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Review

Caspase inhibitors: viral, cellular and chemical

BA Callus*,[1] and DL Vaux[1]

Caspases, key mediators of apoptosis, are a structurally related family of cysteine proteases that cleave their substrates at aspartic acid residues either to cause cell death or to activate cytokines as part of an immune response. They can be controlled upstream by the regulation of signals that lead to zymogen activation, or downstream by inhibitors that prevent them from reaching their substrates. This review specifically looks at caspase inhibitors as distinct from caspase regulators: those produced by the cell itself; those whose genes are carried by viruses; and artificial caspase inhibitors used for research and potentially as therapeutics.
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The first caspase inhibitor was discovered before caspases were termed 'caspases', and before caspases were known to be the key executioners of the cell death program. Caspase 1, initially called interleukin 1β converting enzyme (ICE), was identified not because it was involved in cell death, but because it is responsible for cleaving and thereby activating interleukin 1β, a pro-inflammatory cytokine.[1,2] The first caspase inhibitor was cytokine response modifier A (CrmA), a product of cowpox virus that inhibits ICE activity.[3]

With the cloning of the CED-3, which is essential for programmed cell death in *Caenorhabditis elegans,* and recognition of its similarity to ICE,[4] it became clear that caspases were required for cell death in the worm, and therefore that caspase inhibitors might prevent worm cells from killing themselves. Because the mechanisms of programmed cell death in the worm were similar to those for apoptosis of mammalian cells,[5] caspase inhibitors could presumably prevent the death of mammalian cells as well.

The only caspase inhibitor known at the time, CrmA, was therefore tested for its ability to prevent cell death, and indeed, it was able to inhibit death of mammalian cells triggered by overexpression of caspase 1.[6]

The study of other viruses led to the identification of further caspase inhibitors, and pointed the way to identification of their cellular homologues. As several of the viral caspase inhibitors act through binding of pseudosubstrate loops in the catalytic site, study of viral caspase inhibitors has also provided useful examples for the design of artificial caspase inhibitors.

CrmA

Viruses that bear genes for caspase inhibitors have been positively selected either because the caspase inhibitors prolong host cell viability, increasing the time for viral replication or because caspase inhibition prevents the generation of inflammatory cytokines as part of the host's immune response.

CrmA inhibits caspase 1, thus preventing the generation of active cytokines interleukin 1β and interleukin 18.[7,8] In addition to inhibiting mammalian cell death caused by artificial overexpression of caspase 1, it was later found that CrmA could inhibit apoptosis triggered by tumour necrosis factor (TNF) treatment or ligation of Fas receptors.[9–12] In retrospect, however, it has turned out this was due to inhibition of caspase 8 by CrmA, rather than by inhibition of caspase 1.

In addition to inhibiting caspase 1 with an inhibitory constant (K_i) in the low picomolar (pM) range (Table 1), CrmA can also efficiently inhibit caspase 8 and in humans caspase 10, and can thereby often prevent death of cells signalled by ligation of a subset of the TNF receptor super family members known as 'death receptors' that signal via FADD and caspase 8.[32] CrmA is not a strong inhibitor of other caspases, such as caspase 3, 6 and 7 ($K_i > 100$ nM; Table 1), and is therefore unlikely to be expressed at high enough levels to inhibit these capases *in vivo*.[8]

Although CrmA inhibits caspases, which are cysteine proteases, by structure and mechanism of action it is a member of the serine protease inhibitor (serpin) family, whose other members, as their name implies, inhibit serine proteases, rather than cysteine proteases. The pseudosubstrate residues (P_4-P_1) of CrmA, LVAD, bind to the caspases' active sites (Figure 1a). Unbound CrmA is relatively unstable, but is significantly more stable while bound to caspase 1. It is thought that some CrmA molecules are cleaved and subsequently released from caspase 1, whereas other CrmA molecules are not cleaved but form an irreversibly stable complex.[13,33]

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[1]Department of Biochemistry, La Trobe University, Victoria 3086, Australia
*Corresponding author: BA Callus, Department of Biochemistry, La Trobe University, Victoria 3086, Australia. Tel: +61 3 9479 1669; Fax: +61 3 9479 2467; E-mail: b.callus@latrobe.edu.au
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Abbreviations: ICE, interleukin 1β converting enzyme; CrmA, cytokine response modifier A; TNF, tumour necrosis factor; IAP, inhibitor of apoptosis protein; BIR, baculoviral IAP repeat
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Table 1 Specificity of viral and cellular caspase inhibitors

