ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1993, p. 71–77
0066-4804/93/010071-07$02.00/0

Vol. 37, No. 1

Antiretroviral Activities of Protease Inhibitors against Murine Leukemia Virus and Simian Immunodeficiency Virus in Tissue Culture

PAUL L. BLACK,[1,2]* MARY BETH DOWNS,[3] MARK G. LEWIS,[1]† MICHAEL A. USSERY,[2] GEOFFREY B. DREYER,[4] STEPHEN R. PETTEWAY, JR.,[5] AND DENNIS M. LAMBERT[5]

Southern Research Institute-Frederick Research Center, Frederick, Maryland 21701[1]; Division of Antiviral Drug Products, Center for Drug Evaluation and Research, Food and Drug Administration, HFD-530, 5600 Fishers Lane, Rockville, Maryland 20857[2]; Pathology Division, United States Army Medical Research Institute for Infectious Diseases, Fort Detrick, Frederick, Maryland 21702[3]; and Departments of Medicinal Chemistry[4] and Anti-Infectives,[5] SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406*

Received 13 April 1992/Accepted 19 October 1992

Rationally designed synthetic inhibitors of retroviral proteases inhibit the processing of viral polyproteins in cultures of human immunodeficiency virus type 1 (HIV-1)-infected T lymphocytes and, as a result, inhibit the infectivity of HIV-1 for such cultures. The ability of HIV-1 protease inhibitors to suppress replication of the C-type retrovirus Rauscher murine leukemia virus (R-MuLV) and the HIV-related lentivirus simian immunodeficiency virus (SIV) was examined in plaque reduction assays and syncytium reduction assays, respectively. Three of seven compounds examined blocked production of infectious R-MuLV, with 50% inhibitory concentrations of ≤1 μM. Little or no cellular cytotoxicity was detectable at concentrations up to 100 μM. The same compounds which inhibited the infectivity of HIV-1 also produced activity against SIV and R-MuLV. Electron microscopic examination revealed the presence of many virions with atypical morphologies in cultures treated with the active compounds. Morphometric analysis demonstrated that the active compounds reduced the number of membrane-associated virus particles. These results demonstrate that synthetic peptide analog inhibitors of retroviral proteases significantly inhibit proteolytic processing of the *gag* polyproteins of R-MuLV and SIV and inhibit the replication of these retroviruses. These results are similar to those for inhibition of HIV-1 infectivity by these compounds, and thus, R-MuLV and SIV might be suitable models for the in vivo evaluation of the antiretroviral activities of these protease inhibitors.

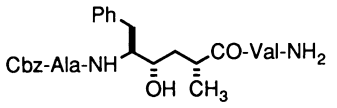
Maturation of C-type retroviruses as well as the lentivirus human immunodeficiency virus type 1 (HIV-1) requires the action of a virally encoded protease (4, 9, 13, 14, 28, 32). Retroviral mRNA is translated into polyproteins which are modified posttranslationally before assembly into immature virions and subsequent release from the infected cell (16, 29). Immature particles are not infectious, have undetectable or low levels of reverse transcriptase (RT) activity, and characteristically appear spherical with annular cores in electron micrographs of sectioned material (8, 18). Activation of the nascent viral protease within the immature virus particle apparently occurs after detachment of immature virions from the cell membrane (16, 22). The activated protease cleaves the retroviral polyproteins at specific sites to produce the functional structural proteins and enzymes of the virus. The acquisition of infectivity is also accompanied by a characteristic change in morphology, from an annular to a dense core (8, 36). Inhibition of the function of retroviral proteases by mutation (9, 13–15, 24, 28, 32) or use of specific inhibitors (18, 19, 21, 30) results in the formation of immature, noninfectious particles, and such studies have established the central roles of these viral enzymes in the process of virus maturation.

Retroviral proteases, which form homodimers in the active state (20, 23, 25, 26, 35), belong to the class of aspartic proteases, all of which contain a pair of highly conserved regions composed of two hydrophobic amino acids followed by the characteristic sequence Asp-Thr/Ser-Gly (12, 16, 20). The overall conservation in tertiary and quaternary structures, especially in the region of the active site of retroviral proteases, leads to similarities in the cleavage site sequences which they recognize (16, 27). These similarities led us to examine the possibility that the infectivities of animal retroviruses might be inhibited by synthetic protease inhibitors which have previously demonstrated activity against HIV-1 (18, 21). If protease inhibitors had similar effects on animal retroviruses, animal retrovirus models could serve as experimental systems for investigating the consequences of viral protease inhibition in vivo and, thus, as a guide for the preclinical development of protease inhibitors for AIDS therapy.

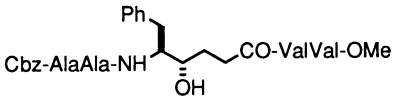
Elucidation of the structure, mechanism of action, and substrate specificity of HIV-1 protease has led to the design of peptide substrate analogs in which the scissile dipeptide linkage is replaced with a nonscissile hydroxyethylene isostere analog (5, 6). Such synthetic peptide analogs inhibit the processing of viral polyproteins in T lymphocytes infected with HIV-1 and reduce viral infectivity (5, 21). In the present studies we examined whether such synthetic protease inhibitors have similar effects on Rauscher murine leukemia virus (R-MuLV), a C-type retrovirus, and simian immunodeficiency virus (SIV), a lentivirus. R-MuLV and SIV both contain the genome organization typical of retroviruses,

* Corresponding author.
† Present address: Henry M. Jackson Foundation, Rockville, MD 20850.

