E64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane] analogues as inhibitors of cysteine proteinases: investigation of S_2 subsite interactions

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A number of epoxysuccinyl amino acid benzyl esters (HO-Eps-AA-OBzl) and benzyl amides (HO-Eps-AA-NHBzl) (where AA represents amino acid) were synthesized as analogues of E64, a naturally occurring inhibitor of cysteine proteinases. These inhibitors were designed to evaluate if selectivity for cathepsin B could be achieved by varying the amino acid on the basis of known substrate specificity. Contrary to the situation with substrates, it was found that variation of the amino acid in the E64 analogues does not lead to major changes in the kinetic parameter $k_{inac.}/K_i$ and that the specificity of these analogues does not parallel that observed for substrates. This is particularly true in the case of the benzyl ester derivatives where the deviation from substrate-like behaviour is more important than with the benzyl amide derivatives. The results suggest that the amide proton of the benzyl amide group in HO-Eps-AA-NHBzl interacts in the S_2 subsite in both cathepsin B and papain and contributes to increase the potency of these inhibitors. The kinetic data also suggest that differences in the orientation of the Cα–Cβ bond of the side chain in the S_2 subsite of the enzyme might explain the differences between substrate and E64 analogue specificities. This hypothesis is supported by the fact that the order of inactivation rates with chloromethane inhibitors (which are believed to be good models of enzyme–substrate interactions) is indeed very similar to that observed with the corresponding amidomethylcoumarin substrates. In conclusion, the information available from S_2–P_2 interactions with substrates cannot be used to enhance the selectivity of the E64 analogues in a rational manner.

INTRODUCTION

Cathepsins B, H, L and S are members of the papain family of cysteine proteinases which can be found in the lysosomes of mammals and are involved in protein turnover within cells [1–3]. The cathepsins are also believed to contribute to several pathological effects of various diseases. Cathepsin B, for example, has been implicated in disease states such as muscular dystrophy [4–6], bone resorption [7–9], pulmonary emphysema [10,11] and tumour invasion [12–16]. Specific cysteine proteinase inhibitors could serve not only as powerful tools in the clarification of the physiological roles of the different lysosomal cathepsins, but also as therapeutic drugs for diseases caused by abnormal elevations of proteolytic activity. Many inhibitors specific to cysteine proteinases are known [17,18]. The challenge, however, is to design an inhibitor specific to a particular member of the cysteine proteinase class.

Information obtained from substrate studies indicates that the interaction in the S_2 subsite is the predominant factor in defining substrate specificity with cysteine proteinases of the papain family [19]. In particular, the S_2 subsite specificity of cathepsin B is markedly different from that of other cysteine proteinases [20]. Cathepsin B is able to hydrolyse substrates with an arginine residue in the P_2 position at a significant rate, whereas these substrates are only very slowly hydrolysed by papain and other cysteine proteinases [20]. On the basis of kinetic studies [21], computer modelling [22,23] and site-directed mutagenesis experiments [23,24], this specificity has been attributed to the existence of an electrostatic interaction between the negatively charged Glu-245 located in the back of the S_2 subsite of cathepsin B and the positive side chain of the arginine at position P_2 of the substrate. The recently determined X-ray crystallographic structure of human liver cathepsin B [25] confirms that Glu-245 is properly located in the S_2 subsite to participate in such an interaction.

As the Glu-245 residue is absent from other cysteine proteinases, a Glu-245–arginine interaction could possibly be exploited to introduce selectivity for cathepsin B in an inhibitor. Amongst the known cysteine proteinase inhibitors, the epoxysuccinyl peptide, E64, has been shown to interact in the S_2 subsite of papain and could serve as a template for selective cathepsin B inhibitor design. A comparison of the papain–E64 crystal structure with that of papain alkylated by Cbz-Phe-Ala-CH_2Cl [22] (papain-CMK, which is believed to represent a good model of an enzyme–substrate intermediate) clearly shows that the two inhibitors bind in the same subsites of papain and interact with the enzyme in a very similar manner. This is schematically presented in Figure 1. It can be seen that both the P_2 side chain of phenylalanine in the CMK inhibitor and the leucine side chain of E64 are located in the S_2 subsite of papain. Another possibly important feature of this region of the enzyme–inhibitor complexes is the existence of two hydrogen bonds between the inhibitors and Gly-66 (interactions 1 and 2 in Figure 1b). These two hydrogen bonds have been shown to be of major importance for substrate hydrolysis by papain [27].

