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Antiviral Activity of Human Immunodeficiency Virus Type 1 Protease Inhibitors in a Single Cycle of Infection: Evidence for a Role of Protease in the Early Phase

KÁROLY NAGY,[1] MATTHEW YOUNG,[1] CHRISTINA BABOONIAN,[2] JAMES MERSON,[3] PETER WHITTLE,[3] AND STEPHEN OROSZLAN[1]*

Laboratory of Molecular Virology and Carcinogenesis, ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201,[1] University of North London, London N7 8DB,[2] and Pfizer Central Research, Sandwich, Kent CT13 9NJ,[3] United Kingdom

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The antiviral activities of two substrate-based inhibitors of human immunodeficiency virus type 1 (HIV-1) protease, UK-88,947 and Ro 31-8959, were studied in acute infections. H9 and HeLaCD4-LTR/β-gal cells were infected either with HIV-1_{IIIB} or a replication-defective virus, HIV-*gpt*(HXB-2). Both inhibitors were capable of blocking early steps of HIV-1 replication if added to cells prior to infection. Partial inhibition was also obtained by addition of inhibitor at the time of or as late as 15 min after infection. The inhibitors were ineffective if added 30 min postinfection. The inhibitory effects were studied by cDNA analysis with PCR followed by Southern blot hybridization and by infectivity assays allowing quantitation of HIV-1 in a single cycle of replication. When UK-88,947-treated H9 cells were coinfected with HIV-1 and human T-cell leukemia virus type I only the replication of HIV-1 was inhibited, demonstrating viral specificity. Pretreating the infectious virus stocks with the inhibitors also prevented replication, indicating that the inhibitors block the action of the viral protease and not a cellular protease. A panel of primer sets was used to analyze cDNA from cell lysates by PCR amplification at 4 and 18 h postinfection. Four hours after infection, viral specific cDNA was detected with all of the four primer pairs used: R/U5, *nef*/U3, 5′ *gag*, and long terminal repeat (LTR)/*gag*. However, after 18 h, only the R/U5 and *nef*/U3 primer pairs and not the 5′ *gag* or LTR/*gag* primer pair were able to allow amplification of cDNA. The results suggest a crucial role of HIV-1 protease in the early phase of viral replication. Although it is not clear what early steps are affected by the protease, it is likely that the target is the NC protein, as referred from our previous reports of the in situ cleavage of the nucleocapsid (NC) protein by the viral protease inside lentiviral capsids. The results suggest that it is not the inhibition of initiation and progression of reverse transcription but the stability of full-size unintegrated cDNA which is affected in the presence of protease inhibitors. Alternatively, the cleavage of the NC protein may be required for the proper formation of preintegration complex and/or for its transport to the nucleus.

The protease (PR) is one of the virally encoded enzymes of human immunodeficiency virus type 1 (HIV-1) that are essential for virus replication. HIV-1 PR functions in the late phase of virus replication by processing of Gag and Gag-Pol polyproteins that are the primary translational products of the genome-size mRNA and assemble with genomic RNA to form the noninfectious immature particles. To produce mature infectious virus, the cleavage of the polyproteins into smaller functional structural units is necessary (32, 43). Therefore, the PR is a potential target for chemotherapy of HIV infection (22).

A large number of specific HIV PR inhibitors with various chemical structures have been reported. Many of them exhibit potent antiviral activity (17, 28, 44, 47). The ability of PR inhibitors to inhibit the processing of Gag and Gag-Pol polyproteins and production of infectious HIV in chronic as well as in acute infections (27, 29) may represent a major advantage over inhibitors of reverse transcriptase (RT), such as zidovudine (AZT) and analogs, which readily block acute infection but are unable to reduce the number of infectious virions produced in a chronically infected cell.

The possible involvement of retroviral PR in the early stages of viral replication cycle was suggested on the basis of in vitro experiments with purified capsids of equine infectious anemia virus (EIAV), a lentivirus related to HIV (36–39). These studies demonstrated a novel retroviral protein processing pathway mediated by the viral PR that involves the regulated in situ cleavage of the mature nucleocapsid (NC) protein assembled with the viral RNA. It was proposed that such a proteolytic event may be required for the progression of virus replication in the early phase of infection. Furthermore, Baboonian et al. (2) observed that a specific inhibitor of HIV-1 PR, UK-88,947, not only inhibited HIV-1 maturation in the late phase but apparently prevented cDNA synthesis when added to the infected cells prior to infection. In contrast, it was reported by others (18) that another potent inhibitor of HIV-1 PR, Ro 31-8959, did not restrict progression of the early steps of HIV-1 replication. These seemingly contradictory observations, however, were made by using either amplification of cDNA by semiquantitative PCR with a single primer pair (5′ *gag*) at a single time point (18 h) after infection (2) or only with a single dose (1 μM) of PR inhibitor (18).

In this communication, we report the results of experiments designed to study acute HIV-1 infection of different cell lines in the presence and absence of UK-88,947 and Ro 31-8959. We

* Corresponding author. Mailing address: Laboratory of Molecular Virology and Carcinogenesis, ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, P.O. Box B, Frederick, MD 21702-1201. Phone: (301) 846-1355. Fax: (301) 846-1666.

used PCR amplification with a panel of oligonucleotide primer pairs recognizing various regions of the viral genome. In addition, we carried out quantitative infectivity assays in a single-cycle infection with HIV-1_{IIIB} and a replication-defective virus, HIV-*gpt*(HXB-2), in the presence and absence of the same inhibitors. Our results demonstrate a definitive dose- and time-dependent antiviral effect of these inhibitors under conditions which do not allow secondary infection by progeny virus. Coinfection of H9 cells with HIV-1 and human T-cell leukemia virus type I (HTLV-I) demonstrated specificity of inhibition.

MATERIALS AND METHODS

Cells and viruses. Cells and viral reagents obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, were as follows: HeLaT4 cells from R. Axel, HeLaCD4-LTR/β-gal cells from M. Emerman, and HIV-*gpt* and HXB2*env* plasmid DNAs from K. Page and D. Littman. HIV-1 clone 4803 DNA was obtained from E. M. Fenyö, and the C8166 cell line was obtained from P. Nara. COS-7 (ATCC CRL-1651), HeLaT4, and HeLaCD4-LTR/β-gal cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. HIV-1_{IIIB} (1.7×10^5 syncytium-forming units per ml; obtained from P. Nara)-infected H9 cells (ATCC CRL-8543) and HTLV-I-producing MT-2 cells (obtained from Y. Adachi) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics.

HIV-1_{IIIB} stocks were prepared from 24-h supernatants of freshly infected H9 cells at 4 to 6 days after infection. Infectious titer was determined by infectivity assays on C8166 cells (2) and on HeLaCD4-LTR/β-gal cells (21). Supernatants with titers of 2.1×10^5 IU/ml were filtered through a 0.45-μm-pore-size filter (Millipore) and stored at −70°C. Viral DNA in stock preparations was assayed by PCR, using the JA4 and JA7 *gag* primers (see below).

HTLV-I stocks were prepared from the culture media of virus-producing cell line MT-2 (31). The relative amounts of viral particles were determined by HTLV-I RT assay (7). Aliquots with 5.8×10^6 cpm/ml were stored at −70°C.

Infectious HIV-*gpt*(HXB-2) was produced by cotransfection of COS-7 cells with the HIV-*gpt* plasmid DNA and HXB-2*env* DNA (33). COS-7 culture supernatants were harvested at 72 h after transfection, filtered, and stored at −70°C. Infectious titer was initially determined by a colony formation assay (33) on HeLaT4 cells and later in an infectivity assay (21) on HeLaCD4-LTR/β-gal cells (multinuclear activation of galactosidase indicator [MAGI] assay; see below). Stocks of HIV-*gpt*(HXB-2) virus preparation with a titer of 1.2×10^4 IU/ml were used for all subsequent infections.

