Caspases: key mediators of apoptosis

Nancy A Thornberry

Recent studies have established that members of the caspase protease family are essential components of a conserved cell death program. Insights into their biological roles, structure and mechanism are enabling investigators to begin to explore the therapeutic potential of caspase inhibition.

Address: Merck Research Laboratories, R80W-250, PO Box 2000, Rahway, NJ 07065, USA.

E-mail: nancy_thornberry@merck.com

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Introduction

Apoptosis is the highly conserved mechanism by which eukaryotic cells commit suicide (for review see [1]). Over the past few years, it has emerged as one of the hottest areas of research in cell biology as the importance of the process in the proper development and physiological well-being of mammals has become recognized. For example, apoptosis is required for normal tissue turnover, for the proper development and maintenance of the immune system, and for the elimination of virus-infected cells. It follows that inopportune or insufficient apoptosis is believed to result in several pathologies for which there are currently no good therapies [2], such as cancer and autoimmune disorders, which may result from a failure of cells to undergo apoptosis, ischemic injury and at least some neurodegenerative disorders, which instead may result from excessive apoptosis. The potential medical impact that would result from the effective treatment of any one of these pathologies is the driving force for investigators in this intensely competitive field.

Apoptosis is an extremely well-ordered process that is characterized by DNA fragmentation, chromatin condensation, membrane blebbing and cell shrinkage. Cells undergoing apoptosis ultimately disassemble into membrane-enclosed vesicles (apoptotic bodies) that are engulfed by neighboring cells and phagocytes, thus preventing an inflammatory response. In the past few years enormous progress has been made in elucidating the biochemical events that contribute to this complex, tightly regulated phenomenon, largely stemming from the pivotal observation in 1993 that CED-3, the product of a gene that is absolutely required for cell death in the nematode *Caenorhabditis elegans*, is related to the mammalian cysteine protease interleukin-1β converting enzyme (ICE, caspase-1) [3]. This finding not only confirmed earlier work suggesting that the mechanism of cell death is largely conserved across species, but also provided the first evidence that proteases are integral to the death program.

Caspase-1 was first identified in 1989 [4,5] as the enzyme responsible for the processing of the pro-inflammatory cytokine pro-interleukin-1β in monocytes. When this protein was first purified and sequenced in 1992 [6,7], it was found to be unrelated to any known protease. The report of the relationship between caspase-1 and CED-3 [3] prompted a frenetic search for other mammalian caspases; to date, ten homologs of human origin have been described. Members of the caspase family clearly have prominent and distinct biological functions in inflammation and apoptosis. For example, caspase-1-deficient mice are defective in the production of several inflammatory cytokines, but they have no major defect in apoptosis [8,9]. In contrast, mice that are deficient in caspase-3, the mammalian homolog most closely related to CED-3, have a profound defect in the apoptosis that occurs during neuronal development [10].

Concerning the biochemical mechanisms that result in the apoptotic phenotype, it appears that diverse signaling pathways are engaged in apoptosis (depending on the stimulus employed and cell type), and they converge upon a common effector pathway that results in hydrolysis of a number of key structural and housekeeping proteins (for review see [11]). In general, cleavage of the target protein occurs at a single site and results in either gain or loss of activity. Caspases appear to be involved in both the effector phase of apoptosis, where they are responsible for most, if not all, of these cleavage events, and in at least some of the upstream signaling pathways, where they become activated and, in turn, activate the caspases involved in the effector phase.

Because of the central role of caspases in important physiological processes, and the possibility that caspase inhibitors may be effective therapeutic agents, these enzymes have been the subject of intense scrutiny. This review summarizes current understanding of the structure, catalytic mechanism, specificity and inhibition of these important biological mediators.

