Aza-Peptide Epoxides: A New Class of Inhibitors Selective for Clan CD Cysteine Proteases

Juliana L. Asgian,[†] Karen Ellis James,[†]
Zhao Zhao Li,[†] Wendy Carter,[‡] Alan J. Barrett,[‡]
Jowita Mikolajczyk,[§] Guy S. Salvesen,[§] and
James C. Powers*,[†]

*School of Chemistry and Biochemistry, Georgia Institute of
Technology, Atlanta, Georgia 30332-0400, MRC Molecular
Enzymology Laboratory, The Babraham Institute,
Babraham, Cambridgeshire CB2 4AT, U.K., and
Program in Apoptosis and Cell Death Research,
The Burnham Institute, 10901 North Torrey Pines Road,
La Jolla, California 92037*

Abstract: Aza-peptide epoxides, a new class of irreversible protease inhibitors, are specific for the clan CD cysteine proteases. The inhibitors have second-order rate constants up to 10^5 M^{-1} s^{-1}, with the most potent epoxides having the *S,S* stereochemistry. The aza-Asn derivatives are effective legumain inhibitors, while the aza-Asp epoxides were specific for caspases. The inhibitors have little or no inhibition with other proteases such as chymotrypsin, papain, or cathepsin B.

Cysteine proteases have been grouped into families and clans by Barrett and Rawlings.[1−3] Most cysteine proteases are members of the papain clan (clan CA), which consists of enzymes such as calpains, cathepsins, and papain. Clan CD is smaller but has several important enzymes, including caspases, legumain, gingipain, clostripain, and separase. The caspases, *c*ysteine *asp*artate specific prote*ases*, are a family of >15 members that are involved in apoptosis (programmed cell death). Caspases are associated with inflammatory disorders (IL-1β processing) and neurodegenerative diseases such as stroke, ALS, Alzheimer's, and Parkinson's. Gingipain, from *Porphyromonas gingivalis*, causes tissue damage in periodontal disease, legumain is associated with antigen processing and immune disorders, while clostripain is involved in bacterial infections. Thus, the design of new specific inhibitors for clan CD cysteine proteases could lead to the development of potential drugs.

A variety of other epoxide inhibitors have been reported in the literature as irreversible inhibitors of cysteine proteases. Epoxysuccinate derivatives related to the natural product E-64 (Figure 1), first isolated from *Aspergillus japonicus*,[4] have been developed as highly reactive inhibitors for many clan CA cysteine proteases including papain, cruzain, and cathepsin B.[5−8] Subsequently, aminoacyl epoxides and α,β-epoxyketones have been developed as inhibitors for serine, threonine,

* To whom correspondence should be addressed. E-mail: james.powers@chemistry.gatech.edu. Phone: 404-894-4038. Fax: 404-894-2295.
† Georgia Institute of Technology.
‡ The Babraham Institute.
§ The Burnham Institute.
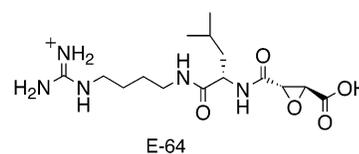
Figure 1. Structure of the natural product E-64.
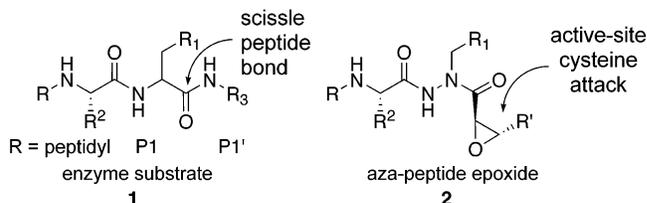
Figure 2. Aza-peptide epoxide design.
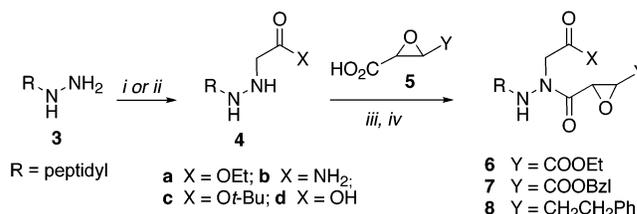
Figure 3. Synthesis of aza-peptide epoxides. Reagents are the following: (i) BrCH$_2$COOEt, NMM, DMF, NH$_3$/MeOH, 0.1 equiv of NaCN, DMF; (ii) BrCH$_2$COO-*t*Bu, NMM DMF; (iii) **3**, EDC, HOBt, DMF or NMM, IBCF, DMF; (iv) TFA (X = O-*t*Bu).

