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An inhibitor of the protease blocks maturation of human and simian immunodeficiency viruses and spread of infection

(acquired immunodeficiency syndrome/anti-viral therapy/peptidomimetic inhibitor)

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ABSTRACT **The activity of the human immunodeficiency virus (HIV) protease is essential for processing of the gag–pol precursor proteins and maturation of infectious virions. We have prepared a peptidomimetic inhibitor, U-75875, that inhibited HIV-1 gag–pol protein processing in an essentially irreversible manner. Noninfectious virus particles produced in the presence of the drug contained gag precursors and were morphologically immature. In human peripheral blood mononuclear cells and in a continuous cell line, U-75875 completely blocked HIV replication; in the latter case, no spread occurred over a period of 4 weeks. U-75875, on a molar basis, was as potent as 3′-azido-3′-deoxythymidine in blocking HIV-1 replication in human lymphocytes and also inhibited HIV-2 and simian immunodeficiency virus proteases, demonstrating that it has broad activity. These results provide further evidence for the therapeutic potential of protease inhibitors in HIV infection.**

The expanding AIDS epidemic and the relentless nature of the disease have created a desperate need for effective anti-viral therapies to control the replication of the human immunodeficiency virus (HIV) in infected patients. One attractive target for specific anti-viral therapy is the protease, encoded by the *pol* gene of HIV (1, 2). This aspartic protease (3–9) cleaves the viral p55 gag precursor into the four structural proteins of the virion core (p17, p24, p8, and p7); additionally, protease activity is required for cleavage of the p160 gag–pol precursor, which yields protease itself, reverse transcriptase (RT), and endonuclease as well as structural proteins (10). Such processing of the HIV gag and gag–pol precursor polyproteins is essential for the maturation of infectious virions (11–14).

Synthetic peptidomimetic HIV-1 protease inhibitors, containing a nonhydrolyzable synthetic replacement of the P_1-P_1' scissile amide bond, bind to the protease, interfere with the processing of the HIV-1 p55 gag precursor in mammalian cells, and block HIV replication in human T lymphocytes (15–17). However, our studies with one of these compounds, U-81749 {Tba-Cha-Ψ[CH(OH)CH_2]-Val-Ile-Amp; where Tba is *tert*-butylacetyl, Cha is cyclohexylalanine, and Amp is 2-aminomethylpyridine}, indicated that protease inhibition in isolated particles was reversed within a few hours by diluting them in drug-free medium (15). The latter property was useful in establishing that the protease was the target of the inhibitor but undesirable for potential antiviral therapy. We sought, therefore, to identify more potent peptidomimetic HIV-1 protease inhibitors with less reversible activities.

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MATERIALS AND METHODS

Cell Lines Used. H9/HTLVIIIB (a gift of M. Robert-Guroff, National Cancer Institute, National Institutes of Health) is a human T-cell line chronically infected with HIV-$1_{HTLVIIIB}$ that continuously produces infectious virions. CEMx174 (a gift of P. Marx, University of California–Davis Primate Center) is a human T-lymphocyte/B-lymphocyte hybrid cell line that is readily infected and killed by HIV or simian immunodeficiency virus (SIV). MT-4 is an uninfected human T-lymphocyte cell line (18, 19) that was obtained from D. Richman through the AIDS Research and Reference Reagent Program, Division of AIDS (National Institute of Allergy and Infectious Diseases). The cell lines were propagated in RPMI 1640 medium (Quality Biologicals, Rockville, MD) supplemented with 10% (vol/vol) fetal calf serum (FCS; GIBCO), 10 mM Hepes (Quality Biologicals), 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (0.1 mg/ml) (RPMI/FCS).

Western Blot Analysis. A volume (200 μl) of 50% (wt/vol) polyethylene glycol (PEG 8000, J. T. Baker) was added to 1 ml of filtered (0.45-μm-pore filter; Costar) culture supernatant. After 1 hr on ice, precipitated virions were pelleted by centrifugation for 10 min at 10,000 × *g*, resuspended in 100 μl of Laemmli sample buffer, and heated to 100°C for 5 min, and 40 μl was applied to a 10% polyacrylamide gel. Proteins were electrophoretically transferred onto nitrocellulose (Schleicher & Schuell) and incubated with serum from either an HIV-1-infected patient or an SIV-infected monkey. Bound IgG antibodies were detected with ^{125}I-labeled protein A (Amersham) and autoradiography.

