

CASPASES: BIOLOGICAL FUNCTION, SPECIFICITY, AND DESIGN OF INHIBITORS

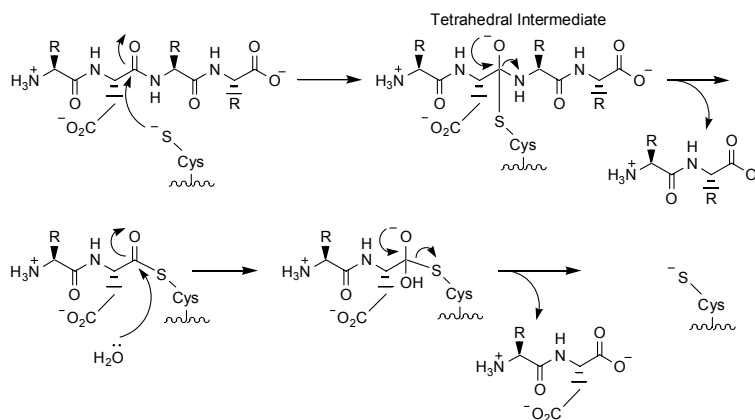
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INTRODUCTION

Caspases represent a unique class of cysteine aspartate-specific proteases because of their substrate specificity and biological functions.¹ Caspases utilize a cysteine thiolate residue of the active site to catalyze the hydrolysis of the peptide substrate at the C-terminus of an aspartic acid residue (Scheme 1).² Nucleophilic attack by the cysteine thiolate initially forms a tetrahedral intermediate that is stabilized by the enzyme active site. This is followed by elimination of the C-terminal portion of the peptide, leaving a thioester in the active site. Subsequent hydrolysis of the thioester releases the N-terminal portion of the substrate peptide and regenerates an available active site to reenter the catalytic cycle.

Scheme 1. Mechanism of a Cysteine Protease



The first caspase isolated, caspase-1, was found to convert a proform of interleukin-1 β (IL-1 β) into its biologically active hormonal form.^{3,4} Thus, it was originally termed interleukin converting enzyme (ICE). In 1993, Yuan and coworkers recognized that caspase-1 was similar in amino acid sequence to CED-3, a protease in the nematode *Caenorhabditis elegans* that plays a key role in programmed cell death (apoptosis).⁵ Subsequent searches for mammalian caspases have revealed thirteen caspases in the human genome, although the exact mechanistic roles of all caspases are not yet known.^{6,7} Agents that activate apoptosis could be active therapeutics in treating diseases where programmed cell death is suppressed, such as cancer proliferation. Alternatively, agents that inhibit apoptosis would also be valuable pharmaceuticals to combat excess cellular death in diseases such as autoimmune disorders and stroke.

BIOLOGICAL FUNCTION

Human caspases can be divided into two classes based on their distinct roles in cellular processes.⁸ Caspases-1, -4, and -5 are involved in cytokine maturation leading to an inflammatory response. The ICE substrate, IL-1 β , is a key signaling protein in inflammation response and inhibition

of these caspases might be a viable treatment for conditions such as rheumatoid arthritis.⁸ The other class of caspases are involved in the signaling of apoptosis in target cells.

All caspases exist in target cells as inactive precursors or zymogens, which for caspases are termed pro-caspases. Activation of pro-caspases by cleavages at the two N-terminal prodomain sites and at the four protease domain cleavage sites converts the zymogen into its active form (Figure 1).⁹ The active form of caspase-1 was originally believed to be a heterodimer of the p10 and p20 subunits, in which the active site was formed between the two subunits, but the crystal structure of caspase-1 revealed that it was actually a homodimer of the two heterodimers.^{2,9}

