

Review

Viral Modulation of Cell Death by Inhibition of Caspases

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Abstract. Caspases are key effectors of the apoptotic process. Some of them play important roles in the immune system, being involved in the proteolytic maturation of the key cytokines, including interleukin 1 β (IL-1 β) and IL-18. The latter directs the production of interferon γ (IFN- γ). Among pathogens, particularly viruses express various modulators of caspases that inhibit their activity by direct binding. By evading the apoptotic process, viruses can better control their production in the infected cell and avoid the attack of the immune system. Targeting the maturation of the key cytokines involved in the initiation of (antiviral) immune response helps to avoid recognition and eradication by the immune system. The three main classes of caspase inhibitors frequently found among viruses include serine proteinase inhibitors (serpins: CrmA/SPI-2), viral IAPs (vIAPs) and p35. Their molecular mechanisms of action, structures and overall influence on cellular physiology are discussed in the review below.

Key words: CrmA; IAP; p35; caspase; granzyme B.

Introduction

Programmed cell death (PCD, apoptosis), a set of ordered events allowing the selective removal of single cells from the system, is essential for the homeostasis of a multicellular organism, for the proper function of the immune system, and as a defense mechanism against viral infections^{37, 69}. The molecular networks that regulate these processes become critical targets for intracellular pathogens, mainly viruses. Thus, it is not surprising that caspases (cysteine-dependent aspar-

tases), enzymes crucial for apoptosis^{18, 45}, are frequently targeted by viral proteins. Caspases are members of the C14 protease family according to the Barrett and Rawlings classification⁵⁹. All caspases are characterized by a nearly absolute specificity to substrates containing aspartic acid in the P₁ or P₁-P'₁ cleavage position and the use of a cysteine side-chain nucleophile to assist hydrolysis of the peptide bond⁷⁵. There are currently 12 known caspases in humans. Caspases-1, -4 and -5 mainly play a role in the regulation of inflammatory response, by proteolytic activation of in-

Abbreviations used: BIR – baculovirus IAP repeat, CARD – caspase recruitment domain, CrmA – cytokine response modifier protein A (SPI-2), DED – death effector domain, IAP – inhibitor of apoptosis protein, NAIP – neuronal IAP, RING – really interesting gene, RSL – reactive site loop, serpin – serine proteinase inhibitor, SPI-2 – serine proteinase inhibitor-2 (CrmA), vIAP – viral IAP, XIAP – X-linked IAP.

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flammatory cytokines. Caspases-2, -3, -6, -7, -8, -9 and -10 are considered to be apoptotic caspases⁵⁰. They process a number of cellular proteins during the apoptotic process. Among these the cleavage and inactivation of poly(ADP-ribose)polymerase-1 is important for maintaining sufficient cellular ATP content and propagation of the apoptotic processes⁴¹. The function of caspase-12 and -14 is not well understood. In addition to the roles in apoptosis and inflammation, the function of caspases in other processes, such as cell cycle regulation, hematopoiesis and signal transduction in the immune system, have been recently proposed⁴².

All caspases are synthesized as inactive zymogens which are activated through proteolytic cleavage. Among the caspase activation pathways, the best described are the death receptor dependent signaling cascades and the mitochondrial/apoptosome-dependent pathways^{35, 45, 63, 64, 82, 91}. The zymogen consists of a protease domain preceded by an N-terminal prodomain of varying length (2–25 kDa), which is cleaved and removed during zymogen activation (Fig. 1). This event is followed by internal cleavage of the protease domain into subunits of approximately 20 and 10 kDa. Some caspases have a short linker between the small and large subunits which is likewise eliminated during activation. All domains are linked by Asp-X bonds, which allows auto- and/or heteroactivation by caspases.

Inflammatory caspases and those initiating apoptosis have longer domains (>100 amino acids), whereas effector caspases, those involved in the execution phase of apoptosis, have shorter domains, of ~30 amino acids or less. Two types of functional elements are found in the prodomains; 1) the caspase recruitment domain (CARD) and 2) the death effector domain (DED). These domains are structurally similar and both are composed of six helix bundles^{10, 57}, but DED domains are primarily hydrophobic whereas CARD domains are hydrophilic. DED domains are found only within caspases-8 and -10, caspases associated with death receptor-triggered cell death. The function of the CARD domain is less specific, as it is found in caspases which are involved in cytokine regulation (caspases-1, -4, -5) as well as caspases which lead to cell death (caspase-2 and -9).