HIV PR inhibitors. The two substrate-based inhibitors, UK-88,947 and Ro 31-8959, used in this study were synthesized in the laboratories of Pfizer Central Research, Sandwich, United Kingdom. UK-88,947 is an inhibitor reported by Baboonian et al. (2), and Ro 31-8959 was synthesized by published methods (16, 40). Inhibitors were dissolved in dimethyl sulfoxide, and 10 mM stocks were stored at −70°C.

Infectivity assays. Cultures of H9 cells (10^6/ml) were infected with HIV-1_{IIIB} at a multiplicity of infection (MOI) of ~0.1 IU for 1 h. After infection, the cells were washed three times in phosphate-buffered saline (PBS), resuspended in culture medium, and maintained at 37°C. When tested, inhibitors were added to the cells at various time points prior to, at the time of, or after infection and were present at all subsequent stages. In some experiments, H9 cell cultures were harvested at 18, 36, and 120 h postinfection for analysis of presence of viral cDNA, then inhibitors were removed from the culture medium by resuspending cells in fresh medium, and cells were further cultured for up to 21 days. Aliquots of culture supernatant were collected at the indicated time points and analyzed for RT activity (34).

Colony formation assay. HeLaT4 cells (2×10^5 cells per well in a six-well plate) were infected with HIV-*gpt*(HXB-2) virus preparation (MOI of 0.1 to 0.2). Virus was allowed to adsorb for 1 h at 37°C, and then fresh culture medium was added. PR inhibitors or AZT were added to the cultures 1 h prior to or 2.5 h after virus infection. Compounds were present at all subsequent stages. Culture medium was replaced by selection medium containing mycophenolic acid (50 μg/ml) 18 h after virus infection (33). Twelve days after infection, colonies of drug-resistant HeLaT4 cells were stained with methanol containing methylene blue (0.25%) and basic fuchsin (0.25%) and counted. Percent inhibition of virus infection by the PR inhibitors was calculated as mean number of colonies in inhibitor-treated wells compared with the number of colonies in untreated wells; 50 and 90% inhibitory concentrations (IC_{50} and IC_{90}) were than calculated by the Hill equation (8), using the Fig. P program (Fig. P Software Corp., Durham, N.C.).

MAGI assay. HeLaCD4-LTR/β-gal cells were seeded in 24-well plates (4×10^4 cells per well) 1 day prior infection. Cultures were infected with an MOI of 0.1 to 0.2 of HIV-*gpt*(HXB-2) or HIV-1_{IIIB} for 1 h at 37°C, and then fresh medium (1 ml per well) was added. PR inhibitors with various concentrations were added to the cultures prior to or after virus infection and were present throughout the experiments. To prevent virus spread, recombinant soluble CD4(rsCD4; 5 μg/ml; DuPont) was included to the medium of HIV-1_{IIIB}-infected cultures 1 h postinfection. Forty hours after infection, infected cells were counted in situ with a light microscope by virtue of their blue color after incubation with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (21). Percent inhibition was calculated as mean numbers of blue cells of inhibitor-treated cultures compared with those of untreated cultures. IC_{50} and IC_{90} values were determined by Hill analysis with 95% confidence limit.

Coinfection of H9 cells with HIV-1 and HTLV-I. H9 cells (10^6/ml) were incubated in culture medium containing UK-88,947 (24 μM) for 1 h prior to infection, and inhibitor was present throughout all subsequent stages. The cell cultures were (i) infected with HTLV-I virus alone (5.8×10^6 cpm/ml) (11), (ii) infected with HIV_{IIIB} alone (MOI of 0.1), or (iii) coinfected with HTLV-I and HIV-1_{IIIB} in the presence of 10 μg of DEAE-dextran per ml for 4 h at 37°C; then cells were washed and cultured at 37°C. Samples of untreated and inhibitor-treated infected H9 cells were collected 18 h postinfection. Synthesis of HIV-1 and/or HTLV-I cDNA was assayed by DNA hybridization analysis after amplification of viral DNAs by PCR.

PCR amplification of cDNA. To analyze viral DNA, H9 cells (10^6/ml) acutely infected with HIV-1_{IIIB} and/or HTLV-I were washed once in PBS and resuspended in 0.1 ml of a lysis buffer (1) (10 mM Tris HCl [pH 8.3], 1 mM EDTA, 0.5% Triton X-100, 0.001% sodium dodecyl sulfate, 300 μg of proteinase K per ml). The samples were incubated at 55°C for 1 h and then boiled for 15 min. These lysates were used directly in PCR amplification as described below.

The primer sets used for HIV DNA amplification were M667 and AA55 (48), which amplify a 140-bp fragment corresponding to sequences in the R/U5 region of long terminal repeat (LTR); AA943 and AA946 (49), which amplify a 218-bp fragment corresponding to sequences in the *nef* gene

that proceed and overlap the U3 region of the 3′ LTR; JA4 (positions 1319 to 1338) and JA7 (positions 1615 to 1596) (1), which amplify a 298-bp fragment corresponding to a conserved sequence in the capsid (CA) region of the *gag* gene; and M667 and M661 (48), which amplify a 200-bp fragment in the LTR/*gag* region including the viral primer binding site. Primers used for HTLV-I DNA amplification were *gag*3 (5′TTATGC CAGACCATCCGGC) and *gag*4 (5′TATCTAGCTGCTGGT TGATGG), which amplify a 119-bp fragment corresponding sequences of the capsid protein region of the HTLV-I *gag* gene (42). These primers were selected in order to monitor the progression of cDNA synthesis in infected cells in the presence and absence of PR inhibitors. The conditions for DNA amplification reactions using HIV-specific primers were those described in the HIV detection kit by Perkin Elmer/Cetus Corp. Typically, DNA from 10^5 cells was used in each amplification reaction. Target DNA sequences were amplified for 35 cycles, each consisting a 1-min denaturation step at 94°C followed by a 1-min annealing at 55°C and a 1-min extension phase at 72°C. An 8-min primer extension at 72°C completed the sequence.

Southern blot hybridization. Amplified DNA resolved on 2.5% agarose gel was transferred to a nylon membrane (PhotoGene; Bethesda Research Laboratories) under capillary conditions and hybridized overnight at 42°C as described by Sambrook et al. (41) with the relevant purified biotinylated DNA probe (BioNick labeling system; Gibco BRL) of either HIV or HTLV. For the HIV-1 probe, a 5.3-kb 5′ fragment of HIV-1 clone 4803 (13) was inserted into a pGEM-5Zf(−) (Promega) vector at the *Sac*I sites, and this construct was amplified in *Escherichia coli* DH10B. For the HTLV-I probe, plasmid pCS95 (6) was used. Hybridized products were visualized by using the PhotoGene chemiluminescence nucleic acid detection system (BRL Life Technologies).

RT assay. HTLV-I and HIV-1 RT activity was determined as previously described (7, 34).