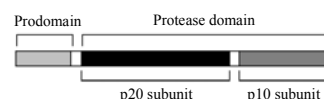


Figure 1. Map of Caspase Zymogen

Initial cleavage of a pro-caspase into its active enzyme is accomplished either by self-catalysis or cleavage by another caspase. Since a caspase can trigger many pro-caspases, a propagation of active caspases, termed a caspase cascade, can occur.¹⁰ In the case of apoptosis, the upstream signals that start such a caspase cascade occur via two distinct pathways (Figure 2). Activation through the mitochondria from an unknown extra-cellular source results in the release of cytochrome c, which recruits caspase-9 through apoptosis protease-activating factor-1 (Apaf-1). Alternatively, a membrane-bound death receptor complex can trigger caspase-8. Both intracellular signaling processes proceed through distinctive sets of initiator caspases but lead to the activation of the same set of effector caspases (caspase-3, -6, and -7). Once the effector caspases are triggered, they proceed to stimulate cellular machinery, such as deoxyribonucleases, which degrade DNA, or cleave cytoskeletal scaffold proteins, and eventually lead to apoptosis.

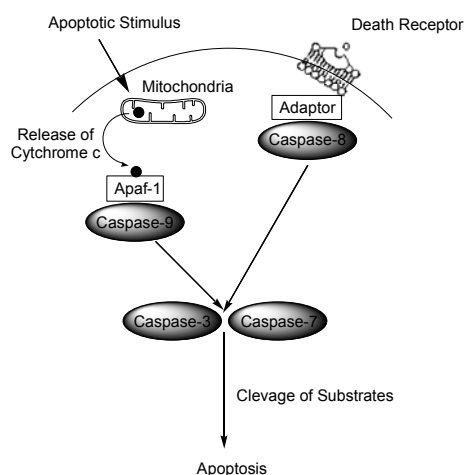


Figure 2. Apoptosis Signaling Pathway

SUBSTRATE SPECIFICITY

One of the most attractive features for caspase inhibition is that these enzymes have high substrate specificity. Peptide substrates are named by designating the amino acid extending from the N-terminal side of the cleaved peptide bond as P1, P2, P3, etc and those extending from the C-terminal side of the hydrolyzed bond as P1', P2', P3', etc (Figure 3).¹¹

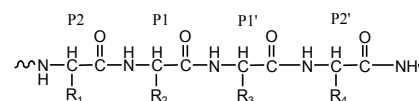
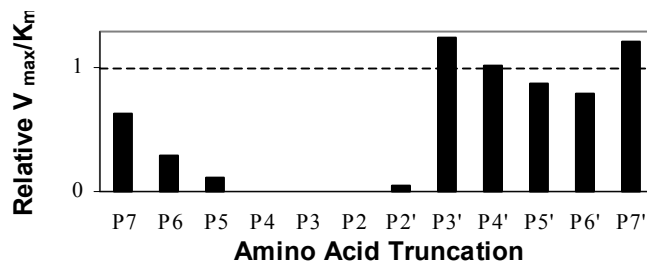


Figure 3. Peptide Nomenclature

Initial studies showed that caspases had very high P1 specificity. The rate of catalysis decreased more than 100-fold when the P1 aspartic acid residue was substituted by any other amino acid.¹² To examine the substrate specificity at other positions, peptides of varying length, based on the natural caspase-1 substrate, pro-II-1 β , were synthesized, and then analyzed to determine their V_{\max}/K_m (Table 1).² Amino acids spanning P1 to P4 proved critical for substrate recognition by caspase-1, as deletions of these residues diminish substrate activity. No significant reduction in catalysis was observed when C-terminal deletions are examined except for the P2' position. It is important to note that the P5 deletion significantly reduced the relative rate of hydrolysis, but placing an acetyl group on the N-terminus of P4 restored optimal activity.

Table 1. Relative V_{\max}/K_m of II-1 β Deletions



PEPTIDE INHIBITION

Peptide Aldehyde Inhibitors

Thornberry and coworkers found that replacement of the natural P2 histidine with alanine increased the rate of catalysis. This indicated that the natural peptide substrate for caspase-1 was not the optimal sequence for caspase-1.² In order to improve further upon natural substrates, a positional scanning synthetic combinatorial library (PS-SCL) was designed to probe the substrate specificity of caspase -1.¹³ Using an aminomethylcoumarin tethered to the C-terminus of each peptide in the library, the PS-SCL was synthesized by scanning with a defined amino acid at P2, while having an equimolar ratio of amino acids at the P3 and P4 sites (Figure 4). Two additional sublibraries were made by defining either P3 or P4 and randomizing the other two positions.

