Kageyama et al., 1993; Mimoto et al., 1992).

2.2. Cells and virus

HeLa-CD4-LTR-β-gal cells (Kimpton and Emerman, 1992) were obtained from Dr Michael Emerman and HLCD4-CAT cells (Ciminale et al., 1990) from Drs Barbara K. Felber and George N. Pavlakis through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD). ACH-2 cells (Clouse et al., 1989) were obtained from Dr Thomas M. Folks of the Center for Diseases Control (Atlanta, GA). HIV-1 (HIV-1_{LAI} strain) stock preparation was purchased from Advanced Biotechnology (Columbia, MD) and its 50% tissue culture infectious dose ($TCID_{50}$) was determined by the endpoint titration method, as previously described (Leland and French, 1988).

2.3. MAGI assay

HeLa-CD4-LTR-β-gal indicator cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS: Hyclone, Logan, UT), 100 U/ml of penicillin G sodium, 100 μg/ml of streptomycin sulfate, 100 μg/ml of G418 sulfate, and 50 μg/ml of hygromycin B. MAGI assays were performed as previously described (Kimpton and Emerman, 1992), with some modifications. Briefly, HeLa-CD4-LTR-β-gal cells were seeded (10^4 cells per well) and cultured in 96-well microculture plates for 24 h and exposed to HIV-1 at a 30 $TCID_{50}$ dose for 2 h at 37°C. Various concentrations of drugs were added to the culture, 2 h prior to, or 2 h after viral exposure. The drugs were continuously present and no cell washing was performed throughout the culture. In 48 h of culture, the cells were fixed with 1% formaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline (PBS) and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Infected cells were counted in situ by virtue of their blue color after incubation with X-Gal under the inverted microscope. A decrease in the number of blue cells/well, therefore, reflects inhibitory activity of the drugs.

2.4. PCR amplification of HIV-1 proviral DNA

HeLa-CD4-LTR-β-gal cells (4×10^5) were exposed to a 30 $TCID_{50}$ of HIV-1, 2 h after the addition of drugs. The cells were harvested in 2 days of culture, washed three times in PBS, resuspended in lysis buffer at a density of 5×10^6 cells/ml, and the cell lysates were subjected to PCR amplification, as previously described (Kageyama et al., 1994). Briefly, a pair of primers, AS61 (5′-ATT GGG CCT GAA AAT CCA TAC AAT-3′) and AS62 (5′-GGC TGT ACT GTC CAT TTA TCA GGA-3′) was employed, which yielded a 581-bp fragment of the *pol*-region of HIV-1. DNA from 2.5×10^4 cells was used for each PCR amplification. Target DNA sequences were amplified for 30 cycles, each consisting a 1-min denaturation step at 94°C, followed by a 1-min annealing at 60°C and a 1-min extension at 72°C. A 10-min primer extension at 72°C completed the sequence. One tenth of the PCR products was loaded in 3% agarose gel (Nu Sieve; FMC BioProducts, Rockland, ME) with 0.5 μg/ml ethidium bromide (Life Technologies, Gaithersberg, MD) and subjected to electrophoresis.

2.5. CAT-ELISA assay

HLCD4-CAT cells were maintained in DMEM supplemented with 10% FCS, 100 U/ml of penicillin G sodium, and 100 μg/ml of streptomycin sulfate. The procedure of exposure of HLCD4-CAT cells to HIV-1 and the drugs is similar to that in the MAGI assay. HLCD4-CAT cells were seeded at 4×10^5 cells in a 60 mm dish, 24 h prior to virus exposure. After the exposure to HIV-1 and drugs, cells were incubated for 2 days in a 5% CO_2 incubator at 37°C and subjected to the assay for the production of chloramphenicol acetyltransferase (CAT) using the CAT-ELISA kit (Boehringer Mannheim, Germany) (Hoffmann and Tynes, 1995) according to the manufacturer's instructions.