Determination of HIV p24 protein by ELISA. H9 cells (10^6), untreated or treated with UK-88,947 (24 μM) for 1 h prior to infection with HIV-1_{IIIB} (equivalent to approximately 9.7 ng of HIV-1 p24 protein), were collected at 5, 10, 30, and 60 min after infection. Cells were washed three times in 1 ml of PBS to remove unbound viral particles. These washing fluids were collected and reserved for subsequent detection of viral p24. Cell pellets were resuspended in 0.3 ml of 0.25% trypsin solution and incubated at 37°C for 3 min. Viral particles bound to the surface of the cells were released by the trypsin treatment and subsequent centrifugation (1,500 × *g*). The resulting cell pellets were suspended in 1 ml of disruption buffer (of the enzyme-linked immunosorbent assay [ELISA] kit); these samples represented the internalized HIV-1. The amount of viral p24 capsid proteins in the washing fluids and cell lysates was quantitatively determined by HIV-1 core profile ELISA (DuPont, Boston, Mass.). Four parallel determinations were averaged and recorded as single data points (coefficient of variation <12% of the means).

RESULTS

Kinetics of HIV cDNA synthesis. To detect virus infection during the initial stages of the viral life cycle, the cDNA synthesis in H9 cells acutely infected with HIV-1_{IIIB} (MOI of 0.1) was analyzed by PCR using *gag*-specific primers JA4 and JA7. The specificity of the PCR product was determined by hybridization with the HIV-1 DNA probe. Figure 1 shows the analysis of PCR-amplified cDNA from cells harvested at various time points postinfection. No cDNA was detected immediately after infection (0 h). As the PCR assay used is able to detect <10 copies of *gag*-containing DNA (data not shown), the absence of specific PCR products at 0 h demonstrates that the input viral preparation did not contain detectable *gag*-specific cDNA. As shown in Fig. 1, *gag*-specific cDNA was first detected 1 h after infection, and the amount of cDNA increased over a period of 8 h and beyond (see also Fig. 2).

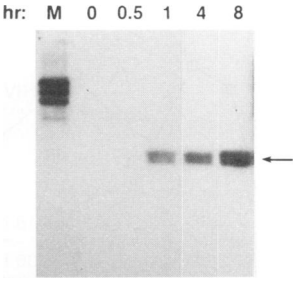

FIG. 1. Kinetics of HIV-1 cDNA synthesis in H9 cells acutely infected with HIV-1_{IIIB} (MOI of 0.1). Shown is hybridization analysis after amplification of 298-bp DNA (←) by PCR using *gag*-specific primers JA4 and JA7. Numbers at the top indicate hours after infection. M, marker DNA, biotinylated φX174 (*Hae*III digested).

Effect of PR inhibitor UK-88,947 on HIV-1_{IIIB} infectivity. To characterize the effect of the PR inhibitor on the course of HIV-1 infection, the synthesis of the cDNA in the presence of UK-88,947 was analyzed in acutely infected H9 cells by Southern hybridization after amplification of cDNA by PCR using the *gag* primer set (JA4-JA7). Initially, we studied the effect of this inhibitor on cDNA synthesis under the conditions described by Baboonian et al. (2). In agreement with this report, we observed a dose-dependent inhibitory effect of UK-88,947 when cell lysates were analyzed 18 h after infection. However, at a concentration of 12 μM (the reported IC_{90}), the results were variable. Therefore, in subsequent experiments, we used this inhibitor at a concentration of 24 μM. When UK-88,947 (24 μM) was added 1 h prior to infection, no cDNA was detected in H9 cells harvested at 18, 36, and 120 h postinfection (Fig. 2). However, cDNA was detected when the inhibitor was added to the cells together with the virus (time 0). Figure 2 also shows that 8 μM AZT inhibited synthesis of *gag*-specific sequences when added to the cells 1 h prior to infection. RT activity was also determined in the culture medium at the time

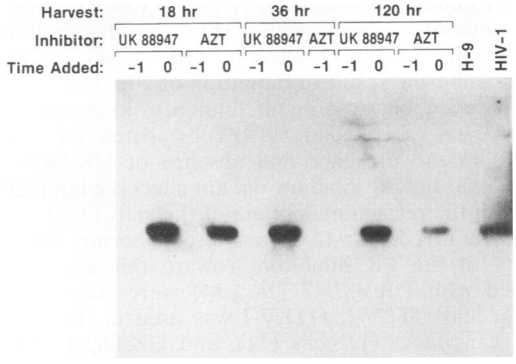

FIG. 2. Hybridization analysis of HIV-1_{IIIB} cDNA synthesis in acutely infected H9 cells treated with UK-88,947 (24 μM) or AZT (8 μM) 1 h prior to (−1 lanes) or at the time of (0 lanes) infection with HIV-1_{IIIB} (MOI of 0.1). Cells were harvested at 18, 36, and 120 h postinfection. DNA of 10^5 cells was subjected to 35 cycles of amplification with *gag*-specific primers JA4 and JA7.

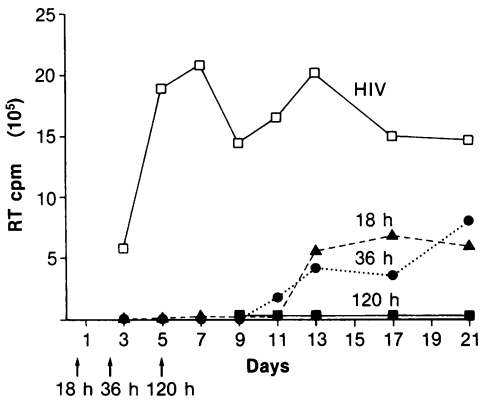

FIG. 3. Reversibility of inhibition of primary HIV infection by early removal of PR inhibitor. UK-88,947 (at a final concentration of 24 μM) was added to cultures of H9 cells 1 h prior to infection with HIV-1_{IIIB} (MOI of 0.1). Inhibitor was removed (↑) from the medium of cultures at 18 h (▲), 36 h (●), and 120 h (■) time points postinfection. □, HIV-1_{IIIB} infection without PR inhibitor treatment. Cells were cultured after removal of inhibitor, and the presence of viral particles in the medium of the cultures was determined by RT assay.

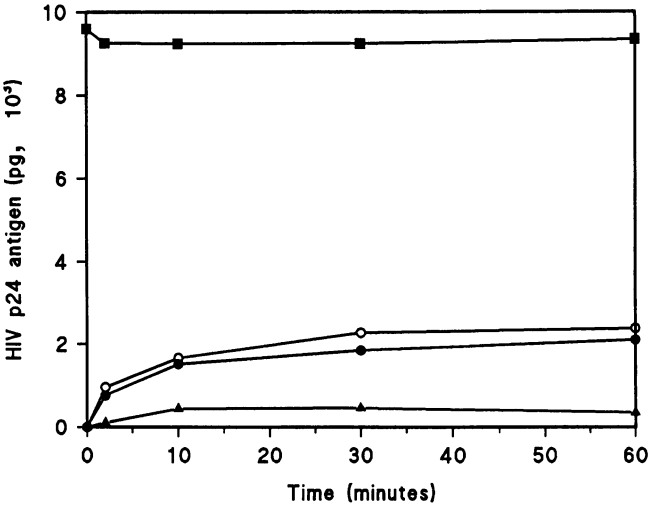

FIG. 4. Kinetics of HIV-1 uptake by H9 cells during acute infection. Cells were treated with UK-88,947 (24 μM) 1 h prior to infection with HIV-1_{IIIB}. Cell samples collected at indicated time points postinfection were washed and treated with 0.25% trypsin for 3 min at 37°C. Data are expressed as picograms of HIV-1 p24 antigen determined by ELISA. Shown are results for internalized p24 in H9 cells untreated (●) and treated (○) with UK-88,947, total amount of p24 recovered from washing fluids and cell lysates (■), and surface-bound p24 removed by trypsin treatment (▲).

of cell harvests (Fig. 2 and 3). No RT activity higher than background was found in the culture medium of inhibitor-treated cells at the time periods indicated. Furthermore, when the inhibitor was removed from the culture medium at 120 h after viral infection, the cultures remained free of RT activity for up to 21 days (Fig. 3). However, when the inhibitor was removed from the cultures at 18 or 36 h postinfection, a low level of virus production was observed after a lag of 7 to 9 days, as shown by RT assays (Fig. 3), indicating the reversibility of the inhibition at the earlier time points.

