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Structure-based design of nonpeptide inhibitors specific for the human immunodeficiency virus 1 protease

R. L. DESJARLAIS*, G. L. SEIBEL, I. D. KUNTZ†, P. S. FURTH, J. C. ALVAREZ, P. R. ORTIZ DE MONTELLANO†, D. L. DECAMP, L. M. BABÉ, AND C. S. CRAIK†

Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, CA 94143-0446

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ABSTRACT **By using a structure-based computer-assisted search, we have found a butyrophenone derivative that is a selective inhibitor of the human immunodeficiency virus 1 (HIV-1) protease. The computer program creates a negative image of the active site cavity using the crystal structure of the HIV-1 protease. This image was compared for steric complementarity with 10,000 molecules of the Cambridge Crystallographic Database. One of the most interesting candidates identified was bromperidol. Haloperidol, a closely related compound and known antipsychotic agent, was chosen for testing. Haloperidol inhibits the HIV-1 and HIV-2 proteases in a concentration-dependent fashion with a K_i of ≈100 μM. It is highly selective, having little inhibitory effect on pepsin activity and no effect on renin at concentrations as high as 5 mM. The hydroxy derivative of haloperidol has a similar effect on HIV-1 protease but a lower potency against the HIV-2 enzyme. Both haloperidol and its hydroxy derivative showed activity against maturation of viral polypeptides in a cell assay system. Although this discovery holds promise for the generation of nonpeptide protease inhibitors, we caution that the serum concentrations of haloperidol in normal use as an antipsychotic agent are <10 ng/ml (0.03 μM). Thus, concentrations required to inhibit the HIV-1 protease are >1000 times higher than the concentrations normally used. Haloperidol is highly toxic at elevated doses and can be life-threatening. Haloperidol is not useful as a treatment for AIDS but may be a useful lead compound for the development of an antiviral pharmaceutical.**

Three-dimensional structures of pharmacologically important macromolecules offer a route to the discovery and improvement of bioactive agents. Traditionally, the search for lead compounds involves random screening (1). Once an active compound is found, derivatives are made and tested. The results of such studies guide subsequent development, often assisted by structure–activity relationships (2). Alternatively, it has been possible to use knowledge of specific enzyme mechanisms or cellular processes to provide starting points for drug discovery (3–5).

We have been particularly interested in using high-resolution receptor structures to design lead compounds. There have been several approaches to this problem. One successful path employs interactive inspection of the structures (6–10). The emphasis in this paper is the complex steric features of macromolecular surfaces (11, 12) at the active site of an enzyme. A set of computer algorithms, called DOCK, has been developed to characterize the shape of invaginations and grooves that form the active sites and recognition surfaces of biological macromolecules (13). The program also searches a data base of small molecules for templates whose shapes are complementary to the macromolecule site (14). These templates normally require modification to achieve good chemical and electrostatic interactions (15). However, the program has been shown to position accurately known cofactors or inhibitors based on shape constraints alone (13).

The solution of the molecular structure of the human immunodeficiency virus 1 (HIV-1) protease (16, 17) and the availability of the coordinates for a complexed form (18) made it possible for us to use the DOCK algorithms to study this therapeutic target. The HIV-1 protease is an aspartyl protease (19) composed of two 99-amino acid monomers (17). The HIV-2 virus encodes a related protease (20). The enzymes are required for viral maturation to process the polypeptides encoded by the viral *gag* and *pol* genes (21). Mutation of the catalytic aspartate of the HIV-1 protease at amino acid 25 leads to loss of proteolytic activity and results in noninfectious virions (19, 22). Peptide-based compounds with submicromolar inhibitory activities against the HIV-1 enzyme, *in vitro*, have been shown to be effective in reducing viral infectivity in cultured T4 cells (4) and in inhibition of gag polyprotein processing (5). However, peptide-based materials are often therapeutically ineffective when orally administered (23), stimulating our interest in the development of nonpeptide agents.