Catalytic mechanism and structure

The two most distinct members of the caspase family, caspase-1 and caspase-3, have been purified from natural sources and cloned, and their structures have been determined using X-ray crystallography [6,12–15]. The results of these studies, together with a comparison of the

Figure 1

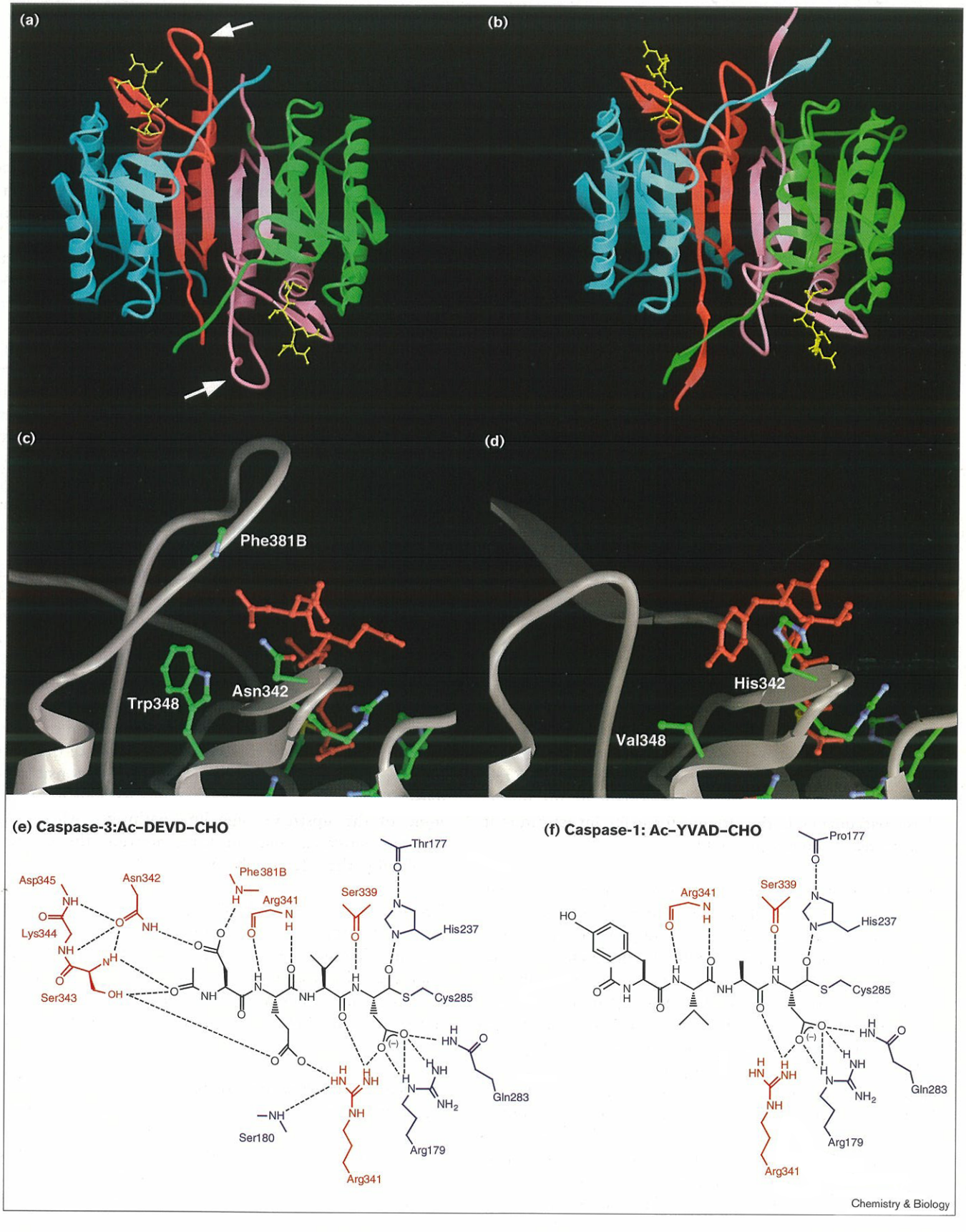
(a)
(b)
(c)
(d)
Phe381B
Trp348
Asn342
His342
Val348
(e) Caspase-3:Ac-DEVD-CHO
Asp345
Asn342
Phe381B
Thr177
Lys344
Arg341
Ser339
His237
Ser343
Cys285
Gln283
Ser180
Arg179
Arg341
(f) Caspase-1: Ac-YVAD-CHO
Pro177
Arg341
Ser339
His237
Cys285
Gln283
Arg179
Arg341
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Figure 1