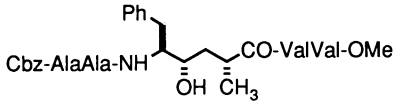
1. SKF 108907



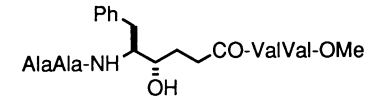
2. SKF 107461



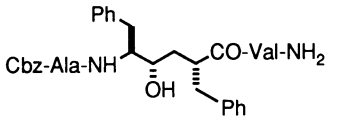
3. SKF 108390



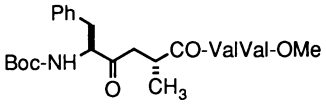
4. SKF 107457



5. SKF 108842



6. SKF 109274



7. SKF 108922

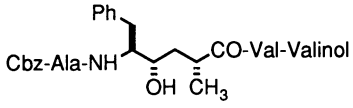


FIG. 1. Structures of protease inhibitors (compounds 1 to 7). Ph, phenyl; Cbz, carbobenzoxy; O Me, O methyl; Boc, *tert*-butlyoxycarbonyl.

5′-*gag-pol-env*-3′ (16). Additionally, SIV, but not R-MuLV, contains some of the regulatory genes found in HIV-1 and other lentiviruses. We report here the antiviral activities in vitro of synthetic peptide analog inhibitors of HIV-1 protease against R-MuLV and SIV at concentrations similar to those that are active against HIV-1.

MATERIALS AND METHODS

Viruses and cell lines. The R-MuLV stock was originally obtained from Ruth Ruprecht (Children's Medical Center, Boston, Mass.). Virus stocks were prepared from the cell-free supernatants of infected SC-1 cells. Titers of infectious virus were determined in a UV-XC plaque assay (see below) and ranged from 10^4 to 10^5 PFU/ml. All virus stocks and cell lines were tested to ensure freedom from mycoplasmas. SC-1 cells (11), originally derived from feral murine embryos, and XC cells (33), a rat tumor cell line induced by Rous sarcoma virus, were obtained from the American Type Culture Collection (Rockville, Md.). The cells were maintained by serial passage in Eagle's minimum essential medium supplemented with 5% fetal bovine serum for SC-1 cells or with 10% fetal bovine serum for XC cells.

The original isolate of SIVsm/pbj was supplied by Harold McClure (Yerkes Primate Center, Atlanta, Ga.). Virus stock was grown as cell-free supernatants, and its titer was determined in AA2 cells (2). Titers ranged between 10^5 and 10^6 syncytium-forming units per ml.

Protease inhibitors. Compounds 1 to 7 (Fig. 1) were prepared by methods similar to those reported previously (5, 6). Inhibitors were characterized by high-pressure liquid chromatography, proton nuclear magnetic resonance, and fast-atom bombardment mass spectrometry. Inhibition constants for compounds 1 to 7 against HIV-1 protease were determined as described previously (5, 6). Peptide analog inhibitors were dissolved in dimethyl sulfoxide at a concentration of 10 mM, diluted in complete tissue culture medium, and tested at a maximum concentration of 20 μM. Protease inhibitors were coded before testing so that evaluations of antiviral activity against R-MuLV and SIV were done in a blinded fashion. 3′-Azido-2′,3′-dideoxythymidine (AZT; generously provided by Burroughs-Wellcome, Research Triangle Park, N.C.) was used as the positive control for the antiviral assays, and it was dissolved directly in tissue culture medium.

UV-XC assay. The UV-XC assay involves a two-stage culture procedure that is used to enumerate the number of PFU (31) as described previously (3). Briefly, subconfluent monolayers of SC-1 cells were infected with appropriate dilutions of virus samples (50 to 100 PFU per well). After 3 days, the cultures were irradiated with a short-wave (254 nm) UV lamp at a total dose of 1,200 ergs/mm^2, and XC cells were added. After 3 more days, the cultures were fixed and stained. Plaques in the cell monolayer were counted with the aid of a dissecting microscope.

Plaque reduction assay for R-MuLV. A modification of the UV-XC assay was used to determine the antiviral activities of the compounds for R-MuLV. Antiviral compounds were added to the culture medium after adsorption of the virus and remained in the culture medium for 3 days. Toxicity was then measured by reduction in uptake of the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. In replicate plates, cells were then irradiated, and XC cells were added as described above. The concentrations for 50% and 90% inhibition of plaque production and 20% cellular cytotoxicity were estimated with the Dose Effect Analysis program of Chou and Chou (Biosoft).

SIVsm/pbj infectivity assay. Virus dilutions were added in duplicate to AA2 cells (10,000 cells per well) in 96-well plates in RPMI 1640 medium supplemented with 10% fetal bovine serum–5% glutamine–0.5% gentamicin–2 μg of polybrene per ml. The compounds and virus were added to the cells to give a total volume of 200 μl. The cultures were held for 10 days, with feeding (100 μl) on day 3 and replacement of 100 μl on day 7. Cultures were observed for syncytium production at days 7 and 10. Wells were scored as positive if any syncytia were observed. Culture supernatants were collected on day 10 for SIV p27 quantitation by using a SIV p27 enzyme-linked immunosorbent assay (Coulter).

