Aza-Peptide Michael Acceptors: A New Class of Inhibitors Specific for Caspases and Other Clan CD Cysteine Proteases

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Abstract: Aza-peptide Michael acceptors are a new class of irreversible inhibitors that are highly potent and specific for clan CD cysteine proteases. The aza-Asp derivatives were specific for caspases, while aza-Asn derivatives were effective legumain inhibitors. Aza-Lys and aza-Orn derivatives were potent inhibitors of gingipain K and clostripain. Aza-peptide Michael acceptors showed no cross reactivity toward papain, cathepsin B, and calpain.

The cysteine proteases caspases, legumain, clostripain, gingipains, and separase, belong to the protease clan CD, which is a small but important group of proteolytic enzymes with a unique α/β fold.[1] Clan CD cysteine proteases are important targets for drug development. Caspases, *c*ysteine *asp*artate specific pro*teases*, are a family with >15 members, 11 of which are found in humans. Some caspases are important mediators of inflammation, whereas others are involved in apoptosis.[2] Excessive neuronal apoptosis leads to a variety of diseases such as stroke, Alzheimer's disease, Huntington's disease, Parkinson's disease, amyotropic lateral sclerosis (ALS), multiple sclerosis (MS), and spinal muscular atrophy.[3] Caspases are recognized as novel therapeutic targets for central nervous diseases in which cell death occurs mainly by an apoptosis mechanism. Legumain, originally identified in leguminous plants[4] and the parasitic blood fluke *Schistosoma mansoni*,[5] has recently been discovered in mammals[6] and is associated with bacterial antigen processing and immune disorders.[7] Gingipains, from *Porphyromonas gingivalis*, cause tissue damage in periodontal disease,[8] while clostripain is involved in bacterial infections.[9] Thus, potent and specific inhibitors of clan CD proteases could lead to the development of potential new drugs.

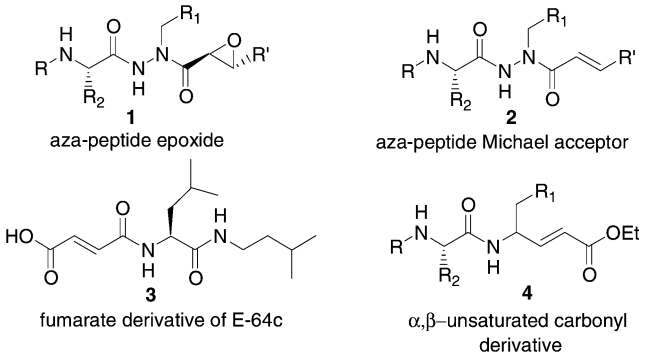

Figure 1. Aza-Peptide Michael acceptor design.

A number of different classes of inhibitors have been developed for cysteine proteases including reversible transition state inhibitors and a variety of irreversible inhibitors.[10] Relatively few inhibitors such as peptide aldehydes, halomethyl ketones, and acyloxymethyl ketones have been tried with clan CD cysteine proteases, mainly with the caspases.[11] The major disadvantage of peptide aldehydes, halomethyl ketones, and acyloxymethyl ketones is their lack of selectivity. It has recently been shown that these inhibitors are not as specific as once claimed.[12] Thus, fluoromethyl and chloromethyl ketones with caspase specific sequences potently inhibit several lysosomal cysteine proteases including cathepsins B, L, and S.

Our laboratory has recently reported aza-peptide epoxide inhibitors (**1**, Figure 1) that are highly specific for clan CD cysteine proteases.[13] Aza-peptide epoxides with caspase specific sequences showed no cross reactivity toward serine proteases or clan CA cysteine proteases such as papain, cathepsin B, and calpain and toward serine proteases.

On the basis of our results with the aza-peptide epoxides, we designed a new class of aza-peptide inhibitors containing Michael acceptors. We refer to this new class of inhibitors as aza-peptide Michael acceptors (**2**, Figure 1). A variety of other inhibitors with electrophilic warheads have been reported as irreversible inhibitors effective for clan CA cysteine proteases. One of the first Michael acceptors described in the literature is the fumarate derivative of E-64c (**3**, Figure 1). This inhibitor contains an α,β-unsaturated carbonyl moiety and was found to be an irreversible inhibitor of cathepsins B, H, and L.[14] Vinyl sulfones and α,β-unsaturated carbonyl derivatives (**4**, Figure 1) have been developed as highly potent inhibitors for exopeptidases such as dipeptidyl peptidase I (DPPI) and many clan CA cysteine endopeptidases including papain, cathepsins B, L, S, and K, calpains, and cruzain.[15,16] The design of vinyl sulfones and α,β-unsaturated carbonyl derivatives was based on the optimal peptide sequence of the target enzyme, where the carbonyl of the scissile bond was replaced by the double bond moiety. In contrast, our aza-peptide Michael acceptor structure resembles the substrate