Electron Microscopy. H9/HTLVIIIB cells (10 ml at 3×10^5 cells per ml), cultured in the presence or absence of 1 μM U-75875 for 24 hr, were washed twice with RPMI/FCS and resuspended in 10 ml of fresh drug-containing medium. After an additional 24 hr, the cells were pelleted and fixed with 2.5% (vol/vol) glutaraldehyde for 1 hr at 4°C. Fixed cell pellets were processed, embedded, sectioned, and examined in the transmission electron microscope at Advanced Biotechnologies (Columbia, MD).

Titration of Infectious HIV-1. MT-4 cells (90 μl of 1.1×10^5 cells per ml) were added to individual wells of flat-bottom 96-well tissue culture plates. Serial 1:10 dilutions (10 μl) of

Abbreviations: HIV, human immunodeficiency virus; PBMC, peripheral blood mononuclear cell; RT, reverse transcriptase; SIV, simian immunodeficiency virus; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Poa, phenoxyacetyl; DMSO, dimethyl sulfoxide; $TCID_{50}$, tissue culture 50% infective dose; FCS, fetal calf serum; Cha, cyclohexylalanine; Amp, 2-aminomethylpyridine; Boc, *tert*-butyloxycarbonyl; Noa, 1-naphthoxyacetyl.

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filtered culture supernatant from H9/HTLVIIIB cells were added to quadruplicate wells. After 6 days, the MT-4 cultures were screened for the presence of RT. The infectivity titers of the H9/HTLVIIIB supernatants were expressed as tissue culture 50% infective dose ($TCID_{50}$) per ml with 1 $TCID_{50}$ corresponding to the amount of supernatant required to infect 50% of the replicate MT-4 cultures.

Infection of Peripheral Blood Mononuclear Cells (PBMCs) with HIV-1. Ficoll/Hypaque-isolated PBMCs were stimulated for 3 days in RPMI/FCS containing phytohemagglutinin (5 μg/ml). The cells were washed and suspended at 10^7 cells per ml in RPMI/FCS, and HIV-1_{LAV} was added at the multiplicity of 0.005 $TCID_{50}$ per cell. After a 2-hr adsorption period, the volume was raised 20-fold with RPMI/FCS supplemented with 10% (vol/vol) interleukin 2-containing conditioned medium (Boehringer Mannheim). The cells were seeded in 24-well tissue culture plates plus drug additions (2.5×10^5 cells/1.25×10^3 $TCID_{50}$ of HIV_{LAV} in a total volume of 1 ml per well). After 3 days, the cells were diluted 1:2 in fresh drug-containing medium. At 6 days, the supernatants were harvested and analyzed for HIV-1 p24 and RT activity.

Viability Assays. The relative numbers of viable cells were determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] oxidation procedure, which has been shown to correlate well with the trypan blue exclusion assay (20). Quadruplicate reactions were initiated by addition of 10 μl of MTT solution [thiazole blue (Sigma); 5 mg/ml in isotonic phosphate-buffered saline] to 100 μl of cell suspensions in 96-well flat-bottom tissue culture plates. After 4 hr at 37°C, 100 μl of 0.01 M HCl containing 10% (wt/vol) SDS was added to each well. Oxidized MTT was allowed to dissolve in medium for 16 hr at 37°C and the absorbance was measured at 590 nm with an ELISA plate reader (V-max; Molecular Devices, Menlo Park, CA). Relative cell numbers were expressed as percent of the MTT value of control wells that received an equal number of cells but no virus or drug.

RT and p24 Assays. Duplicate 5-μl supernatant samples were incubated with 25 μl of [α-^{32}P]dTTP-containing RT reaction mixture (21) for 1.5 hr at 37°C. Samples (10 μl) were spotted on DE81 paper (Whatman) and the air-dried paper was washed with 0.3 M NaCl/0.03 M sodium citrate. Bound radioactivity was measured using a Betascope 603 blot analyzer (Betagen, Waltham, MA) and expressed as cpm/ml of supernatant. HIV p24 was measured from duplicate 180-μl supernatant samples using the DuPont HIV p24 Core Profile ELISA according to the manufacturer's instructions.

