L-696,474, A NOVEL CYTOCHALASIN AS AN INHIBITOR OF HIV-1 PROTEASE

III. BIOLOGICAL ACTIVITY

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L-696,474, an inhibitor of the HIV-1 protease, was discovered in extracts of the fungal culture *Hypoxylon fragiforme* (MF5511; ATCC 20995). L-696,474 is a novel cytochalasin with a molecular weight of 477 and an empirical formula of $C_{30}H_{39}NO_4$. L-696,474 inhibited HIV-1 protease activity with an IC_{50} of 3 μM and the mode of inhibition was competitive with respect to substrate (apparent $K_i = 1$ μM). Furthermore, L-696,474 was not a slow-binding inhibitor. The inhibition due to L-696,474 was also independent of the HIV-1 protease concentration. L-696,474 was inactive against pepsin, another aspartyl protease; stromelysin, a zinc-metalloproteinase; papain, a cysteine-specific protease or human leucocyte elastase, a serine-specific protease. Two other novel cytochalasins (L-697,318 and L-696,475) isolated from the same culture were inactive against the HIV-1 protease. Commercially available cytochalasins B, C, D, E, F, H and J were inactive while cytochalasin A was as active as L-696,474 against the HIV-1 protease.

Human immunodeficiency virus (HIV-1) is a retrovirus that causes acquired immunodeficiency syndrome (AIDS). The major structural and enzymatic proteins of the HIV-1 virus are coded by the *gag*, *pol* and *env* genes. One of the gene products of this virus is a dimeric aspartyl protease that specifically cleaves the precursor forms of the dominant structural and enzymatic proteins to yield mature proteins required to produce infectious virions[1,2]. An active viral protease is essential for the processing, assembly and maturation of infectious viral particles for HIV-1[1,2] and is therefore an appealing target for therapeutic intervention.

To facilitate the discovery of novel inhibitory compounds, an assay to screen microbial metabolites for inhibitors of HIV-1 protease activity was developed. L-696,474 was found in extracts of *Hypoxylon fragiforme* (MF5511; ATCC 20995). The IC_{50} of the purified inhibitor, which is a novel cytochalasin, is 3 μM and the mode of inhibition is competitive with respect to substrate.

Materials and Methods

Methods

Fermentation

The identification and fermentation of the culture that produced L-696,474 was performed as described in an accompanying paper[3].

Isolation of L-696,474

The isolation and structure determination of L-696,474 was performed as described in an accompanying paper[4].

Cloning, Expression and Purification of the HIV-1 Protease

Cloning, expression and purification of the HIV-1 protease was performed as described[5].

Synthesis and Labelling of the Peptide Substrate

The peptide substrate Val-Ser-Gln-Asn-β-naphthylAla-Pro-Ile-Val-Gln-Gly-Arg-Arg was synthesized according to the procedure of MERRIFIELD[6]. The peptide was labeled with [^{3}H]acetic anhydride by dissolving the peptide (20 mg) in 1.5 ml of dimethylformamide. Dimethyl sulfoxide (0.5 ml) was added to clarify the solution. *N*-Methyl morpholine (2 mol per mol of peptide) was added to the peptide solution and the resulting mixture was added to the [^{3}H]acetic anhydride and allowed to react for 16 hours at room temperature. The labeled peptide was purified to homogeneity with reverse phase C18 chromatography and elution with acetonitrile and the purity was confirmed by reverse phase HPLC.

Assay of HIV-1 Protease Activity

Fermentation broth extracts were mixed with 1.05 μM [^{3}H]acetyl-Val-Ser-Gln-Asn-β-naphthylAla-Pro-Ile-Val-Gln-Gly-Arg-Arg and 2 nM HIV-1 protease in final volume of 100 μl containing 100 mM sodium acetate buffer, pH 5.5 and 0.05% bovine serum albumin (BSA). After 60 minutes at 37°C the reaction was stopped with 100 μl of 5% H_3PO_4 and 150 μl of the reaction mixture was loaded onto a 1.0 ml column of Dowex AG50W-X8 resin (H^+ form). The column was washed with 1.85 ml of water and the [^{3}H]acetyl-product was collected into scintillation vials for counting in a liquid scintillation counter.