The structures of members of the caspase family. Structures of **(a)** caspase-3 and **(b)** caspase-1 in complex with tetrapeptide aldehyde inhibitors (yellow). The tetramers are composed of two heterodimeric catalytic domains (blue/red and green/pink). The large (blue/green) and small (red/pink) subunits of the heterodimer both contribute key residues to the active site. The most obvious difference between the two structures is the presence of a surface loop in caspase-3 (indicated with an arrow), not present in caspase-1, that forms one side of the S_4 subsite. A comparison of S_4 in **(c)** caspase-3 and **(d)** caspase-1 reveals the structural basis for the distinct specificities of these two enzymes. Schematic representations of the active sites of **(e)** caspase-3 and **(f)** caspase-1 in complex with aldehyde inhibitors illustrate the extent to which both subunits contribute residues important for binding and catalysis. (a–d) Reproduced with permission from [11].

primary sequences of all of known caspases, suggest that caspases have a number of distinguishing catalytic and structural properties.

The catalytic domain is a heterodimer composed of a large subunit of 17–21 kDa and a smaller subunit of 10–13 kDa. In the crystal structures of caspase-1 and caspase-3 in complex with inhibitors, two heterodimers associate to form a tetramer and at least some evidence suggests that this is the active form of the enzyme in solution (Figure 1) [13]. Within each heterodimer, the two subunits are intimately associated to form a single catalytic domain composed of a central six-stranded β sheet core flanked on either side by α helices. The active site, which lies in a groove on the surface of the protein, is comprised of residues from both large and small subunits.

An analysis of the active site in complex with tetrapeptide-based inhibitors defines those residues that are important for binding and catalysis, and suggests that caspases use a typical cysteine-protease mechanism. Specifically, catalysis involves a catalytic diad composed of a cysteine nucleophile (Cys285) in close proximity to a histidine imidazole group (His237), which is believed to facilitate catalysis via a general acid/base function. The putative oxyanion hole, which may be involved in the stabilization of the oxyanion of the tetrahedral intermediates formed during catalysis, is formed by the backbone amide protons of the active site cysteine residue and Gly238.

Like many other proteases, caspases are synthesized as pro-enzymes that are proteolytically activated to form the mature protein. In addition to the large and small subunits of the heterodimer, the pro-enzyme also has an amino-terminal domain of variable length (2–25 kDa), and, in some cases, a short linker peptide between the large and small subunits. All of these domains are partitioned by Asp–X bonds. As described in detail below, caspases are distinguished among proteases by their stringent specificity for cleavage after aspartic acid residues, suggesting that activation is autocatalytic, mediated by an enzyme with a similar specificity (e.g., another caspase), or both. Evidence that both activation mechanisms occur *in vivo* has been obtained from models of Fas-induced apoptosis.