and cysteine proteases. Unfortunately, E-64 derivatives are ineffective inhibitors of clan CD proteases.[3]

Aza-peptide epoxides are a new class of protease inhibitors, which are highly specific for cysteine proteases of clan CD. Aza-peptide epoxides were designed to closely resemble an extended peptide substrate (**1**, Figure 2) with the placement the carbonyl group of the epoxide moiety in a location identical to that of the carbonyl of the scissile peptide bond in a substrate (**2**, Figure 2). This design allowed the peptide chain of the inhibitor to exactly match that of a good substrate up to the scissile peptide carbonyl group. Conversion of the α-carbon of an amino acid residue into a nitrogen results in the formation of an aza-peptide,[9] which allows the ready synthesis of a variety of derivatives. We also hypothesized that the aza-peptide epoxide binding mode would be opposite that of E-64 and E-64 derivatives, which bind in the reverse direction, relative to the substrate binding mode, in papain and cathepsin B. In addition, we hypothesized that the epoxysuccinate derivatives of aza-peptides would be more reactive than simple α-aminoacyl epoxides.

Aza-peptide epoxides were synthesized from peptidyl hydrazides (**3**, Figure 3). Introduction of the aza-amino acid side chain can be accomplished either by reductive amination with the appropriate aldehyde or by alkylation. The aza-Asn derivatives (**6b**) were obtained by alkylation of **3** with ethyl bromoacetate to form **4a**, followed by ammonolysis to the amide (**4b**). The Asp side chain was introduced by alkylation of **3** with *tert*-butyl bromoacetate to form the substituted hydrazide (**4c**). The substituted hydrazides (**4b** and **4c**) were coupled to the respective epoxysuccinate moiety (**5**) using EDC

Table 1. Second-Order Rate Constants for the Inhibition of Cysteine Proteases by Aza-peptide Epoxides

	inhibitor[a]	EP[b]	caspase-1	caspase-3	caspase-6	caspase-8	legumain	papain	cathepsin B
				k_{obs}/[I] [c] (M^{-1} s^{-1})					
9	PhPr-Val-Ala-AAsp-EP-COOCH₂Ph	trans	36200	58	52	63	21	<10	<10
10	PhPr-Val-Ala-AAsp-EP-CH₂CH₂Ph	trans	6130	<10	<10	<10	NI	NI	NI
11	Cbz-Asp-Glu-Val-AAsp-EP-COOEt	*S,S*		205300	7100	6600	46	NI	NI
12		*R,R*		30560	<10	<10	NI	NI	NI
13	Cbz-Leu-Glu-Thr-AAsp-EP-COOEt	*S,S*		<10	7720	8620	36	NI	NI
14		*R,R*		214	3050	1200	15	NI	NI
15	Cbz-Ala-Ala-AAsn-EP-COOEt	*S,S*		<10	<10	<10	43000	<10	<10
16		*R,R*			NI	NI	25200	<10	NI
17		cis					142	NI	NI
18	Cbz-Leu-Leu-ALeu-EP-COOEt	*S,S*		NI	NI	NI	NI	13	<1
19		*R,R*		NI	NI	NI	NI	21	NI
20		trans					NI	35	NI

[a] PhPr = Ph−CH₂−CH₂−CO−; EP = epoxide; AAsp = aza-Asp; AAsn = aza-Asn; Aleu = aza-Leu; Cbz = Ph-CH₂−CO−. [b] The trans epoxide is a mixture of *S,S* and *R,R*, and the cis epoxide is a mixture of *R,S* and *S,R*. [c] Reference 18. NI = no inhibition.

and HOBt, the mixed anhydride method, or the penta-fluorophenol method to give the desired aza-peptide epoxides (**6b**, **6c**, **7c**, and **8c**). The blocked aza-Asp derivatives were deprotected using TFA to yield **6d**, **7d**, and **8d**.

The Asp aza-peptide inhibitor sequences are based on sequences derived from caspase substrate cleavage sites or obtained by peptide mapping of caspases with libraries of AMC substrates (Table 1). The VAD sequence for caspase-1 is the cleavage sequence in IL-1β.[10] The DEVD and LETD sequences are optimal sequences for caspase-3 and caspase-8, respectively.[11] The VAD aza-peptide epoxides, **9** and **10**, are effective inhibitors of caspase-1, with the epoxysuccinate derivative **9** having a k_{obs}/[I] value of 36 200 M^{-1} s^{-1}. The DEVD aza-peptide inhibitors **11** and **12** were potent inhibitors of caspase-3 (k_{obs}/[I] for **11** is 205 300 M^{-1} s^{-1}), while the LETD epoxides **13** and **14** were potent caspase-6 and -8 inhibitors. The Asn derivatives **15** and **16** were effective and specific legumain inhibitors.[12]

The inhibitors show little cross reactivity with other enzymes in clan CD. The epoxides designed for caspases do not inhibit or poorly inhibit other caspases and legumain. The selectivity of **9** for caspase-1 was >574-fold compared to the other enzymes tested. Similarly, compound **13** was >210-fold more reactive with caspase-6 and -8 relative to the other caspases. The legumain inhibitors, **15** and **16**, had essentially no reactivity toward caspases.