Caspase inhibitor	Source	Target caspase(s)	Strength of inhibition (where known)	Reference
CrmA	Cowpox virus	Caspase 1	K_i 4–10 pM	13–15
		Caspase 8	$K_i < 340$ pM	
		Caspase 10	K_i 4–17 nM	
P35	Baculovirus	Broad spectrum, for example, inhibits caspase 1, 3, 6, 8, 7 and 10	K_is 0.1–9 nM	14,16
P49		Broad spectrum		17,18
		Caspase inhibitor, like p35, but also inhibits initiator caspases DRONC and caspase 9		
OpIAP	Baculovirus	Does not directly inhibit caspase activity		19
DIAP1	*Drosophila*	DRONC	Powerfully inhibits DRONC activation; activity against DrICE may be weak	20–23
		DrICE		
XIAP	Mammalian	Caspase 3	IC50 0.1–2 nM	24–28
		Caspase 7	IC50 1–10 nM	
		Caspase 9	IC50 10 nM	
cIAP1	Mammalian	Does not directly inhibit caspases at physiological concentrations		29
cIAP2	Mammalian	Does not directly inhibit caspases at physiological concentrations		29
ILP-2	Human	Does not directly inhibit caspases at physiological concentrations		28
ML-IAP	Mammalian	Does not directly inhibit caspases at physiological concentrations		30
Survivin	Mammalian	Does not directly inhibit caspases at physiological concentrations		31
NAIP	Mammalian	Does not directly inhibit caspases at physiological concentrations		29
BRUCE	Mammalian	Does not directly inhibit caspases at physiological concentrations		29

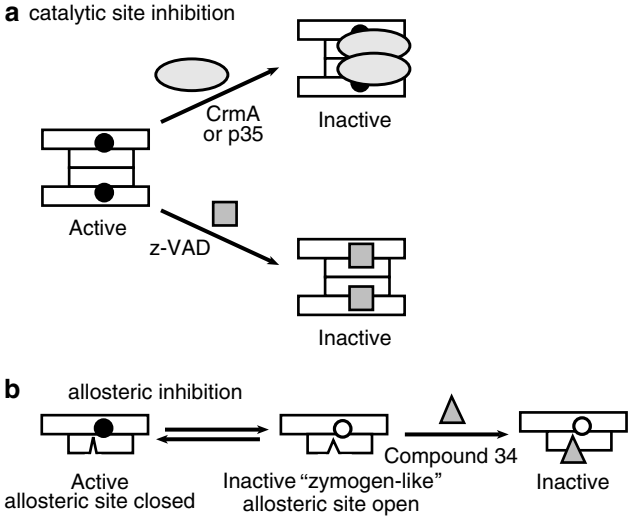

Figure 1 Catalytic site and allosteric site inhibition of caspases. (**a**) The viral inhibitors, CrmA and p35, as well as synthetic peptidic inhibitors for example, z-VAD-fmk, bind as pseudosubstrates to the active catalytic sites (solid circles) of target caspases thereby inhibiting their catalytic activity. (**b**) Following zymogen activation caspases are in equilibrium between active (allosteric site closed) and 'zymogen-like' inactive (allosteric site open) conformations. Compound 34 (triangle) binds to the open allosteric site in the dimeric interface thereby trapping the enzyme in an inactive state. Inactive catalytic sites are represented with open circles. For simplicity only caspase monomers are shown. See text for details

P35

The next caspase inhibitor to be identified was, like CrmA, a viral gene product, but it did not come from viruses that infected mammalian hosts, but from a virus that infects the Alfalfa looper, *Autographa californica*. P35,[34] and the related p49 from *Spodoptera littoralis* nucleopolyhedrovirus[35] inhibit a broad range of caspases.[14,17,36–38] P35 not only inhibits insect caspases, but also inhibits many mammalian caspases, mostly with Kds in the low nanomolar range[14] (Table 1). It is least effective at inhibiting caspases 2 and 9,[39] and is also a relatively weak inhibitor of DRONC, the caspase 9-like initiator caspase from *Drosophila*, whereas p49 seems capable of inhibiting these caspases as well.

