Caspases: A drug discovery perspective
Kevin KW Wang

Address
Department of Neuroscience/Therapeutics
Pierce-Oakes Pharmaceutical Research
2860 Pynchon Road
American
MI 48105
USA
Email: kevin.wang@wcl.com

Current Opinion in Drug Discovery & Development 2002; 5:516-577
© PharmPress Ltd ISSN 1567-6675

Abbreviations
Ap-1 apoptosis-protein-activating factor 1
 CNS central nervous system
 CyRMA cytokine response modifier A
 FADD Fas-associated death domain
 IAP inhibitors of apoptosis proteins
 ICAD inhibitor of caspase-activated DNase
 ICE interleukin-1β-converting enzyme
 MPT mitochondrial permeability transition
 NGF nerve growth factor
 NSAID non-steroidal anti-inflammatory drug
 RAIDD (RIP)-associated ICH-I death domain protein
 SCI spinal cord injury
 TBI traumatic brain injury
 TNR-F/R tumor necrosis factor receptor 1
 TRADD TRAF2-associated death domain
 TRAF-2 TNF-R1-associated factor 2

Recent advances in the elucidation of the biochemical machinery of apoptosis have allowed us to appreciate the significance of unfolded protein in a wide range of human diseases and disorders, such as ischemic stroke, traumatic brain injury, Alzheimer’s disease, drug-induced liver injury and cardiac ischemia. One of the main exciting advances in this area has been the discovery that a family of endonuclease-to-proteases (caspases) plays a central role in the apoptotic cascade. Various caspase inhibitors (peptide or protein) have demonstrated abilities to strongly suppress uncontrolled apoptotic cell death both in vitro and in vivo. Thus, there is potential to exploit the therapeutic values of caspase inhibitors.

Key words: apoptosis, caspase, necrosis, procaspase, predomain

Introduction
Cell death can be characterized into two distinct mechanistic forms: namely, necrosis and apoptosis [1]. Necrosis usually results when cells are subjected to extreme physical stress or chemical challenge to a point where they are beyond repair [2,3] and necrosis is generally associated with massive calcium and sodium influx, cell and mitochondria swelling, and non-specific nuclear DNA and cell membrane rupture. Apoptosis, on the other hand, is a term for gene-directed, programmed cell death that is necessary to dispose of unwanted or damaged cells. It is characterized by nuclear DNA condensation, DNA fragmentation at the nucleosome linkage regions, cell shrinkage, and the formation of apoptotic bodies, which are rapidly recognized and phagocytosed by macrophages or adjacent endothelial cells [2,3].

In the past, unscheduled cell death associated with pathological conditions, such as ischemia, was thought to be necrotic in nature. More recently, however, in part due to our better understanding of the biochemical events that underlie apoptosis, better biochemical and immunological methods to positively identify apoptosis have become available [3]. Consequently, apoptosis has been identified as a component of various pathological conditions. One of the first examples was reported by Linusk and colleagues [4], who identified evidence for apoptosis in a rat focal cerebral ischemia model, based on the presence of DNA laddering. Since then, similar findings have been reported in a global cerebral ischemia model [5], experimental traumatic brain injury (TBI) [6], spinal cord injury (SCI) [7] and various chronic neurodegenerative conditions. It has also been noted in cardiac ischemia [8] and non-steroidal anti-inflammatory agent (NSAID)-induced liver damage [9]. Since apoptosis progresses by a stepwise activation of apoptosis cascade components, it follows that somewhere within the cascade lies one or more excellent targets for therapeutic intervention.

The initial identification of the CED-3 protein, required for apoptosis in the nematode Caenorhabditis elegans, and homologous to the mammalian interleukin-1β-converting (ICE) enzyme [10,11], led to the discovery of a large family of CED-3-related cysteine proteases (referred caspases). Their roles in mediating mammalian apoptosis have now been confirmed [10]. The focus of this review is on the classification of human caspases and their relative role in apoptosis the structure-function relationship of caspase-3 and the current trends in caspase inhibitor discovery and development.