The three-dimensional architecture of active caspases is similar. Caspase-1 was the first to be crystallized^{83, 87}. Later information about caspase-3^{51, 67}, -7⁸⁴ and -8⁴ allowed further definition and refinement of a number of these common features. The basic structural unit is a heterodimer containing one large, ~20 kDa (p20), and one small, ~10 kDa (p10) subunit, which allows definition of the “caspase fold”. Active enzymes contain two heterodimers (p10/p20)₂, aligned together

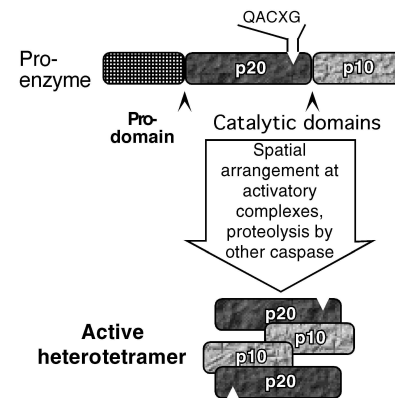


Fig. 1. Mechanism of caspase activation. Apical (initiator) caspases, possess some activity also in their zymogen (proenzyme) form. This activity is sufficient for auto- or heteroproteolysis of apical caspases aggregated at activatory complexes like death inducing signaling complex, or apoptosome. The downstream, effector caspases become activated by caspases that are localized upstream in the enzymatic cascade. Under certain conditions, apical caspases become activated by downstream caspases. This allows the occurrence of amplificatory loops within caspase signaling cascades, and accelerates the apoptotic process

in an antiparallel fashion, with two independent active centers (Fig. 1), both functional in most caspases (except probably caspase-9). The substrate is recognized by a cleft formed by the loop regions of the p10 and p20 subunits. The cleft recognizes a tetrapeptide located N-terminal to the canonical cleavage site Asp-X⁷⁵. The four amino acids to the left of the cleavage site largely define the specificity of caspases, with P₁ (aspartate) being absolutely required in mammals and most other species. After P₁, the P₄ residue is the most important⁷⁹, with the most stringent specificity being for the group II caspases where a P₄ of aspartic acid is found in most protein substrates⁴⁴. The viral caspase inhibitors cytokine response modifier protein A/serine proteinase inhibitor-2 (CrmA/SPI-2), p35 and the inhibitor of apoptosis proteins (IAPs), block the function of already activated (proteolytically-maturated) caspases. Below we will characterize these families of inhibitors with respect to their structure, function and mechanisms of action.

Baculovirus-Derived p35, a Broad-Spectrum Caspase Inhibitor

The insect cellular parasites baculoviruses encode two distinct classes of apoptosis inhibitors, viral IAP (vIAP) and p35^{11, 16}. The p35 family members are notable for their broad inhibitory spectrum, which includes a wide variety of caspases identified from nematodes, insects and mammals^{7, 17}. So far, no cellular homologs

Table 1. Inhibition of mammalian caspases by viral caspase inhibitors (adapted from EKERT et al.¹⁷)

Enzyme	Inhibitory constants (Ki, 10 ⁻⁶ M)	
	p35	CrmA
Caspase-1	9	0.004–0.1
Caspase-2	–	>10 000
Caspase-3	0.1	1600
Caspase-4	–	1
Caspase-5	–	<0.01
Caspase-6	0.4	110
Caspase-7	2	>10 000
Caspase-8	0.5	<0.3–0.95
Caspase-9	–	<2
Caspase-10	7	17