METHODS AND RESULTS

Calculations. The structure of the uncomplexed HIV-1 protease by Wlodawer *et al.* (17) was used in the calculations. No water molecules were included in this set of coordinates. The structure of the complex with MVT-101, the substrate-based inhibitor Ac-Thr-Ile-Ahx-ψ(CH_2-NH)-Ahx-Gln-Arg-NH_2 (where Ahx is 2-aminohexanoic acid), was subsequently obtained (18), and some of our efforts are based on the structure of this complex. The DOCK procedure (version 1.1) has been described (13–15). The first step was the construction of a negative image of the active site from the x-ray coordinates. The molecular surface was generated by the program MS (12). For the HIV-1 protease, the negative image was an approximate cylinder of length 25 Å and diameter 8 Å. It was composed of 34 intersecting spheres whose centers were used with a matching algorithm (13) to determine which small molecule candidates could be placed within the site (for program parameters, see Fig. 2). Our small molecule data base was developed from the Cambridge Structural Database (24, 25) and searched using the methods of DesJarlais *et al.* (14). Before the search, the data base was reduced to ≈10,000 molecules of the most diverse shapes. Each molecule in the Cambridge Structural Database was described by a set of geometric (e.g., principal axes) and topologic (e.g., connectivity indices) parameters. The molecules were clustered in this parameter space, and the first molecule in each cluster was chosen (G.L.S., unpublished data). The DOCK program ranked

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Abbreviations: DMSO, dimethyl sulfoxide; HAL, haloperidol; HIV, human immunodeficiency virus; hSOD, human superoxide dismutase; hydroxy-HAL, hydroxy derivative of haloperidol; IPTG, isopropyl β-D-thiogalactopyranoside.

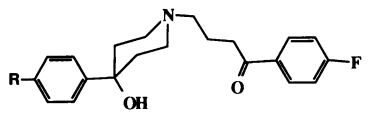
*Present address: SmithKline Beecham Pharmaceuticals, Box 1539, King of Prussia, PA 19406.
†To whom reprint requests should be addressed.

putative ligands based on a simple function of the interatomic distances. This function had three adjustable parameters (see Fig. 2). For each molecule, the program investigated many orientations within the site and saved the orientation that had the highest score. The program was executed on an Iris 4D/70 workstation (Silicon Graphics) and required 10.25 hr of computer time for the search. The top 200 molecules were examined using the interactive graphics package MidasPlus (26). We focused on three criteria to evaluate the molecules as templates for the design of protease inhibitors: (*i*) proximity (within 4 Å) of at least one atom of the template to any of the carboxyl oxygens of the side chains of Asp-25 or Asp-25′ of the protease; (*ii*) potential to form hydrogen bonds to the occluded regions of the protein surface (15); and (*iii*) a modest and flexible synthetic effort not requiring preparation of fused or spiro ring linkages or multiple chiral centers.

Many of the molecules were eliminated by the criteria given above. Of the templates that were seriously considered, our primary candidate was bromperidol (Fig. 1). This molecule was number 51 on the list from DOCK based on its score. Its degree of burial compared favorably with candidates above it on the list. It was of particular interest because its best orientation from the DOCK program placed the hydroxyl group between the active site aspartates, corresponding closely to the position of the hydroxyl group in the cocrystal of a statine-based inhibitor and penicillopepsin (27). Bromperidol is a butyrophenone, a class of molecules used as antipsychotic agents, with well-studied pharmacological properties (28).

The DOCK program proposes specific orientations of a given template molecule in the active site. These proposals cannot be considered as "predictions" since molecular energies are not evaluated. However, they can be used as hypotheses to suggest modifications of the initial template. The highest scoring orientation of bromperidol did not coincide closely with that of the peptide inhibitor in the crystal structure (18) (Fig. 2). The long axis of the bromperidol was at a 45° angle to the backbone direction of the peptide inhibitor. However, the hydroxyl group was placed within 3 Å of the aspartyl groups of the enzyme and one of the phenyl rings of the bromperidol was placed in the same substrate binding pocket as one of the 2-aminohexanoic acid residues of the peptide, suggesting that the compound should act as a competitive inhibitor. In this orientation, a hydrogen bond could be formed to the carbonyl group of Gly-27 if the ketone of bromperidol were reduced to the corresponding alcohol, see below.

