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In vitro inhibition of HIV-1 proteinase by cerulenin

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Retroviruses encode proteinases necessary for the proteolytic processing of the viral *gag* and *gag-pol* precursor proteins. These enzymes have been shown to be structurally and functionally related to aspartyl proteinases such as pepsin and renin. Cerulenin is a naturally occurring antibiotic, commonly used as an inhibitor of fatty acid synthesis. Cerulenin has been observed to inhibit production of Rous sarcoma virus and murine leukaemia virus by infected cells, possibly by interfering with proteolytic processing of viral precursor proteins. We show here that cerulenin inhibits the action of the HIV-1 proteinase in vitro, using 3 substrates: a synthetic heptapeptide (SQNYPIV) which corresponds to the sequence at the HIV-1 *gag* p17/p24 junction, a bacterially expressed *gag* precursor, and purified 66 kDa reverse transcriptase. Inhibition of cleavage by HIV-1 proteinase required preincubation with cerulenin. Cerulenin also inactivates endothiapepsin, a well-characterised fungal aspartyl proteinase, suggesting that the action of cerulenin is a function of the common active site structure of the retroviral and aspartic proteinases. Molecular modelling suggests that cerulenin possesses several of the necessary structural features of an inhibitor of aspartyl proteinases and retroviral proteinases. Although cerulenin itself is cytotoxic and inappropriate for clinical use, it may provide leads for the rational design of inhibitors of the HIV proteinase which could have application in the chemotherapy of AIDS.

Retroviral proteinase inhibitor; Human immunodeficiency virus; Catalytic mechanism

1. INTRODUCTION

Retroviruses translate their proteins as several large precursor proteins, which are cleaved proteolytically, to yield the individual functional proteins [1]. Processing of the *gag* and *gag-pol* polyproteins is due to the action of the virally encoded proteinase [2,3]. In HIV-1, the proteinase itself is encoded as part of a large precursor, $Pr160^{gag-pol}$, produced by a ribosomal frame shift from the *gag* to *pol* reading frames, which avoids the stop codon at the 3′-end of the *gag* reading frame [4].

Analysis of the amino acid sequences for a number of retroviral proteinases, suggested that the retroviral proteinase sequence corresponds to a single domain of an aspartyl proteinase, which would be active as a dimer, and have a very similar active site structure to the pepsin-like aspartic proteinases [5]. This prediction has recently been confirmed by X-ray crystallographic structure determinations for the proteinases from Rous sarcoma virus [6] and HIV-1 [7].

Cerulenin ((2S,3R)2,3-epoxy-4-oxo-7,10-dodecadienoylamide), an inhibitor of fatty acid and sterol synthesis [8], inhibits the fatty acylation of the glycoproteins of vesicular stomatitis virus [9] and significantly affects the production of infectious Rous sarcoma virus [10] and murine leukaemia virus (MuLV) by cells infected in culture [11,12], with an apparent decrease in specific proteolytic cleavage of the $Pr65^{gag}$ precursor protein, possibly due to inhibition of the viral proteinase. While the present work was in progress, Pal et al. reported a similar effect on precursor processing in HIV-1 [13]. Cerulenin has also been found to inhibit processing of antigenic proteins by antigen-presenting cells [14].

Here we demonstrate that cerulenin inhibits in vitro cleavage by recombinant HIV-1 proteinase [15] of HIV-1 *gag* and 66 kDa reverse transcriptase precursor proteins, and of a synthetic peptide containing the sequence at the *gag* p17–p24 junction. A well-characterised pepsin-like aspartic proteinase is also shown to be inactivated by cerulenin and the rate of inactivation is slowed by the presence of an active-site directed competitive inhibitor.

Using the structure of the active site of a retroviral proteinase, we describe the structural features of cerulenin that are responsible for its effect on retroviral and aspartic proteinases, and the implications for the design of novel proteinase inhibitors with clinical potential in the treatment of AIDS.