3. Results

3.1. Three protease inhibitors failed to block HIV-1 infection of HeLa-CD4-LTR-β-gal cells

We first asked whether a battery of protease inhibitors blocked the infectivity of HIV-1 against HeLa-CD4-LTR-β-gal cells in the 2-day multinuclear activation of a galactosidase indicator (MAGI) assay (Kimpton and Emerman, 1992). If HeLa CD4-LTR/β-gal cells carrying the HIV-1 LTR and the β-gal encoding gene are infected with HIV-1, proviral DNA is formed and is integrated into the cellular genome, followed by the expression of viral components. The Tat protein, thus produced, activates the Tar region of the LTR and subsequently the β-gal encoding gene, which is located in the down stream of the LTR inside the cells, producing β-galactosidase. The cells can be counted after staining with X-Gal. Thus, this system allows quantification of the viral infectivity in a single cycle of replication.

When HeLa-CD4-LTR-β-gal cells were exposed to a 30 $TCID_{50}$ of HIV-1, 67 blue cells were produced per well in the absence of drugs (Table 1). When treated with AZT or ddI, either prior to or after viral exposure, less numbers of blue cells were observed in a dose dependent manner. The IC_{50} values of AZT and ddI in MAGI assay were 0.01 μM and 7.1 μM when the cells were exposed to drugs 2 h prior to viral exposure. When the cells were exposed to drugs 2 h after viral exposure, values were 0.02 μM and 9.4 μM, respectively. On the contrary, when HeLa-CD4-LTR-β-gal cells were treated with saquinavir, no significant inhibition was observed at concentrations examined up to 20 μM. Two different protease inhibitors, ritonavir and KNI-272, also failed to block the infection of the HeLa-CD4-LTR-β-gal cells by HIV-1. Generally, all three protease inhibitors started to show some cytotoxicity to HeLa-CD4-LTR-β-gal cells at 10 μM and no protease inhibitors could be examined at levels higher than 20 μM.

3.2. Protease inhibitors failed to block HIV-1 proviral DNA synthesis in HeLa-CD4-LTR-β-gal cells

If the viral protease plays a critical role in the early stages of HIV-1 infection (from viral entry to reverse transcription), the presence of protease inhibitors presumably reduces or blocks the synthesis of proviral DNA in the cells exposed to HIV-1. We therefore asked whether two protease inhibitors (saquinavir and KNI-272) could block proviral DNA synthesis in HeLa-CD4-LTR-β-gal cells exposed to HIV-1 using PCR. The PCR analysis employed was semi-quantitative, in a range of 100–10 000 HIV-1 copies (Fig. 1). In the lysates of cells cultured in the presence of AZT or ddI, no proviral DNA was detected (lanes 3 and 4, respectively). However, neither of the two protease inhibitors tested, reduced or blocked the synthesis of proviral DNA at any concentrations examined: KNI-272 (0.05, 0.5, 5, 10, and 20 μM) and saquinavir (0.05, 0.5, 5, 10, and 20 μM). These data corroborated the findings that these protease inhibitors failed to block the infection of HeLa-CD4-LTR-β-gal cells by HIV-1.

Table 1
Protease inhibitors failed to block HIV-1 infection of HeLa-CD4-LTR-β-gal cells in the MAGI assay

Treatment (μM)	Drug added prior to HIV-1 exposure	Drug added after HIV-1 exposure
No drug (67 ± 10)		
Saquinavir		
0.05	63 ± 9	51 ± 8
0.5	71 ± 7	59 ± 10
5	71 ± 9	69 ± 3
10	77 ± 9	65 ± 10
20	48 ± 0	49 ± 9
Ritonavir		
0.05	50 ± 15	53 ± 10
0.5	54 ± 15	56 ± 7
5	71 ± 7	74 ± 2
10	59 ± 2	62 ± 9
20	58 ± 3	56 ± 6
KNI-272		
0.05	51 ± 12	46 ± 6
0.5	76 ± 13	56 ± 5
5	82 ± 17	46 ± 7
10	58 ± 9	47 ± 7
20	50 ± 10	70 ± 14
AZT		
0.01	36 ± 20	42 ± 6
0.1	10 ± 1	17 ± 6
1	2 ± 1	3 ± 1
10	1 ± 1	1 ± 2
ddI		
0.1	74 ± 4	72 ± 6
1	75 ± 2	75 ± 6
10	30 ± 3	38 ± 8
100	1 ± 1	3 ± 2

Antiviral activity of each drug was determined using HeLa-CD4-LTR-β-gal cells in the MAGI assay. The cells were exposed to a 30 $TCID_{50}$ of $HIV\text{-}1_{LAI}$, treated with each drug, 2 h prior to, or 2 h after viral exposure and cultured for 2 days.
Data shown represent the mean values (± 1 S.D.) of triplicate determinations. When HeLa-CD4-LTR-β-gal cells were not exposed to HIV-1, the number of blue cells detected was 0 ± 1.