Synthetic Methods. The compound selected for preliminary tests was HAL (Fig. 1, I). HAL differs from bromperidol in that it bears a chlorine rather than a bromine substituent on one of the two phenyl rings. The small difference in the size of these two substituents (van der Waals radius: Cl, 1.8 Å; Br, 1.95 Å) is well within the tolerance limits of the search procedures. We, therefore, used HAL, which is available commercially, as the lead molecule for biological evaluation.



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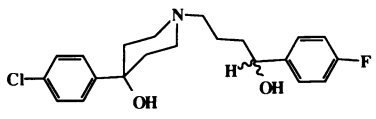

II

FIG. 1. (I) Bromperidol, R = Br; haloperidol (HAL), R = Cl. (II) Hydroxy-haloperidol (hydroxy-HAL).

Commercial HAL (Sigma) was recrystallized from diethyl ether/$CHCl_3$, 4:1 (vol/vol), and the recrystallized material was shown to be pure by its melting point (mp 148.2–149°C; lit. 148.0–149.4°C) (29), by NMR, and by elemental analysis. The purified material was used for all biological work even though little difference was found between the recrystallized and commercial samples. The hydroxy derivative of HAL, hydroxy-HAL (Fig. 1, II), obtained by reduction of HAL with lithium aluminum hydride in diethyl ether/tetrahydrofuran [3:1 (vol/vol)], was crystallized from hexane/methylene chloride [4:1 (vol/vol)] after filtration through silica gel (mp 121–122.5°C). The ^1H NMR, infrared, mass spectrum, and elemental analysis of the crystalline product are consistent with the assigned structure.

Recombinant Protein Preparations. Recombinant HIV-1 protease was expressed and purified from *Escherichia coli* strain D1210 using the pSOD/PR179 expression vector (30). HIV-2 protease was expressed in *Saccharomyces cerevisiae* strain AB110 from the plasmid pHIV2PR115 (31). After reverse-phase HPLC, the homogeneous proteins were refolded as described by Tomasselli *et al.* (32) and stored at −20°C. Concentrations of the enzymes were established by titration with the substrate-based inhibitor Val-Ser-Gln-Asn-Leu-ψ[CH(OH)CH_2]-Val-Ile-Val (32). Stock solutions of HIV-1 and HIV-2 proteases had specific activities on a decapeptide substrate of 23.9 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ and 0.5 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, respectively. Recombinant human renin had a specific activity of 400 Goldblatt units/mg.

Enzymatic Assays. *HIV protease assays.* Both HIV-1 and HIV-2 proteases were assayed against the decapeptide, Ala-Thr-Leu-Asn-Phe-Pro-Ile-Ser-Pro-Trp, corresponding to the HIV-1 C-terminal autoprocessing site (where underlined residues are cleavage sites) (33). The decapeptide was synthesized by conventional solid-state methods. Reactions were carried out and fractionated by HPLC as described (31). Conversion of the decapeptide to the two pentapeptides was quantitated by integration of the peak areas and comparison to product standard curves.

Pepsin assay. Porcine pepsin from Sigma (2×10^{-3} mg/ml) with a specific activity of 0.38 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, was incubated for 1 hr at 37°C with various concentrations of Ala-Thr-Leu-Asn-Phe-Pro-Ile-Ser-Pro-Trp in 0.1 M sodium acetate (pH 4.7) containing 4 mM EDTA and 5% (vol/vol) dimethyl sulfoxide (DMSO). Pepsin also specifically cleaves the Phe-Pro peptide bond. Enzyme velocity was determined for 0.1-ml reaction volumes using the HPLC assay described (31).

Renin assay. Recombinant human renin (1 μg/ml) was assayed with 150 μM porcine angiotensinogen-(1–14) (Sigma) in 0.1 ml of 0.1 M sodium phosphate (pH 6.1) containing 10 mM EDTA and 5% DMSO. After a 15-min incubation at 37°C, the reaction was quenched as described (31). Hydrolysis products (Leu-Val-Tyr-Ser and angiotensin I, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) were separated using the HPLC assay with the absorbance monitored at 220 nm. The peak area of angiotensin I was integrated and compared to a standard curve of human angiotensin I (Sigma).