2. MATERIALS AND METHODS

Cerulenin was purchased from Sigma Chemical Co. and CalBiochem. The synthetic peptide SQNYPIV (Ser-Gln-Asn-Tyr-

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Pro-Ile-Val) was a gift from Dr R. Mertz, Munich. HIV-1 proteinase was expressed in bacteria harbouring a plasmid constructed from the proteinase sequence from HIV-1 (strain BH10) and an MS2 expression vector, and partially purified as described [15,16]. The *gag* substrate was produced as an MS2-*gag* fusion protein as described [15]. Reverse transcriptase, consisting mainly of the 66 kDa form, was isolated and purified as described [17] from a bacterial lysate generously supplied by Dr Gary Tarpley, MI, USA. The *pol* gene was expressed by a p*tac* vector essentially as described [18]. Endothiapepsin was purified as described [19]. The endothiapepsin substrate RALPH (Pro-Pro-Thr-Ile-Phe-Phe(NO_2)-Arg-Leu) was synthesised as described [20]. The aspartic proteinase competitive inhibitor L-363,980 was a gift from Drs Boger and Jacobs of Merck, Sharp and Dohme Research Labs, USA. The inhibitor has an apparent K_i for endothiapepsin and RALPH, of 15 nM.

Peptide cleavage assays were performed in a total volume of 20 μl, containing 2 μl of the heptapeptide SQNYPIV (20 mM), 5 μl of partially purified proteinase (Ultrogel peak fraction, see [16]) in a standard MGTE Buffer (15 mM Mes, pH 6.0, 15% (w/v) glycerol, 0.08% (v/v) Triton X-100, 4 mM EDTA, pH 7.0). Incubation was for 2 h at 37°C. Inhibition by cerulenin was performed by preincubation of the proteinase in the presence of cerulenin in the conditions described above, but from which the peptide substrate was omitted. Cerulenin was dissolved in dimethyl sulphoxide (DMSO), and used at the concentrations indicated in the results. The final concentration of DMSO was 2.5% in the assay. Preincubation was for 30 min at 37°C. The cleavage reaction was started by addition of peptide substrate. Peptide cleavage was analysed by HPLC as described [16] and the integrated areas of the peaks corresponding to the cleavage products and residual substrate were used to calculate the percentage of cleavage shown in the results.

Cleavage of HIV-1 *gag* was performed using bacterial lysate from bacteria expressing the HIV-1 *gag* sequence as part of an MS2-*gag* fusion protein [15]. The inhibition assay was performed by incubation in a total volume of 40 μl of 15 mM Mes, pH 6.0, buffer, 5 μl proteinase and cerulenin (dissolved in DMSO) at the concentrations shown in the results. After preincubation for 30 min at 37°C, 1 μl of bacterial lysate containing *gag* was added, the volume adjusted to 50 μl, and incubated at 37°C for 2 h. Cleavage of *gag* in the bacterial lysate was monitored by immunoblot using a monoclonal antibody recognising the MS2 portion of the MS2-*gag* fusion protein used for *gag* expression [15]. Cleavage assays of purified reverse transcriptase in the presence of cerulenin were performed by an essentially identical procedure but using 3 μl of purified reverse transcriptase as the substrate. Cleavage of p66 to p51 is specifically achieved by the purified HIV-1 proteinase in vitro, which generates approximately equimolar amounts of p66 and p51, and no further cleavage products even during overnight incubation (Moelling et al., unpublished observations). Cleavage of p66 to p51 was monitored by immunoblot using a monoclonal antibody against HIV-1 reverse transcriptase [15].

Inhibition of peptide cleavage by endothiapepsin was performed by preincubating 100 g of endothiapepsin with 5 mg of cerulenin in 1.0 ml of 0.1 M sodium acetate, pH 5.0, containing 30% DMSO. Endothiapepsin activity was assayed by following the cleavage of the chromogenic substrate Pro-Pro-Thr-Ile-Phe-Phe(NO_2)-Arg-Leu spectrophotometrically at 300 nm at pH 5.0 as described [20]. Control samples were incubated simultaneously, including a sample containing the competitive inhibitor L-363,980 at a final concentration of 440 nM.

3. RESULTS

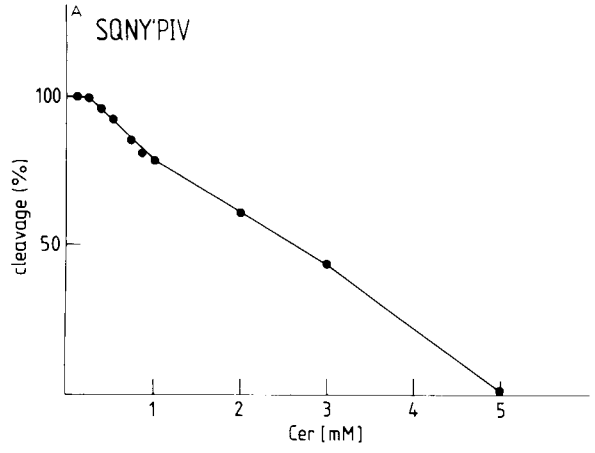
The effect of cerulenin was measured in 3 systems: cleavage of a bacterially expressed *gag* precursor protein, cleavage of the 66 kDa form of HIV-1 reverse transcriptase, and cleavage of a synthetic peptide, SQNYPIV, which is the sequence observed at the junction of p17 and p24 in the HIV-1 *gag* polyprotein. The peptide is cleaved at the Tyr-Pro (YP) bond.