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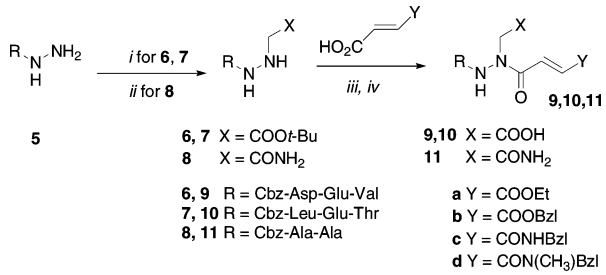
Scheme 1. Synthesis of Aza-Asp and Aza-Asn Michael Acceptors[a]



[a] Reagents and conditions: (i) $BrCH_2COO$-*t*Bu, NMM, DMF; (ii) $BrCH_2COOEt$, NMM, DMF, followed by NH_3/MeOH, 0.1 equiv of NaCN, DMF; (iii) EDC, HOBt, DMF; (iv) TFA (for **6**, **7** X = COO-*t*Bu).

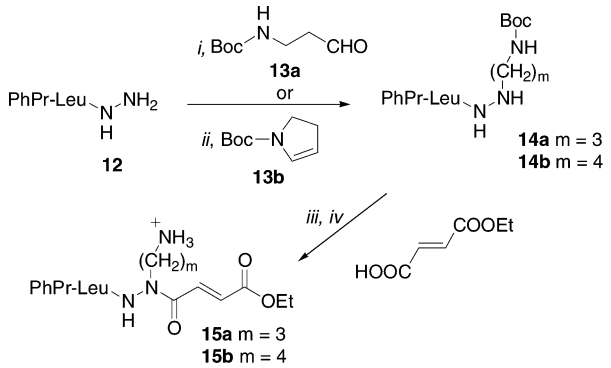
peptide sequence, where the carbonyl group of the double bond moiety replaces the carbonyl group of the scissile bond (Figure 1). Replacement of the α-carbon of the P1 amino acid with a nitrogen results in the formation of the aza-amino acid, which allows the ready synthesis of a variety of derivatives. In the fumarate derivative **3**, the Michael acceptor is on the N-terminus of the amino acid residue, while in our aza-peptide design, the warhead is at the C-terminal end of the inhibitor. In this paper we report that the aza-peptide Michael acceptors are highly potent and specific inhibitors for clan CD cysteine proteases.

We synthesized a variety of peptidyl aza-peptide Michael acceptors with P1 Asp, Asn, Lys, and Orn residues as potential inhibitors for caspases, legumain, gingipain K, and clostripain, respectively. All of the aza-peptide Michael acceptor inhibitors were synthesized from the appropriate peptidyl hydrazide by introduction of the P1 side chain followed by coupling with the desired fumarate (Scheme 1).

Peptidyl hydrazides **5** were obtained by reacting peptidyl methyl esters with hydrazine. The aza-Asp side chain for the caspase specific inhibitors was introduced by alkylation of the peptidyl hydrazide with *tert*-butyl bromoacetate to form the substituted hydrazides **6** or **7**. Likewise, the substituted hydrazide derivative with an aza-Asn side chain at P1 for legumain was obtained by alkylation of the appropriate peptidyl hydrazide with ethyl bromoacetate, followed by ammonolysis to yield the amide **8**. The aza-peptide Michael acceptors targeted for caspases (**9a**−**d**, **10a**, **10b**, and **10d**) were obtained by coupling the substituted hydrazide **6** or **7** with the desired fumarate ester or fumarate monoamide by the EDC/HOBt coupling method, followed by the deprotection of the *tert*-butyl group with TFA. The substituted hydrazide **8** with an Asn side chain was coupled to the desired ester or amide derivatives of fumaric acid to afford the aza-peptide Michael acceptors **11a**−**d** targeting legumain. The aza-Orn and aza-Lys side chains of the gingipain and clostripain specific inhibitors were introduced by reductive amination of the hydrazide **12** with the aldehyde **13a** and the 2-pyrroline (an aldehyde equivalent) **13b**, respectively (Scheme 2).