RESULTS

Structure of U-75875. Thaisrivongs *et al.* (22) have described the design and synthesis of dihydroxyethylene isostere Ψ[CH(OH)CH(OH)] transition-state-analogue inserts. Introduction of such inserts into a dipeptidic cleavage site recognized by human renin yielded potent inhibitors ($K_i < 1$ nM) of this aspartic protease. To extend these results, we investigated the relationship between structure and HIV-1 protease inhibitory activity of compounds of the formula W-Xaa-Cha-Ψ[CH(OH)CH(OH)]-Val-Ile-Amp, where W is an amino-terminal acyl moiety and Xaa represents any amino acid. Relative to U-81749, a HIV-1 protease inhibitor (15), one compound of this class showed an ≈3-fold higher inhibitory potency toward purified HIV-1 protease (compound I; Table 1). Substitution of its amino-terminal Boc-Phe with Poa yielded compound III with an ≈10-fold improved inhibitory potency. However, replacement of the carboxyl termini of compounds I and III with Mba (compounds II and IV, respectively) greatly reduced the potency of protease inhibition. Replacement of the Leu-Ψ[CH(OH)CH(OH)]-Val transition-state insert with Cha-Ψ[CH(OH)CH(OH)]-Val (compound V) resulted in an inhibitor equipotent to compound III. This potency was maintained when Boc-Phe was changed to Poa (compound VI). A further improvement in inhibitory potency was obtained by replacing Poa of compound VI with Noa to give compound VII (U-75875; Fig. 1). Some of these compounds were shown to interfere with the processing of p55 in cells infected with a recombinant vaccinia virus (23) encoding the HIV-1 gag–pol proteins. Moreover, analysis of the HIV-like particles produced in these cells in the presence of U-75875 indicated that the protease inhibition was essentially irreversible. Therefore, U-75875 was chosen for detailed studies with HIV-1.

U-75875 Blocks the Processing of gag and gag–pol Precursor Polyproteins in Infected Cells. We analyzed the inhibition of HIV-1 p55 processing by U-75875 in H9 cells chronically infected with the HTLVIIIB isolate of HIV-1. No differences were observed in the total amounts of immunoreactive proteins recovered from supernatants collected between 48 and 72 hr from cultures that were incubated in the continuous presence of various concentrations of U-75875 (Fig. 2), indicating that this drug did not markedly alter the number of particles released from the infected cells. Significantly, 1 μM U-75875 completely blocked the processing of p55 to p24; at 0.1 μM U-75875 partial inhibition was observed. The inhibition of p55 processing in HIV-1 particles by U-75875 was confirmed using an HIV-1 p24 antigen-capture ELISA that efficiently detects p24 but not p55. HIV-1 particles produced in the presence of 0.1–1 μM U-75875 also were deficient in RT activity (20–40% that of control particles), indicating that proper protease function is required for p160 gag–pol precursor processing and full RT activity (Fig. 2). To investigate the kinetics of the protease inhibition, we repeated the experiment using 1 μM U-75875 and collected supernatants containing particles formed during the following times 0–3,

Table 1. Structure–function relationship of peptidomimetic inhibitors of the HIV-1 protease

Compound	Inhibitor structure	K_i, nM
U-81749	Tba-Cha-Ψ[CH(OH)CH_2]-Val-Ile-Amp	70
I	Boc-Phe-His-Leu-Ψ[CH(OH)CH(OH)]-Val-Ile-Amp	28
II	Boc-Phe-His-Leu-Ψ[CH(OH)CH(OH)]-Val-Mba	360
III	Poa-His-Leu-Ψ[CH(OH)CH(OH)]-Val-Ile-Amp	3
IV	Poa-His-Leu-Ψ[CH(OH)CH(OH)]-Val-Mba	350
V	Boc-Phe-His-Cha-Ψ[CH(OH)CH(OH)]-Val-Ile-Amp	3
VI	Poa-His-Cha-Ψ[CH(OH)CH(OH)]-Val-Ile-Amp	2
VII	Noa-His-Cha-Ψ[CH(OH)CH(OH)]-Val-Ile-Amp	<1