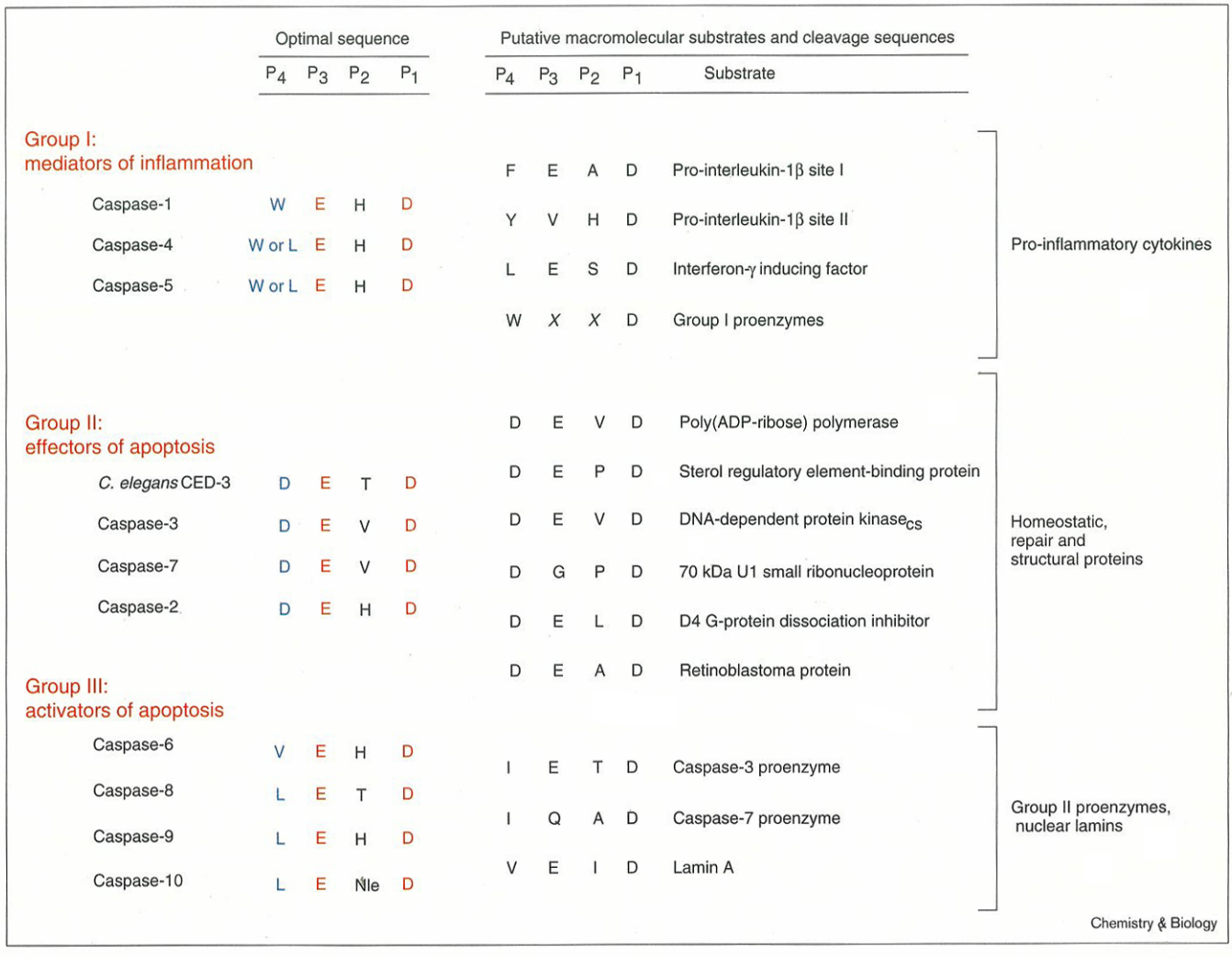
Specificity

Caspases are among the most specific of proteases. They have a near absolute requirement for aspartic acid in the S_1 subsite, such that substitution of any other amino acid (including glutamic acid) leads to > 100-fold reduction in catalytic efficiency [16,17]. This stringent specificity can be attributed to hydrogen-bonding interactions between aspartic acid and three residues of the caspase, Arg179, Gln283 and Arg341, contributed by both subunits (Figure 1e,f). Other than caspases, the only mammalian protease known to have a similar specificity is the cytotoxic lymphocyte-derived serine protease granzyme B, another important mediator of apoptosis (where it has been implicated in the activation of caspases).

