31. NMR data was acquired on a Bruker AM-400 spectrometer at 162 MHz with a 1.2-ml microcell in a 10-mm ^{31}P probe in 5000 scans with 60° pulses and a 1.4-s relaxation delay. Chemical shifts were referenced to an external standard of 85% orthophosphate at 0.0 ppm or to an internal standard of glycerophosphorylcholine set at 0.49 ppm.
32. Diabetes was induced in rats (Sprague-Dawley, 200 g of body weight) by a single tail vein injection of streptozotocin (65 mg per kilogram of body weight) in 100 m*M* citrate buffer (*p*H 4.5). Diabetes was confirmed by monitoring urine glucose, water consumption, and urine output.
33. K. James, A. R. Tachell, P. K. Ray, *J. Chem. Soc. Sect. C. Org. Chem.* **1967**, 2681 (1967).
34. B. S. Szwergold, unpublished observations.
35. We thank I. Rose, F. Matchinsky, and C. Marano for discussions and S. Osman, A. Goll, and S. Singh for technical assistance. Supported in part by NIH grants EY-08223, CA-06927, and CA-41078 and by the Commonwealth of Pennsylvania.

21 July 1989; accepted 1 December 1989

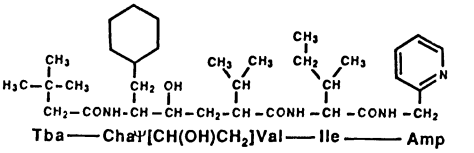


Fig. 1. Chemical structure of U-81749 (Tba-ChaΨ[CH(OH)CH_2]Val-Ile-Amp); see text for abbreviations. All stereocenters are of the *S* configuration.

A Synthetic HIV-1 Protease Inhibitor with Antiviral Activity Arrests HIV-Like Particle Maturation

T. J. McQuade, A. G. Tomasselli, L. Liu, V. Karacostas, B. Moss, T. K. Sawyer, R. L. Heinrikson, W. G. Tarpley

A synthetic peptidemimetic substrate of the human immunodeficiency virus 1 (HIV-1) protease with a nonhydrolyzable pseudodipeptidyl insert at the protease cleavage site was prepared. The peptide U-81749 inhibited recombinant HIV-1 protease in vitro (inhibition constant K_i of 70 nanomolar) and HIV-1 replication in human peripheral blood lymphocytes (inhibitory concentration IC_{50} of 0.1 to 1 micromolar). Moreover, 10 micromolar concentrations of U-81749 significantly inhibited proteolysis of the HIV-1 gag polyprotein (p55) to the mature viral structural proteins p24 and p17 in cells infected with a recombinant vaccinia virus expressing the HIV-1 *gag-pol* genes. The HIV-1 like particles released from inhibitor-treated cells contained almost exclusively p55 and other gag precursors, but not p24. Incubation of HIV-like particles recovered from drug-treated cultures in drug-free medium indicated that inhibition of p55 proteolysis was at least partially reversible, suggesting that U-81749 was present within the particles.

The protease encoded by HIV-1 is required for the processing of the viral polyproteins encoded by the *gag* and *pol* genes into mature virion proteins (*1–3*). This processing involves cleavage of the gag precursor (p55) to form the four structural proteins of the virion core (p17, p24, p8, and p7); in addition, processing of the p160 gag-pol precursor yields these structural proteins as well as enzymes essential for HIV replication [that is, protease and reverse transcriptase, and integration protein (*4*)]. The HIV-1 protease is an aspartic protease that is enzymatically active upon dimerization (*5–11*).

To date the major efforts to identify synthetic compounds combating HIV have focused principally on compounds targeting the viral reverse transcriptase or its cellular receptor, the CD4 molecule (*12–15*). Relatively little information has been reported on compounds specifically designed to inhibit the HIV-1 protease or describing their anti-HIV activities. Therefore we prepared a variety of peptidemimetic HIV-1 protease inhibitors and evaluated their anti-HIV properties. These inhibitors contain a hydroxyethylene isostere (Ψ[CH(OH)CH_2]) as a nonhydrolyzable, synthetic replacement of the scissile amide bond at the P_1-P_1' site. Such chemical modifications have been shown recently by Richards *et al.* (*16*) and Tomasselli *et al.* (*17*) to be key chemical determinants of highly potent inhibitors (such as H-261 and U-85548E) of the protease in vitro. However, we determined experimentally that a direct correlation between protease inhibition in vitro and anti-HIV activity in cellular systems could not be established (that is, U-85548E and H-261–like derivatives do not inhibit HIV replication in cell culture).

