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Apoptosis Review Series

Caspases: potential targets for regulating cell death

A. Philchenkov *

R. E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology,
National Academy of Sciences of Ukraine,
Kiev, Ukraine

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Abstract

While in multicellular organisms all cells inexorably die, there are several different ways provided for the realization of cell death. One of them, apoptosis, represents a universal energy-dependent and tightly regulated physiologic process of cell death in both normal and pathologic tissues. The execution of apoptosis appears to be uniformly mediated through consecutive activation of the members of a caspase family. This review briefly summarizes current knowledge on the molecular mechanisms of caspase activation and the inhibitory components of caspase cascades. The suitability of caspases as a new potential therapeutic target is discussed next. Particular attention is focused on two broad categories of caspase-directed compounds: highly specific caspase inhibitors that distinctly block the progress of apoptosis and caspase activators that selectively induce cell death in a variety of *in vitro* and *in vivo* systems. These agents promise to be useful clinically, either alone or in combination with more conventional therapeutics.

Keywords: apoptosis - caspase - death receptor - mitochondria - cytochrome c - Bcl-2 - apoptosome - DISC - PIDDosome - IAP - endoplasmic reticulum - caspase inhibitor - caspase activator - therapy

* Correspondence to: Dr. Aleksei PHILCHENKOV
Mechanisms of Leukemogenesis Laboratory, R.E. Kavetsky
Institute of Experimental Pathology, Oncology, and Radiobiology,
Vasilkovskaya Str. 45, 03022, Kiev, Ukraine.
Tel.: 380 (44) 2577792, Fax: 380 (44) 2581656
E-mail: apoclub@mail.ru

Introduction

Apoptosis is a physiological mechanism that eliminates excessive, damaged or dangerous cells from an organism without damaging surrounding cells and tissue. Such systematic cell clearance is necessary for both the normal development of a multicellular organism during embryogenesis and the maintenance of tissue homeostasis in adults. In contrast, necrosis predominantly represents a passive form of cell death induced mainly by non-physiological agents and accompanied by autolysis of the cell. Necrosis is frequently initiated by damage to the plasma membrane. Cells may also be eliminated by a number of other mechanisms including: autophagy, mitotic catastrophe, dark cell death, paraptosis, chondroptosis, autoschizis, etc. It is important to recognize that these types of cell death differ not only morphologically, but biochemically as well. The specific manifestation of cell death in each case depends on the type of cell system and on the kind and intensity of stimuli. While the realisation of cell death in most of the above-stated processes is caspase-independent, the existence of various alternative types of cell death should be taken into consideration in designing novel approaches to modulate apoptosis.

Early cell-death studies in nematode *Caenorhabditis elegans* show that 131 of the 1090 cells undergo programmed death in the course of morphogenesis. Extensive genetic analysis revealed *ced-3*, *ced-4*, *ced-9*, and *egl-1* genes whose products regulate this process (reviewed in [1]). The CED-3 and CED-4 proteins induce the death of *C. elegans* cells, whereas CED-9 prevents it. Binding of EGL-1 to CED-9 causes the release of CED-4 protein, which is then free to initiate the lethal action of CED-3 in the nematode. Genes homologous to *ced-3*, *ced-4*, and *ced-9* were also found in mammals. The *ced-3*-like gene encodes the cysteine protease ICE (interleukin-1beta-converting enzyme) which converts the interleukin-1beta precursor into the mature form of this proinflammatory cytokine. Later, several other cysteine proteases were revealed in mammals. All these proteases are comprised of conservative sequence sites for substrate binding and catalysis, and cleave their substrates following certain accessible aspartic acid residue. Therefore, these proteases were named caspases (cysteinyl aspartate-specific proteases). Twelve mammalian caspases presently known are numbered in the chronological order of their identification. The prototype of caspase family, ICE was discovered fifteen years ago and is now referred to as caspase-1 [2].

The aim of this article is to summarize the molecular anatomy of caspase-dependent apoptosis and to give a brief overview of promising new caspase-targeting compounds developed for the therapeutic manipulation of apoptosis.

The nature of the caspase-dependent apoptosis and its regulation

Apoptosis can be initiated by various external or internal signals and executed through several interrelated signaling pathways (Figure 1). At the molecular level, the apoptotic cell death machinery forms a complex cascade of ordered events, controlled by the regulated expression of apoptosis-associated genes and proteins. It is the concerted action of these components that finally results in cell dismantling and in the formation of apoptotic bodies. The key components of this self-destruction machinery are members of the caspase family.