To inhibit caspases, p35 is first cleaved at the exposed reactive site loop P_4-P_1 residues DQMD, but the cleaved polypeptides do not dissociate, as a covalent thiol linkage is formed with the caspase's catalytic cysteine.[14,40,41] Structural studies of p35 have shown the conformation of this loop and the preceding alpha-helix are essential for caspase inhibition.[36,37]

IAPs

The first inhibitor of apoptosis protein (IAP) was discovered as baculoviral gene product that could complement *Autographa californica* nuclear polyhedrosis virus for loss of p35.[42] They, and cellular homologues discovered subsequently, all bear from one to three baculoviral IAP repeats (BIRs), a novel zinc binding fold of about 70 amino acids.[43] Although the first IAP described, OpIAP, is able to inhibit cell death, and able to complement AcNPV for mutation to p35, OpIAP does not directly inhibit the catalytic activity of caspases, but presumably acts upstream to prevent activation of caspase zymogens.[19]

NAIP was the first mammalian IAP to be discovered, but rather than having a role in regulation of cell death, it is a

member of the NACHT-LRR/NOD-LRR/CATERPILLAR/CLAN family[44,45] that appears to function in providing innate immunity to infection by bacteria such as *Leigonella pneumophila*.[46,47] Although some initial reports suggested it might be able to inhibit caspases *in vitro*, subsequent studies have shown NAIP cannot directly inhibit caspases at physiological concentrations.[29]

Similarly, although Survivin, an IAP that acts together with aurora kinase B and the inner centromere protein during mitosis, was initially proposed to inhibit caspases,[48] it is no longer thought to be able to do so.[31]

XIAP, which bears three BIRs, is the most thoroughly characterized mammalian IAP, and also the most potent inhibitor of cell death *in vitro*. In interactions involving BIR2 and the flanking region just N-terminal to it (the BIR1/BIR2 linker region), XIAP can inhibit caspase 3 and caspase 7 with low nanomolar efficiency[24–26] (Table 1, Figure 2a). The main interaction involves insertion of an aspartic acid residue from the region flanking BIR2 into the active site of the caspases, but orientation of the inserted peptide is the reverse of that adopted by caspase substrates when they enter the catalytic site.[26]

As well as inhibiting caspase 3 and 7 by this mechanism, XIAP can also inhibit caspase 9 by a different mechanism, in which the third BIR binds to the processed amino-terminus of monomeric caspase 9, to prevent its dimerization, which is required for caspase activation. In this way, XIAP inhibits caspase 9 with an IC50 of around 10 nM.[27,28]

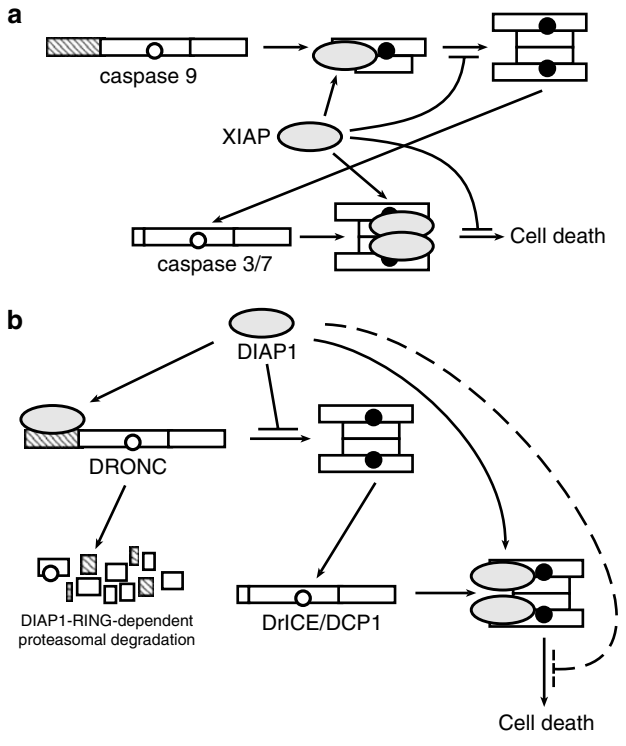

Figure 2 Caspase inhibition by IAPs. (**a**) XIAP can bind to the processed end of the large subunit of caspase 9 thereby preventing its dimerization and subsequent activation. XIAP can also bind to the active catalytic sites (solid circles) of effector caspases, caspase 3/7, thereby blocking their activity. (**b**) DIAP1 neutralizes DRONC by binding to its pro-domain (hatched box) resulting in DIAP1-RING-dependent degradation of DRONC. DIAP1 can also bind to the processed ends of effector caspases, DrICE and DCP1, but may or may not inhibit their activity (indicated with a dashed line). See text for details