We investigated the structure-activity relations of HIV-1 protease inhibitors of the formula W-XaaΨ[CH(OH)CH_2]Yaa-Ile-Z that varied in amino- and carboxyl-terminal functionalization as well as the P_1-P_1' amino acids. On the basis of this strategy we produced U-81749, which contained an amino-terminal *tert*-butylacetyl, a P_1 cyclohexylalanine (Cha), a P_1' Val, and carboxyl-terminal aminomethylpyridine (Tba-ChaΨ-[CH(OH)CH_2]Val-Ile-Amp; Fig. 1). Recombinant HIV-1 protease was competitively inhibited by U-81749 in vitro [K_i = 70 n*M* (*17*)]. Good selectivity for the HIV-1 enzyme was maintained compared with human renin (>100 times lower potency toward renin). Because of U-81749's enhanced aqueous solubility compared to other synthetic gag analogs prepared, we investigated its anti-HIV and protease inhibitory properties in cellular systems.

We evaluated the anti-HIV activity of U-81749 by determining the levels of HIV p24 and HIV RNA in culture supernatants of human peripheral blood lymphocytes (PBLs) 3 and 4 days after HIV infection (*18*). Addition of 1 μ*M* U-81749, a level determined to be nontoxic to PBL proliferation (*19*), to the culture medium immediately after virus addition reduced the levels of HIV p24 ~70% compared with those found in the supernatants of control, infected cells (8 versus 25 and 18 versus 58 ng/ml of p24 at 3 and 4 days after infection, respectively). However, the levels of HIV RNA detected in supernatants from drug-treated cultures were only ~3 pg/ml of RNA at these times compared with control values of 91 and 278 pg/ml of RNA. In the presence of 0.1 μ*M* drug the levels of HIV p24 and RNA were still approximately 25 to 50% lower than those in control supernatants (19 versus 25 and 25 versus 58 ng/ml of p24 at 3 and 4 days after infection, respectively); at lower concentrations of U-81749 (0.001 to 0.01 μ*M*), no significant inhibition was seen.

To investigate whether the HIV protease was the actual target of U-81749 in cells, we studied processing of the HIV p55 and the maturation of HIV-like particles in recombinant virus–infected CV-1 cells (Figs. 2 and 3). As recently described by Karacostas *et al.* (*20*), infection of CV-1 cells with a recombinant vaccinia virus (vVK-1) engineered to express the HIV *gag-pol* genes results in the synthesis and processing of gag-pol precursors. Initially, the most prominent HIV polypeptides synthesized are p55, p46, and p41; with time, however, these proteins are further processed to the mature viral pro-

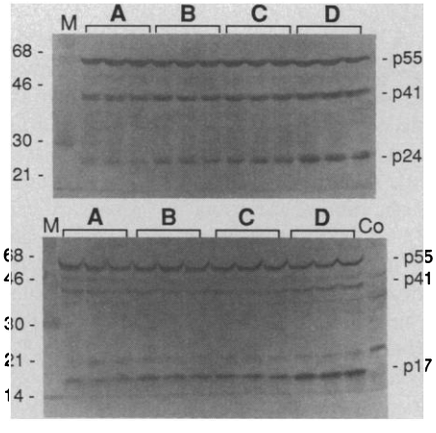
T. J. McQuade and W. G. Tarpley, Infectious Disease Research Unit, The Upjohn Company, Kalamazoo, MI 49001.
A. G. Tomasselli, L. Liu, T. K. Sawyer, R. L. Heinrikson, Biopolymer Research Unit, The Upjohn Company, Kalamazoo, MI 49001.
V. Karacostas and B. Moss, Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892.