Caspase classification and structure-function relationship
Caspases are intracellular cysteine proteases, which generally exist in cells as inactive proenzymes (zymogens). The zymogen prodomain is organized into three regions: an N-terminal 'prepeptide' region, a large subunit region and a C-terminal small subunit region. Zymogenes are transformed into active enzymes by hydrolysis at specific aspartate residues. The prodomain is cleaved into a large subunit and a small subunit; two large and two small subunits can combine to form a heterotetrameric complex, which subsequently cleaves other substrates. Up to 14 human caspases have been identified [10] which are involved in the proteolysis cascade. Caspases can be divided into three categories: (i) initiator caspases, (ii) effector caspases, and (iii) cytoplasmic processing caspases (Table 1).

Initiator caspases are activated via protein-protein association with thrombospondin-bound receptors, such as caspase-8 and caspase-10, or a cytochrome c binding protein, apoptosis-protein-activating factor (Apaf-1), caspase-9. They are acceptors of the initial proapoptotic signal and form a dimer by proteolytically processing and activating the effector caspases (caspase-2, -3, -5, -6, -7, -12 and -14). Caspase-3 proenzyme is a single polypeptide (32 kDa) that exists in most cells, including neurons. It is processed and activated by caspase-8 or 9 to a
heterotetrameric form (17 kDa + 12 kDa), [106] (Figure 1). The three-dimensional crystal structure of caspase-3 has been solved [11]; the substrate binding cleft is lined with residues from the p17 (residues 29 to 75) and p12 (residues 175 to 227) subunits, including the active site Cys^175, which lies within the conserved pentapeptide sequence (residue 161 to 166, Gin-Ala-Cys-Asp-Cys, QACRG) and the His^180 from p17. Effector caspases degrade a subgroup of cellular protein substrates (Table 2) resulting in destruction of cell cycle proteins, DNA repair enzymes, cytokines, and the activation of endonuclease. Caspase-2 is atypical in that it is activated directly via association with the tumor necrosis factor-α (TNFα) receptor coupled adaptor protein pair: receptor-interacting protein (RIP)-associated K6-1 death domain protein (RAIDD). However, it is capable of fulfilling functions as an effector casapse.

The last category, the cytokine processing caspases, primarily function to process and activate interleukins, such as IL-3 and IL-18. This group includes caspase-1, -4, -5, -11, and -13. Although the role of caspase-1 in certain forms of apoptosis can not be ruled out, the present review will focus on the initiator and effector caspases only.

Table 1. Caspase classifications.

<table>
<thead>
<tr>
<th>Category</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initiator caspases</td>
<td>Caspase-8, -9 and -10</td>
</tr>
<tr>
<td>Effector caspases</td>
<td>Caspase-2, -3, -5, -6, -7, -11, -12 and -14</td>
</tr>
<tr>
<td>Cytokine-processing caspases</td>
<td>Caspase-1, -4, -5, -11 and -13</td>
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Table 2. Selected effector caspase substrate.

<table>
<thead>
<tr>
<th>Substrate category</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoskeletal proteins</td>
<td>reseptin, β-actinin, vimentin, talin, actin</td>
</tr>
<tr>
<td>Signal transduction enzymes</td>
<td>CaM-PKIV, CaMKII, PKCε, PKCβ, PP2A(b lakes), PLA, FAK</td>
</tr>
<tr>
<td>Cell cycle proteins</td>
<td>Rb, MDM2, p21, p27</td>
</tr>
<tr>
<td>DNA repairing enzymes</td>
<td>PARP, DNA-PK(cs)</td>
</tr>
<tr>
<td>Apoptosis proteins</td>
<td>Bid-2, Bcl-2, IAP, cypstatin</td>
</tr>
<tr>
<td>Disease-associated proteins</td>
<td>Huntingtin, APP, prelactogen</td>
</tr>
</tbody>
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**Figure 1. Caspase structure schematics.**

The structure and key functional residues are illustrated using caspase-3 as an example. Procaspase-3 has prodomain p17 and p12 subunits, and is activated by a two-step process. In the first step, proteolytic cleavage between p17 and p12 occurs, which is followed by further cleavage between the prodomain and p17, generating the fully activated oligomeric form (p17 + p12). It is believed that caspase-8 and caspase-9 are activated by oligomerization induced by binding to Fas-associated death domain (FADD) and Apo-1, respectively, via their prodomains.
Mitochondria and caspase-9-mediated pathway

