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Organic Azide Inhibitors of Cysteine Proteases

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Cysteine proteases account for 26% of human endopeptidase enzymes and play crucial roles in diseases, immune defense, inflammation, apoptosis, and bone resorption.[1] When overexpressed or unregulated in diseased states, cysteine proteases are validated therapeutic targets,[2] but their inhibitors have not yet succeeded in the clinic,[2] mainly because of a common flaw in their design. Inhibitors of cysteine proteases usually employ a reactive electrophilic group (alkylating agent, aldehyde, nitrile, ketone, α-keto-amide, halo-ketone, vinyl sulfone, etc.)[3] to covalently bond to the enzyme's catalytic cysteinyl sulfur. Although useful tools in vitro, such inhibitors are not druglike[4] because of indiscriminate competing reactions in vivo with other nucleophiles (thiols, amines, etc), resulting in low bioavailability, metabolic instability, and side-effects.[4] Electrophilic isosteres are still thought to be necessary for potent inhibition of cysteine proteases, especially caspases.[5] We now report an alternative use of much less reactive alkyl azide isosteres to confer potent inhibition of cysteine proteases. Inhibition of a proinflammatory interleukin converting enzyme, caspase-1 (ICE),[7] is shown to be competitive, reversible, and selective, relying upon multiple noncovalent interactions for enzyme binding. The new azido isostere is also shown to be effective for inhibiting cathepsins (B, K, S) and other caspases (3, 8), suggesting its use for inhibiting cysteine proteases in general.

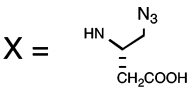
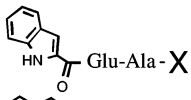
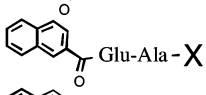
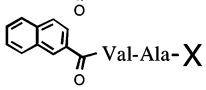
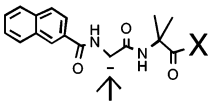
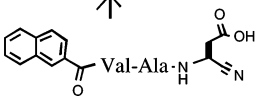
Table 1 shows that replacing the C-terminal aspartic aldehyde in the known caspase-1 inhibitor **1**[8] by the azidomethylene derivative of aspartic acid (X) maintains sub-μM inhibition (**2**, Table 1) of caspase-1. The reduced potency was recovered through sequential variation at P3 and P4 positions (**3**−**5**, Table 1); compound **5** with a 2-naphthoyl substituent at P4 being only 2-fold less potent than **1**. Substitution of Val and Ala in **5** with more hydrophobic amino acids gave **6** (Table 1), with more druglike characteristics (MW = 524—ethyl ester, logP = 2.8, polar surface area = 143 Å^2, H-bond donors/acceptors = 3/10, 13 rotatable bonds)[12] and less susceptibility to proteolytic degradation. By contrast, the nitrile analogue **7** (even at 50 μM) did not inhibit caspase-1.

Azides **2**−**6** are competitive and reversible inhibitors of caspase-1 as shown by Lineweaver−Burk plots of $1/V_o$ versus 1/[S] at varying [I] (Figure S1, Supporting Information (SI)). K_m increased with [I] but V_{max} remained constant. Preincubation of, for example, **5** (50 nM) for 10 min with caspase-1 resulted in complete inhibition. Full enzymatic activity was restored by adding 20× K_m of substrate (Ac-YVAD-AMC, K_m = 14 μM), establishing that inhibition by **5** is competitive and reversible (SI). Inhibitor **5** was also quite selective for caspase-1 (IC_{50} > 50 μM, caspase-3; 2.0μM, caspase-5).