teins p17 and p24. In addition, HIV-like particles are assembled within and released from these cells and undergo subsequent maturation in a process that closely mimics that occurring in a natural HIV-1 infection. We initially analyzed vVK-1–infected CV-1 cell lysates, for gag-pol precursors and p24 at various times after infection by protein immunoblotting. The p24 product was first detected 6 to 9 hours after infection and by 24 hours it accounted for 31 ± 3% of the immunoreactive proteins as determined by densitometric analyses of the immunoblots (Table 1). As shown in Fig. 2 (top panel) and Table 1, addition of 2.5, 5, or 10 μ*M* U-81749 to infected cultures reduced the levels of p24 in cell lysates at this time 32, 42, and 84%, respectively. Similarly, the levels of p17 were also reduced 16, 34, and 49% at these concentrations of U-81749 (Fig. 2, bottom panel, and Table 1). These data suggest that U-81749 inhibits the processing of p55 by the HIV-1 protease in these mammalian cells.

Table 1. U-81749 inhibits HIV-1 gag precursor processing in vVK-1–infected CV-1 cells. The synthesis of HIV gag proteins in vVK-1–infected CV-1 cells treated with U-81749 was investigated by protein immunoblotting as described in Fig. 2. The levels of immunoreactive proteins were quantified by densitometry (Bio-Rad, Model 260) with the accompanying 1-D Analyst Software. The data are presented as the mean percentage of p24 or p17 ± SD, $n = 3$, of the total immunoreactive proteins, normalized to 100%. Inhibition (*I*) refers to percent decrease in p24 and p17 levels relative to nondrug-treated controls.

U-81749 (μ*M*)	p24 (%)	*I* (%)	p17 (%)	*I* (%)
10	5 ± 0.8	84	31 ± 3	49
5	18 ± 0.2	42	40 ± 4	34
2.5	21 ± 0.5	32	51 ± 3	16
0	31 ± 3		61 ± 2	

Fig. 2. U-81749 inhibits processing of HIV-1 p55 to p24 (**top**) and p17 (**bottom**) in vVK-1–infected CV-1 cells. CV-1 cells were seeded at 2×10^5 cells per well in 24-well Costar dishes and infected 6 to 12 hours later with vVK-1 at 5 plaque-forming units (PFU) per cell (*20*); U-81749 dissolved in Dulbecco's minimum essential medium (DMEM) containing 2.5% fetal bovine serum was added to triplicate wells at the indicated final concentrations immediately after virus addition (lanes A, 10 μ*M*; lanes B, 5 μ*M*; lanes C, 2.5 μ*M*; and lanes D, no U-81749; the lane marked Co in the bottom panel contained lysate from control, uninfected CV-1 cells; similar lysates analyzed with sera containing antibodies to p24 did not yield observable bands). Twenty-four hours after infection the culture medium was removed, the monolayer washed with 1 ml of PBS, and the cells lysed by the addition of 0.1 ml of loading buffer (62.5 m*M* tris, *p*H 6.8, 2.3% SDS, 5% β-mercaptoethanol, and 10% glycerol). The cells lysates were collected individually, placed in boiling water for 3 min, and then 0.025 ml of each sample was subjected to electrophoresis on 12% SDS-polyacrylamide gels. The proteins were electroblotted onto nitrocellulose and analyzed by protein immunoblotting as described; the primary antibodies were sheep anti-



body to p24 or sheep antibody to p17 (International Enzyme, Inc., Fallbrook, California) and the secondary antibody in both cases was alkaline-phosphatase–conjugated rabbit antibody to sheep immunoglobulin G. (Kirkegaard & Perry Laboratories, Gaithersburg, Maryland). The indicated protein molecular weights M_r were determined by comparing their migration with standard proteins of known M_r (M lanes).