3.3. Inhibition of CAT production by HLCD4-CAT cells by protease inhibitors

We also employed HLCD4-CAT cells as target cells for HIV-1, in which HIV-1 infection was monitored quantitatively through the Tat-dependent CAT expression (Ciminale et al., 1990). The use of HLCD4-CAT cells carrying the HIV-1 LTR, coupled to the CAT gene, also allows the detection of HIV-1 infection at the early steps, by virtue of their Tat-dependent CAT production, which can be quantified by either enzymatically determining the CAT activity or immunologically determining the amount of CAT produced.

As shown in Table 2, AZT completely blocked the expression of CAT at 1 μM without regard to the timing of drug addition. ddI Also completely blocked CAT expression at 10 μM, when the cells were treated with drug prior to HIV-1 exposure. In contrast, KNI-272 exhibited no significant inhibitory effect on CAT expression at any concentrations tested. Saquinavir produced moderate reduction at 5 and 20 μM but there was no dose response. Ritonavir produced no decrease at 5 μM, although it showed a reduction at 20 μM. The apparent reduction observed with saquinavir and ritonavir was suspected to be, at least in part, due to their inherent toxicity, since no dose re-

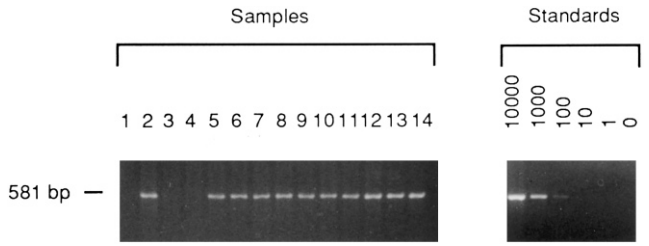

Fig. 1. Saquinavir and KNI-272 failed proviral DNA synthesis in HeLa-CD4-LTR-β-gal cells exposed to HIV-1. HeLa-CD4-LTR-β-gal cells (4×10^5) were exposed to a 30 $TCID_{50}$ of HIV-1_{LAI}, 2 h after drug treatment. Cell lysates were prepared in 2 days of culture and subjected to PCR amplification and gel electrophoresis. Only HIV-1 PCR products (581-bp) are shown. Lane 1: unexposed cells; lane 2: HIV-1_{LAI}-exposed cells; lane 3: 10 μM AZT; lane 4: 100 μM ddI; lanes 5–9: 0.05, 0.5, 5, 10, and 20 μM KNI-272, respectively; lanes 10–14: 0.05, 0.5, 5, 10, and 20 μM saquinavir, respectively. Various numbers of ACH-2 cells served as standards.

sponse was demonstrated and the reduction was seen at high concentrations. Indeed, both drugs showed toxicity to HLCD4-CAT cells even at 10 μM, as assessed under the inverted microscope (data not shown).

4. Discussion

Retroviral protease is primarily thought to play a major role at late stages in retroviral replication. It has been shown, however, that active protease molecules are present and the protease-mediated processing of viral proteins is operational within retrovirus particles after budding from the host cells (von der Helm, 1977; Yoshinaka and Luftig, 1977). There are several reports that retroviral protease plays a role at the early stages of viral replication. Roberts et al. (1990) have reported, that in the case of EIAV, the protease-mediated cleavage of p11 nucleocapsid protein to produce p6 and p4, occurs only when the capsid protein is separated from envelope and other subviral components and protease becomes enzymatically active, suggesting that protease plays a role after viral entry to a target cell (Roberts and Oroszlan, 1989; Roberts et al., 1991). Venaud et al. (1992) subsequently reported that a synthetic anti-HIV peptide analogue, SR41476, efficiently inhibited proviral DNA synthesis in target CEM cells exposed to HIV-1. Baboonian et al. (1991) also reported that a synthetic peptide analogue, UK-88,947, blocked HIV-1 proviral DNA synthesis in H9 cells. More recently, Nagy et al. (1994) reported that two protease inhibitors (UK-88,947 and saquinavir) inhibited HIV-1 infection and proviral DNA synthesis in HeLa-CD4-LTR-β-gal cells exposed to HIV-1. They reported that, as in EIAV, the nucleocapsid protein is cleaved by HIV-1 protease after viral entry into a cell, a process which is required for the proper formation of preintegration complex and/or for its transport to the nucleus.