RT assay for R-MuLV. The procedure for measuring RT activity has been described previously (3), and it monitors incorporation of [^{3}H]dTTP into acid-precipitable DNA with poly(rA) · poly(dT)$_{12-18}$ as the template-primer. The linear range of the assay is 400 to 40,000 RT units, with 1 unit of RT activity defined as the number of picomoles of the dTTP substrate incorporated in 1 h by 1 ml of serum.

Polyprotein processing inhibition assay. Cell lysates for Western blot (immunoblot) analysis were prepared by the harvesting on day 3 of acutely R-MuLV-infected SC-1 cells treated with protease inhibitors. For analysis of SIV polyprotein processing, inhibitors were added to 20,000 SIVsm/pbj-infected AA2 cells at day 1, and 100 μl of medium was

TABLE 1. Effects of protease inhibitors on retrovirus replication[a]

Compound no. and name	HIV-1		R-MuLV infectivity[b,h]		SIV		
	Protease K_i (nM)[c]	Infectivity IC_{50} (μM)[d]	IC_{50} (μM)	IC_{90} (μM)	Syncytium reduction (μM)[e]	p27[f] IC_{50} (μM)	p27[f] IC_{90} (μM)
1. SKF 108907	50[g]	8.0[g]	>20	>20	>20	8.0	>20
2. SKF 17461	48[g]	2.3	18 ± 3.5	>20	10	4.8	8.8
3. SKF 108390	0.7[g]	0.76 ± 0.37[h]	0.82 ± 0.47	1.4 ± 0.67	<0.63	0.63	3.6
4. SKF 107457	4.0[g]	>100	>20	>20	>20	10.2	>20
5. SKF 108842	0.7[g]	0.85 ± 0.32[g]	0.67 ± 0.39	1.1 ± 0.47	1.3	0.13	1.9
6. SKF 109274	>1,000	NT[i]	>20	>20	>20	>20	>20
7. SKF 108922	2.0	0.88	1.0 ± 0.04	1.8 ± 0.03	<0.63	0.077	3.9

[a] IC_{50}, 50% inhibitory concentration; IC_{90}, 90% inhibitory concentration.
[b] The 20% cellular cytotoxicity was >20 μM for all compounds.
[c] Inhibition of recombinant HIV-1 protease in a cell-free system, measured as previously described (5, 6, 21).
[d] Inhibition of RT production by continuous human T-cell lines as described previously (5, 18, 21).
[e] Minimum effective concentration (i.e., concentration at which no syncytia were observed). Reduction of syncytia in AA2 cells by SIVsm/pbj (100 tissue culture infective doses) was quantitated on days 7 and 10 of culture as described in the text. The levels of p27 in culture supernatants were measured on day 10.
[f] Values represent the averages of two separate experiments performed in duplicate wells.
[g] Data were reported previously (5).
[h] Values are means ± standard deviations of four separate determinations.
[i] NT, not tested.

added at day 4. Cells were harvested at day 10. For both R-MuLV- and SIV-infected cells, cell extracts were diluted (10^6 cells per 250 μl) with 2× polyacrylamide gel electrophoresis (PAGE) sample buffer (17) for Western blot analysis. For lysates in sodium dodecyl sulfate-PAGE sample buffer, cell DNA was sheared with a tuberculin syringe before heat inactivation at 90°C for 5 min. Samples (50 μl) were loaded onto 12.5% polyacrylamide gels and electrophoresed at 10 mA per gel overnight. Proteins were transblotted onto 0.4-μm-pore-size nitrocellulose paper (Bio-Rad) as described by Towbin et al. (34), with minor modifications.

Nitrocellulose filters containing transblotted proteins were blocked by incubating in 3% dried milk phosphate-buffered saline for 30 min. Blots were reacted with either goat anti-R-MuLV (1:250; National Cancer Institute Repository) or SIVsm/pbj-infected monkey serum (1:250) in 3% dried milk and were incubated overnight at 4°C. After washing, blots were reacted with a second antibody-alkaline phosphatase conjugate (rabbit anti-goat alkaline phosphatase [1:1,000] or goat anti-human alkaline phosphatase [1:1,000]; Bio-Rad) at room temperature for 2 h. Blots were developed with Nitro Blue Tetrazolium–5-bromo-4-chloro-3-indolylphosphate substrate for 30 min.

Morphometric analysis of virion production. Acutely R-MuLV-infected SC-1 cells were treated with protease inhibitors at concentrations of 20 μM for 3 days. The media were then decanted from the flasks, and cells were fixed in situ with 3% glutaraldehyde–4% paraformaldehyde in cacodylate buffer (pH 7.4). The monolayers were postfixed in osmium tetroxide, stained en bloc with uranyl acetate, dehydrated, and embedded in epoxy resin. Thin sections were cut en face, poststained with uranyl acetate and lead citrate, and examined in a JEOL 100-CX electron microscope.

Thin sections were mounted onto 200-mesh hexagonal grids. At low magnification, two grid spaces were randomly chosen for examination. Electron micrographs (final magnification, ×10,450) of the perimeters of all cells within the grid spaces were taken. The plasmalemmae were measured by using a Videoplan 2 (Carl Zeiss Inc., Thornwood, N.Y.). Virions which were within 0.65 μm of the plasmalemma were counted.

RESULTS

Effect of protease inhibitors on R-MuLV. The activities of the protease inhibitors against HIV-1 protease in cell-free systems, as well as their abilities to inhibit HIV-1 and R-MuLV infectivities in cell culture, are given in Table 1. Compounds 3, 5, and 7 inhibited production of infectious R-MuLV, with 50% inhibitory concentrations of 0.82 μM for compound 3, 0.67 μM for compound 5, and 1.0 μM for compound 7. Compound 2 also displayed dose-dependent antiviral activity at greater than 10-fold higher concentrations. In contrast, compounds 1, 4, and 6 did not reduce the number of PFU. No cellular cytotoxicity was detectable at concentrations up to 100 μM with any of the seven compounds.