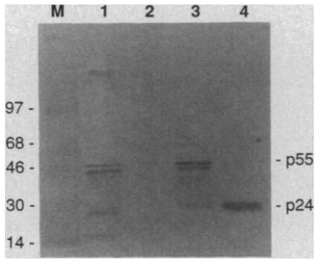


Fig. 3. U-81749 arrests HIV-1–like particle maturation. Flasks containing approximately 1×10^7 CV-1 cells were infected with vVK-1 at 5 plaque-forming units per cell (*20*); 16 hours later the culture medium was removed, clarified by centrifugation at 2000*g* for 5 min, and the particulate material was recovered after centrifugation through a 0.8-ml 20% sucrose cushion at 33,000 rpm in a SW50.1 rotor for 1.5 hours. The pellets (containing the HIV-1–like particles) were resuspended in 0.08 ml of PBS; 0.025-ml samples were analyzed by SDS–polyacrylamide gel electrophoresis and protein immunoblotting as described in the legend to Fig. 2. Lane M, standard protein molecular weight markers; lane 1, vVK-1–infected CV-1 cell lysate; lane 2, particulate fraction from uninfected CV-1 cultures; lane 3, particulate fraction from infected cultures in the presence of 10 μ*M* U-81749; and lane 4, particulate fraction from infected cultures without U-81749 exposure.

Data by Kohl *et al.* (*21*) indicated that cells transfected with HIV proviral DNA encoding a protease mutagenized in the presumed active site produced noninfectious virions composed of p55 but not p24. Thus protease appears to be essential for the maturation of viral particles to infectious virus. Therefore, we determined whether U-81749 prevented the maturation of the HIV-like particles found in the medium of vVK-1–infected cells (Fig. 3). Medium was collected 16 hours after infection of CV-1 cells, and the HIV-like particles were recovered by centrifugation. The protein components of the particles were then analyzed by protein immunoblotting as described above. As shown in Fig. 3, HIV-like particles recovered from control medium contained almost exclusively p24 (>97% of immunoreactive proteins) and no detectable p55 or other gag precursors (Fig. 3, lane 4). In contrast, particles recovered from drug-treated cultures contained almost exclusively p55 and p46 (>90% of the immunoreactive protein) and only trace levels of p24 (<9% of immunoreactive protein; lane 3). Thus maturation of the HIV-1–like particles appeared to be arrested by 10 μ*M* U-81749.

We speculated that blocking of particle maturation might be due to direct inhibition of the protease by U-81749 present in the particle. To test this hypothesis, we isolated particles in the presence of U-81749 and then attempted to wash the drug out. Under these conditions we anticipated that protease might be reactivated, resulting in particle maturation and the appearance of p24. As shown in Fig. 4, HIV-like particles recovered from drug-treated medium contained p55 and gag precursors (p46 and p41) but very little p24 (<2% of the immunoreactive proteins; Fig. 4A, lane 0). However, incubation of these HIV-like particles in phosphate-buffered saline (PBS) without U-81749 at 37°C for 1 to 2 hours resulted in a decrease in p55 levels and the appearance of significant amounts of p24 (about 75% of the immunoreactive proteins after 2 hours, Fig. 4B). In contrast, particles incubated under identical conditions but in PBS containing drug maintained only very low levels of p24 (Fig. 4A, lanes 1 and 2). As expected, particles recovered from cultures not exposed to drug contained exclusively p24 and no detectable p55 or other gag precursors during these incubations (Fig. 4C). Together, these data suggest that U-81749 is present within the HIV-like particle where it directly inhibits the protease, and thus the subsequent maturation of the particle.

Expression of the HIV-1 protease in *Escherichia coli* (*7, 22–24*) and chemical synthesis (*8, 9, 25*) of active enzyme have enabled the development of in vitro assays to facilitate the search for inhibitors. Inhibition of retroviral protase activity, including the HIV-1 protease, has been reported for the general aspartyl proteinase inhibitor, pepstatin A (7–9). Moreover, pepstatin A shows some

