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Review

Caspase inhibitors

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Abstract

Caspases are the key effector molecules of the physiological death process known as apoptosis, although some are involved in activation of cytokines, rather than cell death. They exist in most of our cells as inactive precursors (zymogens) that kill the cell once activated. Caspases can be controlled in two ways. The processing and activation of a caspase can be regulated by molecules such as FADD, APAF-1, Bcl-2 family members, FLIP and IAPs. Active caspases can be controlled by a variety of inhibitors that directly interact with the protease. This review describes the later direct caspase inhibitors that have been identified, products of both viral and cellular genes, and artificial caspase inhibitors that have been developed both as research tools and as pharmaceutical agents to inhibit cell death *in vivo*.

Keywords: caspase; viral inhibitors; peptide inhibitors; IAPs

Abbreviations: crmA, cytokine response modifier A; IAP, inhibitor of apoptosis proteins; ICE, interleukin 1β converting enzyme

Viral and cellular caspase inhibitors

Viruses must co-opt a cell's synthetic machinery in order to reproduce. Therefore a most effective way of preventing viral replication is for an infected cell to kill itself. However, just as apoptosis is used to defend against viruses, viruses carry cell death inhibitors to block this response of their host cells. Several viral cell death inhibitors act by binding to activated caspases.

The first caspase inhibitor to be identified was the Cowpox virus product Cytokine Response Modifier A (CrmA), which was found to inhibit Caspase 1 (Interleukin 1β converting enzyme, or ICE).[1] As crmA was discovered before the role of caspases in apoptosis had been established, it was hypothesized that viruses used CrmA to bind to active Caspase 1 to reduce the defensive inflammatory response triggered by IL-1β.[2] While this is indeed true, with the identification of caspases as the key mediators of apoptosis it also became clear that CrmA could be used to prevent defensive suicide of infected cells, thereby allowing more time for viral replication.[3] These observations also highlighted the close association between inflammation and defensive apoptosis.

CrmA

CrmA can bind to and inhibit Caspase 1 with an inhibitory constant (K_i) of $\sim$10 picomolar and Caspase 8 with a K_i of less than 300 pM (Table 1). In this way crmA can prevent production of mature IL-1β and IL-18 by Caspase 1, and apoptosis triggered by TNF receptor family members, which is mediated by Caspase 8.[4–6] The inhibitory constants indicate crmA is unlikely to inhibit caspases 3, 6, 7 *in vivo*[7,8] whilst there is some discrepancy in results regarding Caspase 10.[8,9] Although it inhibits caspase 9 with a K_i of 2 nM *in vitro*, the fact that lymphocytes from Caspase 9 knock out animals were resistant to dexamethasone, but lymphocytes from transgenic mice expressing crmA were not,[10,11] suggests crmA cannot inhibit Caspase 9 *in vivo*.

These data raise an important point, which is, how much does the *in vitro* determined inhibition constant reflect the physiological role of the caspase inhibitor? The above data might suggest that to be effective *in vivo*, a caspase inhibitor must have a K_i of $\leqslant$1 nM. It should be remembered that the K_i determined *in vitro* may not reflect the activity inside a cell. *In vitro* translated protein may not be properly folded, may lack important post-translational modifications and the physiological relevance of the *in vitro* determined K_i would depend on the relative abundance of the caspase and its inhibitor within the cell.

Structurally, crmA belongs to the serine protease inhibitor (serpin) group, but unlike other serpins, it inhibits caspases, which are cysteine proteases.[1,12] However, like conventional serpins crmA acts as a pseudosubstrate that binds to the active proteases. The pseudosubstrate region of crmA has the residues LVAD (designated P4 - P1). Cleavage of caspase substrates (including crmA) occurs after the aspartate residue (P1).