These precursors **13a** and **13b** were synthesized from the corresponding alcohols 3-(*tert*-butyloxycarbonylamino)propanol and 4-(*tert*-butyloxycarbonylamino)butanol, respectively, by oxidation with iodooxybenzoic acid (IBX) in DMSO. The substituted hydrazides **14a** and **14b** were then coupled to monoethyl fumarate using EDC/HOBt,

Scheme 2. Synthesis of Aza-Orn and Aza-Lys Michael Acceptors[a]



[a] Reagents and conditions: (i) absolute EtOH, followed by $NaBH_3CN$, AcOH, THF; (ii) Tos-OH, absolute EtOH, followed by $NaBH_3CN$, AcOH, THF; (iii) EDC, HOBt, DMF; (iv) TFA, CH_2Cl_2, 0 °C.

and subsequent deprotection with TFA afforded the aza-peptide Michael acceptors **15a** and **15b**.

Kinetic measurements with aza-peptide Michael acceptors demonstrated that these inhibitors are potent and selective inhibitors for all the clan CD cysteine proteases examined. The aza-peptide Michael acceptors with a P1 aza-Asp residue inhibit caspases-3, -6, and -8 with k_2 values in the order of 10^6 M^{-1} s^{-1} (Table 1). The DEVD and LETD sequences are optimal sequences for caspase-3 and caspase-8, respectively.[17] The DEVD inhibitors **9a**−**d** are potent inhibitors of caspase-3 (k_2 values in the range of 1 700 000 M^{-1} s^{-1} to 2 640 000 M^{-1} s^{-1}), while the LETD derivatives **10a**, **10b**, and **10d** are potent inhibitors of caspases-6 and -8 with k_2 values up to 47 600 M^{-1} s^{-1} and 237 000 M^{-1} s^{-1}, respectively. The disubstituted amide analogue **9d** is the most potent compound for caspase-3 with a k_2 value of 2 640 000 M^{-1} s^{-1}. It is also the most selective compound among the caspases-3, -6, and -8, where it inhibits caspase-3 277-fold more potently than caspase-6 and 29-fold more potently than caspase-8. Esters seem to work better with caspases-6 and -8. The ethyl ester analogues **9a** and **10a** are very potent inhibitors of caspase-8 with k_2 values of 273 000 M^{-1} s^{-1} and 237 000 M^{-1} s^{-1}, respectively. We propose that the ethyl ester group is especially favored in the caspase-8 active site, since the prime site is relatively small.[18]

The aza-Asn derivatives **11a**, **11b**, and **11d** are potent inhibitors of *S. mansoni* legumain[19] with IC_{50} values in the nanomolar range (IC_{50} = 31−55 nM, Table 2). Interestingly, the monosubstituted amide analogue **11c** is a weaker inhibitor with an IC_{50} value of 1000 nM, whereas the disubstituted analogue **11d** is almost as potent as the ester analogues (IC_{50} = 55 nM). We suspect that the legumain active site cannot tolerate a hydrogen bond donor at this position in the inhibitor structure.

The aza-peptide Michael acceptors with an aza-Lys or aza-Orn residue at P1 are potent inhibitors of gingipain K and clostripain (Table 3). The aza-Lys derivative **15a** inhibits gingipain K very potently with a k_2 value of 3 280 000 M^{-1} s^{-1}. The aza-Orn derivative **15b** is also a very potent inhibitor of gingipain K, but it is much more selective toward gingipain K, since the clostripain k_2 value is only 788 M^{-1} s^{-1}. Compounds **15a** and **15b** were also tested for activity toward gingipain