Research has focused on mitochondria as important organelles in the initiation phase of apoptosis [12]. One class of apoptosis suppression proteins, called Bcl-2, resides primarily on the outer membrane of mitochondria, where it exists as a homodimer. A subclass of Bcl-2-like proteins, which contains a pro-apoptotic domain called BID (e.g., Bax and Bid), normally reside in the cytosol. However, once triggered by an apoptotic signal (via an as-yet-unknown mechanism), they translocate to the outer membrane of the mitochondria and heterodimerize with BID-2 and negate the pro-apoptotic effects of Bcl-2 [13] (Figure 2). In addition, Bax can form ion channels and thus may alter mitochondrial permeability [13]. The net result appears to be the release of loosely associated cytochrome c from mitochondria, which has been demonstrated in a number of experimental apoptotic systems [14-16]. Cytochrome c, in the presence of dATP, will associate with Apaf-1. The cytochrome c/Apaf-1 complex then associates with the procaspase-9 (casp-9) and induces its dimerization and autolytic activation (17,18) (Figure 2). The activation of effector caspase-3 by caspase-9 has been well-documented [18], and recent studies have demonstrated that caspase-9 knockout mice have severe impairment in neural development as a direct result of improper processing of caspase-3 and neuronal apoptosis [19].

A Bcl-2 family protein, Bax [15], can act downstream from cytochrome c release to inhibit apoptosis [20], since Bcl-2 interacts with the cytochrome c/Apaf-1 complex and prevents its association with procaspase-9 [21]. Bcl-2 subfamily members, such as Bax, can sequester Bcl-xL and thus prevent its protective effects. Also of interest are two internal suppression mechanisms: (i) direct phosphorylation; and (ii) inactivation of Bax and caspase-9 by Akt (protein kinase B) via the prosurvival PI-kinase pathway [22,23,24].

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Figure 2. Mitochondria-mediated and receptor-mediated pathways of apoptosis.

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Two distinct pathways exist for the activation of the caspase cascade. The first pathway begins with Bax or Bcl-2 translocation to mitochondria, which destabilizes them and results in the release of cytochrome c. Cytochrome c then forms a complex with Apaf-1 to activate caspase-9. The second pathway begins with ligand binding to membrane receptors, which transduces a cell death signal. For example, the activated receptor TNF-R1 binds the adapter protein pair, TRADD-FADD, and subsequently caspase-8, inducing its activation. Caspase-8 and -9 converge on the activation of caspase-3 and other effector caspases. Oncotek also exists via Bcl-2 and Bcl-xL cleavage. Activated caspase-3 executes the cell death program by cleaving a number of structural and signal transduction proteins, as well as those that feed toward apoptosis, e.g., Bcl-2, BAD, and LC3.
Mitochondria permeability transition (MPT) has been observed in a range of apoptotic and necrotic models [24]. The MPT pore might facilitate the invasion of the apoptosis cascade, with Bax binding to the pore and inducing MPT directly [25]. The occurrence of MPT under some conditions appears to be a late event in apoptosis. Some MPT blockers, such as bongkrekic acid and cyclosporine A, in combination with the phosphatase 2A inhibitor, aurovertin acid, are effective against some forms of apoptosis and neuronal injury [26].

The death receptor-mediated caspase-8/caspase-3 pathway
Caspase-3 activation can also be triggered by a receptor-mediated pathway. The so-called death receptors include TNFR-1 receptor (TNFR-1), nerve growth factor (NGF) p75 receptor and Fas (FN) which generally cause a death domain. Many cell types express TNFR-1 (55 kDa), which binds to TNFRs, TNFR-1, when activated, couples to the TNF-R1-associated death domain protein (TRADD) (Figure 2). TRADD can interact with TNFR-1-associated factor 2 (TRAF2) or with another adapter protein called Fas-associated death domain protein (FADD). FADD triggers apoptosis by association with procaspase-8, or the more obscure procaspase-10 (10 kDa). Upon binding to FADD (Figure 2), procaspase-8 (85 kDa) appears to autoregulate its activity for its activated form (85-20 kDa). Like caspase-9, TRADD-FADD-caspase-8 interaction induces the autoregulatory activation of caspase-8, which in turn processes the downstream caspase, such as caspase-3 (20-15 kDa) (Figure 2). Thus, FADD-caspase-8 interaction is inessential for essential for TNF-mediated apoptosis [5]. An opposing pathway exists to prevent uncontrolled apoptosis. TRADD/TRAF2 can activate NF-kappaB, nuclear kinase (IKK), which leads to phosphorylation/degradation of I{kappa}B and thus allows NF-kappaB to translocate to the nucleus and transcription of anti-apoptotic genes [25]. The Fas ligand-Fas receptor pathway is similar to that of TNFR-1, except that Fas-induced FADD directly activates caspase-8. In addition, receptor-mediated caspase-8 activation is amplified by the mitochrondria pathway [50]. First, Bid, which when truncated by caspase-8, translocates to mitochondria and helps to release cytochrome c [24,25]. Then, cytochrome c activates caspase-8 to cleave Bid into a 30-kDa fragment, thus activating the mitochondria-mediated pathway [56].