Many serpins undergo a dramatic structural change from a 'stressed' to 'relaxed' conformation when they interact with serine proteases. While some serpins remain in an inhibitory complex with the protease after they are cleaved, it is thought that some CrmA molecules remain intact after binding to Caspase 1 to form an inhibitory complex, while cleaved CrmA polypeptides are released.[12,13]

CrmA has been genetically engineered in order to make variants that are able to inhibit other caspases,[14,15] To do this the tetrapeptide pseudosubstrate region of crmA (LVAD) was replaced with tetrapeptides that are optimal

Table 1 Inhibition of mammalian Caspases by viral and mammalian Caspase inhibitors

	Inhibitory constants (K_i nM)								
	Viral			Cellular					
	crmA[7,8,12]	p35[20,57]	OpIAP[58,59,60]	XIAP[37,39,41]	cIAP1[37,40,41]	cIAP2[37,40,41]	Survivin[38]	NAIP[40]	PI-9/GBI[17]
Caspase 1	0.004–0.01	9.0	inhibitory[a]	non-inhibitory[a,b]	non-inhibitory[a]	non-inhibitory[a]			non-inhibitory[a]
Caspase 2	>10 000	inhibitory[a]	inhibitory[a]						
Caspase 3	1600	0.1	non-inhibitory[a]	0.7	108	35	inhibitory	non-inhibitory	
Caspase 4	1.0								
Caspase 5	<0.1								
Caspase 6	110	0.4		non-inhibitory[a,b]	non-inhibitory[a]	non-inhibitory[a]			
Caspase 7	>10 000	2.0		0.2	42	29	inhibitory	non-inhibitory[a]	
Caspase 8	<0.3–0.95	0.5		non-inhibitory[a,b]	non-inhibitory[a]	non-inhibitory[a]	non-inhibitory[a]		non-inhibitory[a]
Caspase 9	<2.0			inhibitory[a]	inhibitory[a]	inhibitory[a]			
Caspase 10	17	7.0							
Granzyme B	non-inhibitory[a]	non-inhibitory[a]							Ka 1.7×10^6

[a]Assignment as inhibitory or non-inhibitory is based on functional assays such as fluorogenic substrate cleavage, processing of Caspase or cell survival. No K_i available. [b]Unable to inhibit even when present in 50 molar excess to caspase

substrates for the different families of caspases, or with the four residues from the cleavage site of the baculovirus protein p35 (DQMD). These crmA variants showed altered ability to block caspases and protect against cell death. For example, unlike wild-type crmA, crmA.DQMD was able to inhibit *ced-3* mediated deaths in *C. elegans*,[14] reduce growth inhibition of yeast expressing Caspase 3,[16] and protect lymphoid cells from death due to radiation or dexamethasone.[15]

PI-9

The closest mammalian homolog of crmA is the serine protease inhibitor leukocyte elastase inhibitor, but this serpin does not have an acidic residue in its active (P1) site, and cannot inhibit caspases. The cytotoxic lymphocyte serpin proteinase inhibitor 9 (PI-9) (also known as granzyme B inhibitor (GBI)) is also a close relative of crmA, but is unique among vertebrate serpins in having an acidic residue in its pseudosubstrate region. Although in PI-9 this is a glutamate residue rather than the aspartate found in crmA, it raised the possibility that PI-9 might be able to inhibit caspases. In experiments *in vitro* it turned out that PI-9 does not inhibit caspases, but can inhibit granzyme B. Consistent with this, it can protect against granzyme B-mediated apoptosis, but not killing stimulated by the CD95 pathway, which uses Caspase 8.[17,18]

p35

p35 is a baculoviral protein that can block the defensive apoptotic response of insect cells to viral infection.[19] As well as being able to inhibit several insect caspases, p35 can inhibit *C. elegans* CED-3 (Table 3) and mammalian Caspases 1, 3, 6, 7, 8, and 10 with K_i values of less than 10 nM (Table 1), but does not inhibit non-caspase cysteine proteases or serine proteases. After it is cleaved at its P1 residue (Asp87) by the caspase, p35 forms a complex that can be dissociated by SDS.[20] As the cleaved subunits of p35 remain in an inhibitory complex with the caspases, p35 works in a fundamentally different manner to crmA.

p35 is able to inhibit caspases *in vivo*. In *Drosophila*, transgenic expression of p35 was able to prevent apoptotic death of cells in the developing embryo and eye.[21,22] In *C. elegans*, transgenically expressed p35 could inhibit developmental cell death and block *ced-3* induced cleavage of PARP[14] and in mice, transgenic p35 rendered thymocytes resistant to several apoptosis-inducing agents.[23] In experiments analogous to those varying the pseudosubstrate site of crmA, replacement of the p35 caspase substrate sequence (DQMD) with the reactive site of crmA (LVAD) resulted in p35 inhibiting Caspase 1 and Caspase 8 well, but increased the K_i for Caspase 3 20-fold. Further, this mutant became a substrate for Granzyme B.[20]