Table 1. Irreversible Inhibition of Caspases by Aza-Peptide Michael Acceptors

	inhibitor[b]	k_2[a] ($M^{-1}\,s^{-1}$) caspase-3	caspase-6	caspase-8
9a	Cbz-Asp-Glu-Val-AAsp-CH=CH−COOEt[c]	2130000 ± 99100	35600 ± 0	273000 ± 18400
9b	Cbz-Asp-Glu-Val-AAsp-CH=CH−COOBzl	1700000 ± 106000	8470	121000 ± 13700
9c	Cbz-Asp-Glu-Val-AAsp-CH=CH−CONHBzl	1750000 ± 18500	3210 ± 105	78200 ± 8000
9d	Cbz-Asp-Glu-Val-AAsp-CH=CH−CON(CH_3)Bzl	2640000 ± 397000	9500 ± 210	90300 ± 18200
10a	Cbz-Leu-Glu-Thr-AAsp-CH=CH−COOEt	5560 ± 290	18700 ± 1040	237000 ± 52700
10b	Cbz-Leu-Glu-Thr-AAsp-CH=CH−COOBzl[c]	4600 ± 330	47600 ± 2500	98400 ± 9130
10d	Cbz-Leu-Glu-Thr-AAsp-CH=CH−CON(CH_3)Bzl	6000 ± 100	10800 ± 410	169000

[a] Inhibition buffer was 40 mM PIPES, 200 mM NaCl, 0.2% (w/v) CHAPS, sucrose 20% (w/v) and 10 mM DTT, at pH 7.2, with Ac-DEVD-AMC as the substrate. [b] Inhibitors have trans double bonds unless otherwise indicated, Cbz = $PhCH_2OCO$, AAsp = aza-Asp. [c] No cross reactivity with *S. mansoni* legumain, clostripain, gingipain K, papain, cathepsin B, and calpain.

Table 2. Irreversible Inhibition of *S. mansoni* Legumain by Aza-Peptide Michael Acceptors

	inhibitor[a]	IC_{50}[b] (nM)
11a	Cbz-Ala-Ala-AAsn-CH=CH−COOEt[c]	31 ± 25
11b	Cbz-Ala-Ala-AAsn-CH=CH−COOBzl	38
11c	Cbz-Ala-Ala-AAsn-CH=CH−CONHBzl	1000
11d	Cbz-Ala-Ala-AAsn-CH=CH−CON(CH_3)Bzl[b]	55

[a] Inhibitors have trans double bonds unless otherwise indicated, Cbz = $PhCH_2OCO$, AAsn = aza-Asn. [b] Inhibition buffer was 0.1 M citrate-phosphate buffer at pH 6.8 containing 4 mM DTT with Cbz-AAN-AMC as the substrate; irreversible inhibition was measured at 20 min incubation time. [c] No cross reactivity with caspases-3, -6, and -8, clostripain, gingipain K, papain, cathepsin B, and calpain.

Table 3. Irreversible Inhibition of Clostripain and Gingipain K by Aza-Peptide Michael Acceptors

	inhibitor[a]	k_2[b] ($M^{-1}\,s^{-1}$) clostripain	gingipain K
15a	PhPr-Leu-ALys-CH=CH−COOEt	40750 ± 7600	3280000 ± 652000
15b	PhPr-Leu-AOrn-CH=CH−COOEt	788 ± 169	927000 ± 59000

[a] Inhibitors have trans double bonds unless otherwise indicated; no cross reactivity with caspases-3, -6, and -8, *S. mansoni* legumain, papain, cathepsin B, and calpain. PhPr = $PhCH_2CH_2OCO$, ALys = aza-Lys, AOrn = aza-Orn. [b] Inhibition buffer was 20 mM Tris/HCl, 10 mM $CaCl_2$, 0.005% Brij 35, 2 mM DTT at pH 7.6 with Cbz-FR-AMC as the substrate for clostripain and 0.2 M Tris/HCl, 0.1 M NaCl, 5 mM $CaCl_2$, 2 mM DTT at pH 8.0 with Suc-AFK-AMC as the substrate with gingipain K.

R. Both compounds inhibit gingipain R weakly with k_2 values of 289 $M^{-1}\,s^{-1}$ and 32 $M^{-1}\,s^{-1}$.

Aza-peptide Michael acceptors designed with clan CD specific sequences are quite specific and do not inhibit clan CA proteases such as papain, cathepsin B, and calpain (data not shown). The inhibitors show little to no cross reactivity with the other members of clan CD proteases. Thus, the aza-Asp Michael acceptor **9a** does not inhibit clostripain, gingipain K, or legumain. The legumain inhibitors **11a**−**d** have essentially no reactivity toward caspases, clostripain or gingipain K. Likewise, **15a** and **15b** designed for clostripain and gingipain show no cross reactivity with caspases or legumain. Specificity of the inhibitors **15a** and **15b** was also tested with the serine protease trypsin. The inhibitor **15a** is a weak reversible inhibitor of trypsin with a K_i value of 17 μM, whereas **15b** shows no trypsin inhibitory potency.