Inhibition of R-MuLV protease. Cell extracts of inhibitor-treated cells acutely infected with R-MuLV were examined by Western blotting to determine whether these HIV-1 protease inhibitors can also inhibit the processing of R-MuLV *gag* polyproteins. R-MuLV-infected SC-1 cells treated with two of the HIV-1 protease inhibitors, compounds 3 and 5 at 20 or 10 μM, contained greatly reduced amounts of the p30 *gag* protein, indicating that these compounds inhibit *gag* processing or replication of R-MuLV (Fig. 2). Untreated R-MuLV controls contained p30, and uninfected untreated controls contained no p30 band. The protein bands detected in the 50- to 80-kDa range represent host proteins that were reactive with the antibody that we used, since these were detected in uninfected cells extracts. It seems likely that, because of the inhibition of spread of virus to susceptible cells, insufficient levels of viral core proteins were made in the treated cells to detect $Pr65^{gag}$ or its processing intermediates.

Effects of protease inhibitors on SIVsm/pbj. The synthetic protease inhibitors inhibited replication of SIV in assays of syncytium reduction and SIV p27 production at similar concentrations (Table 1). All of the compounds except compounds 4 and 6 were able to block SIV p27 production by 100 tissue culture infective doses. Compounds 3, 5, and 7 were the most potent and completely inhibited syncytium production by SIV at concentrations ≤1.3 μM (Table 1).

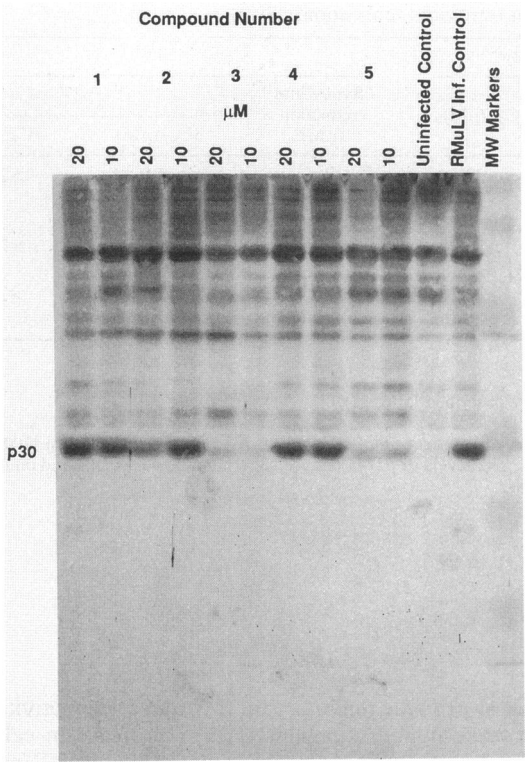


FIG. 2. Inhibition of processing of R-MuLV polyproteins by protease inhibitors as assessed by Western blotting. Lysates of R-MuLV-infected SC-1 cells were prepared 3 days after the initiation of culture, and they were analyzed by Western blotting. Blots were developed with goat antiserum to R-MuLV (1:250; National Cancer Institute Repository) and alkaline phosphate-conjugated second antibody. Compound numbers and concentrations (10 or 20 μM) are indicated, as is the location of the p30 band. MW, molecular weight.

Compound 2 blocked syncytium production by 100 tissue culture infective doses of SIV only at an inhibitor concentration of ≥10 μM (Table 1). With a higher dose of virus (1,000 tissue culture infective doses), compounds 3, 5, and 7, but not compound 2, were able to block syncytium production at ≥10 μM (data not shown).

Inhibition of SIV protease. No SIV-specific p27 protein band was detected in Western blots of extracts of SIV-infected cells treated with compounds 3 and 5 (Fig. 3). This finding suggests that synthesis of the viral *gag* protein was inhibited to undetectable levels. SIV p27 and 40-kDa protein bands were detected only in samples treated with the lowest concentrations of compounds 1 (0.625 and 1.25 μM) and 2 (0.625 μM). Similar bands were observed in the SIV_{mac} control virus marker.

Electron microscopy of R-MuLV-infected cells treated with protease inhibitors. No significant changes were seen in the ultrastructures of cells treated with any of the compounds. Untreated cells were surrounded by numerous virions, as were cells from infected cultures treated with compounds 1, 2, 4, or 6 (data not shown). Cells from cultures treated with AZT or compounds 3, 5, or 7 (data not shown) had greatly reduced numbers of virus particles. Morphometric analysis was used to quantify the reduction in the number of membrane-associated virus particles. When compared with untreated cultures, AZT and compounds 3, 5, and 7 and, to a lesser extent, compound 6 reduced the numbers of membrane-associated virions (Fig. 4). The majority of virions produced by all cultures, except those treated with compound 3, were normal in both size and structure, but abnormal extracellular particles in addition to normal virions were observed in some cultures (Fig. 4). Cultures treated with compound 3 in particular produced extracellular particles with the annular cores characteristic of immature virions, in addition to some other particles with abnormal morphologies. Increased numbers of immature virions with annular cores, similar in appearance to immature HIV-1 particles (8), were observed in cultures treated with AZT and compounds 2, 3, 5, 6, and 7. Additionally, we noted particles lacking a condensed core and particles containing multiple cores. Cultures treated with AZT and compounds 3, 5, and 7 showed a reduction in the production of mature (infectious) particles (Fig. 4).