Labeling of β-Casein

Fifty mg of β-casein was dissolved in 5.0 ml of 40 mM sodium phosphate buffer, pH 7.0. Ten ml of formaldehyde was added, the solution was stirred briefly and kept at room temperature for 20 minutes. Sodium borotritide (12.5 mCi) was added and the solution was stirred as before. The solution was dialysed overnight at 4°C against 40 mM sodium phosphate buffer pH 7.0 with 3 changes (1 liter each) of buffer. The labeled protein had a specific activity of $3 \sim 7 \times 10^6$ cpm/mg protein and was used as a substrate in the pepsin and stromelysin assays.

Assay of Pepsin Activity

Pepsin activity was assayed by incubating pepsin (5 nM) with [^{3}H]β-casein (2 μM) in 100 mM HCl for 30 minutes at 37°C. The reaction was stopped with 10% TCA and TCA-insoluble material was removed by centrifugation. An aliquot of the TCA-soluble supernatant was transferred to scintillation vials for counting in a liquid scintillation counter.

Assay of Papain and Human Leucocyte Elastase (HLE) Activity

Papain and HLE activity were assayed as described by BARRETT and KIRSCHKE[7] and ZIMMERMAN *et al.*[8].

Assay of Stromelysin Activity

Stromelysin activity was assayed by incubating stromelysin (10 nM), isolated from gingival fibroblasts[9], with [^{3}H]β-casein (2 μM) in 100 mM HEPES, 10 mM $CaCl_2$ and 0.05% Brij 35, pH 6.5 for 60 minutes at 37°C. The reaction was stopped with 100 μl 4.2% TCA and TCA-insoluble material was removed by centrifugation. An aliquot of the TCA-soluble supernatant was transferred to scintillation vials for counting in a liquid scintillation counter.

Materials

Sodium [^{3}H]borohydride and [^{3}H]acetic anhydride were obtained from New England Nuclear (Boston, MA). β-Casein, BSA, pepsin, calcium chloride and Brij 35 were obtained from Sigma (St. Louis, MO). DMSO, dimethylformamide and *N*-methyl morpholine were obtained from Aldrich Chemical Co. (Milwaukee, WI). Acetonitrile, methanol, sodium acetate, phosphoric acid, trichloroacetic acid, formaldehyde, sodium phosphate and disposable columns were obtained from Fisher (Somerset, NJ). Dowex AG50W-X8 resin (H^+ form) was obtained from BioRad (Richmond, CA). HEPES was from Boehringher (Indianapolis, IN). All other reagents were of the highest grade possible.

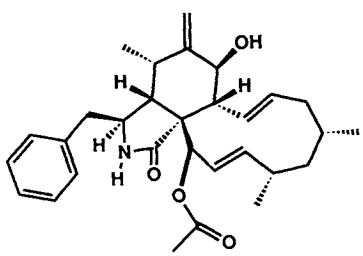
Results and Discussion

The HIV-1 protease cleaves peptides with sequences representing cleavage sites found in the *gag* and

gag-pol gene products[10]. The peptide Val-Ser-Gln-Asn-β-naphthylAla-Pro-Ile-Val is a substrate for the HIV-1 protease and the scissile bond is flanked by the β-naphthylalanine and proline residues[10,11]. The assay described above used the substrate [^{3}H]acetyl-Val-Ser-Gln-Asn-β-naphthylAla-Pro-Ile-Val-Gly-Arg-Arg. The arginine residues allow this substrate to bind tightly to the H^+ form of Dowex AG50-X8 resin and this facilitates the separation of the labeled peptide from the unhydrolyzed substrate and the other product peptide. HIV-1 protease activity was linear with respect to time (up to 120 minutes), substrate and enzyme concentrations (results not shown). Less than 10% of the substrate was hydrolyzed during the incubation period. Also, the resin used to separate the labeled product peptide from unhydrolyzed substrate bound 99.8% of the substrate (results not shown).