K_i determination for inhibition of recombinant HIV-1 protease activity was performed as described (15). Tba, *tert*-butylacetyl; Boc, *tert*-butyloxycarbonyl; Poa, phenoxyacetyl; Noa, 1-naphthoxyacetyl; Amp, 2-aminomethylpyridine; Mba, (2*S*)-methylbutylamine; Leu-Ψ[CH(OH)CH(OH)]-Val, (5*S*)-amino-(3*R*,4*R*)-dihydroxy-(2*R*)-isopropyl-7-methyloctanoyl; Cha-Ψ[CH(OH)CH(OH)]-Val, (5*S*)-amino-6-cyclohexyl-(3*R*,4*R*)-dihydroxy-(2*R*)-isopropylhexanoyl; Cha, cyclohexylalanine. Compound VII has the code name U-75875.

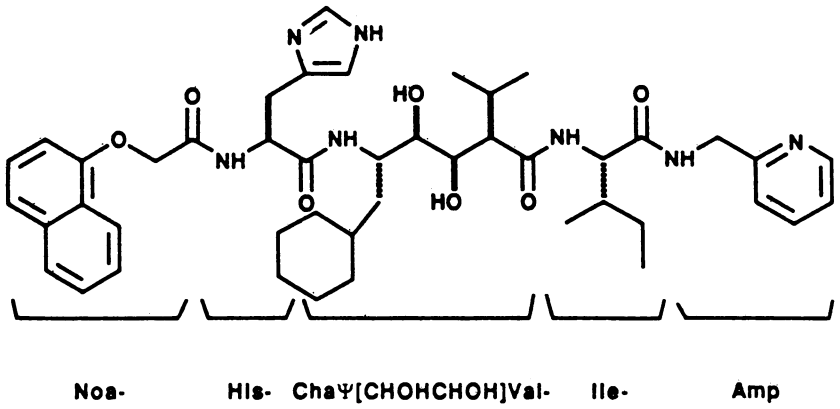

FIG. 1. Chemical structure of U-75875, Noa-His-Cha-Ψ[CH(OH)CH(OH)]-Val-Ile-Amp.

3–6, 6–9, and 24–48 hr. As shown in Fig. 3, inhibition of p55 processing by U-75875 was evident 3 hr after the addition of the compound; by 24 hr the inhibition was almost complete.

Virus Particles Produced in the Presence of U-75875 Are Immature and Noninfectious. To further investigate the effects of inhibition of gag and gag–pol processing, we analyzed the particle morphology and infectivity. Electron microscopic analysis of control H9/HTLVIIIB cells revealed principally mature virions, characterized by a thin envelope structure, both outside the cells and in cytoplasmic vacuoles. In some particles, depending on the plane of the section, dense nucleoids were visible. In contrast and with few exceptions, the particles observed in the cells cultured with 1 μM U-75875 had an immature morphology, characterized by thick ring-shaped nucleoids (Fig. 4). By using a human MT-4 lymphocyte end-point assay, we determined that the untreated H9/HTLVIIIB cells released moderate amounts of infectious HIV particles (titer = 3.2×10^3 $TCID_{50}$ per ml of supernatant). Significantly, supernatants recovered from cells incubated with 1 μM U-75875 contained no detectable infectious HIV (titer < 32 $TCID_{50}$ per ml), despite a comparable total particle number as indicated by the protein analysis of these supernatants shown in Fig. 3, sample at 24–48 hr.

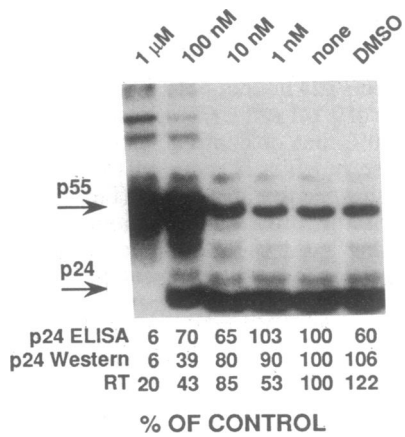

FIG. 2. U-75875 inhibits gag processing in HIV-1 chronically infected cells. H9/HTLVIIIB cells were cultured in the presence of U-75875, as indicated. Controls contained no U-75875 (none) or 0.02% M dimethyl sulfoxide (DMSO). The cells were diluted 1:2 in fresh drug-containing medium at 24 hr and 48 hr. At 72 hr, the supernatants were harvested, the HIV-1 particles were dissociated, and the proteins were electrophoresed on a 10% polyacrylamide gel, transferred onto nitrocellulose, and incubated with antiserum from an HIV-infected patient. Relative amounts of p24 in each sample, as quantified by an antigen-capture ELISA and densitometric analysis of the Western blot, and the relative RT activity are shown at the bottom.