DISCUSSION

The studies described here revealed striking similarities in the in vitro antiviral activities of rationally designed synthetic protease inhibitors against two different animal retroviruses: SIV, a simian lentivirus, and R-MuLV, a murine C-type virus. The same compounds, namely, compounds 3, 5, and 7, were highly active against both viruses in a number of different assay systems. Compound 2 had much less activity against both viruses, and compounds 1, 4, and 6 had little or no activity. Even more striking was their similar activities against HIV-1. As reported previously (5, 18, 21, 22), these same protease inhibitors inhibit the processing of viral polyproteins in cultures of human T lymphocytes chronically infected with HIV-1 and, consequently, the production of infectious virus particles. As shown in Table 1, compounds 3, 5, and 7 had the greatest activities against HIV-1. Thus, the same three protease inhibitors inhibited the infectivities of R-MuLV, SIV, and HIV-1.

It is not surprising that the same compounds were active against both HIV-1 and SIV because they are related lentiviruses which share similar genetic organizations and morphologies. Indeed, several inhibitors related to the compounds listed in Table 1, including compound 4, exhibit similar inhibition constants with the recombinant proteases of HIV-1 and SIV_{mac} (10). Furthermore, the antiviral activities of HIV-1 protease inhibitors in cell culture experiments with SIV_{mac} have also been reported (1). However, the striking similarities in the biological effects of this group of compounds against lentiviruses and murine C-type retroviruses may not have been so predictable. Overall, HIV and the MuLVs display limited sequence homology except for two highly conserved domains, one of which encompasses the protease enzyme's active site (12, 16, 20). In the proteases of these viruses, as in all known retroviral proteases, the active site includes the prototypic Asp-Thr-Gly sequence, from which the aspartic proteases derive their name (12, 16, 20). In all aspartic proteases, this sequence is preceded by two hydrophobic residues, Leu-Leu for HIV and Leu-Val for MuLV (12, 16, 20). As a result of these and other structural similarities, the proteases of HIV and MuLV share similar substrate cleavage sequence specificities (16, 27). A typical cleavage site sequence in the polyprotein precursors of these retroviruses has the sequence Ser (or Thr)-X-Y-Phe (or Tyr)*Pro-Z, in which the Phe-Pro bond is scissile and amino acids X, Y, and Z are commonly hydrophobic residues (16, 27). The results of these studies indicate that the proteases of HIV-1, SIVsm/pbj, and R-MuLV share