A fungal culture (MF5511, ATCC 20995) produced L-696,474 that inhibited HIV-1 protease activity. L-696,474 is a unique cytochalasin with a molecular weight of 477 and an empirical formula of $C_{30}H_{39}NO_4$ (Fig. 1). L-696,474 reduced HIV-1 protease activity in a dose-dependent manner (Fig. 2) with an IC_{50} of

Fig. 1. Chemical structures of L-696,474 (**1**), L-696,475 (**2**), L-697,318 (**3**), cytochalasin H (**4**) and cytochalasin A (**5**).



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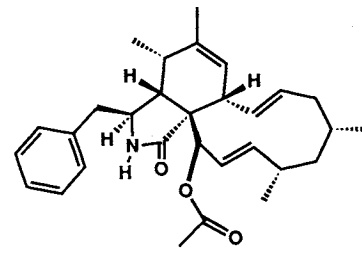

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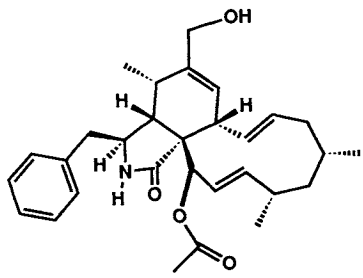

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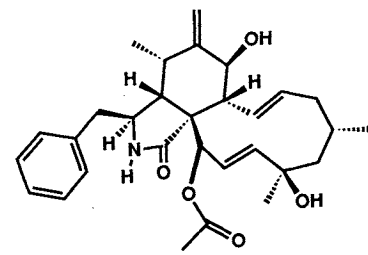

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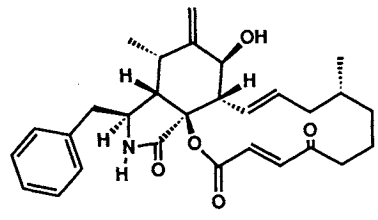

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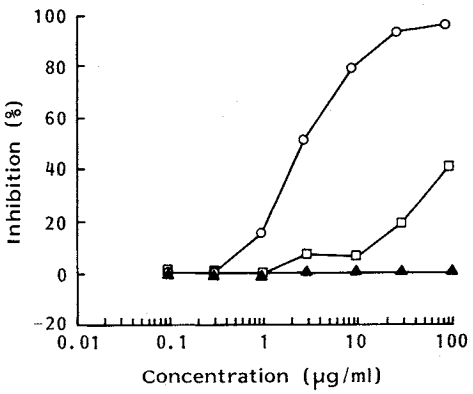
Fig. 2. Effect of various cytochalasins isolated from the fungal culture *Hypoxylon fragiforme* on HIV-1 protease activity.

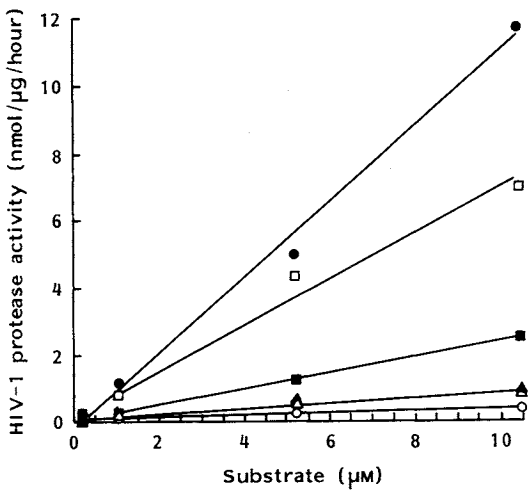
○ L-696,474, □ L-696,475, ▲ L-697,318.