Like XIAP, cIAP1 and cIAP2 have three BIRs and a RING domain. However, although cIAP1 and cIAP2 are able to bind to caspases, they are not able to inhibit them in physiological circumstances, because their K_i are greater than 5 μM,[29] so their ability to inhibit apoptosis is probably indirect rather than by the direct inhibition of caspase activity. Possible mechanisms include: binding to IAP antagonists such as Smac/Diablo, to reduce the amount available to antagonize XIAP, thereby allowing more XIAP molecules to be free to inhibit caspases; or influencing signalling by NF-κB and MAP kinases, or targeting caspases for ubiquitylation and proteasomal degradation.[29,49]

The strongest evidence for a role of IAPs in controlling apoptosis comes not from mammalian experiments but from *Drosophila*. Whereas deletion of genes for XIAP in the mouse has a very minor phenotype,[50] deletion of DIAP1 in the fly results in massive, lethal apoptosis.[51] However, even though they are similar, DIAP1 and XIAP do not act in precisely the same ways. For example, XIAP inhibits caspase 9 activation by binding, via its BIRs, to N terminally processed caspase 9, thereby stopping it from dimerizing. DIAP1, on the other hand, binds to the unprocessed form of DRONC, the *Drosophila* homologue of caspase 9[20] (Figure 2b). Although just the BIRs of XIAP are sufficient to inhibit caspase 9 activation, DIAP1 requires not only its BIRs that bind to DRONC, but also the E3 ligase activity of its RING domain in order to neutralize it.[52] DIAP1 can also bind to processed effector caspases such as DrICE and DCP1 via conserved IAP-binding motifs (IBMs) in the neo-amino-termini of the large caspase subunits.[53] Although some groups report DIAP1 inhibits DrICE,[21] another group suggests that, like cIAP1 and cIAP2, DIAP1 may not inhibit the proteolytic activity of effector caspases even while bound to them.[53]

Other Caspase Regulators

In addition to direct inhibitors of caspase activity, several other regulators of caspases have been described that act upstream by indirect mechanisms, rather than by directly blocking catalytic activity. Although these regulators are not the focus of this review, they remain nonetheless extremely interesting proteins by virtue of their potential to modulate caspase activity. For example, using the yeast two hybrid system with the pro-domain of caspase 8 as bait, McDonald *et al*. reported the identification of caspase 8 and 10 associated RING proteins (CARP1 and CARP2) as inhibitors of apoptosis triggered by death receptor ligation.[54] Their mechanism of action is yet not fully elucidated, but it is likely that CARPs (also known as human Ring Finger homologous to IAP type or hRFIs[55]) do not directly inhibit caspase proteolytic activity, but may prevent dimerization of procaspases, preventing their activation, or, because they bear a C-terminal RING domain, they may ubiquitylate caspases 8 and 10 and target them for degradation by the proteasome. Similarly, in *Drosophila*, proteins such as dBruce, a BIR bearing protein and the RING protein Defense repressor 1 (Dnr1) might also be capable of regulating caspases via as yet undetermined mechanisms.[56,57]

Synthetic Caspase Inhibitors

Synthetic caspase inhibitors have been developed both as research tools, and with the hope that they may eventually be used to prevent cell death in the clinic, where they would be of tremendous benefit if they could reduce cell loss in ischaemic vascular diseases, neurodegenerative diseases, and in organ transplant surgery.

Because caspases are cysteine proteases, any inhibitors that can react with the catalytic cysteine, for example, iodoacetamide, are also capable of inhibiting caspases. However, inhibitors that are specific for caspases are more useful for research, and only these could be used in the clinic.