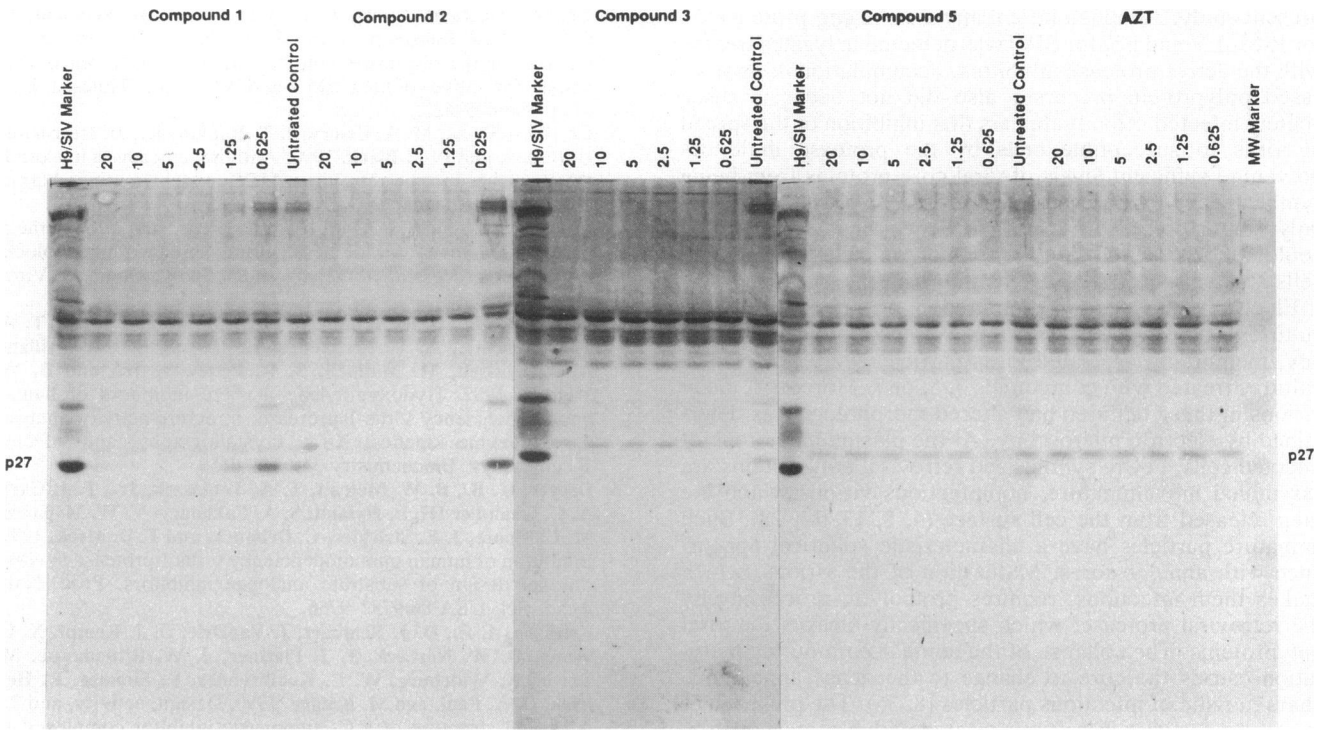


FIG. 3. Inhibition of processing of SIV polyproteins by protease inhibitors as assessed by Western blotting. Lysates of SIV-infected AA2 cells were prepared 10 days after the initiation of culture, and they were analyzed by Western blotting. Blots were developed with monkey serum from infected animals (1:250) and alkaline phosphatase-conjugated second antibody. Compound names and concentrations (0.625 to 20 μM) are indicated, as is the location of the p27 band.

sufficient homologies in their active sites and tertiary conformations that members of this series of peptide analog inhibitors have similar antiviral effects.

In addition to their similar antiviral activities against three different retroviruses, it was notable that the active compounds also lacked cytotoxicity against uninfected murine cells, even at concentrations 100 times greater than those required for antiviral effects. A similar lack of cytotoxicity of similar compounds for uninfected human CEM cells was reported previously by Meek et al. (21, 22) and Lambert et al. (18), who also reported the specificities of closely related protease inhibitors for HIV-1 protease compared with those for the cellular aspartic proteases cathepsin D and renin.

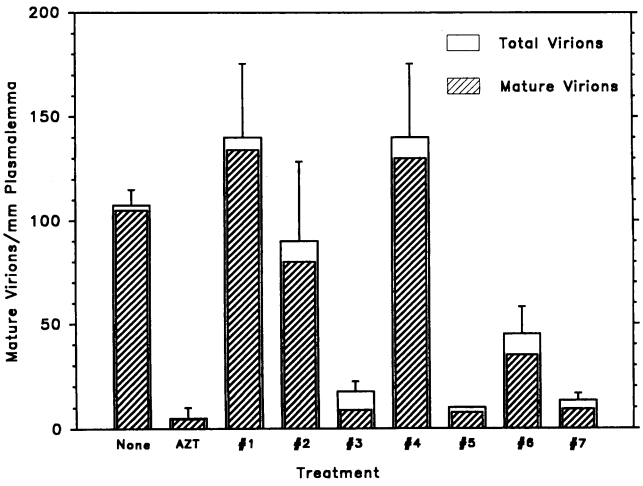


FIG. 4. Total numbers of virions and numbers of mature virions observed within 0.6 μm of the plasmalemmae of cells treated with protease inhibitors. The number of particles with mature morphologies was calculated by multiplying the total number of virions per micrometer of plasmalemma by the percentage of the particles with mature forms. Numbers on the x axis are compound numbers. The values are expressed as mean ± standard deviation of four separate determinations.

Despite the qualitative similarities in function of the peptide analog inhibitors against HIV-1, SIV, and R-MuLV, it cannot be stated unequivocally that the mechanism of action of the protease inhibitors is the same for all three types of retroviruses. Indeed, some discrepancies were noted in the antiviral activities of the less active protease inhibitors. For example, compound 1 had some marginal activity against HIV-1 and SIV but not against R-MuLV, whereas compound 4 had no activity against HIV-1 and R-MuLV but some marginal activity against SIV.

For HIV-1 (5, 18, 21, 22) as well as for the two retroviruses used in the present study, it has been demonstrated that these synthetic protease inhibitors can reduce or eliminate acute infection of naive cells. Furthermore, these protease inhibitors markedly reduced the processing of viral polyproteins in human cell lines chronically infected with HIV-1 (5, 18, 21). In the study described here, processing of viral polyproteins in cells which were acutely infected with SIV or R-MuLV was studied. Because the protease inhibitors interfere with the establishment of a productive viral infection in naive cells, it is not possible to state definitively that inhibition of viral polyprotein processing is responsible for the blocking of R-MuLV and SIV infection seen in the

present study. Although little if any mature *gag* protein (p30 for R-MuLV and p27 for SIV) was detected in lysates treated with the active protease inhibitors, accumulation of unprocessed polyprotein precursor also did not occur in these acutely infected cells. It appears that inhibition of the spread of virus to susceptible cells by the protease inhibitors prevented sufficient levels of viral core proteins from being synthesized in treated cells. Therefore, the presence of viral polyprotein precursors and their processing intermediates could not be detected. Studies with chronically SIV infected cells are in progress to address this issue.

Electron microscopic examination of infected, treated cultures was consistent with this explanation in that few, if any, membrane-associated virus particles were detected in cultures treated with compounds 3, 5, or 7. Moreover, many virions in these cultures had altered morphologies as determined by electron microscopy. At the plasma membrane of infected cells, newly synthesized retroviral polyproteins are assembled into immature, noninfectious virions which are then released from the cell surface (4, 8, 13, 14, 28). Such immature particles have a characteristic spherical appearance with annular cores. Maturation of the virions, which makes them infectious, requires proteolytic processing by the retroviral protease, which specifically cleaves the viral polyproteins. The collapse of the annular core during maturation causes the core to change to the dense appearance characteristic of infectious particles (8, 36). The presence of immature particles in cultures treated with a protease inhibitor indicates that interruption of polyprotein processing by protease inhibitors leads to a block in virus maturation. This observation is consistent with the interpretation that, at the least, protease inhibitors interfere with viral infectivity by preventing processing of retroviral polyproteins.