The aza-peptide Michael acceptors were tested for their reactivity toward thiol nucleophiles such as the thiol DTT contained in the assay buffer conditions. The stability of a representative group of compounds was studied by monitoring the UV spectrum of buffer solutions of the inhibitors at 250 nm, at 25 °C. The buffer solutions contained 10 mM DTT and the pH range was 5.8−7.2. The half-lives ($t_{1/2}$) were obtained from first-order rate plots of $\ln(A_t/A_o)$ versus time. The reaction was followed for up to 15 h. We observed that the ester analogues **9a**, **11a**, **15a**, and **15b** had half-lives of 3−10 min, whereas the monosubstituted amide analogues **6c** and **11c** were less reactive with half-lives of 20−58 min. The caspase specific disubstituted amide analogue **9d** had a half-life of 116 min, whereas the disubstituted amide analogue **11d** designed for legumain was essentially stable when tested over a period of 15 h.

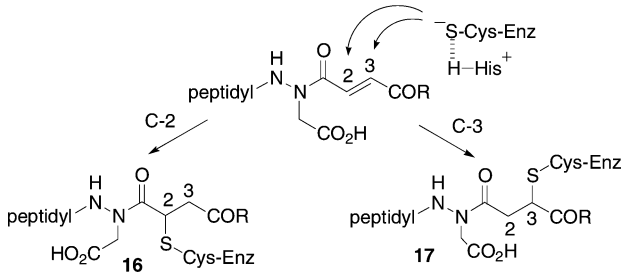


Figure 2. Proposed mechanism of inhibition of clan CD cysteine proteases by aza-peptide Michael acceptors.

The mechanism of inhibition of clan CD proteases by aza-peptide Michael acceptors could involve irreversible thioalkylation of the active site Cys by the Michael acceptor by the two pathways shown in Figure 2. The nucleophilic attack could take place at either the C-2 or C-3 carbon on the double bond moiety forming a thioether adduct **16** or **17**. We favor C-2 attack since the dienyl derivative Cbz-Val-AAsp-CH=CH−CH=CH−CH_3, designed for caspases showed no inhibitory potency toward caspases and was also stable when tested for reactivity with DTT. In addition, it has previously been shown that simple acrylates are more reactive than simple acrylamides to thiol nucleophiles such as glutathione.[20] This also correlates with our observations of the greater stability of aza-peptides containing acrylamides versus acrylates with DTT.

We further investigated the inhibition mechanism by conducting an NMR study of the reaction of the inhibitors with the thiol nucleophile benzylmercaptan. The inhibitor Cbz-Asp-Glu-Val-AAsp-CH=CH−COOEt (**9a**) was reacted with benzylmercaptan, and the reaction was monitored using ^1H NMR over 48 h. The formation of a new signal at 4.05 ppm was observed with the simultaneous disappearance of the vinyl proton signal at 6.63 ppm. The observed spectrum was consistent with a thioether adduct that is formed by the attack of the

benzylmercaptan at the C-2 carbon (**16**, Figure 2). An attack at the C-3 carbon would result in a chemical shift of the C-3 hydrogen at 3.59 ppm (**17**, Figure 2) as predicted by the additivity rules[21] as used in ChemDraw. There was essentially no further change in the spectrum after 48 h, which indicates that probably a stable thioether adduct had formed. The dienyl derivative showed no change in its spectrum when reacted with benzylmercaptan for 48 h. The NMR shift predictions by ChemDraw are generally not infallible; however, the greater reactivity of acrylates, the lack of inhibitory potency of compounds such as the dienyl derivative, and the NMR data supports our hypothesis of a C-2 thioalkylation.

Aza-peptide Michael acceptors designed with the appropriate P1 amino acids are potent and specific inhibitors for clan CD cysteine proteases. The second-order inhibition rate constants are as high as 3 280 000 M^{-1} s^{-1}. The inhibitors with the appropriate peptide sequence for the targeted enzyme do not show any cross reactivity with clan CA cysteine proteases such as papain, cathepsin B, and calpain. There is also little to no cross reactivity toward the other members of clan CD cysteine proteases. Hence, the aza-peptide Michael acceptor design is clearly specific for clan CD cysteine proteases. Currently, we are trying to refine the Michael acceptor inhibitor design[22] in the P′ portion to obtain greater specificity, and we plan to test the caspase specific inhibitors with all other caspases.

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

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