Caspases have an equally stringent specificity for at least four amino acids to the left of the cleavage site [6], and it is clear that primary sequence recognition is a necessary requirement for catalysis. It follows, given the diverse biological behavior of caspases, that the extended amino-acid preferences of members of this family are quite distinct. Recently, an intimate understanding of the tetrapeptide specificities of all ten human caspases was obtained using a positional-scanning combinatorial substrate library (PS-CSL; Figure 2) [18,19]. The results divide these enzymes into three major groups, and indicate that the S_4 subsite is the single most important determinant of specificity among caspases. Group I caspases (1, 4 and 5), all favor hydrophobic amino acids in S_4, with an optimal sequence of Trp–Glu–His–Asp (WEHD). Group II enzymes (2, 3, 7 and *C. elegans* CED-3) have a strict requirement for aspartic acid in S_4, preferring the sequence Asp–Glu–X–Asp (DEXD). Caspases in group III tolerate many amino acids in S_4, but have a marked preference for those with branched, aliphatic sidechains, and an optimal sequence of (Val,Leu)–Glu–X–Asp ((V,L)EXD). Within each group, the specific amino-acid preferences are exceedingly similar, in some cases identical, implying that at least some of these enzymes either have redundant functions, or are cell-type or tissue-specific isoforms.

The structural basis for the distinct specificities of the two most diverse caspases (1 and 3) is evident from a comparison of their crystal structures [13–15]. In general, the tertiary and quaternary structures of the enzymes are quite comparable, as are the positions of the catalytic residues and components of the S_1 subsites. In contrast, there are striking differences in the geometry and chemical composition of S_4 that account for the different specificities of these enzymes and, ultimately, their distinct biological functions (Figure 1c,d). In caspase-1, S_4 is a shallow groove on the surface of the protein that easily accommodates

Figure 2



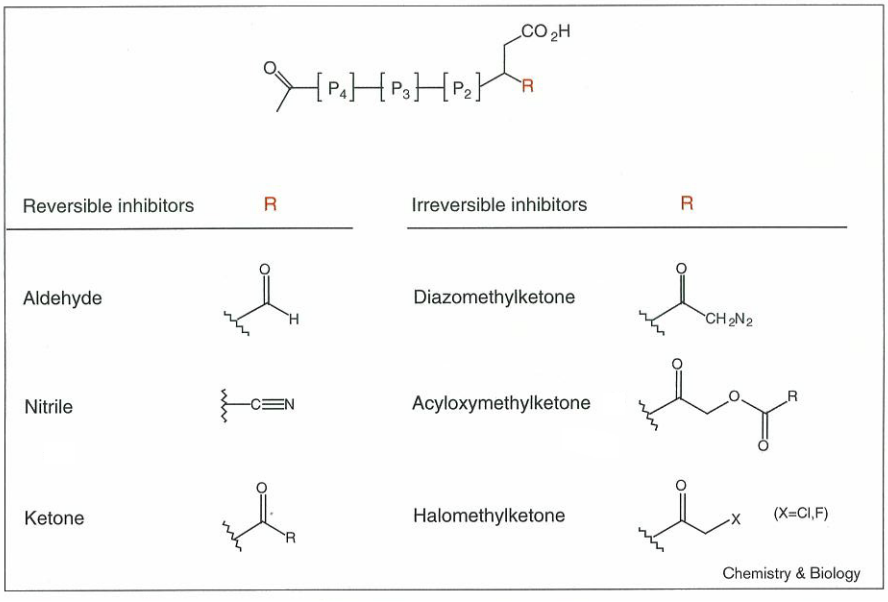
Tetrapeptide specificities define three functional groups. The amino-acid preferences of caspases within each group are exceedingly similar, in some cases identical, implying that at least some of these enzymes have redundant functions. A comparison of tetrapeptide specificities with cleavage sequences in a subset of the known endogenous caspase substrates suggests that group I caspases are mediators of inflammation, where they are responsible for cleavage of several pro-inflammatory cytokines. In contrast, group II and group III caspases appear to function primarily in apoptosis, where they are involved in effector processes and upstream signalling events, respectively.