Analysis of viral entry and kinetics of HIV uptake. Quantitative analysis of the kinetics of HIV p24 uptake during primary infection of H9 cells was carried out to evaluate whether the observed inhibitory effect of UK-88,947 was HIV specific or was due to unspecific inhibition of entry of viral particles. Figure 4 illustrates the time course of virus uptake in untreated and UK-88,947-treated H9 cells.

Consistent with previous observations of others (25, 26), a maximum of 29% (2,100 pg) of the input p24 had been internalized into untreated H9 cells after incubation for 60 min at 37°C. A similar amount, 2,380 pg of p24 (34.5%), was found to have entered inhibitor-treated H9 cells. This finding indicates that the inhibitory effect of UK-88,947 on primary infection is not the result of inhibition of viral entry.

In an independent experiment, immunogold-electron microscopic analyses of H9 (and MT4) cells acutely infected with HIV-1_{IIIB} in the presence and absence of UK-88,947 also suggested that the PR inhibitor did not affect the interaction of HIV-1 with the plasma membrane of the cells (35a).

Selectivity of UK-88,947. It was also important to confirm specificity of the PR inhibitor. Toward this end, H9 cells pretreated with UK-88,947 (24 μM) were coinfected with HIV-1_{IIIB} and HTLV-I. HTLV-I was used in this assay because it can infect H9 cells (11), and UK-88,947 does not inhibit the HTLV-I PR (29a), nor does it affect infection by or maturation of the virus (1a). The synthesis of the cDNA of both HIV and HTLV was studied 18 h postinfection by PCR amplification using the relevant *gag* primer sets (JA4-JA7 and *gag*3-*gag*4, respectively). Figure 5 shows the results of Southern blot hybridization for the detection of amplified cDNA with probes specific for HIV-1 (Fig. 5A) and HTLV-I (Fig. 5B). As expected, in H9 cells infected with either HIV-1 alone (Fig. 5A, lane 1) or HTLV-I alone (Fig. 5B, lane 4), only the specific cDNAs were detected. However, detection of the cDNAs of both HIV-1 and HTLV-I in the coinfected H9 cells (Fig. 5A and B, lanes 3) indicates that infection by both viruses occurred in the same cell culture. In contrast, when H9 cells pretreated

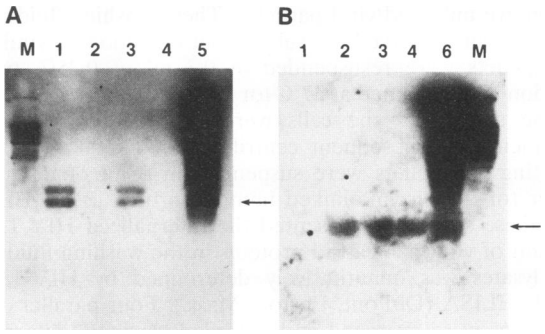

FIG. 5. Hybridization analysis of HIV and HTLV cDNA in H9 cells coinfected with HIV-1_{IIIB} and HTLV-I. Cells were treated with UK-88,947 (24 μM) 1 h prior to infection and harvested 18 h later. DNA of 10^5 cells was amplified by PCR with *gag*-specific primer sets (JA4-JA7 for HIV and *gag*3-*gag*4 for HTLV), electrophoresed in a 2.5% agarose gel, transferred to a nylon membrane, and hybridized with the HIV-1 probe (A) or the HTLV-I probe (B). Arrows indicate the 298-bp HIV-1 *gag* fragment in panel A and the 119-bp HTLV-I *gag* fragment in panel B. Lanes: 1, HIV-1-infected, untreated H9 cells; 2, H9 cells treated with UK-88,947 and doubly infected with HIV-1 and HTLV-I; 3, the same as lane 2 but without PR inhibitor; 4, H9 cells treated with UK-88947 and infected with HTLV-I; 5, HIV DNA; 6, HTLV plasmid DNA; M, DNA marker, biotinylated φX174 (*Hae*III digested).

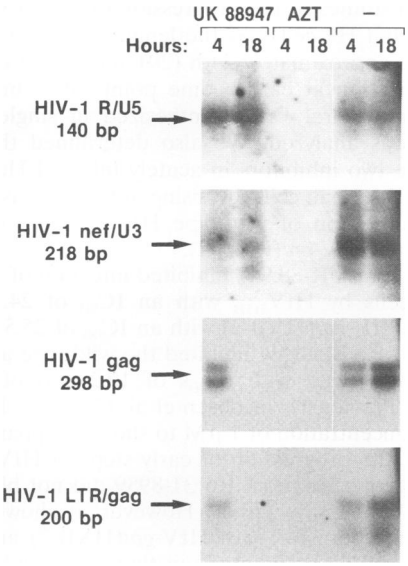

FIG. 6. Hybridization analysis of HIV-1 proviral DNA synthesis in acutely infected H9 cells in the presence of the PR inhibitor UK-88,947. H9 cells were treated with UK-88,947 (24 μM) or AZT (8 μM) 1 h prior to infection with HIV-1_{IIIB} (MOI of 0.1). Cells were harvested at 4- and 18-h time points postinfection, and DNA of 10^5 cells was subjected to 35 cycles of amplification with R/U5-, *nef*/U3-, *gag*-, or LTR/*gag*-specific primers (see Materials and Methods). –, untreated, infected H9 cells. Zero-hour samples were not analyzed for cDNA of input virus by PCR and Southern hybridization, but in the AZT-treated samples, very little (upper-panel 4-h lane) or no cDNA was detected.

with UK-88,947 were doubly infected, no synthesis of HIV-1 cDNA was detected (Fig. 5A, lane 2), whereas HTLV-I cDNA synthesis was not affected (Fig. 5B, lane 2). HeLaT4 cells were also coinfected with HIV-1 and HTLV-I, and UK-88,947 pretreatment resulted in the inhibition of the synthesis of HIV-1 cDNA but not HTLV-I cDNA (data not shown). The untreated, coinfected H9 cells were cultured further, and the presence of HIV-1 and HTLV-I particles in the culture medium was determined by appropriate RT assays (7, 34) up to 19 days, which indicate the establishment of the productive infection. The results showed that the inhibitory activity of UK-88,947 is selective for HIV and that it did not block entry and infectivity of another T-lymphotropic retrovirus, HTLV-I.

PCR amplification of cDNA by using various primers. To obtain a better insight into the early progression of HIV cDNA synthesis and for a more complete characterization of the effects of PR inhibitors, lysates of H9 cells treated with UK-88,947 1 h prior to infection with HIV-1_{IIIB} were analyzed for cDNA by PCR at 4 and 18 h postinfection, using a panel of primer sets. In addition to the *gag*-specific primers (JA4 and JA7) described above, primer sets specific to R/U5 (M667 and AA55), *nef*/U3 (AA943 and, AA946), and LTR/*gag* (M667 and M661) were used. The DNA hybridization results are shown in Fig. 6. At 4 h after infection, cDNA was detected with all four primer pairs. This result indicates that as expected on the basis of the results described above, the virus entered the cell and reverse transcription was initiated in the presence of the PR inhibitor. Surprisingly, after 18 h, only the R/U5- and the *nef*/U3-specific primers were able to detect cDNA. Consistent with experiments described above, the *gag*-specific primers and now the LTR/*gag*-specific primers were unable to amplify cDNA (Fig. 6). These results suggest that by 18 h after infection, the full-size cDNA detected at 4 h in PR inhibitor-treated cells may have been degraded rather than integrated in the presence of UK-88,947. Although a 0-h time point was not included, the observation that very little or no cDNA was detected at any time points in the AZT-treated cells indicates again that the input virus did not contain a detectable amount of cDNA.