of baculovirus p35 have been identified, but the crystal structures of the native p35 and its inhibitory complex have both been determined¹⁹, and search efforts are on the way as ongoing genomic and proteomic analyses of mammalian genomes continue. The inhibitory spectrum of p35 includes nematode CED-3, *Drosophila* Sf-caspase-1, the mammalian caspases-1, -3, -6, -7, -8, -10, (Table 1), and gingipain-K^{7, 17}. The p35 protein acts as a suicide inhibitor of caspases, with a 1:1 stoichiometry similar to that of serpins (see below), but with the inhibitory complex characterized by a distinctive, protected thioester bond between the caspase and p35^{7, 19, 88}. Structural analysis of the inhibitory complex between p35 and caspase-8 reveals a unique, active site configuration that protects the intermediary thioester link from solvent hydrolysis⁸⁸. Protein 35 is cleaved during inhibitory complex formation, and this cleavage is necessary for the stability of the inhibitory complex, but other protein-protein contacts also contribute to the caspase inhibition⁸⁸. The deduced structure of noncomplexed p35 reveals an exposed reactive site loop that includes the caspase cleavage site at the apex aspartate residue¹⁹. Following cleavage of the p35 scissile bond, the interaction between the reactive center loop and the B-sheets of the p35 body stabilizes the assembled inhibitory complex⁹³. The p35/caspase complex is long-lived, such that both components are effectively sequestered and conformational changes resulting from the cleavage of p35 render the complexed p35 energetically more stable than the noncleaved form⁶⁶.

Ectopic expression of p35 inhibits apoptosis in developing *Drosophila* embryos²⁶, *Caenorhabditis elegans*⁸⁹ and mice³¹. Interestingly, p35 does not inhibit the caspase DRONC, which is one of the two known initiator caspases in *Drosophila*, as opposed to the downstream executioner Sf-caspase-1, which is inhibitable by p35^{25, 36}. When expressed constitutively in

transformed insect (Sf9) cells, p35 induces increased resistance to apoptosis caused by actinomycin D or nutrient withdrawal, and also increases the secretion levels of ectopically expressed glycoproteins³⁸. Protein 35 itself does not inhibit the apical caspase that activates Sf-caspase-1, although vIAP appears to be capable of inhibiting this activity^{36, 49}. The *in vitro* inhibition spectrum of p35 is quite impressive, particularly for downstream effector caspases that mediate the execution phase of apoptosis^{74, 81}.

Viral Inhibitor of Apoptosis Proteins

The IAP family of proteins is characterized by a novel domain of approximately 70 amino acids termed the “baculovirus IAP repeat” or BIR^{3, 12}. The first *iap* genes were discovered in baculoviruses (*Cydia pomonella* granulovirus, CpGV*iap*) based on an assay designed to rescue *Autographa californica* nuclear polyhedrosis virus (AcMNPV) deleted for p35^{3, 12}. At least 10 distinct baculoviruses are known to contain one or more *iap* genes. In addition to baculoviruses, IAPs have been found in entomopoxviruses^{1, 2} and African swine fever virus (Fig. 2)⁵⁴.

While up to three BIR domains can occur in cellular IAPs, only one or two are found in viral IAPs. The baculovirus IAPs with demonstrated anti-apoptotic activity, such as those from CpGV and *Orgyia pseudotsugata* (Op) MNPV, usually have two BIR domains^{3, 12}, as do both the IAP-1 and IAP-2 from *Epiphyas postvittana* MNPV (EppoMNPV-1 and -2)⁴⁸. On the other hand, the IAP from African swine fever virus, which

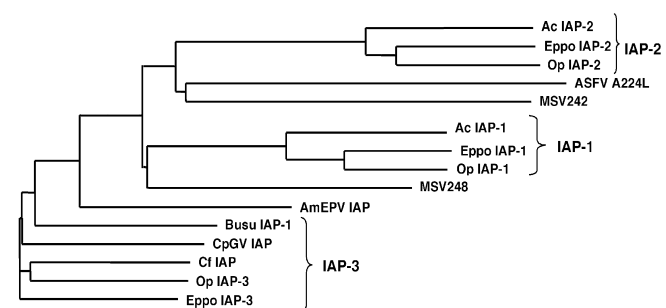


Fig. 2. Phylogenetic analysis of baculovirus, entomopoxvirus and African swine fever virus IAPs. The Cf – *Choristoneura fumiferana* MNPV, baculoviruses Ac – *Autographa californica* MNPV, CpGV – *Cydia pomonella* granulovirus, Eppo – *Epiphyas postvittana* MNPV, Op – *Orgyia pseudotsugata* MNPV, Busu – *Buzura suppressaria* SNPV. Non-baculoviruses include MSV – *Melanoplus sanguinipes* EPV, AmEPV – *Amsacta moorei* EPV, ASFV – African swine fever virus. The tree was generated using Husar software (Heidelberg), based on GCG package (Univ. Wisconsin)