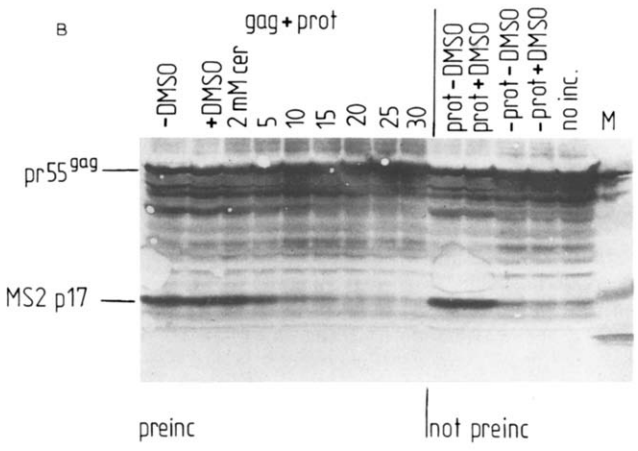
The ability of HIV-1 proteinase to cleave the synthetic substrate SQNYPIV, was progressively reduced by preincubation with increasing concentrations of cerulenin (fig.1A), giving an approximate IC_{50} of 2.5 mM. The activity of the enzyme alone or with DMSO at the concentration used (2.5%), was not reduced over this incubation period (controls not shown).

The effect of cerulenin on HIV-1 proteinase cleavage of the HIV-1 *gag* polyprotein was assayed using an *E. coli* expressed MS2-*gag* fusion protein as substrate, with cleavage monitored by immunoblot using a monoclonal antibody against the MS2 moiety. Protein detected by this method corresponds to the amino-terminal p17 (MA) domain of the *gag* polyprotein. Generation of p17 is significantly decreased by preincubation of the proteinase with cerulenin at 5–10 mM, and completely abolished by 20 mM. Production of several bands which are intermediate in molecular weight between the MS2-$Pr55^{gag}$ and the MS2-$p17^{gag}$ is also abolished.

As a third substrate, the 66 kDa form of HIV-1 reverse transcriptase was used. When purified from a bacterially expressed ptac-*pol* construct by the method of Hansen et al. [17], this predominantly consists of p66 [18] and smaller intermediates, but no p51. Incubation with the HIV-1 proteinase in vitro produces one predominant cleavage product which comigrates with RT-p51 (fig.1C). This suggests that in HIV-1, the proteinase may be responsible for generating the reverse transcriptase heterodimer as described previously in other retroviruses [21]. This cleavage can also be performed by other proteinases [22] suggesting that the p51/RNase H junction has a structure particularly susceptible to proteolysis. Cleavage of the p66-RT to a molecule comigrating with p51, was also significantly inhibited by cerulenin at concentrations around 5 mM (fig.1C) and effectively abolished by 20 mM cerulenin. With the *gag* and *pol* substrates, no cleavage was observed in the absence of proteinase, and cleavage was not inhibited by preincubation of the proteinase alone or with DMSO. With all 3 substrates, inhibition of proteinase activity required preincubation with cerulenin.

To determine whether inhibition by cerulenin is a specific property of retroviral aspartic proteinases or a property of aspartic proteinases in general, we have examined the effect of cerulenin on a well-characterised aspartic proteinase of known structure, endothiapepsin. Incubation of endothiapepsin with cerulenin results in progressive loss of its proteolytic activity against a peptide substrate, with 50% inactivation after 3 h incubation with 5 mg/ml (20 mM) cerulenin (fig.2). When the active-site-directed competitive inhibitor L-363,980 was included in the preincubation, the rate of inactivation was 1.5 times slower.