Antiviral effect of PR inhibitors in a single cycle of infection by wild-type HIV-1_{IIIB} and a replication-defective virus, HIV-*gpt*(HXB-2). Two assay systems were used to quantitate HIV infectivity in a single cycle of infection. First, the effect on primary infection of UK-88,947 as well as Ro 31-8959 was quantitatively determined in the genetic assay system (33) which utilizes the replication-defective HIV-*gpt*(HXB-2) virus. Infection of HeLaT4 cells leads to the formation of drug-resistant colonies after 10 to 12 days under the conditions of selection medium. As described by Page et al. (33), the number of HeLaT4 cell colonies is proportional to the titer of input virus and secondary infection is prevented because the progeny virus is not infectious. Treatment of HeLaT4 cell cultures with UK-88,947 or Ro 31-8959 1 h prior to infection with HIV-*gpt*(HXB-2) resulted in the reduction of the number of drug-resistant (infected) cell colonies in a dose-dependent manner. When added to the cultures 1 h prior to virus, UK-88,947 (6 to 24 μM) inhibited the formation of infected HeLaT4 colonies by 60 to 90%, and 55 to 90% inhibition was obtained with 5 to 20 μM Ro 31-8959. However, the additions of the PR inhibitors to the HeLaT4 cultures 2.5 h after infection did not prevent formation of colonies, i.e., infection (data not shown).

We also studied HIV infectivity in the presence and absence of PR inhibitors in the MAGI assay, which is based on the activation of the integrated β-galactosidase gene of HeLaCD4-LTR/β-gal cells by HIV Tat protein (21). This assay is rapid and can utilize both replication-defective and replication-competent viruses. Results of representative experiments are presented in Table 1. UK-88,947 and Ro 31-8959 effectively inhibited infection with both HIV-*gpt*(HXB-2) and HIV-1_{IIIB} when added to the cells 1 h prior to infection. UK-88,947 had IC_{50}s of 15.8 and 12.6 μM and IC_{90}s of 25.5 and 24.6 μM, respectively, against the two viruses, while Ro 31-8959 had IC_{50}s of 3.1 and 4.3 μM and IC_{90}s of 12.8 and 10.2 μM, respectively. Addition of the compounds 15 min after infection also inhibited progression of replication of HIV-1_{IIIB}, whereas addition 30 min after infection had a minimal inhibitory effect (Table 1).

When the replication-competent HIV-1_{IIIB} was used in the MAGI assay, rsCD4 was added to parallel cultures after virus infection to prevent possible spread of the virus. It should be noted that omission of the rsCD4 did not result in increase of blue (i.e., infected) cells; however, a higher number of multinucleated giant cells was observed, which were counted as one infectious unit as suggested by Kimpton and Emerman (21).

Treatment of extracellular virus with PR inhibitor. The infectious virus stock was incubated at room temperature in medium containing 24 μM UK-88,947 or 10 μM Ro 31-8959, and after 50-fold dilution to reduce the concentrations of UK-88,947 and Ro 31-8959 to 0.48 and 0.2 μM, respectively, infectivity was assayed at an MOI of 0.1 (Fig. 7). As shown in Fig. 7A and B for UK-88,947 and Ro 31-8959, respectively, virus infectivity gradually decreased with time, and 85 to 90% inhibition of infectivity was obtained after 60 min of treatment. It is also shown that the inhibition was partially reversible by allowing the inhibitors taken up by the virus to leach out (Fig. 7 and legend).

TABLE 1. Effects of PR inhibitors UK-88,947 and Ro 31-8959 on a single cycle infection of HeLaCD4-LTR/β-gal cells with HIV-*gpt*(HXB-2) or HIV-1_{IIIB}[a]

Concn[b] (μM)	% Inhibition[c]			
	−1 h		+15 min, HIV-1_{IIIB}	+30 min, HIV-1_{IIIB}
	HIV-*gpt*(HXB-2)	HIV-1_{IIIB}		
UK-88,947				
6	15	34.5	25	1
12	34	48.8	35	5
24	81	88.1	72	8.9
IC_{50}	15.8	12.6	18	
IC_{90}	25.5	24.6		
Ro 31-8959				
1	45.2	18.6	17	1
5	58.3	54.3	48	8
10	75.2	88.6	80	13.2
20	90.1	99.7	80.8	13
IC_{50}	3.1	4.3	5.3	
IC_{90}	12.8	10.2		

[a] HIV-*gpt*(HXB-2) was produced with cotransfection of COS-7 cells with HIV-*gpt* plasmid DNA and HXB-2 *env* DNA (1.2×10^4 IU/ml). HIV-1_{IIIB} was cultured in H9 cells (2.1×10^5 IU/ml). HeLaCD4-LTR/β-gal cells were infected with of each of the viruses at an MOI of 0.1 for 1 h. To prevent virus spread, rsCD4 (5 μg/ml) was added to the cultures 1 h after infection with HIV-1_{IIIB}.

[b] Inhibitor was added to the cultures at the indicated time intervals before or after infection with virus. At 40 h after infection, X-Gal was added and blue foci of treated and untreated cultures were counted. Percent inhibition was calculated as mean number of blue cells in inhibitor-treated cultures compared with the number in untreated cultures. IC values were determined by Hill analysis. When added at −1 h, AZT inhibited HIV-1_{IIIB} infection with IC_{50} and IC_{90} of 0.2 and 2.3 μM, respectively.

[c] Stock solutions were made in dimethyl sulfoxide. No cytotoxicity was seen even at the highest concentration of dimethyl sulfoxide (0.2%) and inhibitors. The 50% toxic dose of UK-88,947 was 72 μM, and that of Ro 31-8959 was 40 μM.

DISCUSSION

The results presented in this report demonstrate the antiviral activity of HIV-1 PR inhibitors in a single cycle of infection and suggest that the HIV-1 PR has an important function in the early steps (from entry to integration) of viral replication. Specific inhibitors of HIV-1 PR, UK-88,947 and Ro 31-8959, were used in studies of the progression of cDNA synthesis in acutely infected H9 cells. As the length of one cycle of HIV replication is approximately 24 h (20), harvesting the cells for PCR amplification at earlier time points after infection ensured that only viral DNA synthesized in single round of replication was analyzed. We also determined the antiviral effects of the two inhibitors in acutely infected HeLaT4, H9, and HeLaCD4/β-gal cells by using infectivity assays which allowed quantitation of wild-type HIV-1 and a replication-defective virus, HIV-*gpt*(HXB-2).