IAPs

Inhibitor of apoptosis (IAP) proteins were identified by Lois Miller and colleagues as baculoviral products that could suppress apoptosis of cells infected with a p35-deleted baculoviral strain.[24] Subsequently genetic and sequence based experiments identified a group of cellular IAP homologs in yeasts, *C. elegans*, *Drosophila* and vertebrates, as well as in a number of other viruses (reviewed in Uren *et al*[25] and LaCasse *et al*[26]). All members of this family bear from one to three baculoviral IAP repeats (BIRs), which mediate all interactions between IAPs and other proteins described to date.

Although there is evidence implicating many IAPs in the regulation of apoptosis, for several, such as the IAP in *Autographa californica* NPV, one of the two IAPs in *Orgyia pseudosugata* NPV, no such role has been demonstrated,

and the nature of their true function has not been determined. One group of IAPs, including those from the yeasts *S. pombe* and *S. cerevisiae*, the mammalian protein Survivin, and the IAPs from *C. elegans* (*bir1* and *bir2*), have BIRs that form a structural sub-group.[25] Emerging evidence suggests these IAPs may function in cytokinesis rather than in regulation of apoptosis.[27–29]

Precisely how the anti-apoptotic IAPs function is not known with certainty. Genetic and biochemical experiments in insect systems suggest some IAPs can act via interactions with apoptosis activating proteins such as *grim*, *reaper* and *hid*.[30–35] Recent data suggest *grim*, *reaper* and *hid* are upstream of the IAPs, and that the inhibition of the IAP-caspase interaction is the mechanism by which *grim*, *reaper* and *hid* induce apoptosis.[36] However, there is also biochemical evidence that insect and vertebrate IAPs can inhibit apoptosis by directly binding to the activated caspases themselves. *Drosophila* DIAP1 can inhibit mammalian Caspase 3 (Table 3). In this review of caspase inhibitors we will only discuss the latter activity.

The mammalian IAPs XIAP (MIHA, hILP), MIHB (c-IAP1), MIHC (c-IAP2), NAIP and Survivin have all been reported to be able to bind to and inhibit caspases, but the inhibitory constants of XIAP and Survivin are considerably lower than the other IAPs (Table 1) which may imply more potent (or more physiological) function *in vivo*. XIAP and Survivin are reported to inhibit Caspases 3 and 7 with K_i's of 0.7 and 0.2 nM respectively,[37,38] which is similar to the K_i of crmA for Caspase 8. Deletion analysis of XIAP suggests its middle BIR, BIR2, is necessary and sufficient to bind to and inhibit activate Caspases 3 and 7.[39]

MIHB, MIHC and NAIP on the other hand have not been found to inhibit any caspases with a K_i of less than 29 nM.[40] The fact that this is three times higher than the K_i of crmA for Caspase 9 (which appears to be insufficient for it to inhibit caspase 9 mediated apoptosis *in vivo*) suggests these IAPs mainly act in some other way. Consistent with this notion, MIHB and MIHC were required to be at 100–1000-fold molar excess in order to inhibit Caspases 3 and 7 by more than 50%,[40] and these levels probably never occur *in vivo*.

Survivin is reported to inhibit caspases as potently as XIAP,[38] which is able to block apoptosis triggered by ligation of CD95 (Fas/APO-1), dexamethasone or Bax expression.[41] Curiously, like most transformed cell lines, Jurkat T cells express abundant Survivin,[42] but remain sensitive to killing by ligation of CD95, dexamethasone and Bax expression. As Survivin is expressed in cells that are rapidly dividing (i.e. embryonal tissues and tumor lines but not in adult tissues) and is able to associate with tubulin, Survivin's function may not be to regulate apoptosis but to mediate events during cell division.[27]

When apoptosis is inhibited by XIAP, MIHB or MIHC, the cells do not accumulate processed caspases complexed with the IAPs, but are found to have increased amounts of pro-caspases.[37,40,43] The initial suggestion that this may be due to the IAPs binding Caspase 3 as it became activated, thereby preventing autocatalysis of the remaining pro-Caspase 3 seems not to be correct as activated Caspase 3 is not capable of activating pro-Caspase 3.[44] The finding that the IAPs can also bind pro-Caspase 9[41] may provide the answer to this puzzle, as by doing so they would prevent processing of other, downstream caspases, including Caspase 3. To date the association of IAPs with pro-caspases has not been confirmed in intact cells.