Mechanisms of caspase activation

Caspases are synthesized as a single-chain of inactive zymogens, consisting of four domains: a NH_2-terminal prodomain of variable length, a large subunit with molecular weight of about 20 kDa, a small subunit (~10 kDa), and a linker region connecting these catalytic subunits. The linker region is missing in some family members. Proteolytic cleavage of the caspase precursors results in the separation of large and small subunits with the production of a heterotetrameric complex (the active enzyme) consisting of two large and two small subunits [3]. Caspases differ in the length and in the amino acid sequence of their NH_2-terminal prodomain which is either short (20-30 amino acid residues) or long (Table 1). The long prodomain (more than 90 amino acid residues) contains one of two modular regions essential for the interaction with adaptor proteins. These modules contain death effector domains (DED) and caspase recruitment domains (CARD). Hydrophobic protein interactions are mainly

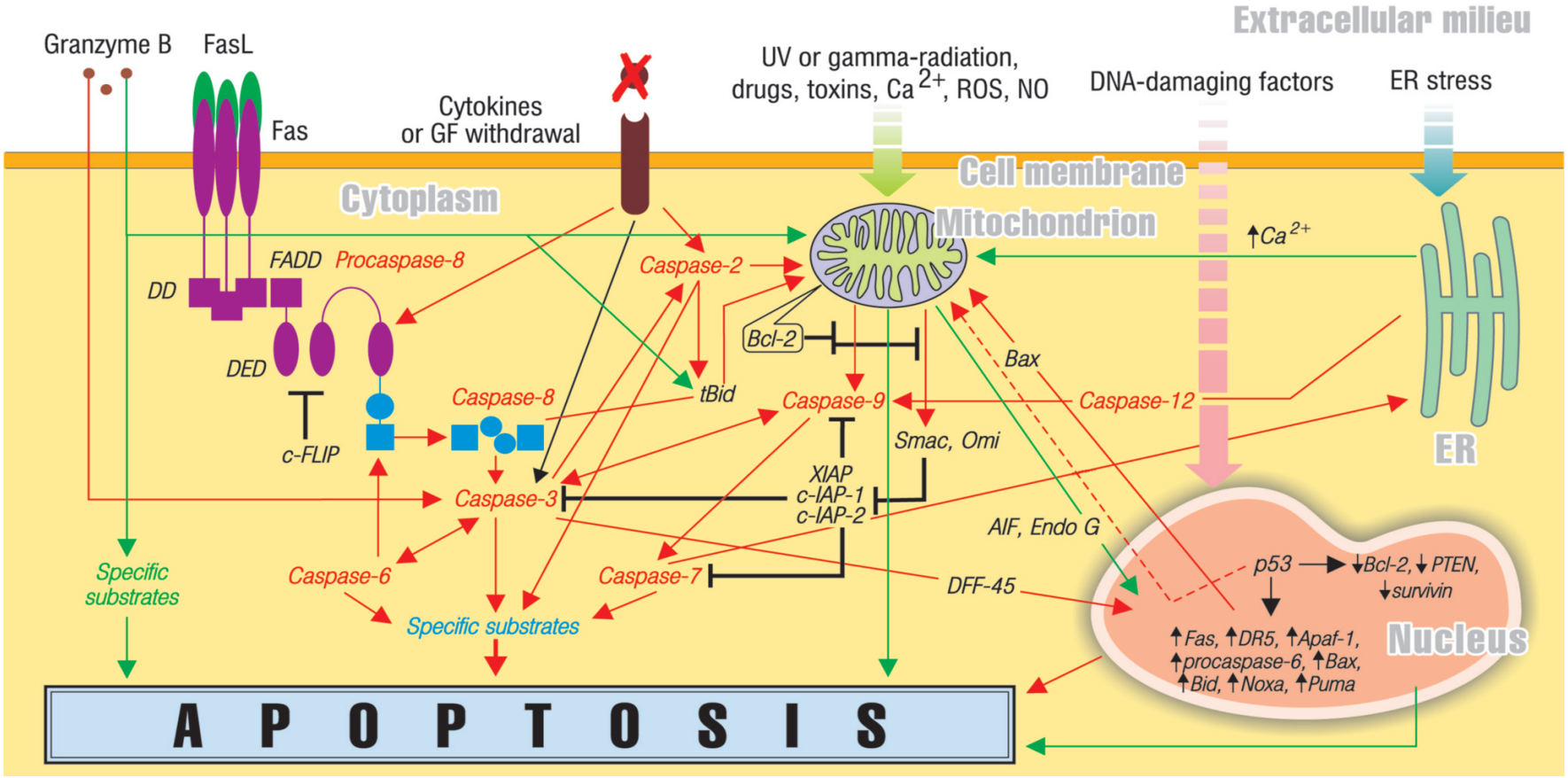

Fig. 1 A schematic diagram showing the basic caspase-dependent (marked in red), and caspase-independent (marked in green) apoptosis signal transduction pathways. Death receptor (e.g. Fas) ligation results in formation of a multiprotein complex DISC that includes the receptor, adaptor (e.g. FADD) and procaspase-8. Upon recruitment by FADD, procaspase oligomerization drives its own activation. Caspase-8 then activates effector caspases such as caspase-3. The release of cytochrome *c* from mitochondrial intermembrane space results in caspase-3 activation via formation of the cytochrome *c*/Apaf-1/caspase-9-containing apoptosome complex. The caspase-activated DNase (CAD in mouse, DFF-40 - human analogue of CAD), responsible for the internucleosomal DNA fragmentation is usually complexed in the cytoplasm with its inhibitor (ICAD in mouse, DFF-45 - human analogue of ICAD). The cleavage of the DFF-45 by caspase-3, liberates DFF-40 resulting in its nuclear transfer and subsequent internucleosomal DNA fragmentation. DFF-45 is depicted in cytoplasm. Meanwhile, there is no consensus concerning its cytosolic or nuclear localization. Activated caspase-8 or granzyme B can cleave a pro-apoptotic BH3-only Bcl-2 family member Bid. Truncated form of Bid (tBid) is then able to activate the caspase-9-dependent mitochondrial pathway, tBid action being prevented by Bcl-2. Upon increasing the intracellular Ca^{2+} content, or induction of ER stress, calpain or caspase-7 translocates to the ER surface where they process the procaspase-12 with further activation of caspase-9 and caspase-3. Moreover, Ca^{2+} efflux might trigger secondary activation of mitochondria following ER stress responses. Growth factor (GF) deprivation can activate caspase-2 that generates a highly active tBid stimulating the efflux of cytochrome *c* from the mitochondria. Also, an active caspase-2 is able