contains only one BIR, is also active⁵⁵. Other viruses that encode IAP proteins with only one BIR include those from the entomopoxviruses^{1, 2} and the Chilo iridescent virus IAP, a member of the iridovirus family³². The defining BIR core structural motif (Cx₂Cx₆Wx₃Dx₅Hx₆C) reveals an unusual spacing of histidine and cystine residues which produces a novel zinc-binding fold^{27, 80}. These structural studies suggest that the 70-residue core region forms four short α -helices linked by a number of loops. Within the BIR core are interactions between the hydrophobic residues and a zinc atom coordinated by three cysteines and the histidine. The surface of the BIR contains a large number of hydrophobic regions and conserved charged amino acids, which may participate in interactions of the IAPs with other proteins. While a BIR domain is required for inhibition of apoptosis, not all BIR domains have an anti-apoptotic function.

Some of the vIAPs also contain a really interesting gene (RING) finger domain. The RING domain²⁰ has been associated with cellular ubiquitination reactions⁹⁰ and, functionally, the RING domains can be involved in control of apoptosis under some conditions but are not required in others, indicating an environment-specific function¹⁴. Additional levels of complexity in cellular proteins is provided in certain of the human cellular IAPs which can additionally contain a CARD³⁹, a ubiquitin-conjugating domain such as found in BRUCE, an unusually large (528 kDa) BIR, containing protein²⁴, or a NACHT domain. NACHT refers to a novel NTPase domain found in neuronal IAP (NIAP)³⁴. The focus of this review is on the vIAP proteins and we only discuss cellular IAPs within the context of understanding the function and mechanism of action of vIAPs as caspase inhibitors.

The classical definition of an IAP protein is that it contains a BIR element and functions to inhibit apoptosis. Mammalian IAPs typically contain two or more of these elements. Studies with a variety of IAPs suggest that they inhibit caspases-3, -7 and -9, but not caspases-1, -6, -8, -10^{15, 68}. The minimum number of BIR domains required for caspase inhibition is one⁷⁸. The human X-linked IAP (XIAP) protein, which contains three BIR domains, illustrates several interesting features relevant to caspase inhibition and BIR function. Inhibition of caspases-3 and -7 and concordant anti-apoptotic activity was localized to the second BIR of XIAP⁷⁸, whereas the third BIR and RING domain is required for inhibition of caspase-9^{13, 76}. These results suggest that BIR domains within the same or different proteins are not functionally equivalent and may have additional functions other than to interact with caspases.

IAPs bind to susceptible caspases at either a 1:1 or 2:1 ratio, indicative perhaps of the presence of two active sites per active caspase macromolecule⁶⁸. Like p35 and CrmA-like serine proteinase inhibitors (serpins), all the IAPs competitively inhibit caspase, but only the IAPs inhibit through a mechanism which does not require peptide bond hydrolysis⁶⁸. It has been proposed¹⁴ that IAPs could still function as competitive inhibitors of caspases in much the same way as the Kunitz, Kazal and Eglin families of serine protease inhibitors, which contain structural loops that conform and adapt to the catalytic pocket of their respective proteases⁵. In all cases, a loop region of the inhibitor binds to the catalytic groove of the protease without scissile bond formation. Recently, a crystallographic analysis of the second BIR of the XIAP protein in complex with caspases-3 or -7 showed, perhaps surprisingly, that the BIR domain has almost no direct role in inhibition, as all important inhibitory interactions are made by the flexible region which precedes the BIR domain (Fig. 3)^{29, 65}.

Instead of direct and intimate contact, a model for inhibition has been articulated whereby the main function of the BIR domain may be to align the inhibitor and stabilize inhibitory actions mediated by what are defined as the “hook”, “line” and “sinker” regions located within the upstream N-terminal inter-domain linker region⁶⁵. The BIR and relevant adjacent regions can be linearly depicted as “N-terminal interdomain region-hook-line-sinker-BIR-C-terminus”. This model then proposes that the substrate pocket on the caspase consists of subpockets designated S₄, S₃, S₂, S₁ and S'₁. The “hook” region interacts with the S₁ and S'₁ exosites.

