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# LEUPEPTIN INHIBITS THE C3/5 CONVERTASE CVFBb OF THE COMPLEMENT SYSTEM

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**Using a new fluorescent assay of C3/5 convertase activity, the protease inhibitor leupeptin was shown to inhibit CVFBb with a $K_i$ of 40 $\mu$M.**

Leupeptin, a bacterially-derived inhibitor of tryptic proteases [1], has been demonstrated to affect the complement system. For example, the complement enzyme $C\overline{1s}$ is one of the proteases which leupeptin inhibits [2]. In addition, leupeptin has been demonstrated to block complement-mediated lysis of paroxysmal nocturnal hemoglobinuria erythrocytes (PNH cells) [3], although the mechanism by which it does this is not known. Its inhibition of lysis of PNH cells cannot be due to its inhibition of the classical complement pathway enzyme $C\overline{1s}$ as leupeptin has been shown to inhibit lysis under conditions in which only the alternative complement pathway, not the classical pathway, was involved [3].

The alternative pathway of complement activation involves two enzymes, Factor D and C3bBb [4]. C3bBb cuts the complement protein C3 at the peptide bond following arginine in the sequence Leu-Ala-Arg [5]. As leupeptin is a tripeptide with a similar sequence

Leupeptin Ac Leu Leu Arg

Abbreviations: AMC, 7-amino methyl coumarin; CVFBb, complex of *Naja naja* cobra venom factor and activated factor B; Boc, *t*-butyloxycarbonyl.

| Peptide Substrate | Boc Leu Gly Arg AMC |
|---|---|
| C3 | ------ Gly Leu Ala Arg ------ |
| C5 | ------ Gln Leu Gly Arg ------ |

= Sequence comparison of leupeptin and C3/5 convertase substrates.

with an inhibitory aldehyde function rather than an amide bond on the carboxyl side of arginine [1], it seemed likely that leupeptin would inhibit C3bBb. We have investigated this question using CVFBb, a stable analogue of C3bBb [4], and report here that leupeptin does inhibit this enzyme.

Human Bb, stabilized by CVF prepared as described [6] was a generous gift from O. Götze. Leupeptin was purchased from both Peninsula Laboratories and United States Biochemicals. CVFBb activity was assayed by generation of free amino methyl coumarin from Boc-Leu-Gly-Arg-AMC [7]. To a cuvette containing an appropriate concentration of substrate, with or without leupeptin, in 0.01 M $NaKHPO_4$, pH 7.4/0.14 M NaCl, 13 nM CVFBb were added. Incubations were at 37°C for about 10 min; the increase in fluorescence (excitation 380 emission 460) was monitored continuously in an Aminco-Bowman SPF 125 spectrofluorimeter. Velocities were calculated directly from the slopes; lines based on the data were drawn, and slopes and intercepts calculated, using a least-squares program designed by Dr. Preston Hensley of Georgetown University to deter-

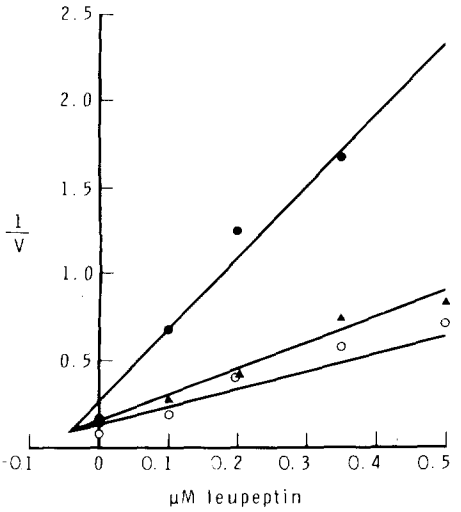


Fig. 1. Dixon plot for inhibition by leupeptin in CVFBb cleavage of three different concentrations of substrate Boc-Leu-Gly-Arg-AMC. ●———●, 24 μM; ▲———▲, 72 μM and ●———●, 120 μM

mine the intercept of a set of lines.

Data for five different concentrations of inhibitor acting on three different concentrations of substrate are plotted by the method of Dixon [8] in Fig. 1. The location of the intersection of the three lines indicates competitive or mixed-type inhibition with a $K_i$ of 40 μM.

These data confirm the prediction, based upon both the observed inhibition of lysis of PNH cells by leupeptin, and its sequence similarity to C3, that leupeptin would inhibit the alternative complement pathway convertase CVFBb. However, at 40 μM, the $K_i$ is lower than might have been expected, indicating that leupeptin is an especially good inhibitor of the enzyme. This $K_i$ is value of the same order of magnitude as that for the microbial product called 'complestatin', which inhibits via a different mechanism [9]. In contrast, the $K_i$ of value leupeptin for C1s is about 400 μM [10,2].

This low $K_i$ is surprising, as Takada et al. [11] reported that leupeptin at a concentration of 1 mM was unable to inhibit the alternative pathway when it was activated by rabbit erythrocytes in diluted serum [11]. A high concentration of leupeptin, 30 mM, was used to inhibit the alternative pathway-mediated lysis of PNH cells in diluted serum [4]. The apparent discrepancy between these results and the low $K_i$ for pure CVFBb acting on a pure substrate dissolved in buffer can be explained by considering the involvement of other proteins in serum. It is likely that 1 mM leupeptin, used by Takada et al. [11] may have bound to the many enzymes in serum which it is known to inhibit, making it less available to inhibit the alternative pathway convertase; however Takada et al. were able to inhibit the classical pathway under similar conditions [11] in spite of the fact that the $K_i$ for $\overline{C1s}$ is much higher. The unique problem with the alternative pathway probably results from the presence of C3 in serum. C3, the substrate for $\overline{C3bBb}$, participates in a positive feedback loop. That is, when it is cleaved the resultant C3b can lead to the formation of more C3bBb which can in turn cleave more C3. It may be extremely difficult to inhibit an enzyme under such conditions as the enzyme 'fights back', that is at every moment in which it is free from the inhibitor and able to cut its substrate, it can generate yet another molecule of enzyme, which now must be inhibited as well, or it will in turn generate still more enzyme.

This comparison of the relative effectiveness of an inhibitor acting on a pure enzyme compared to an enzyme in serum proves instructive as we try to extrapolate results back and forth between the two systems. To design an effective inhibitor of the C3-cleaving enzyme these data point to the need for either much tighter noncovalent binding or rapid covalent inactivation due to the involvement of this enzyme in the alternative pathway positive feedback loop.

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