In the present study, however, we failed to reproduce such data when we employed three protease inhibitors (two substrate-based inhibitors and one symmetry-based inhibitor) and two different reporter cell lines (HeLa-CD4-LTR-β-gal and HLCD4-CAT cells), which allowed quantitation of virus infection at the early steps in a single cycle of HIV-1 replication. Contrary to the data reported by Nagy et al. (1994), all protease inhibitors examined did not block HIV-1 infection when the cells were treated with drugs 2 h before and after viral exposure. Nagy et al. (1994) used saquinavir and UK-88,947, while we employed saquinavir and two additional inhibitors, ritonavir and KNI-272. It is not clear as to why these apparently opposite observations were made in the same assay, using the same target cells. The failure of inhibition of proviral DNA synthesis by protease inhibitors in HeLa-CD4-LTR-β-gal cells exposed to HIV-1 is also in disagreement with data reported by Nagy et al. (1994) and Baboonian et al. (1991).

In our quantitative PCR, the amount of proviral DNA produced in the cells was all comparable, strongly suggesting that both inhibitors, saquinavir

and KNI-272, failed to block the proviral DNA synthesis in HeLa-CD4-LTR-β-gal cells. It should be noted, that the proviral DNA amount was determined within the density range of the standard samples and it is unlikely that the comparable DNA amount was due to a template excess in the samples examined (Fig. 1). In this respect, Jacobsen et al. (1992) reported saquinavir failed to block the synthesis of proviral DNA, its integration into cellular DNA and its transcription in a one-step, acute infection of MT-4 cells, and concluded that viral protease does not play a role in the early stages of HIV-1 infection. More recently, Kaplan et al. (1996) also concluded that HIV-1 protease does not play a role early after infection, when they employed conditional HIV-1 protease mutants as probes. Our present data are in agreement with these two reports.

In conclusion, contrary to previous reports (Baboonian et al., 1991; Nagy et al., 1994), our data showed that HIV-1 protease does not play a major role in the early stages of HIV-1 replication, although a possibility that the viral protease is operational after viral entry cannot be absolutely precluded.

Table 2
Amounts of CAT produced by HLCD4-CAT cells exposed to HIV-1_{LAI} and protease inhibitors

Treatment (μM)	Drug added prior to HIV-1 exposure	Drug added after HIV-1 exposure
No drug 142 ± 15 (pg/ml)		
Saquinavir		
0.5	104 ± 5	100 ± 10
5	78 ± 0	81 ± 18
20	66 ± 28	90 ± 5
Ritonavir		
0.5	78 ± 0	107 ± 19
5	136 ± 0	136 ± 15
20	77 ± 12	70 ± 33
KNI-272		
0.5	117 ± 4	125 ± 8
5	162 ± 0	166 ± 7
20	159 ± 10	117 ± 4
AZT		
0.1	16 ± 22	16 ± 22
1	0 ± 0	0 ± 0
10	0 ± 0	0 ± 0
ddI		
1	82 ± 6	100 ± 10
10	0 ± 0	47 ± 0
100	0 ± 0	0 ± 0

The amount of CAT was determined using the CAT-ELISA kit. HLCD4-CAT cells were exposed to a 100 $TCID_{50}$ of HIV-1_{LAI}. The drugs were added to culture, 2 h prior to, or 2 h after the viral exposure. On day 2 of culture, cell lysates were prepared and subjected to CAT-ELISA.
Data shown represent the mean values (± 1 S.D.) of duplicate determinations.

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