U-75875 Blocks the Spread of HIV Infection in Human Lymphocytes. From the experiments described above, we concluded that in the presence of U-75875, HIV-infected cells produce immature noninfectious virions. To study the efficiency with which the protease inhibitor was able to block the spread of HIV, we infected primary cultures of PBMCs with $HIV\text{-}1_{LAV}$ and then added various U-75875 concentrations. Under these conditions, HIV killed about 85% of the CD4-positive cells within 6 days in cultures without drug. Significantly, 1 μM U-75875 completely blocked the spread of HIV-1 infection, as measured by supernatant RT and p24 (Fig. 5). A separate experiment directly comparing the antiviral potency of U-75875 to that of 3′-azido-3′-deoxythymidine as well as to U-81749 indicated that, on a molar basis, U-75875 was as potent as 3′-azido-3′-deoxythymidine and considerably more potent than U-81749 (data not shown). Additionally U-75875 displayed no nonspecific toxicity on PBMCs even at 10 μM (the highest concentration tested; data not shown).

The above experiments were of limited duration since, even in the presence of interleukin 2, PBMCs can only be maintained in culture for a defined period. To study the anti-HIV effect of U-75875 over a longer time, we infected continuously growing CEMx174 cells with $HIV\text{-}1_{LAV}$ and

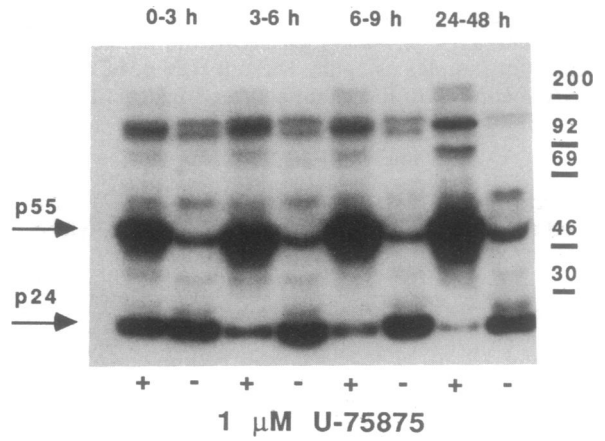

FIG. 3. Kinetics of the HIV-1 protease inhibition by U-75875. H9/HTLVIIIB cells were incubated in the presence (+) or absence (−) of 1 μM U-75875. Supernatants were harvested and the cells were resuspended in fresh drug-containing medium at 3-hr intervals. A supernatant between 24 and 48 hr was obtained from a separate experiment in which H9/HTLVIIIB cells were incubated for 48 hr in the presence or absence of 1 μM U-75875 for 24 hr with a wash and medium change at 24 hr. Supernatants were processed and analyzed for gag processing on a protein immunoblot, as described in Fig. 2. Molecular masses in kDa are shown to the right.