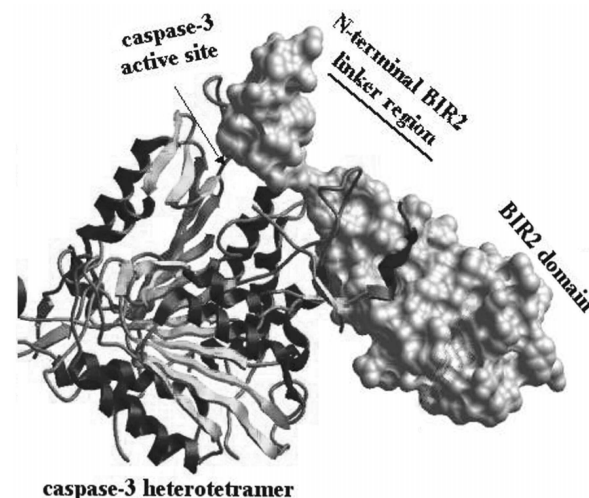


Fig. 3. Crystal structure of the BIR2-linker region of XIAP bound to caspase-3. The BIR2 inhibitor makes contacts with the caspase surface through its BIR domain; however, most of the caspase-3 contacts occur through the BIR2-linker region⁶⁵. This linker region lies across the substrate-binding cleft of the caspase-sterically preventing substrate binding

From the hook, two peptide bonds (the “line”) stretch across the binding cleft (exosites S_2 and S_3) and connect to the “sinker” and the BIR domain. The sinker and part of the BIR domain interact with only S_4 , with most of the interaction coming from the sinker region. Most of the inhibitory interactions stem from the most distal hook region, which prevents catalysis through steric blockage of substrate access. While most of the structural insight has come from the human XIAP, it seems likely that the overall mechanism for caspase inhibition is likely to be conserved for the vIAPs.

Poxvirus-Encoded Inhibitor of Caspase CrmA

The CrmA protein of cowpox virus (CPV) earned its name because deletion of the gene strongly modified growth morphology and immune response to the virus when tested on the chorioallantoic membrane of embryonated chicken eggs⁵⁶. It is a member of the serpin superfamily and is classified as a member of Clade N⁷¹. In other orthopoxviruses, such as vaccinia, ectromelia and rabbitpox viruses, this gene is known as SPI-2. The CrmA protein acts as an inhibitory serpin and achieves inhibition of proteinase activity through acting as a suicide substrate.

The cleaved form of CrmA has recently been crystallized^{62, 72}. CrmA has been defined as a minimal serpin because it lacks the entire D-helix, half of the A-helix and a small portion of the E-helix, all of which are highly conserved throughout the serpin superfamily. Despite these deficiencies, the overall serpin architecture and fold³⁰ have been maintained. Since the D-helix missing in CrmA is responsible for interactions with other molecules, it has been proposed that CrmA is not regulated by external cofactors⁶².

All inhibitory serpins possess a metastable, energy-rich conformation that is required for their inhibitory activity⁸⁶. The reactive site loop (RSL), the region of the serpin, which directly interacts with the proteinase, consists of approximately 17 amino acid residues, is flexible and exposed, thus facilitating the accession of the respective catalytic center. The accepted nomenclature for serpins²¹ defines an approximate RSL length of 17 amino acids, where the P_1 and P'_1 residues are those cleaved during the scissile bond formation reaction with the proteinase. The P_1 residue in particular is very important in defining susceptibility and specificity towards a particular proteinase. Inhibitory serpins first form a noncovalent Michaelis-like complex through interactions with amino-acid residues which flank the scissile bond (P_1 - P'_1). In the case of the caspases, attack

by the caspase active site cysteine (rather than serine, typical of most proteases) leads to a covalent thiol ester linkage between the active site cysteine of the proteinase and the P_1 residue. Cleavage of the P_1 - P'_1 serpin peptide bond is typical of the initial stages of ester or thiolester bond hydrolysis. The P_1 residue of CrmA is aspartic acid, a rather unusual P_1 residue for serpins, but critical for the interaction with caspases. The RSL insertion leads to a profound translocation of the proteinase with a concomitant distortion of the active site. Proteinase inactivation results from compression and restraint of the proteinase against the body of the serpin, which in turn is dependent on the length of the RSL. The energetics of the process derive from the fact that the cleaved loop-inserted conformation is less energy rich than the native structure of the serpin. The end result of the conformational rearrangement is the kinetic trapping of the acyl intermediate, as the deacylation step leading to hydrolysis is slowed by 6–8 orders of magnitude. Practically, the half-life of such trapped intermediates may be hours or even weeks.