In order to alter the selectivity of E64 in a rational manner, more information is needed regarding the significance of the S_2 subsite interactions. E64 analogues with modified peptide portions have been reported previously. However, the interpretation of the data is questionable as the assays were carried out in terms of IC_{50} values [28–32] which are not appropriate for the characterization of irreversible inhibitors. In the present study,

Abbreviations used: E64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; Eps, epoxysuccinyl; Bzl, benzyl; Cbz, benzyloxycarbonyl; Mec, methylcoumarin.

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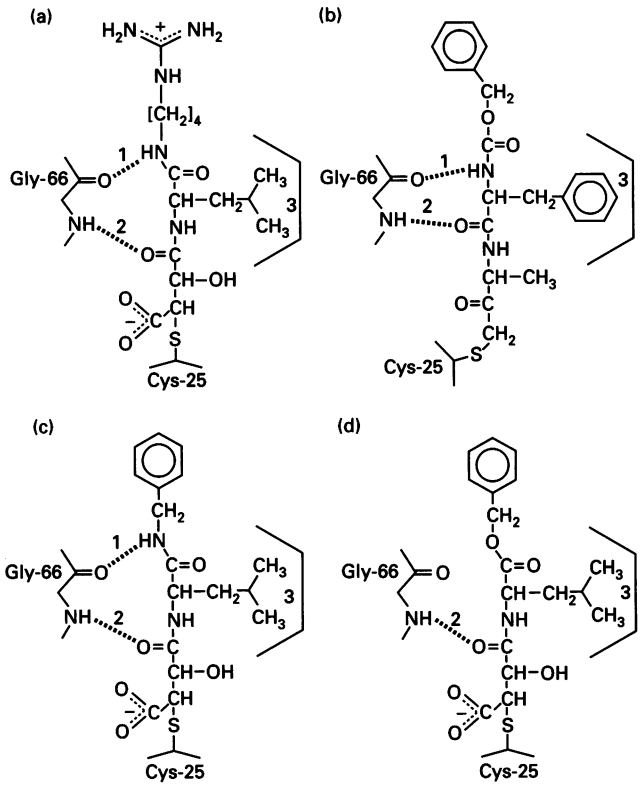
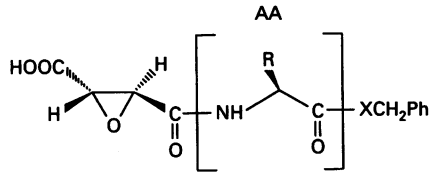


Figure 1 Schematic representation of the interaction of various inhibitors in the S_2 subsite of papain

(**a**) E64 (from the crystal structure [26]); (**b**) Cbz-Phe-Ala-CH_2Cl (from the crystal structure [22]); (**c**) proposed binding mode of HO-Eps-AA-NHBzl; (**d**) proposed binding mode of HO-Eps-AA-OBzl. Interactions in the S_2 subsite are represented by numbers (1 and 2, hydrogen bonds between main-chain amide and carbonyl groups of the inhibitor and residue Gly-66 of papain; 3, interaction from the side chain of the inhibitor).



AA = Phe, Leu, Arg, D-Phe; (1) X = O; (2) X = NH

the significance of the interaction involving the amino acid side chain has been investigated through the use of both benzyl ester (1) and benzyl amide (2) derivatives of E64 where the amino acid (AA) has been varied. In the case of the benzyl amide derivatives (HO-Eps-AA-NHBzl), it is assumed to a first approximation that the two hydrogen bonds with Gly-66 could be maintained in the complex with papain or cathepsin B (Figure 1c). However, with benzyl esters (HO-Eps-AA-OBzl), one hydrogen bond is necessarily absent (Figure 1d), and this can be used to evaluate the importance of this interaction for the E64 analogues. The substrate-like peptidyl chloromethane derivatives were also characterized in order to evaluate S_2–P_2 interactions with this class of inhibitor.