to contribute to the mitochondrial pathway by promoting Bax translocation to this organelle. Serine protease granzyme B, released by cytotoxic T cells or NK cells, can induce apoptosis via either direct activation of caspase-3 or the alternative caspase-independent pathway. Activation/activity of caspases is negatively regulated by IAPs, c-FLIP and anti-apoptotic members of the Bcl-2 family (e.g. Bcl-2). Smac and Omi promote caspase activation by neutralizing the inhibitory effect of IAPs, while apoptosis inducing factor (AIF) and endonuclease G cause non-caspase dependent nuclear DNA fragmentation. The dotted line marks the p53 mediated apoptosis initiation pathways non-related to its transcriptional activity. See text for other abbreviations.

Table 1. Structural and functional characteristics of cysteine endopeptidases of the caspase family[a]

Enzyme	Map position of gene	Size of enzyme precursor (kDa)	Prodomain type	Active subunits (kDa)	Adaptor proteins	Caspase-activating complex	Caspase proteolytic specificities
Apoptotic initiator caspases							
Caspase-2	7q34-q35	51	Long, with CARD region	19/12	PIDD, RAIDD, PACAP, DEFCAP	PIDDosome?	VDVAD[b]
Caspase-8	2q33-q34	55	Long, with two DED-regions	18/11	FADD, DEDAF, ASC	DISC	(L/V/D)E(T/V/I)D[c]
Caspase-9	1p36.1-p36.3	45	Long, with CARD region	17/10	Apaf-1, Nod-1, PACAP	Apoptosome	(L/V/I)EHD
Caspase-10	2q33-q34	55	Long, with two DED-regions	17/12	FADD, DEDAF	DISC	(I/V/L)EXD
Caspase-12[d]	#9[e]	50	Long, with CARD region	20/10	TRAF-2		Unknown
Apoptotic effector caspases							
Caspase-3	4q35	32	Short	17/12	NA[f]		DE(V/I)D
Caspase-6	4q25	34	Short	18/11	NA		(T/V/I)E(H/V/I)D
Caspase-7	10q25.1-q25.2	35	Short	20/12	NA		DE(V/I)D
Inflammatory caspases							
Caspase-1	11q22.2-q22.3	45	Long, with CARD region	20/10	ASC, NALP1, CARDIAK, CARD-8, Ipaf, Nod-1	Inflammasome	(W/Y/F)EHD
Caspase-4	11q22.2-q22.3	43	Long, with CARD region	20/10	Unknown		(W/L)EHD
Caspase-5	11q22.2-q22.3	48	Long, with CARD region	20/10	ASC, NALP1	Inflammasome	(W/L/F)EHD
Caspase-11[d]	#9	42	Long, with CARD region	20/10	Unknown		(V/I/P/L)EHD
Other mammalian caspases							
Caspase-14	19p13.1	30	Short	20/10	NA		
Invertebrate caspases[g]							
Ced-3		56	Long, with CARD region	17/14	Ced-4, RAIDD		DEXD
Dcp-1[h]	58E3-59F4	36	Short	22/13	NA		DEVD
Dronc[h]	67C4-67C5	50	Long, with CARD region	20/14	DARK[i]		VDVAD

[a] Adapted from Ref. 3 with modifications.
[b] The sequence of amino acid residues is presented in P_4-P_1 direction; the proteolysis occurs after the aspartic acid residue in the P_1 position.
[c] In parentheses each amino acid possibility is listed.
[d] Detected in murine cells.
[e] The *pseudo-caspase-12* gene is localized on human chromosome 11q22.3.
[f] NA - not applicable.
[g] Only several caspases are given as an example.
[h] The *Drosophila* caspases.
[i] A *Drosophila* homologue of Apaf-1

achieved via DED-DED contacts, whereas electrostatic interactions occur through CARD-CARD contacts.