hydrophobic amino acids, explaining the relatively promiscuous nature of this subsite. In caspase-3, S_4 is significantly smaller and contains a network of hydrogen bonds that serve to stabilize the P_4–Asp of its substrates. With regard to S_3, the preference of all caspases for glutamic acid in this position is explained by the observation that one of the arginine residues (Arg341) involved in stabilization of the P_1 aspartic acid of ligands is appropriately positioned to form a salt link with glutamic acid in P_3 (Figure 1e).

It is clear from several lines of evidence that the substrate specificity observed for tetrapeptides also extends to macromolecules. Most compelling, in cases where endogenous substrates for particular caspases are known, the tetrapeptide sequence at the cleavage sites are similar or identical to the optimal sequence determined by PS–CSL. Additionally, for caspase-1 and caspase-3, the k_{cat}/K_m for cleavage of tetrapeptide substrates ($> 10^6\ M^{-1}\ s^{-1}$) is greater than or equal to the corresponding rate of cleavage of their macromolecular substrates. Consequently, the tetrapeptide specificities provide important clues about the biological functions and relationships between these enzymes. Based on these results, and those from numerous biochemical and genetic studies, functional relationships between these enzymes can be tentatively assigned. Group I caspases function primarily as mediators of inflammation, where they are involved in the proteolytic activation of pro-inflammatory cytokines. Group II caspases, and probably caspase-6, are mediators in the effector phase of apoptosis, where they are responsible for cleavage of key

Figure 3

Peptide-based inhibitors. Several classes of reversible and irreversible peptide-based inhibitors have been identified for caspases using strategies that have proven successful for inhibition of other cysteine proteases. The most potent contain a tetrapeptide that is optimal for the enzyme of interest. Aldehyde, nitriles and ketones form covalent adducts with the catalytic cysteine (thiohemiacetal, thioimidate and thiohemiketal, respectively) that are readily hydrolyzed and function as fully reversible inhibitors. The irreversible inhibitors that have been described are α-substituted ketones; inactivation is believed to proceed through formation of a thiohemiketal, with subsequent displacement of the α substituent by the active-site cysteine to form a thiomethylketone.



structural and homeostatic proteins. Group III caspases, with the possible exception of caspase-6, are involved in signaling pathways where they function as upstream activators of the effector caspases.

Inhibitors

Elegant work in the area of cysteine protease inhibition has resulted in the identification of several classes of electrophiles that form both reversible and irreversible adducts with the active-site cysteine residue (for reviews see [20,21]). Reversible inhibitors include aldehydes, ketones, and nitriles, whereas irreversible inhibitors are generally α-substituted ketones, and include diazomethylketones, halomethylketones and acyloxymethylketones. All of these inhibitor strategies have proven successful in efforts to identify caspase inhibitors (Figure 3).

The most potent inhibitors contain a peptide that is optimal for its target caspase. For example, the tetrapeptide aldehyde Ac–WEHD–CHO, containing the optimal recognition sequence for caspase-1, has a dissociation constant (K_i) for this enzyme of 56 pM [18]. Similarly, the K for inhibition of caspase-3 by Ac–DEVD–CHO is 230 pM [12]. Surprisingly, aldehydes do not appear to bind in a transition-state conformation, in which the oxyanion of the thiohemiacetal is stabilized in the putative oxyanion hole; instead, the oxyanion participates in a hydrogen-bond network involving the catalytic histidine residue (Figure 1e,f).

Although aldehydes are the most potent reversible peptide-based inhibitors that have been described for these enzymes, others, most notably ketones, are remarkably efficient; a tetrapeptide phenylpentyl ketone inhibitor of caspase-1 has a dissociation constant of 18.5 nM [22]. The use of ketones also allows specificity- and affinity-enhancing substituents to be accommodated on both sides of the carbonyl group.