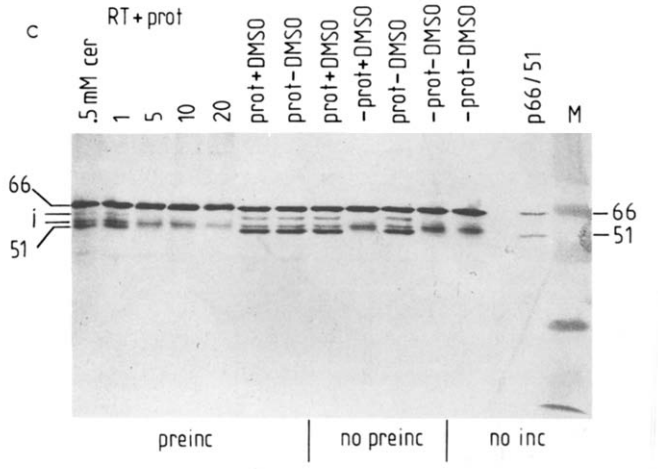


Fig.1. (A) Cleavage of the synthetic heptapeptide (SQNYPIV) corresponding to the p17/p24 junction of HIV-1 *gag*, by the partially purified HIV-1 proteinase, in the presence of the indicated concentrations of cerulenin. Cleavage was monitored by HPLC and the areas of the peaks corresponding to substrate and products were integrated to calculate the percentage cleavage. (B) Cleavage of bacterially expressed MS2-*gag* fusion protein (indicated as $pr55^{gag}$), by the partially purified HIV-1 proteinase, in the presence of the indicated concentrations of cerulenin. Cleavage was analysed by immunoblotting using a monoclonal antibody against the MS2 moiety of the MS2-p17 fusion protein. Control reactions were performed by using the proteinase with and without DMSO (+/− DMSO), with and without preincubation, and by omitting the proteinase itself. M indicates marker proteins, from top to bottom: 92, 68, 43, 34 kDa. (C) Cleavage of the purified p66 reverse transcriptase to p51 by the partially purified HIV-1 proteinase, in the presence of the indicated concentrations of cerulenin. Reaction products were analysed by immunoblot using a monoclonal antibody against the p66/p51 RT. Control reactions are as indicated and explained in fig.1B. p66/51 indicates a purified RT consisting of the naturally occurring heterodimer. M indicates marker proteins (see fig.1B); i indicates intermediates between p66 and p51.

4. STRUCTURAL BASIS OF PROTEINASE INHIBITION BY CERULENIN

Inactivation of retroviral and pepsin-like aspartic proteinases by cerulenin, suggests that cerulenin is acting via the same mechanism in both. There is very little sequence homology between these two types of enzymes, except in the regions which form the active sites, which are virtually identical [6]. It seems reasonable to argue that cerulenin's inhibitory action occurs at the active site of both types of enzymes, rather than by some allosteric mechanism. This is supported by the observation that the rate of inactivation by cerulenin can be slowed by the presence of an active-site directed competitive inhibitor.

The structure of the cerulenin molecule (fig.3A), shows several features which explain its activity as an aspartyl proteinase inhibitor. The presence of an epoxide function in cerulenin is reminiscent of a similar function in a known aspartyl proteinase inhibitor, EPNP [23] (1,2-epoxy-3-nitrophenoxy-propane)

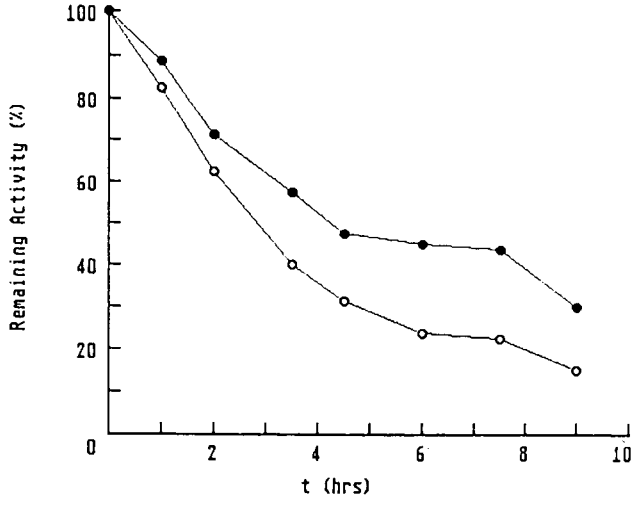


Fig.2. Percentage remaining activity of endothiapepsin for cleavage of chromogenic substrate after incubation with cerulenin at 5 mg/ml, as a function of time. Open circles are incubation with cerulenin alone; closed circles are incubation with cerulenin and the competitive inhibitor L-363,980.