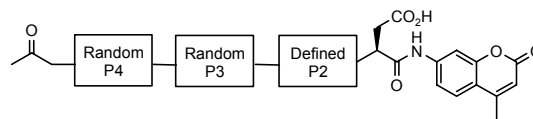


Figure 4. A PS-SCL Sub-library to Determine the Amino Acid Preference of P2

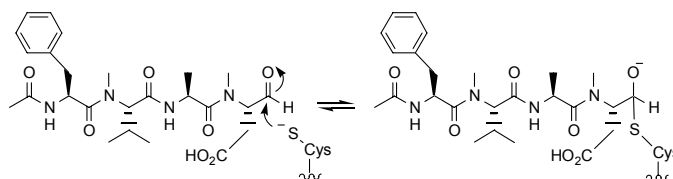
Through this approach catalytic activity was easily monitored through the fluorescence of the free aminomethylcoumarin. The optimal tetrapeptide proved to be WEHD and not YVAD as previously determined.

Subsequent analysis of other caspases showed that they could be subdivided into three classes according to their specificity (Table 2).¹⁴ It is apparent that some caspases may be redundant in the human genome; alternatively, they might be expressed in different tissues. In order to examine the role of individual caspase *in vivo*, caspase selective inhibitors need to be developed.

Table 2. Caspase Substrate Sequence Determined by PS-SCL

Caspase	Sequence	Caspase	Sequence	Caspase	Sequence
4	(W/L) EHD	2	DEHD	6	VEHD
5	(W/L) EHD	3	DEVD	8	LETD
1	WEHD	7	DEVD	9	LEHD

Based on the PS-SCL, several peptides having an aldehyde C-terminus were tested as caspase inhibitors. These were to mimic the enzyme tetrahedral intermediate by forming a stable hemithioacetal with the active site (Scheme 2).¹³ A peptide aldehyde containing the optimal WEHD amino acid sequence inhibited caspase-1 with a K_i of 56 pM, compared to a K_i of 760 pM for the original YVAD peptide aldehyde.

Scheme 2. Mechanism of a Peptide Aldehyde Inhibitor**Irreversible Peptide Inhibitors**

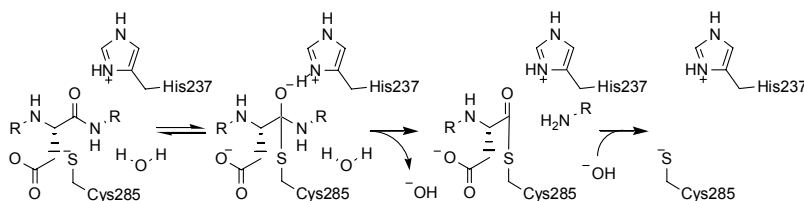
A series of peptide methyl ketones having various leaving groups at the α position were prepared as irreversible inhibitors with the expectation that the active site thiolate would displace the leaving groups (Table 3).¹⁵ All of the peptides proved to be potent, irreversible inhibitors of caspase-1; however, there was no significant dependence of the inactivation rate constant on the pK_a of the leaving group. To explain this unexpected finding, Brady and co-workers performed kinetic studies and found that some of these inhibitors formed a tight complex in which the thiolate actually attacked the ketone carbonyl to form a very stable tetrahedral intermediate. Only after breakdown of this intermediate did the thiolate displace the leaving group.¹⁶

Brady and coworkers used this analogy to propose a mechanism for peptide hydrolysis (Scheme 3).¹⁷ Numerous crystal structures of inhibitors complexed with caspase-1 show that the imidazole of histidine 237 is properly positioned to stabilize the oxyanion of the tetrahedral intermediate. Attack of thiolate on the amide carbonyl forms a thiohemiacetal adduct which is then stabilized by the protonated imidazole. Finally, to account for the stabilization of negative charge on the amine leaving group, a water molecule is invoked to hydrogen bond to a backbone amide nitrogen of the enzyme and protonate the departing amine via general acid catalysis.