We found that UK-88,947 inhibited infection of HeLaCD4-LTR/β-gal cells by HIV_{IIIB} with an IC_{90} of 24.6 μM and infection by HIV-*gpt*(HXB-2) with an IC_{90} of 25.5 μM in the MAGI assay. Ro 31-8959 inhibited the wild-type and replication-defective viruses with IC_{90}s of 10.2 and of 12.8 μM, respectively. Previously, Jacobsen et al. (18) used Ro 31-8959 at a single concentration of 1 μM to show an apparent lack of inhibition of the progression of early steps of HIV-1 replication. At this concentration, Ro 31-8959 did not block HIV-1 infection in our assays either. However, it showed a dose-dependent inhibition of both HIV-*gpt*(HXB-2) and HIV_{IIIB} (Table 1). It should be pointed out that although both inhibitors showed substantial antiviral activity when added to HeLaCD4-LTR/β-gal cells 15 min postinfection, complete inhibition could not be reached even with the highest doses used if added at this time point (Table 1) or at the time of infection (data not shown). The uptake of the PR inhibitors by the cell is both dose and time dependent, and it is apparently slower than the receptor-mediated entry of the virus. Therefore, in order to determine IC_{90}s and evaluate progression of cDNA synthesis by PCR, the inhibitors were added 1 h prior to infection. It is also significant that in the experiments in which the infectious virus stocks were pretreated with the inhibitors, pretreatment of the cells was not necessary to obtain nearly complete (~90%) inhibition (Fig. 7). These results demonstrate that the PR inhibitors were capable of penetrating the virus and that tightly bound inhibitor (it is assumed that the inhibitor is bound to the PR inside the particle) entered the cells rapidly, together with the virus. Pretreatment of the infectious extracellular virus was done at the approximate inhibitor concentrations (24 μM UK-88,947 and 10 μM Ro

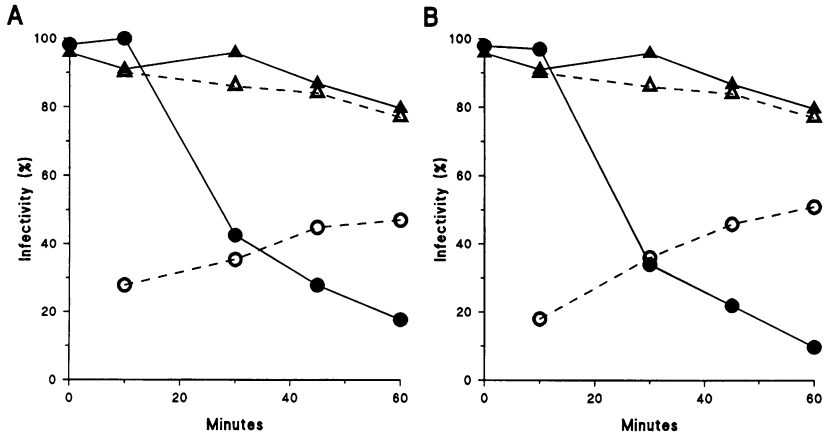

FIG. 7. Treatment of extracellular HIV-1 with PR inhibitors. HIV-1_{IIIB} (10^5 IU/ml) in culture medium was incubated in presence or absence of 24 μM UK-88947 (A) or 10 μM Ro 31-8959 (B) at room temperature. Aliquots were taken at the indicated time points, and infectivity was determined in the MAGI assay. Samples were diluted 50-fold as added to HeLaCD4-LTR/β-gal cells (MOI of 0.1; 0.48 μM UK-88,947 or 0.2 μM Ro 31-8959): ●, inhibitor-treated virus; ▲, untreated virus (MOI of 0.1). After 60 min of incubation, the virus suspensions with and without inhibitor were diluted 1:10 with inhibitor-free medium and incubated further. Infectivity of the samples taken at the indicated times was assayed (MOI of 0.1) again at final inhibitor concentrations of 0.48 μM (A) and 0.2 μM (B): ○, inhibitor-treated virus; △, untreated virus.

31-8959) that were required to give ~90% inhibition in the assays when the cells were pretreated. However, at the time of infection with an MOI of 0.1, the inhibitors had been diluted 50-fold as added to cells. Although under this condition, the inhibitor concentrations for the cells were not higher than 0.48 μM UK-88,947 and 0.2 μM Ro 31-8959, the infectivity was almost completely blocked.

The source of virus used in our assays was the 24-h supernatant from chronically infected H9 cells. One could speculate that the observed inhibitory effects after treatment of the extracellular virus with PR inhibitors were primarily the results of preventing a late maturation step rather than an early event of replication. However, this is unlikely. During the preparation of this report, Kaplan et al. (19) reported that 86% of the extracellular virus produced under similar conditions by CEM cells were morphologically normal mature particles. These authors also observed that HIV-1 of such supernatants from inhibitor treated cells showed a reduction of infectivity unproportionate to the processing of precursor polyproteins. At the inhibitor concentration at which the infectivity titer was reduced by about 80%, approximately two-thirds of the particles appeared to contain fully processed CA protein p24. On the basis of our earlier proposal (36–38), Kaplan et al. (19) considered the possibility "that residual inhibitor carried in the treated virus stock inhibited the protease which caused a failure to cleave the NC protein after infection."

The results obtained when the virus was pretreated indicate that the effects of inhibitors are on the viral PR and not on some cellular protease. Furthermore, the specificity of UK-88,947 was demonstrated by the fact that replication of HTLV-I was not affected by this inhibitor and when inhibitor-treated H9 cells were coinfected with HIV-1 and HTLV-I, only HIV-1 replication was inhibited. This finding together with the results of the experiments on virus uptake (p24 ELISA) and the cDNA analyses (Fig. 6) provided convincing evidence that the effects of the PR inhibitors are not due to unspecific inhibition of virus entry.

Although it is not clear what steps in the early phase of the viral life cycle are affected by the PR inhibitors, analysis of the cDNA synthesized in cells treated with UK-88,947 (Fig. 6) shows that the initiation of reverse transcription is not affected. Similar results have been obtained from in vitro endogenous RT assays with the EIAV cores (38). The fact that the LTR/*gag* primer set was able to amplify cDNA 4 h after infection indicates that reverse transcription has progressed beyond the synthesis of the positive-strand strong-stop DNA and the positive-strand jump and that synthesis has continued at least into the positive-strand *gag* sequence. It has previously been shown that these virus sequences can be detected 1 to 4 h after infection with HIV-1 (15, 20). The lack of any detectable (*gag* sequence) cDNA 18 h after infection in cells treated with the PR inhibitor suggests that the cDNA transcribed in these cells is not integration-competent, stable cDNA. If this DNA is not integrated into the host genome, it may become a target for degradation by cellular nucleases. Kim et al. (20) have suggested that integration of viral cDNA may be the limiting factor to the efficiency of a productive infection. They also reported a decrease in unintegrated linear cDNA detected between 12 and 24 h postinfection and suggested that this may be due to degradation of the reverse transcripts. In addition, the amount of HIV-1 DNA harvested from acutely infected quiescent peripheral blood lymphocytes appears to decrease with time, with an apparent half-life of 1 day (48).

On the basis of in vitro experiments with capsids of EIAV, we have proposed that the PR cleaves the NC protein in the early stages of the viral replication cycle (36–39). In fact, these in vitro experiments have been extended to show that the PR cleavage of the NC protein which occurs during the progression of endogenous reverse transcription is required for the formation of a large-molecular-weight protein-DNA complex (47a). Thus, it seems that the target of the PR in the early phase of viral replication is the NC protein and that its cleavage may be necessary not for reverse transcription per se as previously suggested (2) but for subsequent events, such as the formation of a properly assembled cDNA-protein complex, formation of the preintegration complex, and/or its transport to the nucleus.