EXPERIMENTAL

Materials

The substrate Cbz-Phe-Arg-NH-Mec and the inhibitor E64 were purchased from IAF Biochem International, Laval, Quebec, Canada. Peptidyl chloromethane inhibitors (Cbz-Phe-Ala-CH_2Cl, Cbz-Leu-Ala-CH_2Cl, Cbz-Arg-Ala-CH_2Cl) as well as the substrates Cbz-Arg-Arg-NH-Mec, Cbz-Leu-Arg-NH-Mec and Cbz-D-Phe-Arg-NH-Mec were purchased from Enzyme Systems Products, Dublin, CA, U.S.A. The other epoxide inhibitors were synthesized by methods already described [33]. Papain (crystallized suspension in sodium acetate) was obtained from Sigma Chemical Co., further purified by the method of Sluyterman and Wijdenes [34] and stored as the mercurated form. The enzyme was activated on the day of the experiment with 2-mercaptoethanol followed by gel filtration on Sephadex G-15. The concentration of active papain was determined as previously described [35,36]. Cathepsin B, isolated and purified from human liver, was a generous gift from Dr. Sadiq Hasnain (Institute of Biological Sciences, National Research Council of Canada). This enzyme was activated by incubation for 30 min in 20 mM dithiothreitol and the concentration determined by active-site titration with commercial E64.

Kinetic studies

Typical assay buffer contained 50 mM phosphate buffer, 200 mM NaCl and 5 mM EDTA. Cathepsin B assays also contained 0.5 mM dithiothreitol. All kinetic experiments were performed at 25 °C. Acetonitrile (10%, v/v, h.p.l.c. grade) was used in all assays to increase substrate and inhibitor solubility. The pH was adjusted to 6.5 for papain reactions and 6.0 for cathepsin B. Inactivation of cysteine proteases was monitored by a continuous assay as described by Tian and Tsou [37] using the substrate Cbz-Phe-Arg-NH-Mec. Hydrolysis was followed until the enzyme was completely inactivated (typically 5–30 min), and the first-order rate constant for inactivation ($k_{obs.}$) was obtained by non-linear regression of the fluorescence versus time data. As saturating conditions of inhibitor could not be reached, only the value of $k_{inac.}/K_i$ could be obtained by linear regression of $1/k_{obs.}$ versus 1/[I] plots.

RESULTS AND DISCUSSION

All of the epoxysuccinyl amino acid benzyl amides and esters were synthesized by coupling the amino acid benzyl amide or ester to the monoethyl ester of the epoxysuccinate as synthesized by the method of Mori and Iwasawa [38]. The benzyl amides of the amino acids were synthesized by coupling the *t*-butoxycarbonyl-protected amino acid with benzylamine followed by removal of the *t*-butoxycarbonyl protecting group with trifluoroacetic acid. If the benzyl ester of the amino acid was not commercially available, it was made by allowing the appropriately protected caesium salt of the required amino acid to react with benzyl bromide [39]. The ethyl ester (EtO-Eps-AA-XBzl, X = O, NH) was then cleaved using pig liver esterase [40] to give the corresponding acid derivatives (HO-Eps-AA-XBzl, X = O, NH) of the epoxysuccinyl amino acid benzyl amides and esters.

There is very little difference between the inhibitory activity of E64 and HO-Eps-Leu-NHBzl towards cathepsin B and papain, confirming that the agmatine portion of E64 can be replaced by the benzyl amide function without a major effect on the rate of inactivation. A clear result that emerges from the data presented in Table 1 is that, unlike the situation with substrates (Table 2), variation of the amino acid in the E64 analogue does not lead to major changes in the kinetic parameter $k_{inac.}/K_i$, and, in addition, the specificity observed for the substrates is not reflected in the E64 analogues. With papain, the preference for Phe over Arg in

Table 1 Second-order rate constants for inactivation of cathepsin B and papain by the E64 analogues

The corresponding $k_{inac.}/K_i$ values for E64 are $(27 \pm 1) \times 10^3$ $M^{-1} \cdot s^{-1}$ and $(374.7 \pm 0.2) \times 10^3$ $M^{-1} \cdot s^{-1}$ with cathepsin B and papain respectively. All amino acids were L unless otherwise specified. The values in parentheses represent the ratio of $k_{inac.}/K_i$ for Phe over AA.