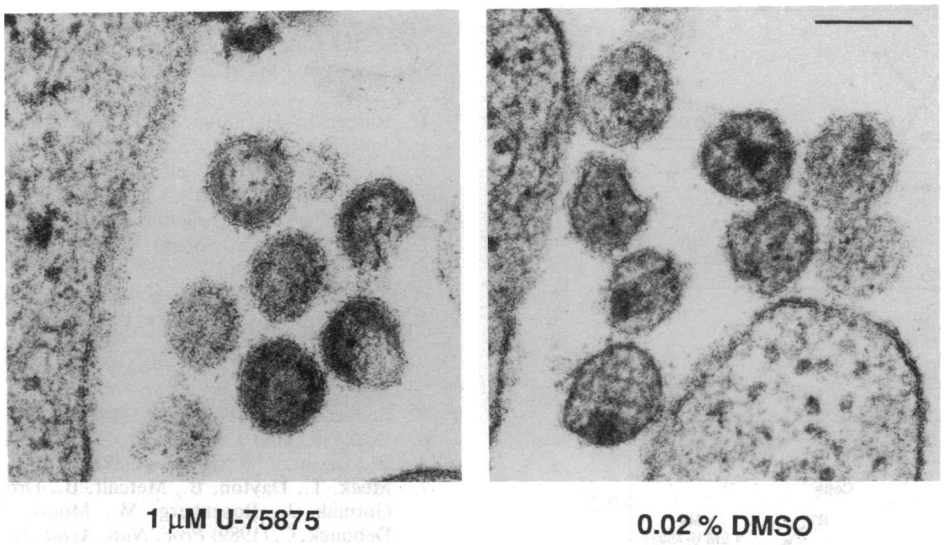

FIG. 4. Electron microscopic analysis of HIV-1 particles produced in the presence of U-75875. H9/HTLVIIIB cells were cultured for 48 hr in the presence of 1 μM U-75875 or a corresponding concentration of the carrier DMSO. Washed cells were pelleted, fixed, embedded, sectioned, and examined with a transmission electron microscope. (Bar = 100 nm.)

cultured them in the presence of U-75875. HIV spread was monitored by cell death and the appearance of RT in culture supernatants. Without drug or at 10 nM U-75875, HIV spread through the culture killing all the cells within 8 days; at 0.1 μM, virus spread was delayed by 2 days. In the presence of 1 μM U-75875, however, no HIV spread occurred over a 4-week period, as judged by complete protection against virus-induced cell death and lack of RT activity in culture medium (Fig. 6).

The homology of the HIV-2 and SIV proteases with the HIV-1 enzyme (24) and the ability of U-75875 to inhibit HIV-2 protease *in vitro* (K_i, 30 nM) led us to investigate the effects of U-75875 on the spread of SIV_{mac251} in CEMx174 cells. As with HIV-1 infection, SIV spread was completely blocked during the 4-week period of the experiment whereas untreated cells were rapidly killed by the virus (Fig. 6). In addition, U-875875 inhibited HIV-2 maturation in CEMx174 cells chronically infected with HIV-2 (data not shown).

DISCUSSION

HIV-1 protease belongs to the mechanistic class of aspartic proteases (3–9). Earlier studies with other members of this family, such as human renin or pepsin, showed that synthetic peptidomimetic compounds containing a hydroxyethylene isosteric insert as a nonhydrolyzable synthetic replacement of the P_1-P_1' scissile amide bond are able to inhibit the catalytic function of such proteases (25, 26). Consequently, peptidomimetic HIV-1 protease inhibitors were prepared and found to inhibit the processing of the HIV-1 p55 gag precursor