Based on their proapoptotic functions, the caspases have been divided into two groups: initiators and effectors. First-group initiator (or apical) caspases (caspases-2, -8, -9, -10, and, probably, -11) activate the second-group of caspases (caspases-3, -6, and -7). The effector (or downstream) caspases are able to directly degrade multiple substrates including the structural and regulatory proteins in the cell nucleus, cytoplasm, and cytoskeleton. In some cases, initiator caspases can also function as effector caspases; this activity helps to amplify a suicide signal in the cell whose death pathways have been only weakly initiated. Furthermore, the activation of effector caspases can not only be caused by initiator caspases, but also by other, non-caspase proteases, including cathepsins, calpains, and granzymes (see [4] and references therein). Caspase-1 and caspase-4, -5 have similar structures and are predominantly involved in the maturation of proinflammatory cytokines. However, significant bodies of experimental evidence exist, that indicate a redundant/accessory role of these caspases in apoptosis.

The caspase proteolytic signaling cascades are interconnected and due to overlapping substrate specificity they are also partially redundant. As a result, the apoptotic signal can be significantly amplified. A number of cellular and viral caspase inhibitors exist that may prevent both, initiation and amplification of the apoptotic signal within the proteolytic cascade. Perhaps the best defined caspase triggering cascade is the receptor mediated pathway. It is initiated by the binding of death ligands (belonging to the tumor necrosis factor [TNF]/nerve growth factor [NGF] superfamily) to the respective death receptor. To date, at least eight human members of the death receptor family have been identified: TNF-R1, Fas (Apo-1, CD95), DR-3 (Apo-3, WSL-1, TRAMP), DR-4 (TRAIL-R1), DR-5 (TRAIL-R2), DR-6, EDA-R (ectodermal dysplasia receptor) and NGF-R [5]. All death-inducing receptors contain a so called "death domain" (DD) in their cytoplasmic tail, which is a conserved stretch of about 80 amino acids. This structure is critical for engaging downstream molecules of the apoptotic cascade. Fas is the best characterized member of death receptor family. Binding of either the Fas ligand (FasL) or an agonistic antibody induces aggregation of Fas upon which this activated receptor recruits the adaptor protein FADD and the initiator caspase-8 to a multiprotein complex, DISC (Death Inducing Signaling Complex), that is essential for the initiation of apoptotic cascade.

Alternatively, caspase cascade may be initiated in a receptor-independent manner by a variety of stimuli, including chemotherapeutic agents. Proapoptotic signals can originate in the nucleus as well as in various cellular organelles including mitochondria, the endoplasmic reticulum (ER), lysosomes, and the Golgi complex [6]. In the majority of these organelles, excluding perhaps mitochondria, the triggering mechanisms and underlying molecular networks are not known in detail, however some basic phenomena are now quite clear. The nuclear protein p53 is a central link in the cellular mechanisms activated upon DNA injury that promotes apoptosis through transcriptional activation/repression of various apoptosis-associated genes (Figure 1). Moreover, some pathways rely on p53-induced initiation of apoptosis and do not depend on p53 transcriptional activity [7].

Many apoptotic stimuli that induce metabolic stress in cell organelles will eventually converge on the mitochondria/apoptosome death pathway. Various inducers of apoptosis can directly or indirectly influence the permeability of the outer mitochondrial membrane ultimately leading to the release of cytochrome *c*. The cytoplasmic efflux of cytochrome *c* is the key event in the activation of the mitochondria/apoptosome-dependent (intrinsic) death pathway. Bcl-2 (the B-cell lymphoma gene 2) family proteins are the major regulators of this pathway [8]. The ratio between prosurvival (Bcl-2, Bcl-x_L, Bcl-w etc.) and proapoptotic (Bax, Bak, Noxa etc.) Bcl-2 family members determines cell life and cell death decisions. Their expression level and activation stage can strongly influence the release of a number of apoptogenic molecules like cytochrome *c*, procaspase-2, -3, -9, the apoptosis inducing factor (AIF), endonuclease G, Smac/DIABLO (second mitochondria derived activator of caspase)/direct IAP binding protein with low pI), Omi/HtrA2 (high temperature requirement A2), and many others from the mitochondrial intermembrane space. All these factors are released via Bax/Bak

channels or non-specific pores in mitochondrial membranes.

The activation of procaspase-9 (initiator of the intrinsic/apoptosome pathway) facilitated by Apaf-1 and cytochrome *c* in the presence of dATP or ATP is perhaps the most important consequence of cytochrome *c* discharge. Cytochrome *c*, combining with Apaf-1, procaspase-9 and dATP or ATP, generates a supramolecular complex with a molecular weight of approximately 700-1400 kDa referred to as the apoptosome. Upon formation of this complex, caspase-9 is activated and then triggers the processing and activation of the downstream caspases-3 and -7 that culminates in apoptotic cell death [3].