Among irreversible caspase inhibitors, acyloxymethylketones and their derivatives are by far the most well explored, and the most interesting. These compounds, which were first described as potent Cathepsin B inhibitors [23,24], are highly efficient caspase inactivators, having typical second-order rate constants for inactivation of $1 \times 10^6\ M^{-1}\ s^{-1}$ (for examples see [25]). Despite their reactivity with caspases, acyloxymethylketones and their derivatives are relatively inert towards other biological nucleophiles, making them excellent probes for biological functions of caspases *in vivo* and in whole-cell models of apoptosis.

Efforts to make nonpeptide counterparts of these inhibitor classes have exploited the results of structural studies that suggested that hydrogen bonds involving P_1–NH, P_3–NH, and P_3–CO of tetrapeptide inhibitors are important for binding, whereas the P_2–NH is dispensable. These observations have led to the identification of nonpeptidyl inhibitors in which the P_3 amino acid is replaced with a pyridone derivative with no significant adverse effect on potency [26]. More recently, this work as been extended to the identification of pyridazinodiazepines as potent, P_2–P_3 peptidomimetic caspase inhibitors [27].

In fact, inhibition of caspases is not only a lucrative prospect for chemists and biologists interested in treatment

of disease, but it is also favorable for viruses. Both apoptosis and inflammation are triggered in response to viral infection, and inhibition of these processes facilitates replication of the pathogen. It is therefore not surprising that several viral caspase inhibitors have been identified. Cytokine response modifier A (CrmA) is a 38 kDa serpin from cowpox virus that has been shown to be a potent inhibitor of group I, and some group III, caspases, having K_i values ranging from 10 pM to 20 nM [28,29] (N.A.T. and M. Garcia-Calvo, unpublished observations). This selectivity suggests that cowpox virus facilitates infection through both inhibition of the host inflammatory response and inhibition of apoptosis. A baculovirus gene product, p35, also appears to attenuate apoptosis via caspase inhibition [30]. Finally, members of the IAP (inhibitor of apoptosis) gene family encode potent inhibitors of the effectors caspase-3 and caspase-7 [31]. In the case of the IAPs, both viral and mammalian homologs have been described. Evidence for the importance of the human homolog, NAIP, has been obtained in studies of spinal muscular atrophy, which is associated with a mutation in the gene encoding this protein [32]. Taken together, the existence of multiple endogenous inhibitors of caspases, both viral and human, are a testament to the importance of these enzymes in maintaining our physiological well-being.

Perspectives

Knowledge of the structure, catalytic mechanism, and specificities of the caspases has led to the design of potent and selective inhibitors. A number of these compounds have been shown to be efficacious in animal models of diseases involving apoptosis and/or inflammation, which is encouraging news for investigators exploring the therapeutic potential of caspase inhibitors. In this regard, it is noteworthy that, in contrast to their potency *in vitro*, none of the reported inhibitors have good efficacy ($IC_{50} < 1\ \mu M$) in whole-cell models of apoptosis and inflammation, so improving the physical properties of such compounds is a major challenge in this area.

Attenuation of inflammation via selective caspase-1 inhibition continues to be a key objective of several laboratories. Similarly, the increasing evidence that excessive apoptosis may be a major contributor to a number of serious disorders, and the identification of the caspases involved in this process, has resulted in several new research initiatives. At least initially, the use of caspase inhibitors may be limited to the treatment of acute disorders, given the importance of apoptosis in the maintenance of normal immune function, the destruction of potentially tumorogenic cells and many other essential physiological processes. Hopefully, the prospects for treatment of chronic diseases will improve as more is learned about the biology of these enzymes, and with improvements in our abilities to target specific tissues and design selective inhibitors that have appropriate physical properties for long term use *in vivo*.

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