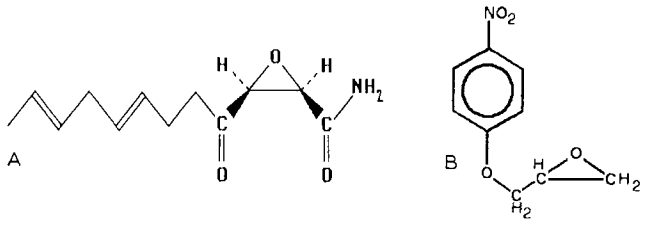


Fig.3. (A) Structure of cerulenin ((2S,3R)2,3-epoxy-4-oxo-7,10-dodecadienoylamide). (B) Structure of EPNP (1,2-epoxy-3-nitrophenoxy propane), a classic covalent inactivator of aspartic proteinases.

(fig.3B), which irreversibly inactivates aspartyl proteinases by esterification of the two active-site aspartic acid residues [24], and has been shown to inactivate recombinant HIV-1 proteinase [25]. The rate of inactivation of endothiapepsin by cerulenin is comparable to that observed for its inactivation by EPNP.

Crystal structures of complexes between aspartyl proteinases and peptide-based inhibitors [26–28] show certain hydrogen bonding contacts to be critical for the correct positioning of the inhibitor (and presumably the substrate) relative to the active-site residues (fig.4). Interestingly, the cerulenin molecule possesses several of the groups necessary for formation of these hydrogen bonds. When model-built into the active site of the crystal structure of endothiapepsin [29,30], the terminal amide group of cerulenin can form both of the hydrogen bonds which serve to position the P1 and P1′ residues of a substrate such that the scissile bond is correctly orientated relative to the catalytic aspartic acid residues. When positioned in this way, the 4-oxo group of cerulenin occupies the same position as the carbonyl group of the scissile peptide bond of a substrate, with the reactive 2,3-epoxide group close to the position of the amide nitrogen of the substrate scissile peptide bond (fig.5A,B). The result of any of the catalytic mechanisms proposed for aspartic proteinases [31–33] on cerulenin thus positioned, would be the esterification of one of the catalytic active-site aspartate residues, resulting in the inactivation of the enzyme. A covalent mechanism for proteinase inhibition by cerulenin, is entirely consistent with our observation that inhibition requires preincubation with the enzyme,

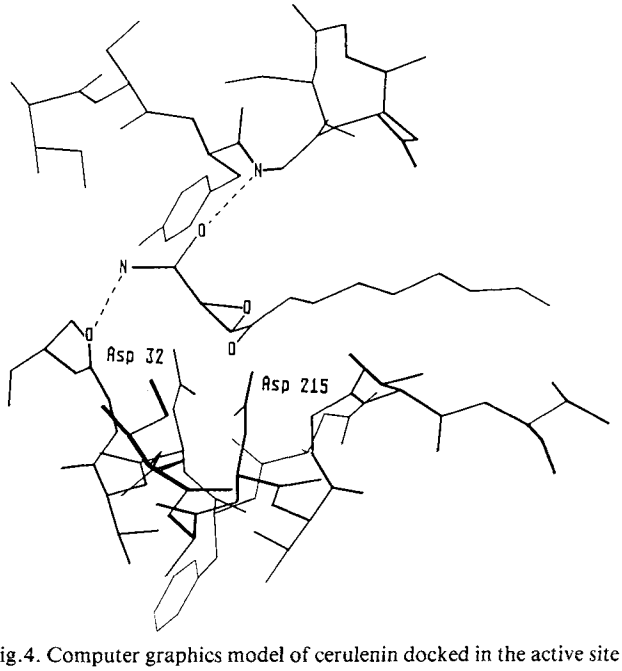


Fig.4. Computer graphics model of cerulenin docked in the active site of endothiapepsin. The dotted lines indicate possible hydrogen bonds between the enzyme and groups on the cerulenin molecule. The epoxide ring is positioned such that it could react with the carboxyl side chain of one of the catalytic aspartates, 32 and 215. The lipophilic tail, which probably helps cerulenin to cross the cell membrane, lies in the substrate-binding cleft interacting with the residues that form the hydrophobic P1 (and possibly P3) binding site.