HIV-1 protease assays were performed in the presence of L-696,474, L-696,475 or L-697,318 at the indicated concentrations. After 60 minutes at 37°C the assays were stopped and processed as described in the Methods section.

Fig. 3. Effect of L-696,474 on HIV-1 protease activity at various substrate concentrations.

○ No enzyme, ● 0 μM L-696,474, □ 2.1 μM L-696,474, ■ 6.3 μM L-696,474, ▲ 21 μM L-696,474, △ 63 μM L-696,474.



HIV-1 protease assays were performed at the indicated substrate and L-696,474 concentrations. The HIV-1 protease concentration was 2 nM. After 60 minutes at 37°C the assays were stopped and processed as described in the Methods section. The data in Fig. 3 were analyzed using a computer program (Enzfitter, R. J. Leatherbarrow, Biosoft, Cambridge, UK).

3 μM. The K_i of L-696,474 was determined by varying the substrate and L-696,474 concentrations (Fig. 3). The mode of inhibition was competitive with respect to substrate with an apparent K_i of 1 μM. Two other novel cytochalasins (L-697,318 and L-696,475, Fig. 1) isolated from the same culture were inactive against the HIV-1 protease.

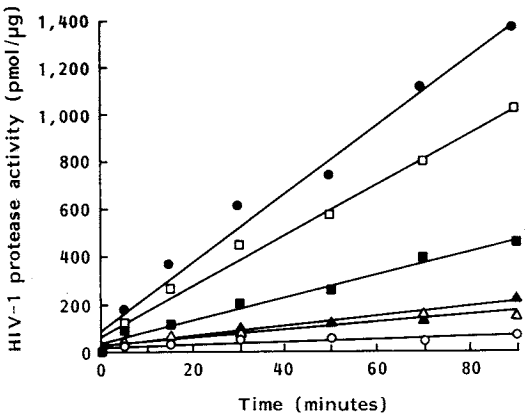
To determine whether L-696,474 was a slow-binding inhibitor of the HIV-1 protease the experiment presented in Fig. 4 was perfomed. L-696,474 was incubated with the HIV-1 protease in the presence of substrate for varying periods of time. Product formation (in the presence and absence of L-696,474) was linear over a 90-minute period. The results indicate that similar levels of inhibition of the HIV-1 protease were obtained irrespective of the time of incubation of the protease with L-696,474 suggesting that L-696,474 binds rapidly to the protease.

When enzyme activity was measured as a function of the HIV-1 protease concentration in the presence of various concentrations of L-696,474 the data presented in Fig. 5 were obtained. As expected, increasing concentrations of HIV-1 protease (in the absence of inhibitor) produced a linear increase in the amount of product formed. Increasing amounts of L-696,474 decreased product formation. The HIV-1 protease activity in the presence of L-696,474 was proportional to the enzyme concentration, but the slopes of the lines (in the presence of L-696,474) were less than in the absence of inhibitor. The percent inhibition (due to L-696,474) was the same at any concentration of enzyme and depended entirely upon the concentration of the inhibitor since the substrate concentration was constant. This, as well as the fact that the curves all intersect at the origin also supports the premise that L-696,474 is a competitive inhibitor[1,2].

The HIV-1 protease is a 99-amino acid aspartyl acid protease[1,2]. To determine whether L-696,474 was specific for the HIV-1 protease L-696,474 was tested against pepsin, stromelysin, papain and human

Fig. 4. Effect of L-696,474 on HIV-1 protease activity for various periods of time.