Modeling of **5** in the substrate-binding active site of caspase-1 using GOLD (Figure 1) showed that the naphthoyl substituent (P4), and side chains of Val (P3), Ala (P2), and pseudo-aspartate (P1) of **5** occupied expected sites (Figure 1A: S4−S1, respectively) in caspase-1 (crystal structure of (Ac-YVAD-H)-enzyme complex, pdb: 1ICE[13]). Inhibitor **5** also occupies very similar conformational space as **1** (Figure 1B), its *backbone* atoms (C, N, O) superimposing well (rmsd 0.37 Å) on the corresponding atoms of **1** in its crystal structure with caspase-1. The backbone adopts the classic extended strand recognized by most proteases.[14] Corresponding *side* chains also align closely. The P1 carboxylate side chain of **5** is important, as it is in **1**,[15] since conversion to the amide reduced caspase-1 inhibition 50-fold (IC_{50} 230 nM). This supports analogous binding for **5** and **1** at the S1 site in caspase-1.

By contrast, the azide of **5** is in a different position to the aldehyde of **1**. The azidomethylene carbon of **5** is 1.9 Å from the aldehyde carbon of **1**, and equidistant from the active site thiolate (Cys 285-S...central azide nitrogen, 3.8 Å) and imidazole (His 237-NH...terminal azide nitrogen, 3.5 Å). The azido substituent does not extend to the S1′ subsite of enzyme. Thus the model supports noncovalent interactions with enzyme. Given that azide has

Table 1. Azidomethylene Based Inhibitors of Caspase-1

	X = 	IC_{50} (nM)	M_r	Log P[a]
1	Ac-Tyr-Val-Ala-Asp-H	2 ± 0.4	492	-0.7
2	Ac-Tyr-Val-Ala-X	226 ± 32	519	0.2
3		75 ± 10	487	0.4
4		40 ± 5	498	1.2
5		4.6 ± 0.5	468	2.1
6		7.9 ± 0.75	496	2.8
7		> 50000	438	1.9

[a] log P (pH 7.4) refers to the mono/diethyl ester derivatives of the P1/P3 Asp/Glu side-chain carboxlates.

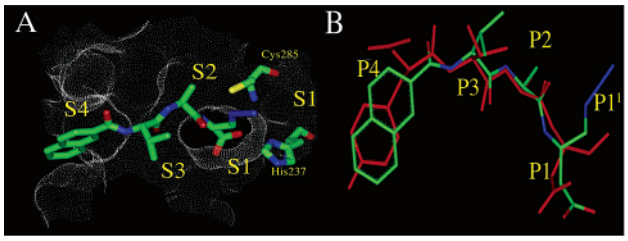


Figure 1. (A) Modeled **5** docked in the active site of caspase-1 (pdb 1ICE); (B) backbone (C, N, O atoms) superimposition of **5** (green) on enzyme-bound structure[13] of Ac−YVAD-H (red).

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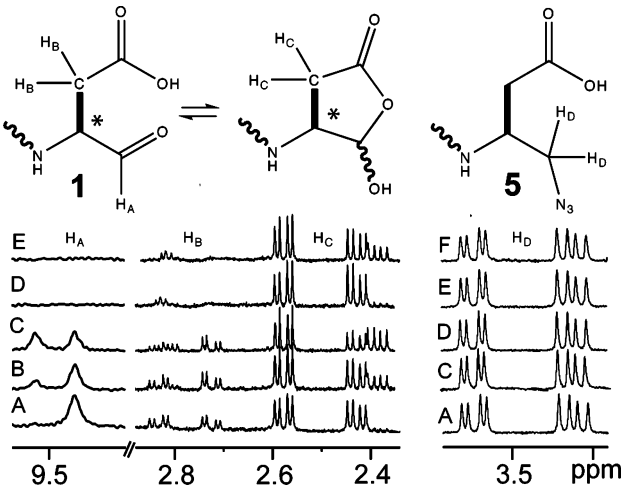


Figure 2. Selected ^{1}H NMR signals for aldehyde **1** (left panel) and azide **5** (right panel) in H_2O/D_2O (9:1, phosphate buffer pH 7.4, 37 °C) at 5 min (A), 3 h (B), 24 h (C) or with 10-fold Ac−Cys for 5 min (D), 3 h (E), or 3 weeks (F). New signals in traces B, C, and E were ascribed to respective protons of *D*-isomer. **5** remained unchanged under all these conditions (right panel).