This hypothesis also implies that not only the PR but also the NC protein itself and/or its proteolytic fragments may have an active role in the early phase of virus replication. The activity of the NC protein in the early steps has been suggested by studies carried out in vivo (4, 10, 14, 30, 35) and in vitro (23). We have shown that the in situ cleavage of EIAV NC protein inside the capsid is ordered such that the first cleavage occurs within the first zinc finger, followed by the second cleavage within the second zinc finger (36). Interestingly, it has been shown that the two zinc fingers in the HIV-1 NC protein and Rous sarcoma virus NC protein are not functionally equivalent (4, 10, 14). In fact, replacing the second zinc finger of the HIV-1 NC protein with the first motif (pNC1/1) resulted in a virus which was defective in replication, yet RNA packaging and virus maturation were not significantly impaired (14). The fact that this mutant retains the double zinc finger structure of the wild-type virus suggests that the amino acid sequence of the second finger is important for efficient replication of the virus (in the early phase). It is possible that the defect in the replication of the pNC1/1 mutant is due to aberrant proteolytic processing during the early phase of replication.

The exact role of the NC protein in the early phase of replication has not been determined. Therefore, it is hard to speculate on what the function of NC cleavage by the protease would be. It has been reported (9) that the HIV-1 NC protein, as well as synthetic fragments of the NC protein similar to that which would be generated by proteolytic cleavage of the Phe-Asn scissile bond (45) in the first zinc finger, is capable of binding RNA, unwinding tRNA, and promoting RNA annealing and cDNA synthesis, while the synthetic fragments which are similar to the products of the putative second cleavage event are no longer capable of unwinding tRNA and promoting cDNA synthesis. In addition, it has been shown that the NC protein binds to strong-stop cDNA and promotes annealing of this cDNA to HIV-1 RNA (23). The proteolytic processing of the NC protein may play a regulatory role in which the initial cleavage would activate or enhance the activity of the NC protein and the second cleavage would inactivate the protein.

It is also possible that the cleavage of the NC protein is necessary (i) to allow a metamorphosis of the viral core structure to form the preintegration DNA-protein complex or (ii) for transport and protein-driven nuclear localization. Preintegration complexes have been isolated from HIV-1, murine leukemia virus, and avian leukosis virus (3, 5, 12, 24, 46). They have been shown to contain DNA, integrase, and some Gag protein. Future studies should be focused on further characterization of these complexes to fully define their protein compositions and determine whether the NC protein or its proteolytic fragment(s) is present in these structures. In this report, we provided evidence for antiviral activity of HIV-1 PR inhibitors and the role of Pr in the early phase. Their significance deserves further study.

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REFERENCES

1. **Albert, J., and E. M. Fenyö.** 1990. Simple, sensitive, and specific detection of human immunodeficiency virus type 1 in clinical specimens by polymerase chain reaction with nested primers. J. Clin. Microbiol. **28:**1560–1564.
1a. **Baboonian, C.** Unpublished data.
2. **Baboonian, C., A. Dalgleish, L. Bountiff, J. Gross, S. Oroszlan, G. Rickett, C. Smith-Burchnell, P. Troke, and J. Merson.** 1991. HIV-1 proteinase is required for synthesis of *pro*-viral DNA. Biochem. Biophys. Res. Commun. **179:**17–24.
3. **Bowerman, B., P. O. Brown, J. M. Bishop, and H. E. Varmus.** 1989. A nucleoprotein complex mediates the integration of retroviral DNA. Genes Dev. **3:**469–478.
4. **Bowles, N. E., P. Damay, and P.-F. Spahr.** 1993. Effect of rearrangements and duplications of the Cys-His motifs of Rous sarcoma virus nucleocapsid protein. J. Virol. **67:**623–631.
5. **Bukrinsky, M. I., N. Sharova, T. L. McDonald, T. Pushkarskaya, W. G. Tarpley, and M. Stevenson.** 1993. Association of integrase, matrix, and reverse transcriptase antigens of human immunodeficiency virus type 1 with viral nucleic acids following acute infection. Proc. Natl. Acad. Sci. USA **90:**6125–6129.
6. **Ciminale, V., G. N. Pavlakis, D. Derse, C. P. Cunningham, and B. K. Felber.** 1992. Complex splicing in the human T-cell leukemia virus (HTLV) family of retroviruses: novel mRNAs and proteins produced by HTLV type I. J. Virol. **66:**1737–1745.
7. **Clapham, P., K. Nagy, R. Cheingsong-Popov, M. Exley, and R. A. Weiss.** 1983. Productive infection and cell-free transmission of human T-cell leukemia virus in a nonlymphoid cell line. Science **222:**1125–1127.
8. **Debyser, Z., A.-M. Vandamme, R. Pauwels, M. Baba, J. Desmyter, and E. DeClercq.** 1992. Kinetics of inhibition of endogenous human immunodeficiency virus type 1 reverse transcription by 2′,3′-dideoxynucleoside 5′-triphosphate, tetrahydroimidazo-[4,5,1-*jk*][1,4]-benzodiazepin-2(1*H*)-thione, and 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine derivatives. J. Biol. Chem. **267:**11769–11776.
9. **DeRocquigny, H., C. Gabus, and A. Vincent, M.-C. Fournié-Zaluski, B. Roques, and J.-L. Darlix.** Viral RNA annealing activities of human immunodeficiency virus type 1 nucleocapsid protein require only peptide domains outside the zinc fingers. Proc. Natl. Acad. Sci. USA **89:**6472–6476.
10. **Dorfman, T., J. Luban, S. P. Goff, W. A. Haseltine, and H. G. Göttlinger.** 1993. Mapping of functionally important residues of a cysteine-histidine box in the human immunodeficiency virus type 1 nucleocapsid protein. J. Virol. **67:**6159–6169.
11. **Fan, N., J. Gavalchin, B. Paul, K. H. Wells, M. J. Lane, and B. J. Poiesz.** 1992. Infection of peripheral blood mononuclear cells and cell lines by cell-free human T-cell lymphoma/leukemia virus type I. J. Clin. Microbiol. **30:**905–910.
12. **Farnet, C. M., and W. A. Haseltine.** 1991. Determination of viral proteins present in the human immunodeficiency virus type 1 preintegration complex. J. Virol. **65:**1910–1915.
13. **Fredriksson, R., P. Stalhanske, A. Von Gegerfelt, B. Lind, P. Aman, E. Rassart, and E. M. Fenyö.** 1991. Biological characterization of infectious molecular clones derived from a human immunodeficiency virus type-1 isolate with rapid/high replicative capacity. Virology **181:**55–61.
14. **Gorelick, R. J., D. J. Chabot, A. Rein, L. E. Henderson, and L. O. Arthur.** 1993. The two zinc fingers in the human immunodeficiency virus type 1 nucleocapsid protein are not functionally equivalent. J. Virol. **67:**4027–4036.
15. **Hewlett, I. K., S. J. Geyer, C. A. Hawthorne, M. Ruta, and J. S. Epstein.** 1991. Kinetics of early HIV-1 gene expression in infected H9 cells assessed by PCR. Oncogene **6:**491–493.
16. **Hoffmann-LaRoche, A. G.** European patents EP 346 847, 1989, and EP 432 695, 1991.
17. **Huff, J. R.** 1991. HIV protease. A novel chemotherapeutic target for AIDS. J. Med. Chem. **34:**2305–2314.
18. **Jacobsen, H., L. Ahlborn-Laake, R. Gugel, and J. Mous.** 1992. Progression of early steps of human immunodeficiency virus type 1 replication in the presence of an inhibitor of viral protease. J. Virol. **66:**5087–5091.
19. **Kaplan, A. H., J. A. Zack, M. Knigge, D. A. Paul, D. J. Kempf, D. W. Norbeck, and R. Swanstrom.** 1993. Partial inhibition of the human immunodeficiency virus type 1 protease results in aberrant virus assembly and the formation of noninfectious particles. J. Virol. **67:**4050–4055.
20. **Kim, S. Y., R. Byrn, J. Groopman, and D. Baltimore.** 1989. Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: evidence for differential gene expression. J. Virol. **63:**3708–3713.
21. **Kimpton, J., and M. Emerman.** 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated β-galactosidase gene. J. Virol. **66:**2232–2239.
22. **Kräusslich, H.-G., S. Oroszlan, and E. Wimmer (ed.).** 1989. Current communications in molecular biology: viral proteinases as targets for chemotherapy. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
23. **Lapadat-Tapolsky, M., H. De Rocquigny, D. V. Gent, B. Roques, R. Plasterk, and J.-L. Darlix.** 1993. Interactions between HIV-1 nucleocapsid protein and viral DNA may have important functions in the viral life cycle. Nucleic Acids Res. **21:**831–839.
24. **Lee, Y. M. H., and J. M. Coffin.** 1991. Relationship of avian retrovirus DNA synthesis to integration in vitro. Mol. Cell. Biol. **11:**1419–1430.
25. **Lu, S., S. D. Putney, and H. L. Robinson.** 1992. Human immunodeficiency virus type 1 entry into T cells: more-rapid escape from an anti-V3 loop than from an antireceptor antibody. J. Virol. **66:**2547–2550.
26. **Ma, X. Y., K. Sakai, F. Sinangil, E. Golub, and D. J. Volsky.** 1990. Interaction of a noncytopathic human immunodeficiency virus type 1 (HIV-1) with T cells: efficient virus entry followed by delayed expression of its RNA and protein. Virology **176:**184–194.
27. **McQuade, T. J., A. G. Tomasselli, L. Liu, V. Karacostas, B. Moss, T. K. Sawyer, R. L. Heinrikson, and W. G. Tarpley.** 1990. A synthetic HIV-1 protease inhibitor with antiviral activity arrests HIV-like particle maturation. Science **247:**454–456.
28. **Meek, T. D.** 1992. Inhibitors of HIV-1 protease. J. Enzyme Inhib. **6:**65–98.
29. **Meek, T. D., D. M. Lambert, G. B. Dreyer, T. J. Carr, T. A. Tomaszek, Jr., M. L. Moore, J. E. Strickler, C. Debouck, L. J. Hyland, T. J. Matthews, B. W. Metcals, and S. R. Petteway.** 1990. Inhibition of HIV-1 protease in infected T-lymphocytes by synthetic peptide analogues. Nature (London) **343:**90–92.
29a. **Menendez-Arias, L., and S. Oroszlan.** Unpublished data.
30. **Meric, C., and S. P. Goff.** 1989. Characterization of Moloney murine leukemia virus mutants with single-amino-acid substitutions in the Cys-His box of the nucleocapsid protein. J. Virol. **63:**1558–1568.
31. **Miyoshi, I., I. Kubonishi, S. Yoshimoto, T. Akagi, Y. Ohtsuki, Y. Shiraishi, K. Nagota, and Y. Hinuma.** 1981. Type C virus particles in a cord T-cell line derived by co-cultivating normal human cord leukocytes and human leukemic T cells. Nature (London) **294:**770–771.
32. **Oroszlan, S., and R. B. Luftig.** 1990. Retroviral proteinases. Curr. Top. Microbiol. Immunol. **157:**153–185.
33. **Page, K. A., N. R. Landau, and D. R. Littman.** 1990. Construction and use of a human immunodeficiency virus vector for analysis of virus infectivity. J. Virol. **64:**5270–5276.
34. **Popovic, M., M. G. Sarngadharan, E. Read, and R. C. Gallo.** 1984. Detection isolation and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science **224:**497–500.
35. **Rice, W. G., C. A. Schaefer, L. Graham, M. Bu, J. S. McDougal, S. L. Orloff, F. Villinger, M. Young, S. Oroszlan, M. R. Fesen, Y. Pommier, J. Mendeleyev, and E. Kun.** 1993. The site of antiviral