Table 3. Leaving Group pK_a vs $K_{obs}/[I]$

X	pK_a	$K_{obs}/[I]$ ($M^{-1} s^{-1}$)
	0.58	900 000
	1.22	1 200 000
	3.35	710 000
	5.53	1 100 000
	7.16	1 300 000

Scheme 3. Revised Mechanism of Caspase-1



SMALL MOLECULE INHIBITORS

Peptide Mimetic

Using an N-methyl scan of the substrate amides of the peptide backbone, Mullican and coworkers sought to determine which substrate amides have strong hydrogen bonds with the active site of caspase-1.¹⁸ They observed a dramatic decrease in inhibition when N-methyl amides were placed on the P1 and P3 amino acid residues of a potent peptide aldehyde inhibitor, and thus postulated that these amides form strong hydrogen bonds with the active site (Figure 5).

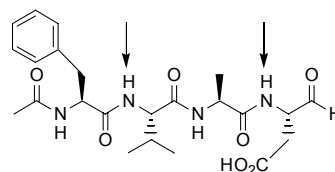


Figure 5. Hydrogen Bond Donors on Caspase-1 Inhibitor

To design a scaffold that utilized these significant hydrogen bonds, Dolle and coworkers used a pyridazinodiazepine as a template for a structure-activity relationship study (SAR).¹⁹ The most potent irreversible inhibitor (Figure 6) had an inactivation rate constant comparable to the irreversible peptide inhibitors in Table 3. Lauffer and Mullican also utilized a similar scaffold to achieve proper hydrogen bonding with the active site, but utilized benzodiazepene as a scaffold (Scheme 4).²⁰

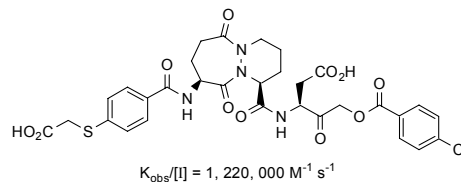
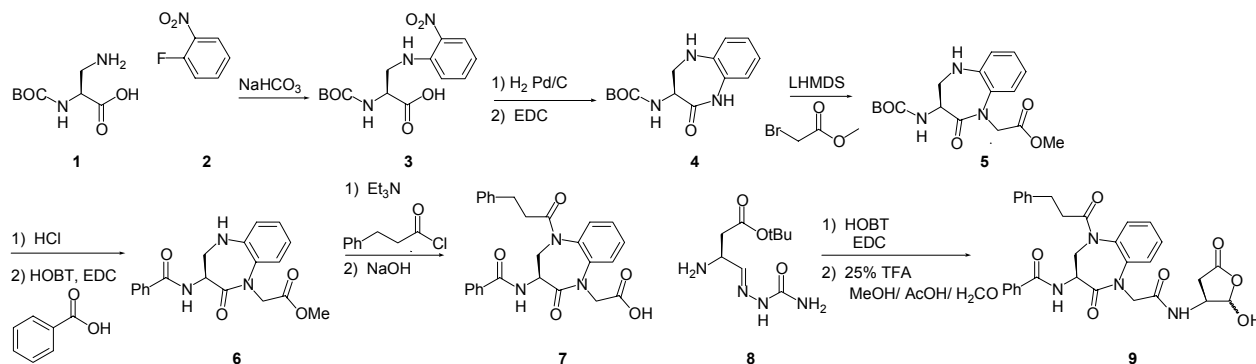