Caspase-2 is structurally and phylogenetically related to caspase-9 and both caspase-2 and -9 bind specifically to proapoptotic caspase adaptor protein PACAP (see Table 1). Although caspase-2 is unable to initiate the processing of procaspases on its own, it stimulates the efflux of cytochrome *c* (and other proapoptotic mediators) from mitochondria by increasing pro-apoptotic activity of Bid (a BH3-only member of Bcl-2 family of proteins), which in turn will promote the activation of caspase-9 [9]. Also, recent findings indicate that caspase-2 is activated early in the apoptotic response to DNA-damage agents and neurotrophic deprivation. Upon DNA damage, caspase-2 is recruited into a large protein complex, the PIDDosome, containing the death domain-containing protein PIDD and the adaptor protein RAIDD [10]. Whether PIDDosome formation is indispensable for other cases of caspase 2-mediated cell death is still unknown.

As mentioned above, the proteolytic processing and activation of initiator procaspases occurs within large multicomponent complexes like DISC, apoptosome and PIDDosome. The involvement of adaptor molecules helps procaspases to align with proper spacing within these protein complexes. Several adaptor proteins (RAIDD, FADD, Apaf-1, PIDD, CARDIAK, DEFCAP, PACAP, DEDAF, Nod1, Ipaf, ASC, CARD-8) have been found in vertebrates and one (Ced-4) in nematodes (Table 1). Nevertheless, it should be noted that some adaptor proteins like FADD can provide a bifunctional switch for cell survival or cell death decisions. Formation of multiprotein signaling complexes may not be the sole mechanism of apical caspase activation. Recently, Chang et al. [11] have shown that the aggregation of multiple procaspase-9 molecules can induce their activation without the apoptosome. It appears that oligomerization represents an alternative mechanism for the activation of all procaspases with long prodomains.

Mechanisms that govern the induction of apoptosis in organelles like ER, Golgi apparatus, or lysosomes are much less clear. Upon increasing the intracellular Ca^{2+} content or inducing ER stress (changes in Ca^{2+} metabolism and accumulation of the unfolded proteins in the ER) calpain or caspase-7 translocate to the ER surface where they process the precursors of caspase-12 with further activation of downstream caspase-9 and caspase-3, as summarized in a recent review [12]. Some Golgi-resident proteins are cleaved during apoptosis facilitating the disassembly of the Golgi apparatus. The identification of golgin-160 as the unique substrate for caspase-2 suggests that caspase-2 may also induce apoptosis independently of the mitochondria/apoptosome pathway [13]. Upon apoptosis induction, lysosomal enzymes, in particular proteases of the cathepsin family may enter the cytosol, facilitating cytochrome *c* release from the mitochondria [14].

Endogenous caspase inhibitors

Both procaspase activation and caspase activity in the cell are tightly controlled. Naturally occurring direct caspase inhibitors include seven members of the mammalian IAP (inhibitors of apoptosis) family of proteins, c-FLIP (FADD-like ICE-inhibitory protein), BAR (bifunctional apoptosis regulator) and ARC (apoptosis repressor with CARD). Some basic properties of these negative caspase regulators are shown in Table 2. It should be noted that expression of caspase inhibitors except for XIAP, and to a lesser extent c-IAP1 and c-IAP2, are tissue-specific. All the IAP family proteins share a specific BIR (baculoviral IAP repeat) region of about 70 amino acid residues required to provide the antiapoptotic effect. All IAPs (except for ILP-2) can preferentially bind to active forms of caspase-3, and -7 but not to their precursors [15]. XIAP, ILP-2 and livin can also inhibit the initiator caspase-9.

At the same time, c-FLIP, BAR and ARC prevent transduction of the proapoptotic signal from death receptors targeting another apical caspase,

Table 2. Basic properties of some endogenous caspase inhibitors.

Protein	Length of protein (in amino acids)	Molecular weight (kDa)	Domain structures	Caspase specificity	Preferential expression
NAIP (BIRC1)	1403	156	BIR1, BIR2, BIR3, NOD	Caspase-3 and -7	Liver, placenta, spinal cord and brain
c-IAP1 (BIRC2)	604	70	BIR1, BIR2, BIR3, CARD, RING	Caspase-3 and -7	Ubiquitous; with highest levels in thymus, testis and ovary
c-IAP2 (BIRC3)	618	68	BIR1, BIR2, BIR3, CARD, RING	Caspase-3 and -7	Ubiquitous; with highest levels in spleen and thymus
XIAP (BIRC4)	497	57	BIR1, BIR2, BIR3, RING	Caspase-3, -7 and -9	Ubiquitous
Survivin (BIRC5)	142	17	BIR	Caspase-3 and -7	Embryonic tissue, transformed cell lines, and solid tumors
Livin (BIRC7)	298	31	BIR, RING	Caspase-3, -7 and -9	Embryonic tissue, some adult tissues, and malignant cell lines (colon and lung cancer, melanoma)
ILP-2 (BIRC8)	236	25	BIR, RING	Caspase-9	Testis
c-FLIPL (I-FLICE)	480	55	DED, DED, pseudo-caspase domain	Caspase-8 and -10	Muscle, lymphoid tissues and testis; also detectable in melanoma cell lines and malignant melanoma tumors
ARC	338	30	CARD	Caspase-2 and -8	Heart and skeletal muscle
BAR	450	46	RING, SAM, DED	Caspase-8	Brain

caspase-8. Intriguingly, BAR is able to inhibit cell death in response to a broad range of cell death stimuli inducing apoptotic pathways via mitochondria, death receptors, or as a result of ER stress [16]. Therefore, individual caspase inhibitors contribute to cell death machinery in cell-type as well as in signaling cascade-specific manner (see Figure 1).