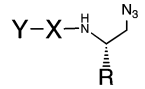
resonance forms with positive and negative charge on central and terminal nitrogens, respectively, noncovalent electrostatic interactions between azide and cysteinyl-S or imidazole-N, might be possible as proposed for sulfonyl azide inhibitors of COX-2.[16]

Azides are widely used in organic synthesis,[9] but are remarkably stable in biological environments. For example, azides are orthogonal protecting groups in vivo,[10] components of drugs, like the anti-HIV drug AZT (only ∼1% is reduced to the amine metabolite by cytochrome P_{450} after oral administration to rats[11]), and azido-sugars are well tolerated in mice (300 mg/kg/day, 7 days).[10] Azides **5** and **6** were completely stable in aqueous solutions (>1 month, 37 °C), serum (24 h, 37 °C, pH 7.2), assay buffer (14 h, pH 7.2, 10 mM dithiothreitol) ± enzyme, or 20 mM glutathione (pH 7.2, 9, 11), as monitored quantitatively by LCMS. By contrast, aldehydes such as **1** are unstable in aqueous solutions (Figure 2, left panel), existing initially as a mixture of aldehyde and cyclic hemi-acetal (A) followed by rapid aspartate racemization (B, C) in phosphate buffer ($t_{1/2}$ = 4 h, 37 °C, pH 7.4). No aldehyde is detectable within minutes in 10 mM Ac−Cys (D, E) or 20 mM glutathione. Azide **5** is very stable under the same conditions (Figure 2, right panel). Under harsher chemical, less biologically relevant, conditions, the azide of **5** can be reduced (Pd/C/H_2) to the amine, which is a 1000× less potent caspase-1 inhibitor (IC_{50} = 5 μM, SI).

Azides have not previously been reported as components of protease inhibitors. Preliminary results here suggest that the azidomethylene group can be used to produce potent inhibitors not only of caspase-1, but also of other cysteine proteases. For example, aldehydes Ac-DEVD-H[17] and Cbz-Leu-Nle-H,[18] were systematically converted into nanomolar inhibitors (Table 2) of other caspases (3, 8) and cathepsins (B, K, S) and are being further optimized for potency, selectivity and bioavailability.

Many cysteine protease inhibitors with reactive electrophiles, like aldehydes, ketones, and acyloxyketones, have failed clinical trials.[2] Two caspase-1 inhibitors with a masked aldehyde, pralnacasan and VX-765, have similarly faltered in clinical trials for arthritis. Very few noncovalent inhibitors are known for cysteine proteases,[6] so this demonstration that the azidomethylene unit is much more inert than aldehydes but still confers potent, competitive, and reversible caspase-1 inhibition may be a valuable discovery toward new, less reactive and/or noncovalent, inhibitors of caspases, cathepsins, and other cysteine proteases.

Table 2. Azidomethylene Inhibitors of Other Cysteine Proteases



Inhibitor	Y	X	R	Enzyme	IC_{50}(nM)
8	Ac-Asp-Glu	Leu	CH_2COOH	Casp-3	390 ± 34
9	Cbz-Glu	Leu	CH_2COOH	Casp-8	162 ± 20
10	2-naphthoyl	Leu	$(CH_2)_3CH_3$	Cat K	1.2 ± 0.3
11	1-naphthoyl	Leu	$(CH_2)_3CH_3$	Cat S	18 ± 4.3
12	Biphenoyl	Phe	$CH_2CH(CH_3)_2$	Cat B	58.3 ± 6.1

Acknowledgment. The Australian funding agencies ARC and NHMRC are gratefully acknowledged for financial support.

Supporting Information Available: Synthesis and characterization data (rpHPLC retention times, ^{1}H NMR spectra, high-resolution MS data) for **1**−**12**, enzyme assays, and stabilities. This material is available free of charge via the Internet at http://pubs.acs.org.

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