Figure 6. An Inhibitor of Caspase-1

Scheme 4. Synthesis of a Benzodiazepene as a Caspase Inhibitor



Nucleophilic aromatic substitution of propionic acid **1** onto a fluoronitrobenzene (**2**) yielded the substituted nitrobenzene **3**. Subsequent reduction of the nitro group and cyclization of the free amine

resulted in formation of the benzodiazapene core (**4**). Regioselective N-alkylation of **4** with α -bromo methyl acetate yielded **5**. Deprotection of the Boc group under acidic conditions followed by coupling of the free amine to benzoic acid resulted in formation of benzamide **6**. Acylation of the secondary amine of the core benzodiazepine with 3-phenylpropionyl chloride followed by hydrolysis with base resulted in formation of **7**. EDC coupling of the carboxylic acid of **7** with the semicarbazone **8** gave the final benzodiazapene aldehyde derivative (**9**) as a single diastereomer after hydrolysis of the t-butyl ester. The peptide mimetic (**9**) was a potent inhibitor of caspase-1 with a K_i of 90 nM.

Non-Selective Inhibitors Based on Isatin

A class of non-peptide inhibitors using an isatin core, initially designed for inhibition of the serine protease chymotrypsin, was used by Lee and coworkers as a template for caspase inhibitor design (Table 4).²¹ A relationship between σ_m value of R and the IC_{50} of the isatin core was observed, where

Table 4. Effect of σ_m on IC_{50} of Isatin Inhibitor

R	σ_m	IC_{50} (μ M)
	-0.1	>50
	0	>50
	0.35	7
	0.36	15
	0.56	0.6
	0.71	1

the 50% inhibition concentration (IC_{50}) decreased significantly as substitution became electron-withdrawing. Presumably, the electron withdrawing capability of R enables the ketone to be more susceptible to nucleophilic attack by the active site cysteine residue. Selection of the isatin substituent (R) was based on its electronic properties, and molecular modeling was used to optimize steric interactions with caspase-3. A library was designed using a N,N-dialkylisatin sulfonamide core structure. The most potent hit proved to be an excellent inhibitor of caspase-3, but unfortunately it was also a potent inhibitor of caspase-7 and somewhat less effective inhibitor of caspase-9 (Table 5).

Table 5. A Potent Isatin Inhibitor

Caspase	K_i (nM)
3	1.2
6	6
9	120

Selective Inhibitors Based on Thiomethylketones

Head and coworkers utilized a thiomethylketone template (Table 6) as the basis for a combinatorial library of selective irreversible caspase inhibition of caspase-3 and caspase-8.²² A virtual combinatorial library was designed using the crystal structure of caspase-3 and a homology model of caspase-8 with the intention of achieving selective substrate design. About 150 compounds passed the parameters set up by Head

Table 6. Selective Inhibitors of Caspase 3 and 8

R	R'	Caspase-3 IC_{50} (μ M)	Caspase-8 IC_{50} (μ M)
		6.0	>25
		>25	7.3

and coworkers to characterize the differences between the S2 binding pockets of these two caspases. After visual inspection of the virtual hits, 12 R groups were selected with a bias towards size and predicted binding mode in the computational model. Alternatively, the R' leaving groups of the thiomethylketone were chosen based on availability and ease of synthesis. After screening of the combinatorial library, two compounds were found to have very good selectivity for the two caspases surveyed (Table 6).

CONCLUSION

The substrate specificity of caspases for their peptide substrates has been thoroughly investigated. Analysis of the specificity of each caspase reveals that caspases are redundant in their recognition of peptide substrates. Peptide inhibitors were found to be potent caspase inhibitors, but these inhibitors are poor pharmaceuticals due to lack of cell permeability *in vivo*.

Many pharmaceutical companies are developing small molecule inhibitors for medicinal purposes. These companies are also trying to develop small molecule inhibitors that are selective for each caspase to determine the mechanistic role that each caspase plays in apoptosis. Even though some inhibitors have been developed that possess moderate selectivity between caspases, the quest for highly selective caspase inhibitors in the future will be a demanding challenge for the medicinal chemist.

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