Synthetic caspase inhibitors

As inappropriate apoptosis has been implicated in many diseases, including ischemic vascular diseases (heart attacks, stroke) and degenerative diseases (Alzheimer's disease, motor neurone disease) there has been a tremendous effort to develop caspase inhibitors for pharmacological use. Such synthetic caspase inhibitors are of course also useful for analysis of caspase activity in experimental models.

Caspases are one of about 20 families of cysteine proteases. Some of the cysteine protease inhibitors such as iodoacetamide react with the catalytic cysteine in caspases, and can therefore inhibit them, but these inhibitors are not specific for caspases.

A number of specific caspase inhibitors have been developed based upon the substrate cleavage sites of the caspases. These peptides act as pseudosubstrates for active caspases and are therefore competitive inhibitors. They range from those containing a single aspartate residue (e.g. Boc-aspartyl(OMe)-fluoromethylketone: Boc-Asp-FMK) to trimers (e.g. Benzyloxycarbonyl-val-ala-asp (OMe) fluoromethylketone: z-VAD-FMK) to tetramers (e.g. YVAD-FMK).

The chemical mechanism of action of the synthetic inhibitors is determined by the chemical groups to which the peptides are linked. Linking the appropriate peptide to fluoro- or chloro-methyl ketones (-CMK, -FMK) groups produces irreversible, competitive inhibitors. A thiomethyl ketone II forms with the active site cysteine resulting in inactivation of the enzyme.[45] The halomethyl ketones are highly reactive and may not be entirely specific for caspases. Peptides linked to aldehyde groups (-CHO) (or nitriles or ketones) act as reversible inhibitors. These act as caspase substrates without chemically altering the protease. The rate limiting step for both classes of inhibitor seems to be the enzyme-inhibitor association (reviewed in Thornberry *et al*[46]). While potent *in vitro*, the activity of peptide caspase inhibitors in intact cells is limited by their membrane permeability. The -FMK adducts are much more permeable than the aldehyde based inhibitors, which enter cells poorly, and do not inhibit any caspases in intact cells at concentrations less than 1 μM. This is probably because the -FMK derivative are prepared as methyl esters of the P1 aspartic acid. In general the shorter peptides are more permeable than the tetramers.

The peptide caspase inhibitors are able to block caspase activity *in vitro* with K_i's down to the high picomolar–low nanomolar range (Table 2). The halomethyl ketone-linked peptide YVAD has similar kinetics to the aldehyde linked peptide.[45] Ac-WEHD-CHO, Ac-DEVD-CHO, Ac-YVAD-CHO, t-butoxycarbonyl-IETD-CHO, and t-butoxycarbonyl-AEVD-CHO display a wide range of selectivity and

potencies against caspases, with dissociation constants ranging from 75 pM to >10 μM.[8] The results obtained with peptide-based inhibitors are in accord with those predicted from the substrate specificity studies.[47]

Although these protease inhibitors are more specific than general cysteine protease inhibitors such as iodoacetamide, z-VAD-FMK, z-DEVD-FMK and Ac-YVAD-CMK are all capable of efficiently inhibiting other proteases, such as cathepsin B, both *in vitro* and in tissue culture at concentrations that are generally used to demonstrate the involvement of caspases.[48] YVAD-CHO is probably the most specific of the commonly used caspase inhibitors as it only potently inhibits Caspase.[8,49] The other caspase inhibitors do not target individual caspases. For example, while it is not usually regarded as a broad range caspase inhibitor and most potently inhibits Caspase 3, DEVD-CHO also strongly inhibits Caspases 7 and 8.[8,49] z-VAD-CHO can strongly inhibit Caspases 1, 3, 5, 7, 8, and 9, but is not a good inhibitor of Caspase 2.[8]

In vivo use

While the peptide based caspase inhibitors are first generation drugs that have been useful in cell extracts and in tissue culture, a number of experiments describe their use *in vivo*, and suggest that caspase inhibitors will one day be valuable drugs in reducing cell death in acute situations, such as heart attacks and stroke, and possibly in chronic diseases characterized by cell death, such as neurodegenerative disease. These caspase inhibitors might also be useful in reducing cell death in organs awaiting transplant, or to reduce apoptosis of normal cells caused by chemotherapeutic drugs used to treat cancer.