Negative regulators of IAPs

The antideath function of the IAP family proteins can be cancelled by specific inhibitors. After the initiation of apoptosis, Smac/DIABLO and Omi/HtrA2 proteins are released from mitochondria to the cytosol along with cytochrome *c*. Both Smac and Omi contain an IAP binding motifs (IBMs) through which they bind to IAPs and release IAPs bound caspases [17]. The binding of Smac/DIABLO or Omi/HtrA2 to the XIAP protein countermands the caspase-inhibitory effect of the latter that promotes the activation of caspases. The overexpression of Smac or Omi/HtrA2 in the cells increases their sensitivity to induction of apoptosis by UV radiation [18, 19]. These findings confirm the capacity of these mitochondrial proteins to function as endogenous activators of apoptosis.

Caspase-based therapeutics

In the last few years, a sizable effort has been made to understand what extent the disorders in apoptotic pathways contribute to various disease conditions. The role of apoptosis dysregulation in various pathologies have been described elsewhere in much detail [20-22], and the evidence linking apoptosis to these pathologies will not be reviewed here again. Many of these alterations are related or associated

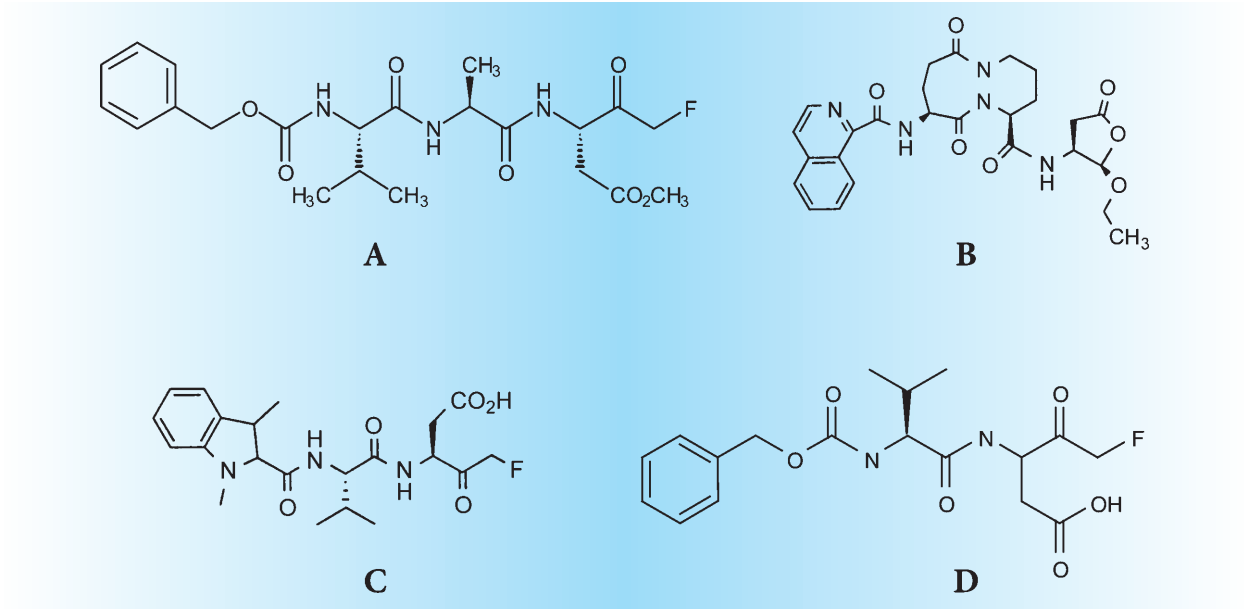

Fig. 2 Example of caspase inhibitors. A - pan-caspase inhibitor z-VAD.fmk; B - selective caspase-1 inhibitor VX-740; C - pan-caspase inhibitor IND-1965; D - pan-caspase inhibitor MX1013.

with caspase activation in the affected cells. The excessive caspase activity may trigger a variety of disease states (e.g., ischaemic heart disease, stroke, neurodegenerative diseases, and sepsis). In contrast, the functional deficiency of caspases may facilitate the development of cancer and autoimmune diseases.

Below, I will restrict discussion to the subjects related to the recent attempts in generating novel reagents for modulating the activity of caspases with therapeutic purposes.

Caspase inhibitors

Numerous studies over the last years have indicated that caspases are attractive targets for therapeutic intervention in a wide variety of diseases characterized by excessive apoptosis. Promising attempts to preserve cell viability after acute injury are focused on synthetic inhibitors of caspases. Typically, these inhibitors represent modified tetra- or tripeptide pseudosubstrates comprising of the cleavage sequence of the caspase target coupled with an aldehyde or ketone group. Ketones are irreversible inhibitors that covalently bind to the cysteine at the active enzymatic center of caspases, whereas aldehydes are reversible/competitive caspases inhibitors. Some examples of designed caspase inhibitors are presented on Figure 2.