Inhibitor assays. Stock solutions of HAL and hydroxy-HAL at 20 mM were prepared in DMSO. Compounds were added to buffer solutions containing additional DMSO to give a final concentration of 5%. Control reaction mixtures contained 5% DMSO only. Enzymes were preincubated with inhibitor for 5 min at 25°C, followed by addition of substrate to initiate the reaction.

Enzymatic Inhibition. The effect of HAL on HIV-1 protease hydrolytic activity was examined with various concentrations of the decapeptide substrate. Each data point was done in triplicate and initial enzyme rates were fit to the Michaelis–Menten equation using a nonlinear regression program ("Enzfitter" from Biosoft). Under the indicated assay conditions, the K_m for the decapeptide substrate was 2.5 ±

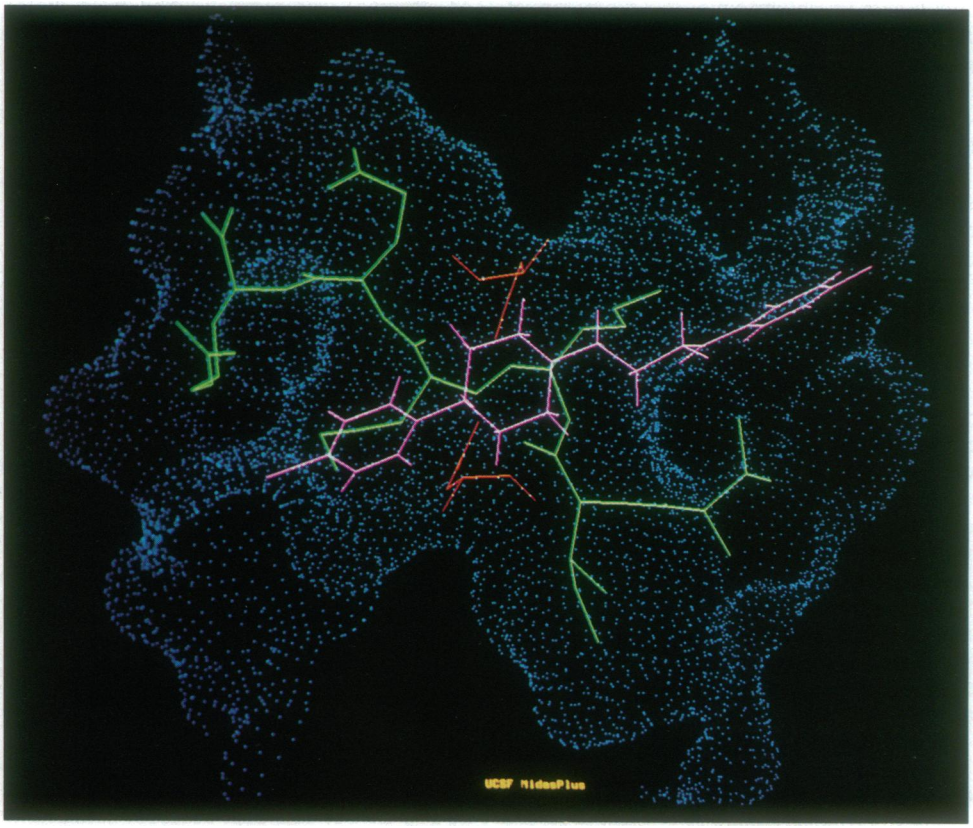

FIG. 2. Bromperidol in HIV-1 active site. This orientation is the highest scoring one for bromperidol in the uncomplexed HIV conformation (17). In this figure the bromperidol (violet) has been placed in the same orientation in the active site (blue) of the protease–MVT-101 complex (18). The substrate-based inhibitor, MVT-101 [Ac-Thr-Ile-Ahx-ψ(CH_2-NH)-Ahx-Gln-Arg-NH_2] is also shown (green). The active site aspartyl side chains are shown in red. Program parameters for DOCK, version 1.1 were: MATCH: dislim = 2.0 Å, nodlim = 8; SCORE: concut = 2.4 Å, dmin = 3.5 Å, discut = 5.0 Å.