The first reports of *in vitro* use of caspase inhibitory drugs were in models of hepatitic in which lever damage was induced by anti-CD95 antibodies. Intravenous injections of z-VAD-FMK prevented signs of liver damage and allowed all mice to recover from a treatment that was otherwise fatal within 3 h.[50] Another group found that mice pretreated with z-VAD-FMK were protected from liver injury caused by ligation of CD95 or from TNFα induced liver injury.[51] YVAD-CMK was also reported to inhibit hepatic apoptosis induced by an anti-CD95 antibody or TNF administration.[52]

In a myocardial ischemia model intravenously administered zVAD-FMK reduced damage to heart muscle in rats subjected to a 30-min coronary occlusion followed by reperfusion. Treatment correlated with a decrease in cardiomyocyte apoptosis.[53] In another study of a middle cerebral artery occlusion/reperfusion model, neuronal damage decreased after intraventricular injections of z-VAD-FMK, YVAD-CMK, or DEVD-FMK. There was reduced tissue damage, brain swelling, and behavioral deficits.[54] When given intrathecally 3 h following cerebral hypoxia-ischemia, Boc-Asp-FMK provided significant neuroprotection.[55]

In a model of bacterial meningitis, z-VAD-FMK inhibited neuronal cell death in the hippocampus and reduced the inflammatory infiltrate into the cerebral spinal fluid. In this model it was demonstrated that the host response to pneumococcal infection was the principal contributor to the pathophysiology.[56]

In interpreting all the above results it should be remembered that the caspase inhibitors used were generally the halomethyl ketone inhibitors which have a broad spectrum of activity and may potently inhibit multiple caspases, including those involved in inflammatory reactions and other biological processes as well as apoptosis. For instance, the use of z-VAD-FMK in bacterial meningitis produced a protective effect similar to dampening the host immune response alone. In this instance, the caspase inhibitor seems to be acting, in part at least, as an anti-inflammatory drug.[56] Further, the potent effect of YVAD-CMK in inhibiting CD95 mediated hepatic injury is presumably via its effect on Caspase 1 which is not required for CD95-mediated apoptosis.[52]

While such clinical effects seem promising and legitimize caspases as potential therapeutic targets, major issues of drug delivery, specificity and permeability among others loom as significant hurdles, and these difficulties will be even greater if the caspase inhibitors are to be used for chronic degenerative diseases. Nevertheless progress in the field has been extraordinarily rapid to date, and with the enormous resources of most of the major pharmaceutical companies being brought to bear, further rapid advances should be expected.

Table 2 Inhibitory constants of synthetic caspase inhibitors for mammalian Caspases

	Inhibitory constants (K_i nM)		
	z-VAD-FMK[8]	Ac-DEVD-CHO[8,44,49]	Ac-YVAD-CHO[8,49]
Caspase 1	2.5[a]	15–18	0.76
Caspase 2	2400[a]	1710	>10 000
Caspase 3	43[a]	0.23–2.2	>10 000
Caspase 4	130[a]	132	362
Caspase 5	5.3[a]	205	163
Caspase 6	98[a]	31	>10 000
Caspase 7	39[a]	1.6	>10 000
Caspase 8	2.5[a]	0.92	352
Caspase 9	3.9[a]	60	970
Caspase 10		12	408

[a]Value shown is $t_{1/2}$ at 1 μM in seconds

Table 3 Inhibition of other Caspases by *Drosophila* IAPs and p35. Inhibition is based on functional assays. drICE and DCP1 are *Drosophila* caspases. CED3 is a *C. elegans* caspase. sfCasp1 is an insect caspase from Spodoptera frugiperda

	Inhibitor		
	DIAP1[58,61]	DIAP2[58]	p35[14,62]
drICE	Inhibits	non-inhibitory	
sfCasp1		non-inhibitory	Inhibits
CED3			Inhibits
DCP1	Inhibits		
Caspase 3[a]	Inhibits (functionally equivalent to XIAP)		(see Table 1)

[a]Mammalian caspase

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