The feasibility for using chemical caspase inhibitors as cytoprotective drugs has been widely proven in animal models of various disease conditions associated with inappropriate apoptosis. For example, novel caspase inhibitors IDN-1965 and IDN-6556 (both developed by the Idun Pharmaceuticals Inc.; http://www.idun.com) may reduce the lesion volume under such pathological states as heart and liver injury [22, 24]. Furthermore, injection of pan-caspase inhibitors was shown to significantly decrease the level of apoptosis in animals with acute lung injury, nephrotoxic nephritis, subarachnoid hemorrhage, and heart failure after a myocardial infarction [25-28]. It is important to note that in some of the above-mentioned models the reduced inflammation and prolongation of the survival rate in diseased animals was also evident. Caspase inhibitors were also highly protective in a sepsis rat model [29]. More recently, a dipeptide broad-spectrum caspase inhibitor MX1013 (Maxim Pharmaceuticals Inc.; http://www.maxim.com) was also shown to be systemically efficacious in three animal models of apoptosis, including Fas-mediated liver damage, brain ischemia/reperfusion injury, and acute myocardial infarction [30].

Thus, preclinical tests involving these broad-spectrum caspase inhibitors suggest that they may have potential to become leads for the development of new pharmaco-active, caspases-modulating drugs.

RNA interference is a newly discovered and exciting technique allowing for selective induction of cognate mRNA degradation. For that purpose, a so-called small interfering RNAs (a short double-stranded RNA oligonucleotides known as siRNAs) complementary to target messenger RNA molecules have been applied. Zender *et al.* [31] have shown that siRNA-mediated inhibition of *caspase-8* gene expression in the liver prevents Fas-mediated apoptosis *in vitro* and *in vivo*. However, use of siRNA as a therapeutic tool in patients with acute liver failure deserves further study.

Caspase activators

Selective activation of caspases or at least lowering their activation threshold might help to combat cancer and other diseases in which apoptosis deficiency had been attributed to pathogenesis. Diverse strategies designed to activate caspases and to stimulate apoptosis are currently under preclinical study.

Pharmacological activation of caspases using small molecules might prove to be one of such effective approaches to kill cancer cells or at least to reverse the resistance against anticancer drugs. Caspase-3 is kept in an inactive stage by an intramolecular electrostatic interaction facilitated by a triplet of aspartic acid molecules termed the "safety-catch" [32]. Attempts have been made to design small pharmacologically active molecules capable of lowering the threshold of activation, or even activating the caspase on its own. Maxim Pharmaceutical Inc. (http://www.maxim.com) has developed a pharmacologically-active caspase activator MX-2060. A comparable approach towards selective activation of caspase-3 by "small molecules" is followed by Merck Frosst (http://www.merckfrosst.ca). "Small molecule" caspase activators are peptides containing arginine-glycine-aspartate (RGD) motif. They exhibit marked proapoptotic properties and can directly induce auto-processing (auto-activation) of pro-caspase-3 [33].

Another apoptosis-triggering approach is based on fusion proteins that contain effector caspases. The chimeric proteins, called immunocasp-3 or immunocasp-6 that comprise of a single-chain anti-erbB2/HER2 antibody, and an active caspase-3 or caspase-6 molecule have been recently generated [34, 35]. Regardless of the method of injection (intramuscular, intratumoral or intravenous), significant tumor regression in mouse xenografts of HER2-positive tumor cells and prolonged animal survival were observed upon overexpression of chimeric *immunocasp-3* or *immunocasp-6* gene. Other authors have shown that specific binding between intracellular antibody-caspase-3 fusion proteins and a respective multivalent antigen results in autoactivation of caspase-3 due to the close proximity of caspase-3 molecules. Such an autoactivation of caspase-3 triggers apoptosis and irreversibly leads to the killing of CHO cells transfected with antibody-caspase-3 fusion protein-expressing plasmid [36]. Caspase-3 fused with antibodies directed towards extra- or intracellular tumor-specific proteins seems to provide a compelling rationale for selective induction of apoptosis in tumor cells.

Several gene therapy approaches have been aimed at replacing the defective caspases or their upstream activators in tumor cells by their normal counterparts. A variety of replication-incompetent adenoviral vectors carrying different caspase genes, including *caspase-3*, *-6*, *-8* and *-9* have been generated and their antitumor activity has been tested (for comprehensive overview see [37]). Both *in vitro* and *in vivo* studies demonstrated antitumor activity of these vectors. The genes coding for effector caspases are preferably used in such constructs since apoptosis induced by the effector caspases is independent of the upstream initiating pathways. Yang et al. [38] demonstrated that an expression vector consisting of autocatalytic reverse *caspase-3* gene under survivin promoter combined with hypoxia-responsive element from the vascular endothelial growth factor gene promoter triggers apoptosis in human tumor cells, but not in normal cells.