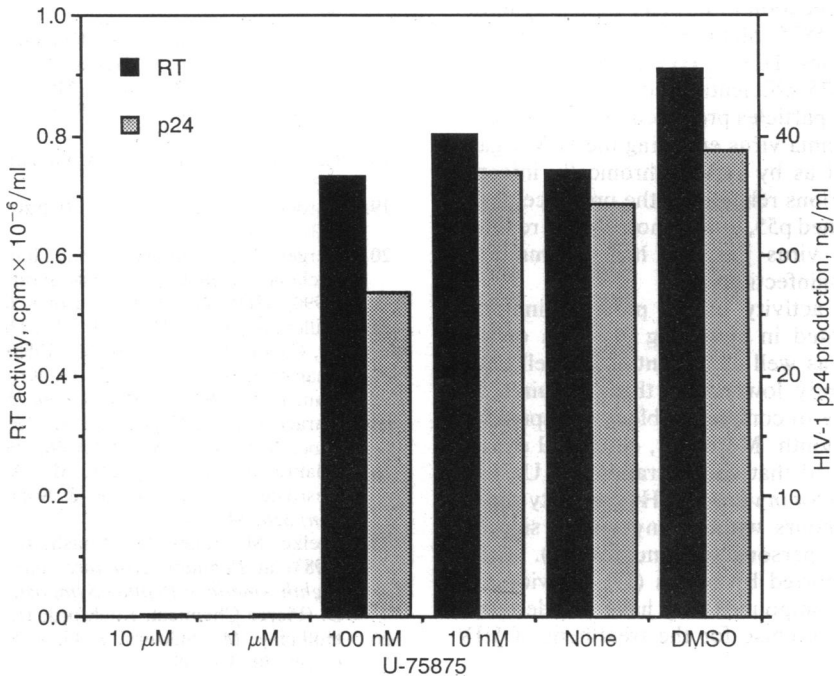

FIG. 5. U-75875 inhibits HIV-1 replication in infected human PBMCs. Phytohemagglutinin-stimulated PBMCs were infected with $HIV\text{-}1_{LAV}$ at a multiplicity of 0.005 and cultured in the presence of U-75875 as indicated. After 6 days, supernatants were harvested and analyzed for HIV-1 p24 (stippled bars) and RT (solid bars).

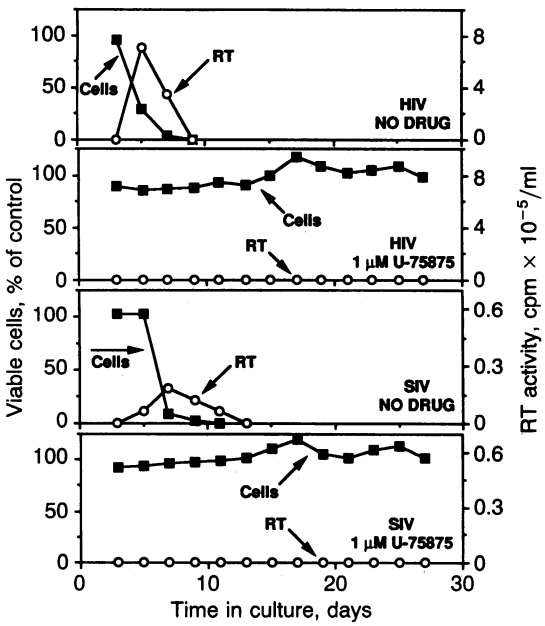

FIG. 6. U-75875 inhibits HIV-1 and SIV spread in human CEMx174 cells. CEMx174 cells (2×10^5 cells) were seeded with 1×10^3 $TCID_{50}$ $HIV\text{-}1_{LAV}$ or SIV_{mac251} (0.005 $TCID_{50}$ per cell) and 1 μM U-75875, or a corresponding concentration of the DMSO solvent, in 24-well tissue culture plates in duplicates in a total volume of 1 ml per well. On day 3 and every 2 days thereafter, samples were removed for HIV RT and cell viability assays. At each time point, the incubations were continued by transferring 200 μl of each cell suspension to new wells containing 800 μl of fresh medium and the corresponding drugs at the original concentrations. Relative cell numbers were expressed as percent of the MTT value obtained in control wells, which received an initial input of 2×10^5 CEMx174 cells but no virus or drug.

protein to the p24 and p17 core proteins (15, 16). In this communication, we describe an inhibitor, U-75875, that on a molar basis is equipotent to 3′-azido-3′-deoxythymidine and considerably more potent than the protease inhibitor U-81749 (15) in blocking HIV replication in primary human lymphocytes. Significantly, U-75875 inhibited maturation of three related primate lentiviruses: HIV-1, HIV-2, and SIV_{mac}.

We found that U-75875 efficiently and irreversibly prevented gag processing in particles produced by cells infected with a recombinant vaccinia virus encoding the HIV-1 gag–pol polyproteins as well as by HIV-1 chronically infected human T cells. Thus, virions released in the presence of the protease inhibitor contained p55, little or no p24, and reduced RT. In addition, these virus particles had an immature appearance and were noninfectious.

The strong anti-HIV activity of the protease inhibitor U-75875 was demonstrated in spreading infection experiments. In primary cells as well as in continuous cell lines, concentrations significantly lower than those required for PBMC toxicity were able to completely block the spread of infection for at least 1 month. Moreover, our initial experiments in rodents indicated that concentrations of U-75875 exceeding those required for *in vitro* anti-HIV activity can be maintained for several hours without any visible signs of toxicity (M. J. Ruwart, personal communication). These results, and findings reported by others (17), provide evidence that this class of compounds may have a wide therapeutic margin and hold promise for the treatment of HIV infection *in vivo*.

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