0.44 mM, V_{max} was 23.9 ± 2.3 μmol·min^{-1}·mg^{-1}, and k_{cat} was 514 min^{-1}. The Dixon plot shown in Fig. 3 yielded a K_i of 100 ± 20 μM. The appearance of the Dixon plot is comparable to that obtained with the transition state analogue, pepstatin, that inhibits HIV-1 protease in a partially noncompetitive fashion (19). The HIV-2 enzyme exhibited a similar Dixon plot with a K_i of ≈100 μM (data not shown).

HAL inhibited HIV-1 protease in a concentration-dependent manner. The extent of inhibition was independent of the incubation period, indicating rapid binding. HIV-1 protease was 50% inhibited by 0.25 mM HAL and ≈90% inhibited at 2.0 mM. HIV-1 protease was inhibited to a similar extent by hydroxy-HAL, but HIV-2 protease was less affected by hydroxy-HAL (Table 1).

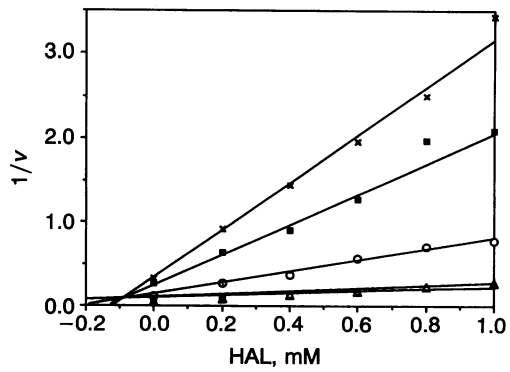

FIG. 3. Dixon plot of the inhibition of HIV-1 protease peptide hydrolysis by HAL. Purified HIV-1 protease (4 × 10^{-4} mg/ml) was preincubated with HAL in 50 mM sodium acetate (pH 5.5) containing 5 mM dithiothreitol, 1 mM EDTA, 1 M NaCl, and 5% DMSO. After 5 min, the substrate peptide was added to give a final substrate concentration of 0.2 (×), 0.3 (■), 0.6 (○), 1.0 (△), and 3.8 (□) mM. The assay solutions were incubated for 1 hr at 37°C and enzyme activity was determined by quantitation of the hydrolysis products on HPLC. Units for v are μmol·min^{-1}·mg^{-1}.

The effect of HAL and hydroxy-HAL on other aspartyl proteases was evaluated. The expected specificity of HAL for the active site of the viral protease was confirmed by its inability to inhibit human renin at concentrations as high as 5 mM (data not shown). Pepsin was 55% inhibited by 1 mM HAL and 30% inhibited by 1 mM hydroxy-HAL.

Assay for Polyprotein Processing by HIV-1 Protease in Bacteria. *E. coli* strain D1210 harboring plasmid pSOD/PR179 (30) was grown at 37°C in Luria broth containing ampicillin (100 μg/ml). Cultures were grown to OD_{650} = 0.4 at which time isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 200 μM for induction and 5-ml samples of the cultures were removed. HAL, hydroxy-HAL, and cerulenin (Sigma) were dissolved in DMSO to 50 mg/ml. The appropriate volumes of these stock solutions were added within 5 min of induction to achieve a final concentration of 50 μM. The cultures were returned to the orbital shaker at 37°C and 1-ml samples were collected at 15, 30, 60, and 120 min. The OD_{650} of each culture was determined and equivalent concentrations of cells (0.2 unit at OD_{650}) were pelleted by centrifugation. The cell pellets were resuspended in 30 μl of 1× Laemmli sample buffer (34), heated at 95°C for 10 min, and passed repeatedly through a syringe needle to shear the chromosomal DNA, and the

Table 1. Relative enzyme activity in the presence of HAL or hydroxy-HAL

Protease	Relative enzyme activity, %	
	HAL	Hydroxy-HAL
Renin	100	100
Pepsin	72	84
HIV-1	26	20
HIV-2	30	63

Enzymes were assayed in the presence of 0.5 mM HAL or hydroxy-HAL. Relative enzyme activity is based on activity of each enzyme in the absence of inhibitor.

sample was clarified by centrifugation. The supernatant was then loaded onto a 12.5–17.5% gradient polyacrylamide gel containing SDS and subjected to electrophoresis. The gels were immunoblotted and probed with antibodies to the HIV-1 protease as described (30).