	$10^{-3} \times k_{inac.}/K_i$ ($M^{-1} \cdot s^{-1}$)	
AA	Cathepsin B	Papain
HO-Eps-AA-OBzl		
Phe	0.91 ± 0.08 (1)	13 ± 1 (1)
Leu	24.7 ± 0.8 (0.04)	52.5 ± 0.8 (0.25)
Arg	8.3 ± 0.5 (0.11)	3.0 ± 0.3 (4.3)
D-Phe	0.54 ± 0.02 (1.7)	8 ± 1 (1.6)
HO-Eps-AA-NHBzl		
Phe	9 ± 1 (1)	43 ± 2 (1)
Leu	37.6 ± 0.8 (0.24)	90 ± 2 (0.48)
Arg	4.5 ± 0.2 (2)	0.95 ± 0.07 (45)
D-Phe	0.689 ± 0.002 (13)	3.7 ± 0.2 (12)

Table 2 Comparison of kinetic parameters for substrate hydrolysis and enzyme inactivation by 'substrate-like' chloromethane inhibitors

All amino acids were L unless otherwise specified. The ratio of cathepsin B/papain has been normalized to 1 for AA = Phe. The values in parentheses represent the ratio of $k_{inac.}/K_i$ or $k_{cat.}/K_m$ for Phe over AA.

(a) Substrate Cbz-AA-Arg-NH-Mec

	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)		Normalized ratio
AA	Cathepsin B	Papain	cathepsin B/papain
Phe	47.1 ± 5.2* (1)	464 ± 44* (1)	1
Leu	12.8 ± 0.5 (3.7)	86 ± 12 (5.3)	1.5
Arg	13.1 ± 7.6* (3.6)	0.51 ± 0.02* (902)	250
D-Phe	0.95 ± 0.21 (50)	3.5 ± 0.2 (135)	2.7

(b) Inhibitor Cbz-AA-Ala-CH_2Cl

	$10^{-3} \times k_{inac.}/K_i$ ($M^{-1} \cdot s^{-1}$)		Normalized ratio
AA	Cathepsin B	Papain	cathepsin B/papain
Phe	345 ± 1 (1)	527 ± 12 (1)	1
Leu	202 ± 22 (1.7)	51.2 ± 0.1 (10)	6.0
Arg	163 ± 30 (2.1)	1.1 ± 0.1 (480)	230

* Data taken from [17].

P_2 is very high ($k_{cat.}/K_m$ is 910-fold higher with Cbz-Phe-Arg-NH-Mec than with Cbz-Arg-Arg-NH-Mec) whereas both substrates are hydrolysed at similar rates with cathepsin B (ratio of $k_{cat.}/K_m$ for Phe over Arg is 3.6 for cathepsin B). As shown in Table 1, HO-Eps-Arg-OBzl is only 4.3-fold less potent towards papain than HO-Eps-Phe-OBzl. The inhibitor HO-Eps-Arg-OBzl does display selectivity for cathepsin B as expected, but the preference over papain is only 2.8-fold, whereas with the substrate Cbz-Arg-Arg-NH-Mec, $k_{cat.}/K_m$ is 26-fold higher with cathepsin B than with papain. In addition the leucine derivatives are better inhibitors for both cathepsin B and papain than the phenylalanine derivatives. From these observations, it is obvious that the specificity of the E64 analogues does not parallel that of substrates.

Comparison of the kinetic parameters obtained for the benzyl esters with those obtained with the benzyl amides allows us to evaluate the contribution of the amide proton of the benzyl amide group to the inhibitory activity of these analogues. The benzyl amide derivatives display in many cases higher values of $k_{inac.}/K_i$ than the corresponding benzyl esters. In addition, the specificity of these inhibitors is somewhat closer to that of substrates. In particular, HO-Eps-Phe-NHBzl is now a significantly better inhibitor than HO-Eps-Arg-NHBzl towards papain ($k_{inac.}/K_i$ 45-fold higher). As shown in Figure 1c, the benzyl amide analogues can form an additional hydrogen bond with Gly-66 in papain (or cathepsin B), and the existence of such an interaction could be responsible for the differences in inhibitory activity between benzyl ester and benzyl amide derivatives. Our results suggest that the hydrogen bond between the amide proton of the inhibitor and the carbonyl oxygen of Gly-66 on the enzyme contributes to the binding and specificity of this class of inhibitor.