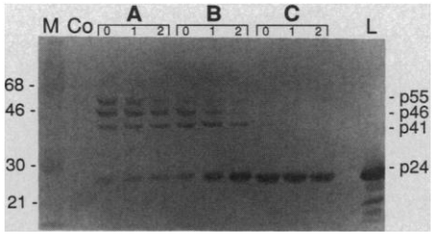


Fig. 4. U-81749 inhibition of HIV-1–like particle maturation is partially reversible. HIV-1–like particles were recovered from the medium of vVK-1–infected CV-1 cells as described in the legend to Fig. 3. The particulate fractions were resuspended in PBS with or without 10 μ*M* U-81749 (lanes 0) and then incubated at 37°C for 1 or 2 hours (lanes 1 and 2, respectively). Samples were taken for protein immunoblot analysis with sera containing antibodies to p24 as described in Fig. 2. (**A**) Particulate fraction from infected cultures exposed to 10 μ*M* drug and incubated with the drug. (**B**) Particulate fraction from infected cultures exposed to 10 μ*M* drug and subsequently incubated in the absence of drug. (**C**) Particulate fraction from cultures without drug and incubated without drug. Lane Co, particulate fraction from uninfected cultures; lane L, control HIV-1 lysate indicating p24 (Scripps Laboratories, San Diego, California); and lane M, standard protein molecular weight markers.

anti-HIV activity in H9 cells at the relatively high concentration of 100 μ*M* (*26*). In this report, we present the structure of U-81749, a synthetic compound specifically designed to inhibit the HIV-1 protease which exhibits potent anti-HIV activity in acutely infected PBLs (ED_{50} 0.1 to 1 μ*M*). Our data suggest that U-81749 inhibits protease activity in these cells, resulting in an inhibition of viral maturation and a reduction in the levels of infectious virus. These findings provide evidence that a chemical compound, specifically targeted against the HIV-1 protease, can inhibit viral replication in cell culture. Although the observed reversibility of U-81749's protease inhibition might be expected to reduce or preclude its anti-HIV activity in vivo, the general nontoxic nature of this type of peptidemimetic compound to animals (*27*) suggests that nontoxic U-81749 derivatives with potentially irreversible protease inhibition might be obtained. Taken together, the above data support the concept of the protease as an attractive target for the development of anti-HIV agents.

REFERENCES AND NOTES

1. L. Ratner *et al.*, *Nature* **313**, 277 (1985).
2. S. Wain-Hobson *et al.*, *Cell* **40**, 9 (1985).
3. L. H. Pearl and W. R. Taylor, *Nature* **329**, 351 (1987).
4. T. Jacks *et al.*, *ibid.* **331**, 280 (1988).
5. M. A. Navia *et al.*, *ibid.* **337**, 615 (1989).
6. A. Wlodawer *et al.*, *Science* **245**, 616 (1989).
7. S. Seelmeier, H. Schmidt, V. Turk, K. von der Helm, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6612 (1988).
8. J. Schneider and S. B. H. Kent, *Cell* **54**, 363 (1988).
9. R. F. Nutt *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7129 (1988).
10. T. D. Meek *et al.*, *ibid.* **86**, 1841 (1989).
11. P. L. Darke *et al.*, *J. Biol. Chem.* **264**, 2307 (1989).
12. S. Broder, in *Human Retroviruses, Cancer, and AIDS*, D. Bolognesi, Ed. (Liss, New York, 1988), p. 365.
13. D. D. Richman *et al.*, *N. Engl. J. Med.* **317**, 192 (1987).
14. J. D. Lifson *et al.*, *Science* **241**, 712 (1988).
15. P. L. Nara, K. M. Hwang, D. M. Rausch, J. D. Lifson, L. E. Eiden, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7139 (1989).
16. A. D. Richards *et al.*, *FEBS Lett.* **247**, 113 (1989).
17. A. G. Tomasselli *et al.*, *Biochemistry* **29**, 264 (1990).
18. The HIV infectivity experiments were conducted in primary cultures of human peripheral blood lymphocytes (PBLs) with HTLV-IIIB. Cultures were infected in triplicate with HIV, U-81749 added, and 3 to 4 days after infection the levels of HIV p24 synthesized and released were quantified by an enzyme-linked immunosorbent assay (ELISA) [R. Maiolini and R. Masseyeff, *J. Immunol. Methods* **8**, 223 (1975)] using a monoclonal capture antibody to HIV p24 (DuPont, Wilmington, DE) and recombinant HIV p24 calibration standard (MicroGenSys, West Haven, CT). The amounts of HIV RNA synthesized in these infected cultures were also determined by hybridization analysis with a HIV-specific $^{-32}$P-labeled probe. The absolute HIV RNA levels were determined by normalizing hybridization values to values obtained from a standard preparation of HIV RNA hybridized in parallel.
19. The proliferation of mitogen-stimulated PBLs 4 days after exposure to U-81749 was quantified relative to nondrug-treated controls to ensure that the infectivity experiments were conducted at noncytotoxic concentrations of drug. At 1 μ*M* U-81749 did not significantly inhibit PBL proliferation (<10% inhibition) and proliferation was reduced <40% at 10 μ*M*.
20. V. Karacostas, K. Nagashima, M. Gonda, B. Moss, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
21. N. E. Kohl *et al.*, *ibid.* **85**, 4686 (1988).
22. M. C. Graves *et al.*, *ibid.*, p. 2449.
23. C. Debouck *et al.*, *ibid.* **84**, 8903 (1987).
24. J. Mous *et al.*, *J. Virol.* **62**, 1433 (1988).
25. T. D. Copeland and S. Oroszlan, *Gene Anal. Tech.* **5**, 109 (1988).
26. K. von der Helm *et al.*, *FEBS Lett.* **247**, 349 (1989).
27. T. J. Kakuk, personal communication.