In summary, the results presented here, combined with the results of previous studies (5, 18, 21, 22) of the activities of these compounds against HIV-1, demonstrate that the selected viral protease inhibitors have broad spectra of activity. The results of the present study indicate that murine and simian retrovirus models may be suitable systems for the preclinical development of some protease inhibitors. Convenient, reliable, and meaningful animal models should prove to be of great value in optimizing conditions for administration of these agents. Even though R-MuLV does not represent a model for HIV pathogenesis, issues affecting agent selection, such as route of delivery, formulation, pharmacokinetics, and bioavailability, can readily be addressed in such systems.

ACKNOWLEDGMENTS

This work was supported in part by an NCDDG from NIAID, 1U01A125617, and by the U.S. Army Medical Research Institute for Infectious Diseases. We express our appreciation for the excellent technical assistance of Katherine M. McKinnon and Sharon L. Wooden of Southern Research Institute (for the R-MuLV infectivity inhibition assays), Thomas J. Carr and Emilio Cacciavillani (for synthetic chemistry), Thomas D. Meek and T. A. Tomaszek (for the protease K_i values) of the Department of Medicinal Chemistry, SmithKline Beecham, and Annabellee Fernandez and H. Ron Bartus (for Western blot and HIV infectivity inhibition assays) of the Department of Anti-Infectives, SmithKline Beecham.

REFERENCES

1. **Ashorn, P., T. J. McQuade, S. Thaisrivongs, A. G. Tommasselli, W. G. Tarpley, and B. Moss.** 1990. An inhibitor of the protease blocks maturation of human and simian immunodeficiency viruses and spread of infection. Proc. Natl. Acad. Sci. USA **87**:7472–7476.
2. **Chaffee, S., J. M. Leeds, T. J. Matthews, K. J. Weinhold, M. Skinner, D. P. Bolognesi, and M. S. Hershfield.** 1988. Phenotypic variation in the response to the human immunodeficiency virus among derivatives of the CEM T and WIL-2 B cell lines. J. Exp. Med. **168**:605–621.
3. **Chirigos, M. A., M. A. Ussery, J. T. Rankin, Jr., D. Herrmann, U. Bicker, and P. L. Black.** 1990. Antiviral efficacy of imexon in the Rauscher murine retrovirus AIDS model. Immunopharmacol. Immunotoxicol. **12**:1–21.
4. **Crawford, S., and S. P. Goff.** 1985. A deletion mutation in the 5′ part of *pol* gene of Moloney murine leukemia virus blocks proteolytic processing of the *gag* and *pol* polyproteins. J. Virol. **53**:899–907.
5. **Dreyer, G. B., D. M. Lambert, T. D. Meek, T. J. Carr, T. A. Tomaszek, Jr., A. V. Fernandez, H. Bartus, E. Cacciavillani, A. M. Hassell, M. Minnich, S. R. Petteway, Jr., and B. W. Metcalf.** 1992. Hydoxyethylene isostere inhibitors of human immunodeficiency virus-1 protease: structure-activity analysis using enzyme kinetics, X-ray crystallography, and infected T-cell assays. Biochemistry **31**:6646–6659.
6. **Dreyer, G. B., B. W. Metcalf, T. A. Tomaszek, Jr., T. J. Carr, A. C. Chandler III, L. Hyland, S. A. Fakhoury, V. W. Magaard, M. L. Moore, J. E. Strickler, C. Debouck, and T. D. Meek.** 1989. Inhibition of human immunodeficiency virus 1 protease *in vitro*: rational design of substrate analogue inhibitors. Proc. Natl. Acad. Sci. USA **86**:9752–9756.
7. **Erickson, J. E., D. J. Neidhart, J. VanDrie, D. J. Kempf, X. C. Wang, D. W. Norbeck, J. J. Plattner, J. W. Rittenhouse, M. Turon, N. Wideburg, W. E. Kohlbrenner, R. Simmer, R. Helfrich, D. A. Paul, and M. Knigge.** 1990. Design, activity, and 2.8 Å crystal structure of a C_2 symmetric inhibitor complexed to HIV-1 protease. Science **249**:527–533.
8. **Gonda, M. A., F. Wong-Staal, R. C. Gallo, J. E. Clements, O. Narayan, and R. V. Gilden.** 1985. Sequence homology and morphologic similarity of HTLV-III and visna virus, a pathogenic lentivirus. Science **227**:173–177.
9. **Gottlinger, H. G., J. G. Sodroski, and W. A. Haseltine.** 1989. Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **86**:5781–5785.
10. **Grant, S. K., I. C. Deckman, M. D. Minnich, J. Culp, S. Franklin, G. B. Dreyer, T. A. Tomaszek, Jr., C. Debouck, and T. D. Meek.** 1991. Purification and biochemical characterization of recombinant simian immunodeficiency virus protease and comparison to human immunodeficiency virus type 1 protease. Biochemistry **30**:8424–8434.
11. **Hartley, J. W., and W. P. Rowe.** 1975. Clonal cell lines from a feral mouse embryo which lack host-range restrictions for murine leukemia viruses. Virology **65**:128–134.
12. **Katoh, I., T. Yasunaga, Y. Ikawa, and Y. Yoshinaka.** 1987. Inhibition of retroviral protease activity by an aspartyl proteinase inhibitor. Nature (London) **329**:654–656.
13. **Katoh, I., Y. Yoshinaka, A. Rein, M. Shibuya, T. Odaka, and S. Oroszlan.** 1985. Murine leukemia virus maturation: protease region required for conversion from "immature" to "mature" core form and for virus infectivity. Virology **145**:280–292.
14. **Kohl, N. E., E. A. Emini, W. A. Schleif, L. J. Davis, J. C. Heimbach, R. A. Dixon, E. M. Scolnick, and I. S. Sigal.** 1988. Active human immunodeficiency virus protease is required for viral infectivity. Proc. Natl. Acad. Sci. USA **85**:4686–4690.
15. **Kramer, R. A., M. D. Schnaber, A. M. Skalka, K. Ganguly, F. Wong-Staal, and E. P. Reddy.** 1986. HTLV-III gag protein is processed in yeast cells by the virus pol-protease. Science **231**:1580–1584.
16. **Kräusslich, H.-G., and E. Wimmer.** 1988. Viral proteinases. Annu. Rev. Biochem. **57**:701–754.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Lambert, D. M., S. R. Petteway, Jr., C. E. McDanal, T. K. Hart, J. J. Leary, G. B. Dreyer, T. D. Meek, P. J. Bugelski, D. P. Bolognesi, B. W. Metcalf, and T. J. Matthews.** 1992. Human immunodeficiency virus type 1 protease inhibitors irreversibly