As mentioned previously, the activation of caspase-8 takes place via a different pathway. Activated TNFR-1 could associate with the adapter proteins RACK1, which associates with a complex of protein and caspase-8 and FADD, which activates caspase-8 (Figure 2). The activation of caspase-8 apparently occurs in two steps, firstly the proteolytic processing that cleaves (IETD-Glu26 to separate the p20 and p12 subunits, although they are similarly processed in the cytosol). A second processed form is then released. The N-terminal between D2 and E6 forms the p32 subunit to form a p32+ + p12 subunit, with an active fragment towards protein [38]. This second active fragment specifically determines caspase activity as well as apoptosis. As the p32 subunit in the F2l and F4 positions, which forms specific oligosaccharides with several protein kinases (PKC), is believed to be an essential component of the interaction of the substrate, leaving them as a fingerprint for the activation. The first caspase-3 subunit is then identified with poly(A,D)D-choline polymers (PAPR), with a major cleavage site of DEVD, which conforms to DDB domain. Mitochondria-mediated apoptosis can be regulated by the pro-apoptotic and anti-apoptotic Bcl-2 family, involving calpain inhibition protein calpains, degradation of ICAD, thus unlocking the activity of the Dras CASO [60] (Figure 2); and (c) proteins involved in pathological conditions, such as premature 1 and 2 and amyloid precursor protein (APP), which is involved in the Alzheimer's disease. Huntington and other glutarimide expansion proteins (in Huntington's disease and other CAG repeat diseases [44-44,45-46]). It should be noted that caspase-3, which is in the body, is essential for the function of the different cell types. Some caspase-3 activity has been demonstrated in the central nervous system (CNS) [51]. Caspase-3 knockout mice showed normal development, except for their neural system, in which an extensive number of neurons were found [55]. Taken together, these data suggest that caspase-3 might have a unique role in neural apoptosis and may be exploited therapeutically. Caspase-3 activation has been demonstrated in excisional NOA/kidney and oxygen-glucose deprivation challenged cardioblasts, neurons, 2-methyl-4-phenylpyridinium (MPP+) toxicity to cerebellar granule neurons, and in an in vivo model of DBA/174,47,52.

Caspase inhibitors
A number of inhibitors of caspases are available [28]. A major design strategy has focused on mimicking a mimic of the aspartic protease, found in the I family of many endogenous substrates. General caspase inhibitors (Figure 3), such as celastrol (Cel-CLC) (2,3-dihydrobenzene (H, Z-DBCh), Boc-Asp-(O-methylester) (2,2-thio-Asp- methyl), Z-VA-DCV (8) and 7-VA-DCV have been reported. This design has also been used for selective inhibitors such
as the caspase-1-selective Ac-YVAD-CHO (4), the caspase-3/7-selective Ac-DEVD-CHO (5), Ac-DEVD-flk1 and the caspase-8/9-selective Ac-IETD-flk1 (28,47,52,53,54). Protocols for procaspase inhibitors are commercially available in cell penetrating versions, formed by esterifying the side chain carboxylic acids of Asp and Glu residues. Whilst these are indeed more cell permeable, a possible trade-off might be a decrease of either potency and/or selectivity should the water bonds remain partially or wholly intact inside the cells.