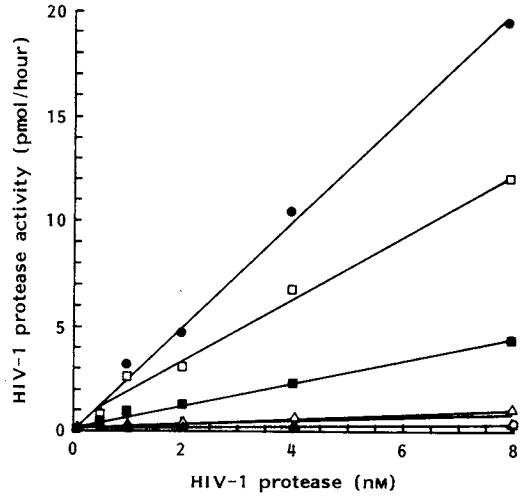
○ No enzyme, ● 0 μM L-696,474, □ 2.1 μM L-696,474, ■ 6.3 μM L-696,474, ▲ 21 μM L-696,474, △ 63 μM L-696,474.



HIV-1 protease assays were performed for the indicated time points and at the indicated L-696,474 concentrations. The HIV-1 protease and ^{3}H-substrate concentrations were 2 nM and 1.05 μM, respectively. The assays were stopped and processed as described in the Methods section.

Fig. 5. Effect of L-696,474 on HIV-1 protease activity at various protease concentrations.

○ Blank, ● 0 μM L-696,474, □ 2.1 μM L-696,474, ■ 6.3 μM L-696,474, ▲ 21 μM L-696,474, △ 63 μM L-696,474.



HIV-1 protease assays were performed at the indicated HIV-1 protease and L-696,474 concentrations. The ^{3}H-substrate concentration was 1.05 μM. After 60 minutes at 37°C the assays were stopped and processed as described in the Methods section.

leucocyte elastase and was found to be inactive against stromelysin, papain and human leucocyte elastase (results not shown). L-696,474 inhibited pepsin activity with an IC_{50} of 52 μM. These data suggest that while L-696,474 could inhibit a related aspartyl acid protease it was a more selective inhibitor of the HIV-1 protease.

To establish whether other known cytochalasins could modulate HIV-1 protease activity, cytochalasins B, C, D, E, F, H and J were tested and found to be inactive (results not shown). Cytochalasin A inhibited HIV-1 protease activity with an IC_{50} of 3 μM and the inhibition was competitive with respect to substrate (P. DARKE, personal communication). L-696,474 is similar to cytochalasin H (Fig. 1), differing by one less hydroxyl group at position C-18, but cytochalasin H had no effect on HIV-1 protease activity. A further indication of the tight structure-activity relationship that exists in this series of compounds is evident from the lack of activity of the closely related isomeric (L-697,318) and the deshydroxy (L-696,475) forms of L-696,474. Cytochalasin A, which contains a 14-membered lactone ring while L-696,474 contains a 11-membered carbocyclic ring, was as effective in inhibiting the HIV-1 protease as L-696,474. By inspection, the two compounds are quite dissimilar and activity of either molecule may depend on the relative conformation of these inhibitors at the active site of the protease.

The active HIV-1 protease is composed of two monomers, each contributing one of the two conserved aspartates to the active site[13,14]. It is likely that L-696,474 and cytochalasin A inhibit the HIV-1 protease by binding to the dimeric form of the enzyme. The alternative possibility that either compound binds to the monomeric form of the enzyme to prevent dimer formation can be discounted since there is no evidence to suggest that, under the conditions of the assay, there is an appreciable amount of the monomeric form

of the enzyme. Recently, an active single monomeric form of the HIV-1 protease was developed[15]. L-696,474 was tested against this form of the HIV-1 protease and found to inhibit the monomeric form of the protease as well as it inhibited the dimeric form of the protease (P. DARKE, personal communication). The identification of a novel, nonpeptide, natural product inhibitor of the HIV-1 protease is encouraging in that this, and other, unique and structurally diverse molecules would be useful chemical entities on which to base a medicinal chemistry effort to develop new compounds that may be more efficacious in the treatment and management of AIDS.

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