block infectivity of purified virions from chronically infected cells. Antimicrob. Agents Chemother. **36:**982–988.

19. **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.
20. **Meek, T. D., B. D. Dayton, B. W. Metcalf, G. B. Dreyer, J. E. Strickler, J. G. Gorniak, M. Rosenberg, M. L. Moore, V. W. Magaard, and C. Debouck.** 1989. Human immunodeficiency virus 1 protease expressed in *Escherichia coli* behaves as a dimeric aspartic protease. Proc. Natl. Acad. Sci. USA **86:**1841–1845.
21. **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. Metcalf, and S. R. Petteway.** 1990. Inhibition of HIV-1 protease in infected T-lymphocytes by synthetic peptide analogues. Nature (London) **343:**90–92.
22. **Meek, T. D., D. M. Lambert, B. W. Metcalf, S. R. Petteway, Jr., and G. B. Dreyer.** 1990. HIV-1 protease as a target for potential anti-AIDS drugs, p. 225–256. *In* E. DeClercq (ed.), Design of anti-AIDS drugs, vol. 14. Pharmaco Chemistry Library. Elsevier, Amsterdam.
23. **Miller, M., M. Jaskolski, J. K. Rao, J. Leis, and A. Wlodawer.** 1989. Crystal structure of a retroviral protease proves relationship to aspartic protease family. Nature (London) **337:**576–579.
24. **Mous, J., E. P. Heimer, and S. F. LeGrice.** 1988. Processing protease and reverse transcriptase from human immunodeficiency virus type I polyprotein in *Escherichia coli*. J. Virol. **62:**1433–1436.
25. **Navia, M. A., P. M. Fitzgerald, B. M. McKeever, C. T. Leu, J. C. Heimbach, W. K. Herber, I. S. Sigal, P. L. Darke, and J. P. Springer.** 1989. Three-dimensional structure of aspartyl protease from human immune deficiency virus HIV-1. Nature (London) **337:**615–620.
26. **Pearl, L. H., and W. R. Taylor.** 1987. A structural model for the retroviral proteases. Nature (London) **329:**351–354.
27. **Pearl, L. H., and W. R. Taylor.** 1987. Sequence specificity of retroviral proteases. Nature (London) **338:**482.
28. **Peng, C., B. K. Ho, T. W. Chang, and N. T. Chang.** 1989. Role of human immunodeficiency virus type 1-specific protease in core protein maturation and viral infectivity. J. Virol. **63:**2550–2556.
29. **Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. Doran, J. A. Rafalski, E. A. Whitehorn, K. Baumeister, L. Ivanoff, S. R. Petteway, Jr., M. L. Pearson, J. A. Lautenberger, T. S. Papis, J. Ghrayeh, N. T. Chang, R. C. Gallo, and F. Wong-Staal.** 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature (London) **313:**277–284.
30. **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:**358–361.
31. **Rowe, W. P., W. E. Pugh, and J. W. Hartley.** 1970. Plaque assay techniques for murine leukemia viruses. Virology **42:**1136–1139.
32. **Seelmeier, S., H. Schmidt, V. Turk, and K. von der Helm.** 1988. Human immunodeficiency virus has an aspartic-type protease that can be inhibited by pepstatin A. Proc. Natl. Acad. Sci. USA **85:**6612–6616.
33. **Simkovic, D., N. Valentova, and V. Thurzo.** 1962. *In vitro* cultivation of rat sarcoma XC cells containing Rous virus. Folia Biol. (Prague) **8:**221–229.
34. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
35. **Wlodawer, A., M. Miller, M. Jaskolski, B. K. Sathyanarayana, E. Baldwin, I. T. Weber, L. M. Selk, L. Clawson, J. Schneider, and S. B. Kent.** 1989. Conserved folding in retroviral proteases: crystal structure of a synthetic HIV-1 protease. Science **245:**616–621.
36. **Yoshinaka, Y., and R. B. Luftig.** 1977. Murine leukemia virus morphogenesis: cleavage of p70 *in vitro* can be accompanied by a shift from a concentrically coiled internal strand ("immature") to a collapsed ("mature") form of the virus core. Proc. Natl. Acad. Sci. USA **74:**3446–3450.