Inhibition of HIV-1 Protease Maturation in Bacteria. The bacterial expression system for the HIV-1 protease involves a fusion protein with human superoxide dismutase (hSOD). The hSOD-protease polyprotein consisted of the 154 amino acids of hSOD, followed by 5 amino acids encoded by a synthetic linker, the 55 N-terminal amino acids of the *pol* reading frame (amino acids 2–56), the 99 amino acids of the protease, and finally the first 24 amino acids of the reverse transcriptase (amino acids 156–180) (Fig. 4*A*). This 38-kDa fusion protein contained the naturally occurring protease-specific cleavage sites at the N terminus of the protease and at the protease–reverse transcriptase junctions. Efficient autoprocessing by the protease was observed *in vivo* resulting in the detection of the 11-kDa mature protease. Various polypeptides corresponding to processing intermediates were also observed (35 and 13.6 kDa). Low levels of expression were detected before induction, and an ≈100-fold increase in expression was observed within 5 min of addition of IPTG. Significant autoprocessing of the protease began within 5 min of induction. Addition of HAL, hydroxy-HAL, and cerulenin to the cultures at 500 μM, resulted in the detection of a larger amount of 38- and 35-kDa precursors (Fig. 4*B*) when compared to the untreated samples, indicating that all three compounds inhibit HIV-1 protease autoprocessing. The HAL-treated samples showed 5- to 10-fold more protein in the precursor bands within 15 min. The effect of cerulenin was delayed and was not significant until 2 hr after its addition. The results with cerulenin are in agreement with earlier studies showing that the antibiotic specifically inhibits the HIV protease *in vitro* (35) and in chronically infected T lymphocytes (36). The delayed effect reported here in the bacterial assay is in agreement with the published *in vitro* kinetics (37). We did not see a concomitant decrease in the mature 11-kDa protease band for any inhibitor because the high levels of expression and processing of the mature protease saturated the immunoblot assay in the 11- and 14-kDa region of the blot. Such high levels of HIV-1 protease are very unlikely in virus-infected cells. Previous work (38) suggests <10 molecules of mature protease per cell. This value is much lower than our estimate of ≈200 molecules of protease per bacterial cell at the peak of expression. Conditions can be achieved that show a decrease in the amount of processed protease in the presence of HAL (data not shown). However, under these conditions the 38- and 35-kDa precursors cannot be detected easily.

DISCUSSION

To date, no drugs have been developed solely through knowledge of the receptor structure. The techniques described in this paper offer an innovative route to lead compounds and constitute an important step in rational drug design. The computer program DOCK will work with any structural data base. Thus, one can easily select the compounds to be searched so that they have specific characteristics (e.g., contain particular functional groups, are known drugs, do not include peptide moieties, etc.). Furthermore, DOCK can be used to develop an initial model for the location of the ligand in the site. Our first attempt to use the model was the synthesis of the hydroxy-HAL. Increased binding was not seen. This could be due to errors in the model or a balancing of desolvation and binding free energy (39) and will require crystallographic studies for a definitive answer.

Since the retroviral protease is essential for viral replication, it is an important target for the therapeutic treatment of AIDS. Most of the reported inhibitors of HIV-1 protease have been produced by replacement of the scissile peptide bond of a substrate with either a tetrahedral intermediate isostere or a reduced peptide bond isostere (4, 5, 32, 40, 41). Generally, peptide-based drugs have been plagued by lack of oral activity, insufficient duration of action, lack of specificity, and inability to cross the blood–brain barrier (42). The antifungal antibiotic cerulenin and related epoxy compounds have been presented as nonpeptide inhibitors of HIV-1 protease (35, 36). However, inhibition of *de novo* fatty acid and sterol synthesis by cerulenin results in pronounced *in vitro* toxicity (36). Based on the pharmacology of HAL, second-generation derivatives hold promise as nonpeptide antiviral agents for treatment of HIV-1 and HIV-2 infection. Useful therapeutics may be obtained by enhancing binding to HIV protease while preserving the advantages of HAL, which does not inhibit renin, can be orally administered, penetrates the central nervous system, and is long-acting (43).