action of 3-nitrosobenzamide on the infectivity process of human immunodeficiency virus in human lymphocytes. Proc. Natl. Acad. Sci. USA **90:**9721–9724.

35a. **Risco, C., et al.** Unpublished data.

36. **Roberts, M. M., T. D. Copeland, and S. Oroszlan.** 1991. *In situ* processing of a retroviral nucleocapsid protein by the viral proteinase. Protein Eng. **4:**695–700.

37. **Roberts, M. M., and S. Oroszlan.** 1989. The preparation and biochemical characterization of intact capsids of equine infectious anemia virus. Biochem. Biophys. Res. Commun. **160:**486–494.

38. **Roberts, M. M., and S. Oroszlan.** 1990. The action of retroviral protease in various phases of virus replication, p. 131–139. *In* L. Pearl (ed.), Retroviral proteases, Control of Maturation and Morphogenesis. Macmillan Press, London.

39. **Roberts, M. M., E. Volker, T. D. Copeland, K. Nagashima, M. B. Cassell, C. J. Briggs, and S. Oroszlan.** 1991. Regulated proteolytic processing within mature retroviral capsids, p. 273–280. *In* A. Kumar (ed.), Advances in molecular biology and targeted treatment for AIDS. Plenum Publishing Co., New York.

40. **Roberts, N. A., J. A. Martin, D. Kinchington, A. V. Broadhurst, J. C. Craig, I. B. Duncan, S. A. Galpin, B. K. Handa, J. Kay, A. Kröhn, R. W. Lambert, J. H. Merrett, J. S. Mills, K. E. B. Parkes, S. Redshaw, A. J. Ritchie, D. L. Taylor, G. J. Thomas, and P. J. Machin.** 1990. Rational design of peptide-based HIV proteinase inhibitors. Science **248:**358–261.

41. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

42. **Seiki, M., S. Hattori, Y. Hirayama, and M. Yoshida.** 1983. Human adult T-cell leukemia virus: complete nucleotide sequence of the provirus genome integrated in leukemia cell DNA. Proc. Natl. Acad. Sci. USA **80:**3618–3622.

43. **Skalka, A. M.** 1989. Retroviral proteases: first glimpse at the anatomy of a processing machine. Cell **56:**911–913.

44. **Tomaselli, A. G., W. F. Howe, T. K. Sawyer, A. Wlodawer, and R. L. Heinrikson.** 1991. The complexities of AIDS: an assessment of the HIV protease as a therapeutic target. Chim. Oggi **9:**6–27.

45. **Tözsér, J., D. Friedman, I. T. Weber, I. Bláha, and S. Oroszlan.** 1993. Studies on the substrate specificity of the proteinase of equine infectious anemia virus using oligopeptide substrates. Biochemistry **32:**3347–3353.

46. **Varmus, H., and P. Brown.** 1989. Retroviruses, p. 53–108. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.

47. **Wlodawer, A., and J. W. Erickson.** 1993. Structure-based inhibitors of HIV-1 protease. Annu. Rev. Biochem. **62:**543–585.

47a. **Young, M., and S. Oroszlan.** Unpublished data.

48. **Zack, J. A., S. J. Arrigo, S. R. Weitsman, A. S. Go, A. Haislip, and I. S. Y. Chen.** 1990. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell **61:**213–222.

49. **Zack, J. A., A. M. Haislip, P. Krogstad, and I. S. Y. Chen.** 1992. Incompletely reverse-transcribed human immunodeficiency virus type 1 genomes in quiescent cells can function as intermediates in the retroviral life cycle. J. Virol. **66:**1717–1725.