Aza-peptide epoxides were specifically designed with the appropriate sequences for clan CA proteases such as papain, cathepsin B, and calpains. Only one sequence, **18**, is shown in Table 1. This inhibitor and similar structures (not shown) were tested for their ability to inhibit clan CA cysteine proteases. They showed little to no inhibition of papain, cathepsin B, and chymotrypsin. Compound **18**, which was designed as a proteasome inhibitor,[13] does not inhibit the proteasome or caspases and poorly inhibits papain and cathepsin B. Clearly, this design is specific for clan CD, since aza-peptide epoxides do not work well with clan CA proteases.

The stereochemistry at the epoxide moiety plays an important role in the potency of the inhibitor. The order of reactivity for the epoxide stereochemistry is *S,S* > *R,R* > trans > cis. Stereochemistry is very important with the caspase inhibitors **11** and **12** where the *S,S* isomer is 6.7-fold more reactive with caspase-3 and over
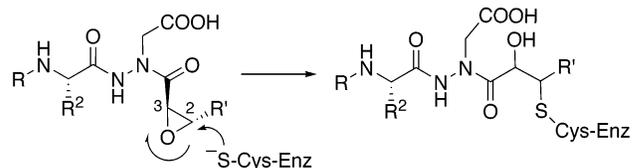
Figure 4. Mechanism of inhibition of caspases by aza-peptide epoxides.

600-fold more reactive with caspase-6 and caspase-8. Stereochemistry is less significant (2- to 8-fold difference) with the caspase-6, caspase-8 (**13** and **14**), and legumain inhibitors (**15** and **16**). The more potent form of E-64 also possesses *S,S* stereochemistry at the epoxide moiety, while the *R,R* isomer is less reactive toward cysteine proteases.[5]

X-ray crystal structures of caspase-1 inhibited by PhPr-Val-Ala-AAsp-EP-COOCH₂Ph and PhPr-Val-Ala-AAsp-EP-CH₂CH₂Ph have been determined (Ron Rubin, unpublished results). Nucleophilic attack by the active site Cys 285 of caspase-1 occurs at the C-2 position of the epoxide ring (Figure 4, R′ = COOBzl or CH₂CH₂-Ph). This site of attack was very surprising because we expected the point of attack to be the C-3 position. In the case of transition-state inhibitors such as peptide aldehydes, the active site cysteine adds to the aldehyde carbonyl group that is equivalent to the scissile peptide carbonyl group in a substrate. Clearly the active site cysteine can attack functional groups quite distant from the side chain of the P1 aza-amino acid residue.

The selectivity of aza-peptide epoxides for clan CD cysteine proteases is a novel feature of these inhibitors. Other epoxide inhibitors, such as E-64 derivatives, are highly reactive for clan CA cysteine proteases and poorly reactive or nonreactive with clan CD cysteine proteases. Enzymes in clan CD and clan CA have different polypeptide folds and varying active site topologies.[14−17] The caspase substrate binding region is quite open and shallow with several binding pockets, while papain has a canyon-like binding site. The more open active site in the caspases along with a strict specificity at P1 residue allows the aza-Asp residue to bind in the S1 pocket and positions the epoxide close to His 237 and Cys 285 where covalent bond formation can occur. In papain the active site is more constricted (crowded) and specificity is determined by interaction with the S2 subsite. A loop structure on the wall of the canyon, which contains the active site histidine, restricts possible binding modes of

inhibitors. We propose that the aza-peptide expoxides cannot bind in a suitable orientation for effective irreversible inhibition because of the inability of the fairly rigid aza-peptide epoxysuccinate moiety to bind properly near the catalytic residues of papain and other clan CA cysteine proteases.

Aza-peptide epoxides have the advantage of being easily extended in the P′ direction, allowing interactions with the S′ subsites of the enzyme. Currently, we are trying to refine the P′ portion of the inhibitors to obtain greater specificity. We are also extending this class of inhibitors to gingipain and clostripain.

Supporting Information Available: Synthetic procedures, final product characterizations, and enzyme assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM025581C