A number of specific and broad-spectrum peptide caspase inhibitors have been developed, the first based upon caspase substrates, for example, the YVHD cleavage site in pro-IL1β.[58] These inhibitors act as pseudosubstrates for active caspases (Figure 1a) and are therefore competitive inhibitors. Peptides have been linked to chemical groups that serve to block the amino acids and to improve cell permeability, stability and efficacy. These peptide inhibitors range from a single *O*-methyl-aspartate residue (e.g. benzyloxycarbonyl-aspartyl(OMe)-fluoromethylketone: Boc-Asp-FMK) to tri-peptides (e.g. Boc-val-ala-asp(OMe)-fluoromethylketone: z-VAD-FMK) and tetrapeptides (e.g. YVAD-FMK).

Peptides linked to aldehydes (or nitriles or ketones) are reversible inhibitors (e.g. Ac-DEVD-CHO, Ac-YVAD-CHO) and bind to the catalytic site, but do not irreversibly chemically alter the enzyme. Peptides linked to leaving groups such as halomethylketones (chloro or fluoro), acylomethyl ketones and (phosphinyloxy) methyl ketones are irreversible inhibitors. It has been proposed that irreversible inhibitors (e.g. Ac-DVAD-FMK) bind via a thioester linkage to a transition state oxyanion and the carbonyl oxygen occupies an oxyanion hole in the caspase transition state.[59,60] Peptide inhibitors display a wide range of specificity and potency against caspase activity with dissociation constants ranging from pM to low micromolar (μM) concentrations (Table 2).

Most synthetic caspase inhibitors are hydrophobic and not very permeable, and could cause nonspecific toxic effects when added at concentrations required to inhibit intracellular caspases. The addition of a carboxy-terminal O-phenoxy group together with an amino-terminal quinolyl group has been reported to significantly improve the efficacy of peptide inhibitors. For example, quinolyl-valyl-*O*-methylaspartyl-[2,6-difluorophenoxy]-methylketone (Q-VD-OPh) effectively inhibits caspase activity at nearly one-tenth the dose required for inhibition by Boc-D-FMK.[61] The improved leaving properties of the carboxy terminal O-phenoxy group makes this inhibitor more reactive with the active cysteine in caspases, explaining the increased effectiveness of Q-VD-OPh compared with inhibitors linked to fluoromethylketones. The amino terminal quinolyl group alone also decreases the dose of peptide inhibitor required for effect, most likely by increasing cell permeability.

Derivatives of caspase inhibitors as well as caspase substrates have been used as reagents to assay caspase activity. Most commonly caspase substrates are made in which a colorimetric or fluorescent chemical group is linked to the aspartic acid residue of a peptidic caspase substrate so that colour or fluorescence is produced upon substrate cleavage. For example, in DEVD-AFC and DEVD-AMC the AFC (7-amino-4-trifluoromethyl coumarin) and AMC (amino-4-methyl coumarin) groups fluoresce following cleavage of the peptide from the substituted coumarin, whereas conjugation to p-nitroanilide (e.g. DEVD-pNA) generates a substrate that allows caspase activity to be determined colorimetrically.

Sometimes fluorescent groups (e.g. FITC) are linked to the amino-terminal end of an irreversible peptidic caspase inhibitor, for example, as in FITC-DEVD-FMK. Results using these reagents have to be interpreted with care, because they do not give quantitative signals, and their fluorescence is the same in the presence or absence of active caspases. Rather than becoming fluorescent when they bind to an active caspase, these inhibitors gain specificity because they remain bound to active caspases, whereas the unbound inhibitors are washed away. However, because dead cells are much more permeable than those that are alive, it is possible for much more of the reagent to enter them, so a bright fluorescent signal might be indicative of increased uptake, rather than caspase activity. Indeed, the apparent caspase activity in dying yeast[67] is most likely an artifact owing to the increased permeability of dead cells.[68]

Until the effects of synthetic caspase inhibitors are confirmed in cells and ultimately *in vivo*, caution should be exercised when assigning an inhibitor's caspase specificity solely on *in vitro* determined K_is, particularly when the K_is are derived from cleavage assays that utilize non-physiological,