The *crmA* gene was originally discovered as being required for the development of red, hemorrhagic pocks on the chorioallantoic membrane of CPV-infected embryonated chicken eggs⁵⁶. Deletion of the CrmA gene of CPV led to the production of white, rather than red, pocks, which were characterized by the development of an intense inflammatory cell influx into the developing lesion^{28, 56}. Subsequently, CrmA was shown to be a potent inhibitor of interleukin 1β (IL- 1β) activation via caspase-1⁶⁰. SPI-2/CrMA is also likely to be a physiological inhibitor of caspases-8 and possibly -10⁹². It is the activity against both caspases which is responsible for the well-documented anti-apoptotic activity of CrmA in a variety of experimental settings, such as following withdrawal of serum⁵², withdrawal of nerve growth factor⁷⁰, death receptor ligation⁴³ and detachment from the extracellular matrix⁶. In a similar fashion, and under more natural conditions, deletion of CrmA from CPV leads to the induction of apoptosis in infected swine kidney cells⁶¹. CrmA also inhibits the serine proteinases granzyme B and the protease E of *Streptomyces griesus*^{33, 58}. Thus, this viral serpin is classified as a cross-class inhibitor, being active against both thio- and serine-proteinases. The serpin P_1 residue plays the key role in determining specificity, but other interactions are also extremely important. CrmA is an excellent inhibitor of caspases-1 and -8, and a weak inhibitor of caspase-3 and other caspases (Table 1)⁹². When the RSL amino acids P_4 - P_1 (Leu-Val-Ala-Asp) were changed to those of the baculovirus anti-apoptotic protein p35 (Asp-Gln-Met-Asp), lymphoid cells were

protected against radiation and dexamethasone-induced apoptotic death mediated by direct activation of caspase-3.

Following the discovery that CrmA was a potent inhibitor of caspase-1, a member of the expanding caspase family, it was anticipated that CrmA would act as a powerful regulator of IL-1 β or apoptosis during natural infections. IL-1 β regulates functions associated with inflammation and immune processes. In addition to cleavage of pro-IL-1 β , caspase-1 also cleaves pro-IL-18, a cytokine structurally related to IL-1 β . Both precursors are cleaved by caspase-1 to yield active cytokine^{22, 23}. One role of IL-18 is to control the levels of IFN- γ , which orchestrates a cells' antiviral defence. Despite the numerous examples of CrmA regulating apoptosis when expressed ectopically, within the context of a natural infection, data indicating CrmA control of apoptosis during virus infection is very limited. It has been clearly demonstrated that induction of apoptosis in CPV-infected swine kidney cells occurs readily in the absence of CrmA⁶¹, and that CrmA functions to control caspase induction in these cells^{46, 77}. It was also suggested that CrmA has an effect on Fas/APO-1 and granule-mediated killing by cytotoxic T lymphocytes (CTL)⁴⁷. However, more recently, it appeared that inhibition of CTL-mediated lysis of target cells is limited to alloreactive but not MHC-restricted CTLs⁵³.

Conclusions

By learning to modulate apoptosis, viruses increase their influence over their developmental cycle. By evading caspases, for instance, viruses gain twice; 1) inhibition of apoptotic caspases allows prolonged viral production by an infected cell, and 2) by blocking inflammatory caspases, viruses avoid launching of the specific immune response and subsequent counterattack of the host. The three viral inhibitors described here are the best-studied examples of virus-encoded caspase inhibitors, but other examples have also been reported in the literature. For example, the adenovirus 14.7 kDa protein and the UL35 protein of human cytomegalovirus (denoted vICA) have both been reported to bind and inhibit caspase-8^{8, 73}. However, the analyses of these proteins are less advanced. Caspase inhibition is not the sole mechanism used by viruses to modulate cell fate; viruses may, for example, influence cell physiology that prompts the induction of gene expression and shift the delicate physiologic balance towards conditions favoring a pro- or antiapoptotic state^{9, 40, 85}. It is

likely that further apoptosis modulators will continue to be discovered, as manipulation of cell death and hindering of the immune response are of major benefit for these primitive cellular parasites.

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