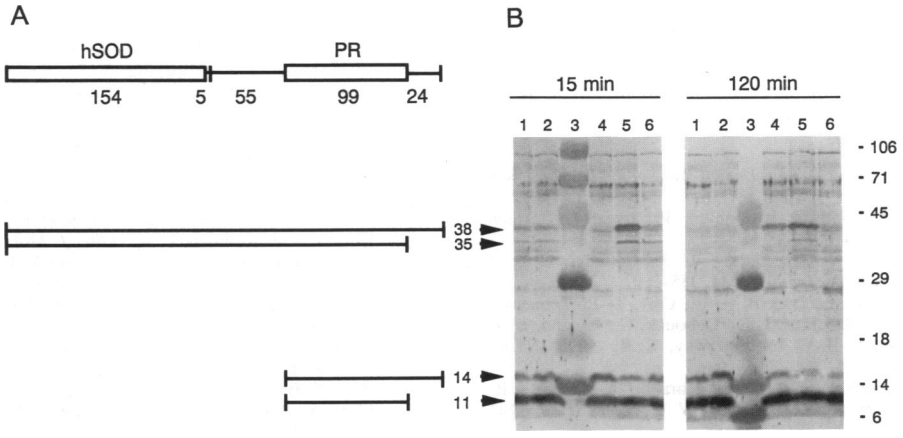

FIG. 4. Measurement of HIV-1 protease autoproteolytic activity in *E. coli*. (*A*) The polypeptide products obtained from the expression of plasmid pSOD/PR179 upon IPTG induction. The 38-kDa fusion protein is autoprocessed by the HIV-1 protease to yield 35- and 14-kDa intermediates as well as the 11-kDa mature protease. Sizes in amino acids are shown. (*B*) *E. coli* extracts (0.2 OD_{650} unit per well) were separated by SDS/PAGE and blotted onto nitrocellulose. The blots were probed with antibodies to HIV-1 protease. Cerulenin, HAL, and hydroxy-HAL were added to a final concentration of 500 μM within 5 min of IPTG addition (200 μM) to the cultures. The samples were incubated for 15 min and 120 min. Samples containing DMSO at the same concentration used with the inhibitors, as well as untreated cells are shown. The migration of prestained molecular mass standards (BRL) is recorded on the right of the figure and other molecular masses are shown on the left in kDa. Lanes: 1, DMSO; 2, untreated; 3, markers; 4, cerulenin; 5, HAL; 6, hydroxy-HAL.

We have used an *E. coli* HIV-1 protease expression system to assess the antiproteolytic potential of the inhibitors *in vivo*. When the protease is expressed in *E. coli* as a fusion protein with hSOD, the 38-kDa polypeptide precursor is autoprocessed *in situ* to release the mature 11-kDa protease in analogy with the maturation of the gag–pol polyprotein expressed in HIV-1-infected cells. Thus, it can serve as an amenable *in vivo* system for the screening compounds with inhibitory properties for the HIV-1 protease. Bacterial systems have been used (44) to determine the toxicity and carcinogenic index of various compounds. Addition of HAL, hydroxy-HAL, and cerulenin resulted in a large amount of the precursor proteins (38 and 35 kDa) compared to the untreated samples (Fig. 4*B*). The inhibition of proteolytic processing in bacterial cells was not complete. However, if maturation is reduced in infected cells, the presence of some p55 gag polyproteins in packaged virions would drastically reduce their infectivity *in vivo*. Even partial proteolytic inhibition could hinder viral proliferation (45).

Caveat. The serum concentrations of HAL in its normal use as an antipsychotic agent are <10 ng/ml (0.03 μM) (43, 46). The concentrations required to inhibit the HIV-1 protease are thus >1000 times larger than the concentrations normally used for antipsychotic therapy. HAL is highly toxic, particularly at elevated doses. Its toxic effects can be life-threatening and include extrapyramidal neurologic symptoms characteristic of Parkinson disease, neuroleptic malignant syndrome, tardive dyskinesia, hypotension, tachycardia, and a variety of other serious effects. In spite of its oral activity and ability to cross the blood–brain barrier, HAL, itself, is not useful as a treatment for AIDS.

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