Another promising strategy in gene therapy of cancer is based on the development of caspase constructs with inducible caspase-1, -3 or -9 molecules that are activated "on demand" *in vivo* by addition of cell-permeable chemical inducers of dimerization (so called dimerizers) specific for the given construct [39]. The approach has already been suc-

cessfully tested in different experimental models. For example, synthetic activation of inducible caspases is sufficient for induction of apoptosis in resistant glioma cells [40]. In addition, controlled activation of an inducible *caspase-9* gene in neovascular endothelial cells in the tumor using the described above approach is being tested as a novel tumor-directed anti-angiogenic therapy [41].

Blocking gene expression of caspase inhibitors using antisense oligonucleotides or antisense RNAs is potentially another powerful strategy for cancer therapeutics. The antisense oligonucleotides down-regulating genes of caspase inhibitors overexpressed in cancer, such as survivin, XIAP, and FLIP have been shown to directly facilitate apoptosis in several tumor models [42-44]. This approach is now exploited by Isis Pharmaceuticals (http://www.isip.com) and Abbott Laboratories (http://abbott.com) with the aim of developing clinically-applicable antisense-based strategies. Williams et al. [45] have shown that siRNA-mediated disruption of survivin mRNA severely reduced colon tumor growth using both in vitro and in vivo xenograft models. A similar experiment based on siRNA technology provides the direct evidence that the intracellular interference with FLIP or Omi/HtrA2 expression resensitizes human tumor cells to diverse proapoptotic stimuli [46, 47]. Taken together, these studies suggest that either direct or indirect caspase modulators have also attracted significant attention as potential targets for therapeutic intervention.

Combined therapy

The near future of cancer therapy will most likely rely on the combined application of apoptosis-sensitizing strategies described above, and conventional radio- and chemotherapy. Significant attention has been given to the combined treatment of tumor cells by caspase-cascade-targeting agents and ionizing radiation. For example, ribozyme-mediated inhibition of survivin expression renders human melanoma cells more susceptible to γ-irradiation [48]. Similarly, overexpression of caspase-9 coupled with radiation has a synergistic effect on the inhibition of glioma invasion [49]. The combination of agents that activate caspases and/or inactivate caspase inhibitors with classical chemotherapeutics will be also more effective than single-agent protocols. Potentiation of drug-induced apoptosis by various caspase-cascade-targeting agents has been demonstrated in various malignancies including glioma, melanoma, breast, prostate, colon, lung, bladder, and ovarian cancer (see [37] for recent review). For instance, anti-survivin oligonucleotides may enhance the efficacy of rituximab (anti-CD20 monoclonal antibody) in aggressive non-Hodgkin's lymphomas [50] whereas hTERT/rev-caspase-6 gene therapy sensitizes malignant glioma cells to the cytotoxic effects of paclitaxel [51]. Moreover, the use of human telomerase reverse transcriptase (hTERT) gene promoter system allows us to enhance the tumor-specificity of this therapy since the expression of telomerase is largely restricted to neoplastic cells. Hence, the combined approaches mentioned above appear to have potential as a novel therapy of cancer when coupled with cancer selective targeting techniques.

Conclusion

The recent achievements in understanding the biochemical and molecular mechanisms of caspase-dependent cell death have inspired an increased interest of biologists and clinical researchers in this subject. The vital role of caspases in cell death propagation have been demonstrated in caspase knockout mice. Moreover, in the last few years, abnormalities in expression or activity of caspases have been identified in a large number of acute pathologies and chronic disorders. Therefore, these proteases seem to be potential targets for therapeutic interventions. However, in fact such treatments have not yet been clinically available. One possible explanation for such a discrepancy is the relatively low specificity of caspase-modulating (inhibitors and activators) compounds presently available. Moreover, most of them are not tissue selective. It is unlikely that inhibition of a single caspase would be sufficient for effective prevention of apoptosis. Pan-caspase inhibitors seem to be more effective with this aim. Nevertheless, caspase inhibitors with a broad spectrum of activity may also inhibit cysteine proteases of other families which are indispensable for cell survival. In addition, caspases are involved in many cellular processes besides apop-

tosis [52, 53]. Therefore chronic inhibition of their activity may result in undesirable side-effects. It is also far from being clear whether the cells with inhibited apoptotic machinery are able to survive. Again, it should not be excluded that the prolonged survival of such cells will promote their malignant transformation. And finally, the increasing efficacy of the therapeutic outcome using caspase inhibitors is possible only when they are combined with other substances that are capable of blocking the various forms of non-caspase dependent apoptosis. Despite all these potential shortcomings, the scope of compounds now entering the early stages of clinical testing (VX-740, VX-765 (both developed by the Vertex Pharmaceuticals Inc.; http://www.vpharm.com) and above-mentioned IDN-6556) will be extended soon and dozens of the novel clinically active drugs for the therapeutic or prophylactic manipulation of apoptosis will become available.

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