Table 2 Specificity of chemical caspase inhibitors

Caspase inhibitor	Source	Target caspase(s)	Strength of inhibition (where known)	Reference
Q-VD-Oph	Synthetic peptidic	Caspases 1, 3, 8 & 9	IC50 25–400 nM	61
DEVD-CHO	Synthetic peptidic	Strong inhibitor of caspase 3 and caspase 7	$K_i < 2$ nM	15
zVAD-FMK	Synthetic peptidic	Inhibits all caspases, but inhibits caspase 2 very weakly		15
IDN-6556	Synthetic non-peptide, Idun (Pfizer)	Broad spectrum irreversible caspase inhibition	IC50 25 nM	62
Pralnacasan	Synthetic non-peptide (Vertex)	Caspase 1		63,64
M867	Synthetic non-peptide (Merck-Frosst)	Reversible, selective effector caspase inhibitor	K_i 1.4 nM (casp 3) K_i 8.9 nM (casp 7)	65
Compound 34	Synthetic non-peptide (Sunesis)	Targets allosteric site rather than active site	Inhibits caspase 1 and caspase 7 and possibly others	66

peptide-based substrates. Although *in vitro* derived K_is do provide a strong indication of whether an inhibitor is likely to block particular caspases at physiological concentrations, they do not give any indication of possible off-target effects on other enzymes. Ideally, when peptidic caspase inhibitors are used, control experiments should be performed with a compound with the same warhead group, but with a glutamic acid residue in place of the aspartic acid (P1) residue.

Drugs

Modified peptides generally do not make good pharmaceuticals, owing to problems such as proteolysis and poor cellular permeability. In order to develop drugs to inhibit cell death when it occurs inappropriately, such that caused by ischaemic vascular disease or in neurodegenerative disease, pharmaceutical companies have developed non-peptide or peptido-mimetic caspase inhibitors. Most of these act like the peptidic caspase inhibitors, that is, they target the caspase's catalytic sites. Recently, researchers at Sunesis[66] described a different approach, namely to target a cysteine in the caspase's allosteric site so that the drug prevents the caspase from adopting the active conformation (Figure 1b). Their Compound 34 was found in a screen for molecules that formed a thiol linkage with a cysteine in the dimeric interface of caspase 1, and it acts in a way reminiscent of the BIR3 domain of XIAP, which inhibits caspase 9 by binding to the dimer interface to prevent dimerization and activation.

M867,[65] produced by Merck-Frosst, is a potent reversible caspase inhibitor selective for effector caspases 3 and 7. Although it was able to block many of the manifestations of apoptosis when administered *in vivo*, it was ineffective at preventing cell death. These results suggest that irreversible inhibitors, and ones that tackle initiator caspases (e.g. caspase 8, caspase 9) or have broad-spectrum activity, would be more effective *in vivo*.

Idun, recently acquired by Pfizer, has produced a number of broad-spectrum caspase inhibitors (IDN-8066, -7503, -7436, -1965, -6556). IDN-6556 is a potent, irreversible, broad-spectrum caspase inhibitor that strongly inhibited Fas-induced apoptosis *in vitro*, as well as inhibiting liver injury in the mouse *in vivo*. In a Phase I clinical trial, oral IDN-6556 was able to reduce levels of liver enzymes in the blood in patients with liver disease, presumably by reducing apoptosis of liver cells.[69] It is now is in Phase II trials in patients undergoing liver transplantation, with the hope that it will reduce cell death owing to ischaemia–reperfusion injury.[62]

Idun also has described small oxoazepinoindoline caspase inhibitors, IDN-5370 and the more permeable IDN-7866, which prevented apoptosis and promoted the survival of neurons exposed to apoptotic stimuli.[70] IDN-5370 was reported to significantly reduce cortical infarct size in rats subjected to permanent middle cerebral artery occlusion.

Vertex produced an orally active non-peptide caspase 1 inhibitor, pralnacasan, and has completed a Phase IIa clinical trial in osteoarthritis.[71] Unfortunately, a phase IIb trial had to be halted because animal toxicology studies showed liver abnormalities after a 9-month exposure to high-doses.

Conclusions

Our understanding of the mechanisms of action of caspase inhibitors continues to rapidly expand, and new pharmaceutical caspase inhibitors are being developed. Although some pharmaceutical companies appear to have ceased their caspase inhibitor projects, and no caspase inhibitors have yet reached phase III trials, there are still grounds to be optimistic that caspase inhibitors will eventually be developed that have wide application in the clinic. In the mean time, study of both synthetic and natural caspase inhibitors has provided many new biological insights, some of which may also turn out to have clinical implications. Study of IAPs, for example, has prompted the development of IAP antagonists that show great promise as inducers of cell death, rather than as cell death inhibitors.

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