These early caspase inhibitors inhibited apoptosis in culture [47,52,54], although whether the caspase-inhibited cells will survive or their death will merely be delayed remains a concern. In vivo data show that Z-VAD-flk, Z-DEVD-flk (6) and Z-DCD (7) and Z-DCB (8) offer significant and sustained protection against ischemia-induced neuronal apoptosis in several focal-ischemic brain injury models (28,57,58). The key strategy in caspase inhibitor design has been to preserve the vital N1-P1 (Asp) interaction, whilst using peptidomimetic and computer-aided drug design [50,61] to improve chemical and physico-chemical stability, potency and selectivity towards subgroups of caspases. Several pharmaceutical companies, e.g. Vertex Pharmaceuticals, have patent applications for caspase-1 and/or other caspase inhibitors [10,105]. The potent caspase-1 inhibitor, BI-9504, VD-15898 (9) Vertex Pharmaceuticals Inc. (Figure 3), with an IC₅₀ = 0.5 nM, is being developed by Hoechst Marion Roussel as the cyclic-DEI prodrg, HMR 3480/VX-710 (8), Figure 3) currently in phase II clinical trials for rheumatoid arthritis.

Natural caspase inhibitor proteins exist, such as the croupox viral protein cytoplasmic response modifier A (CrmA) and the baculovirus protein p35 [28,47]. CrmA is potent against caspase-1, -6 and -9, but not caspase-3, -7 or -10. There is a group of endogenous caspase inhibitor proteins called inhibitors of apoptosis proteins (IAPs). These are present in a wide range of organisms, from insects (Drosophila) to humans (cIAP1, cIAP2) and the homodimeric X-linked XIAP that apparently directly inhibits caspase-3 and -7 [64-67]. A mammalian gene encoding for a neuronal apoptosis inhibitory protein (NAIP) is homologous to the IAPs [65]. The NAIP gene is partially deletions in individuals with spinal muscular atrophy (SMA), a disorder that is manifested by inappropriate apoptosis of normally occurring motor neurons [66]. This points to the

Figure 3. Commonly used caspase inhibitors.
anti-apoptosis nature of NAIP. Overexpression of XIAP potentially inhibits apoptosis of granule neurons in culture [67]. Neuronal apoptosis due to NAIP has been shown to cause ischemic damage in the rat hippocampus [68]. Now that gene therapy approaches are becoming more feasible, exposing out-of-control pro-apoptotic proteins might be a viable strategy to suppress caspase activation.

Caspase-3 expression has recently been shown to be induced under various injury-related conditions, e.g. in rat kidney after ischemia-reperfusion injury [69], in the CA1 region in a rat global forebrain ischemia model [70], and in the hippocampus after transient cerebral ischemia [71]. Increased caspase expression was also found in cells undergoing apoptosis [72]. Since procaspase-3 is present in resting cells, increased caspase-3, while probably non-essential, would contribute apoptosis. Caspase-3 mRNA is elevated in a global ischemia model [73] and is activated in PC12 cell apoptosis [74]. It remains to be seen if suppressing induction of caspases is a viable therapeutic strategy.

Perspective and prospective

In summary, it is apparent that the inhibition of caspases offers tremendous potential, since it is rare that a single family of proteins can contribute drug targets for such a large number of diverse diseases or disorders. Clearly, more in vivo mechanistic and efficacy studies with more potent and caspase species-specific inhibitors are needed to realize their true therapeutic value. However, one also needs to bear in mind that apoptosis occurs physiologically and almost constantly in adult humans and even more so during development (e.g. in immature brain). Chronic suppression of caspases obviously adds to risk, so it could promote tumorogenesis, suppression of tissue development or even regeneration. Thus apoptosis inhibitors might be better suited for use in short-term treatment paradigms against acute pathological conditions such as cerebral ischemia and cardiac ischemia. Extended treatment with a caspase inhibitor for chronic diseases, such as Alzheimer's disease, might result in serious and unwanted side effects or toxicity. However, it might be possible to partially suppress caspase activity to achieve a benefit-risk scenario. Further preclinical studies are clearly needed to address these issues.

Acknowledgements

I would like to thank my colleagues past and present (including Dr. Kim McGirr, Ms. Rathi Noush, Dr. Razaq Razaq, Dr. Reza (Pemantur, Dr. Ismail Hajjimohammadza and Dr. Pei-Wu Yen) and my collaborators Dr. Richard Gilbertson, Dr. Ronald Hayes and Dr. Margaret Gaggy for their original contributions. I would like to give a special thank you to Dr. Catherine Kosian for her advice on caspase inhibitors. I would also like to apologize to the many researchers whose work I have not cited due to space restriction.

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