To explain the differences in specificity observed between the E64 analogues and the substrates, a closer comparison of the binding modes of E64 [26] and Cbz-Phe-Ala-CH_2Cl [22] at the active site of papain is necessary (Figure 1). Even though both inhibitors bind in the S subsites of papain, specific differences can be observed in the S_2 subsite region. As the epoxysuccinyl moiety is linked to leucine via its amino group, the direction of the peptide portion in E64 is opposite to that found in the chloromethane inhibitors. The reverse arrangement of the peptide bond will cause a large difference in the orientation of the Cα–Cβ bond of the L-amino acids in the S_2 subsite between the two types of inhibitors. The D-isomers (HO-Eps-D-Phe-NHBzl and HO-Eps-D-Phe-OBzl) were synthesized, and, as shown in Table 1, there is very little difference between the L-Phe and D-Phe stereoisomers for the inactivation of papain by the benzyl ester analogues ($k_{inac.}/K_i = 0.91 \times 10^3$ and 0.54×10^3 $M^{-1} \cdot s^{-1}$ for the L-Phe and D-Phe stereoisomers respectively) contrary to that observed for substrates (which exhibit large differences in the values of $k_{cat.}/K_m$ for the L and D isomers). These results would seem to confirm that side-chain orientations and interactions in the S_2 subsite are different between substrates and the benzyl ester analogues of E64. With the benzyl amide derivatives HO-Eps-AA-NHBzl, the L-Phe analogue is a 12-fold more potent inhibitor of papain than the D-Phe compound. Even though this preference for L-Phe over D-Phe is still much less than the 135-fold preference observed for the corresponding amidomethylcoumarin substrates (Table 2), the increase in specificity for L-Phe over D-Phe in the benzyl amide compared with the benzyl ester analogues suggests once again that the benzyl amide analogues can form an extra hydrogen bond with Gly-66 in papain (interaction 1 in Figure 1c) and that the interactions with Gly-66 help in defining the S_2 subsite specificity of this class of inhibitors. Similar observations can be made from the data obtained with cathepsin B. The interdependence of S_2 subsite specificity and hydrogen-bonding interactions with Gly-66 has been noted previously in the hydrolysis of esters by papain and is largely due to an entropic effect [27]. Maintaining the hydrogen bonds to Gly-66 in an inhibitor allows a maximization of the binding energy that can be obtained from the interactions of the P_2 side chain by reducing the unfavourable entropic penalty. This will in turn ensure that the maximum inhibitor selectivity can be achieved on the proper choice of the P_2 side chain of an inhibitor.

The crystal structure of papain complexes with peptidyl chloromethane inhibitors is often used to provide structural information about enzyme–substrate interactions, as the binding mode of these inhibitors to papain is believed to bear relevance to that of substrates during the catalytic process [22]. Therefore

the specificity of these inhibitors should closely parallel that of substrates. This was confirmed by measuring the kinetic parameters for a series of three chloromethane inhibitors (Cbz-AA-Ala-CH_2Cl; AA = Phe, Leu, Arg) with papain and cathepsin B (Table 2). Unlike the epoxysuccinyl E64 analogues, the order of inactivation rates with the chloromethanes is very similar to that of the amidomethylcoumarin substrates (Phe > Leu ≫ Arg for papain; Phe > Leu ≅ Arg for cathepsin B). It should also be noted that Cbz-Arg-Ala-CH_2Cl is a selective inhibitor of cathepsin B (ratio of $k_{inac.}/K_i$ for cathepsin B over papain = 150).

From our results, one cannot exclude the possibility that the epoxysuccinyl amino acid benzyl esters and benzyl amides used in this study bind to cysteine proteinases in a manner different from that of E64 (e.g. binding in the S′ subsites). However, based on the similarity between the chemical structures of the epoxysuccinyl amino acid derivatives of E64, we believe that their binding mode is essentially the same as that of E64 in subsites S_1 and S_2 of the enzyme. The main difference between the E64-type inhibitors and the substrate-like chloromethanes is that the chemically reactive epoxysuccinyl group in the former is attached to the amino group of the amino acid residue whereas, in the latter, the chloromethane function is at the C-terminal part of the peptide. Thus the amino acid residue in the epoxysuccinyl derivatives binds in the reverse direction when compared with chloromethanes and substrates. Consequently, the interactions in the S_2 subsite are affected. In addition, modifications in the hydrogen-bonding interactions involving E64 and E64 analogues with residue Gly-66 lead to greater perturbations in S_2 subsite specificity for this class of inhibitors. For these reasons, the specificity of E64 analogues cannot be altered in a rational manner by using the information available from S_2–P_2 interactions with substrates. This information can be used, however, with substrate-like inhibitors such as the chloromethanes, as exemplified by the cathepsin B selectivity obtained with the inhibitor Cbz-Arg-Ala-CH_2Cl.

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