16 November 1989; accepted 4 January 1990

Intercellular Adhesion Molecule–1 (ICAM-1) in the Pathogenesis of Asthma

CRAIG D. WEGNER,* ROBERT H. GUNDEL, PATRICIA REILLY, NANCY HAYNES, L. GORDON LETTS, ROBERT ROTHLEIN

Airway eosinophilia, epithelial desquamation, and hyperresponsiveness are characteristics of the airway inflammation underlying bronchial asthma. The contribution of intercellular adhesion molecule–1 (ICAM-1) to eosinophil migration and airway responsiveness was studied. ICAM-1 partially mediated eosinophil adhesion to endothelium in vitro and was upregulated on inflamed bronchial endothelium in vivo. ICAM-1 expression was also upregulated on inflamed airway epithelium in vitro and in vivo. In a primate model of asthma, a monoclonal antibody to ICAM-1 attenuated airway eosinophilia and hyperresponsiveness. Thus, antagonism of ICAM-1 may provide a therapeutic approach to reducing airway inflammation, hyperresponsiveness, and asthma symptoms.

A MAJOR CHARACTERISTIC OF ASTHma is the extreme (10 to 1000 times normal) sensitivity of the bronchi to inhaled agents (*1, 2*). The severity of this "airway hyperresponsiveness" correlates with the intensity of asthmatic symptoms (*2–4*), diurnal variations in airway caliber (*5*), and therapy required (*2, 6*). Although the underlying pathogenetic mechanisms are not known, many studies suggest that eosinophil infiltration and desquamation of the bronchial epithelium are involved (*7, 8*). Since eosinophil-derived mediators damage airway epithelial cells in vitro, these two events may be linked (*9*). We investigated a mechanism by which eosinophils enter the airways and contribute to airway hyperresponsiveness in primates.

Departments of Pharmacology and Immunology, Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT 06877.

*To whom correspondence should be addressed.

Adhesion of leukocytes to microvascular endothelium is essential for their migration into inflamed tissues. This was demonstrated in a group of patients who, because of an inherited genetic defect, are deficient in the expression of the CD18 family of leukocyte adhesion receptors (*10*). Infected tissues in severely affected patients contain little or no neutrophils. A ligand for some (*11, 12*) of these adhesion receptors is intercellular adhesion molecule–1 (ICAM-1). ICAM-1 was shown to be upregulated on endothelium and skin epithelium both in vitro and in vivo 4 to 24 hours after an inflammatory stimulus (*13–15*). In addition, monoclonal antibodies (MAbs) to ICAM-1 attenuate neutrophil adhesion to endothelium and inhibit neutrophil transendothelial migration in vitro (*12, 15*) and in vivo (*16*). Thus, ICAM-1 appears to be required for neutrophil migration into inflamed tissues.

In this report, we investigated (i) the