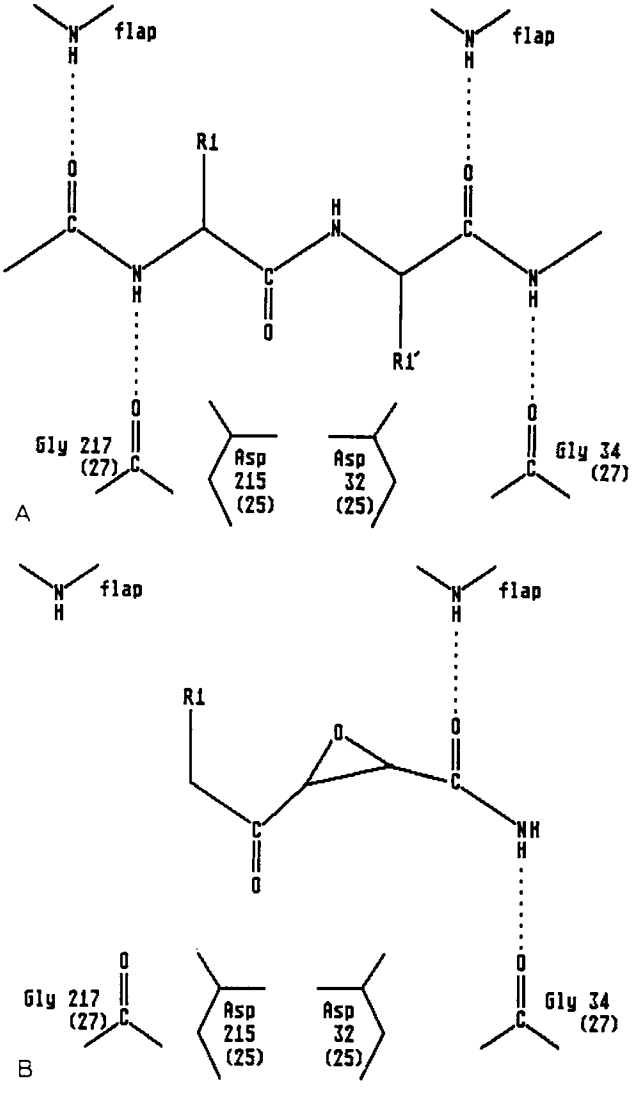


Fig.5. Comparison of the interactions of a peptide substrate (A) and cerulenin (B), with groups in the active site of an aspartic or retroviral proteinase. The numbers in brackets refer to the residues in the dimeric HIV-1 proteinase which correspond to the catalytic aspartates (32 and 215), and the conserved glycines (34 and 217) of the archetypal pepsin family.

and that the degree of inhibition depends on time of incubation as well as inhibitor concentration. Cerulenin is perhaps better described as a retroviral proteinase *'inactivator'*.

The very high degree of similarity between the active-site structure of endothiapepsin and that of the retroviral proteinases (A. Wlodawer, personal communication), suggests that cerulenin would bind in a virtually identical manner in the HIV-1 proteinase to the model presented here.

5. CONCLUSION

In light of our in vitro observations, it is probable that a major component of cerulenin's inhibition of *gag* cleavage in infected cell culture, is due to inactivation of the viral proteinase. However, at higher concentrations, cerulenin appears to inhibit processing of the *env* precursor [11,13], which is not cleaved by the viral proteinase. It is possible that at these higher levels, cerulenin is inhibiting cellular aspartic proteinases such as cathepsins D and E, which may be involved in *env* processing. The inhibition by cerulenin of antigen processing in antigen presenting cells [14], may be due to a similar effect.

Cerulenin displays a broad range of effects, inhibiting proteinase activity, glycosylation, and fatty acylation. This lack of specificity, and a degree of cytotoxicity, make cerulenin itself an inappropriate candidate for clinical use. However, cerulenin's anti-proteinase activity will allow the design of proteinase inactivators incorporating some of the structural features of cerulenin.

Additional note: After submission of this manuscript, Blumenstein et al. [34] reported inhibition of HIV-1 proteinase cleavage of small peptides by cerulenin, and several synthetic analogues. In their system, cleavage of peptide was only evaluated after incubation of enzyme, substrate and inhibitor for 16 h and longer. In this system they did not observe the need for preincubation with inhibitor, which we find with our much shorter incubations with substrate.

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