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Apoptosis is a physiological cell suicide program that is critical for the development and maintenance of healthy tissues. Regulation of programmed cell death allows the organism to control the cell number and the tissue size, and to protect itself from rogue cells that threaten homeostasis. The changed activity of numerous genes influences switching of cells to a self-destruction program. Apoptosis requires co-ordinated action and fine tuning of a set of proteins that are either regulators or executors of the process. Cancer, autoimmune diseases, immunodeficiency disease, reperfusion injury and neurodegenerative disorders are characterised by disregulation of apoptosis. Modulation of the expression and activation of the key molecular components of the apoptotic process has emerged as an attractive therapeutic strategy for many diseases.

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There are two distinct types of cell death, death by injury and death by suicide. Cells that are damaged by injury, such as mechanical damage or exposure to toxic chemicals, undergo a series of changes characterised by swelling of cells and their organelles, leakage of cell content and inflammation of the surrounding tissues. In other words, cells die by necrosis. In contrast, apoptosis is an organised, genetically directed process, which leads to cell death. Thereby, the term »programmed cell death« has been established as a synonym for apoptosis. Cells dying by apoptosis share unique morphological features, distinct from autolytic, degenerative cell changes observed during necrosis.

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MORPHOLOGY OF APOPTOSIS

Morphological changes of an apoptotic cell might be easily detected under the microscope. Some of these changes can be seen even by light microscopy using specific dyes, while other can be detected only by electron microscopy.

The dying cell, as observed by light microscopy, starts to sever its attachments to other cells and to extracellular matrix, and to round up. It starts to show protrusion from the plasma membrane, referred to as blebs (1). Staining DNA with certain dyes allows observation of the condensation of the cell nucleus, which usually starts as a condensed ring along the nuclear envelope. The condensed nucleus can disassemble into several fragments. The entire cell condenses and is reorganised into apoptotic bodies, which are membrane-bound vesicles varying in size and composition, containing the entire cell content in various combinations, such as cytosolic elements, organelles or parts of condensed nuclei (2). Additional changes have been described by electron microscopy. Condensation or swelling of mitochondria (3), dilatation of endoplasmic reticulum (ER) (4), vacuolisation of cytoplasm (5) and loss of plasma membrane microvilli (6) have been observed. At a certain point, apoptosis affects all compartments and organelles in a dying cell.

Phagocytosis of apoptotic cells or apoptotic bodies is an integral feature of apoptosis. Uptake and degradation of apoptotic cells is rapid and can be conducted both by professional phagocytes such as macrophages and non-specialised cells, like epithelial cells (2). It seems that apoptotic cells could be digested by a lysosomal pathway, organised inside the phagocytosing cell, although a link to autophagy, a cell-autonomous autodigestion of cellular components through the lysosomal pathway, has also been suggested (7).

BIOCHEMISTRY OF APOPTOSIS

There are three different mechanisms by which a cell commits suicide: one is generated by signals arising within the cell, another is triggered by death activators binding to receptors at the cell surface, and the third might be induced by dangerous reactive oxygen species (ROS).

Apoptotic process, either induced by signals arising within the cell or triggered by the activation of death receptors at the cell surface, is characterised by the activation of a family of intracellular cysteine proteases, called caspases (8, 9). However, the third mechanism of apoptosis initiation and execution is caspase-independent. Members of the calpain family of Ca^{2+}/calmodulin-activated proteases seem to be able to cleave the same substrates as caspase (10). In addition, particular endonucleases and chromatin-modifying factors (endonuclease G and apoptosis-inducing factor, AIF) can recapitulate some of the nuclear changes that are typical of the caspase-dependent apoptotic process (11, 12).

Caspases

Most of the morphological changes observed during apoptotic cell death are caused by caspases. Therefore, these proteases are considered to be the central executioners of the apoptotic pathway. Caspases have been subdivided into subfamilies based on their substrate preference, extent of sequence identity and structural similarities.

Caspases cleave substrates at Asp-Xxx bonds, and their distinct substrate specificity is determined by the four residues amino-terminal to the cleavage site (8, 13). Caspases cleave their protein substrates usually at one or, occasionally, at a few positions in the primary sequence. In most cases, this cleavage can result in inactivation of the target protein, but the target protein could also be activated due to the cleavage of the negative regulatory domain or due to the inactivation of the regulatory subunit. Although numerous caspase substrates have been identified, the characteristic features of apoptosis can be currently explained only by caspase-mediated cleavage of a few of them.

DNA ladder nuclease, the enzyme that cuts genomic DNA between nucleosomes generating DNA fragments of approximately 180 bp, is responsible for the characteristic DNA ladder pattern observed after DNA electrophoresis. This DNA ladder nuclease, also called caspase-activated DNase or DNA fragmentation factor (CAD/DFF40), pre-exists in living cells as an inactive complex with an inhibitory regulatory subunit ICAD/DFF45. Caspase-3 cleaves off the inhibitory subunit leading to the activation of the catalytic subunit, and thus, to the internucleosomal DNA fragmentation (14–16). Caspases were also found responsible for the cleavage of nuclear lamins, leading to nuclear shrinking and budding (17, 18).

On the other hand, cleavage of proteins participating in the organisation of focal adhesion, such as focal adhesion kinase (FAK), may contribute to the detachment of apoptotic cells from the surrounding matrix (19).

Loss of the overall cell shape is caused by cleavage of cytoskeletal proteins. It has been shown that actin reorganisation is required for the formation of apoptotic blebs and ring-like structures along the cell periphery (20). p21-Activated kinase 2 (PAK2) is activated by cleavage occurring between regulatory and catalytic subunits, and activated PAK2 participates in the remodelling of actin cytoskeleton leading to apoptotic blebbing (21). In addition, other cytoskeleton-associated proteins, such as gelsolin, myosin light chain kinase (MLCK), Rho proteins, and heat shock protein 27 (hsp27) might also have an important role in apoptotic blebbing.

Caspases implicated in apoptosis are divided into two groups, initiator (upstream) and effector (downstream) caspases (22). All of the known caspases are synthesised as zymogens, enzymatically inert proteins. These inactive procaspases contain three domains, N-terminal prodomain, p20 and p10 domains. An activated caspase is a hetero-tetramer containing two p20/p10 heterodimers and two active sites (22). Most caspases are activated by proteolytic cleavage of the zymogen between p20 and p10 domains or between the prodomain and p20 domain. This cleavage occurs at the Asp-Xxx site, which is a caspase substrate site, suggesting the possibility of autoactivation. Indeed, the simplest way of activating procaspase is to expose it to another, previously activated caspase molecule. This cascade model of caspase activation could be applied for the activation of downstream effector caspases, such as caspase-3, -6, and -7. However, this model, responsible for the majority of substrate cleavage observed during apoptosis, cannot

explain the activation of upstream caspases (23). Initiator caspases are activated in response to pro-apoptotic stimuli and are responsible for the activation of effector caspases.

The cell death pathway initiated through engagement of certain members of the tumour necrosis factor receptor family (or plasma membrane-associated death receptors) leads to activation of caspase-8. Upon ligand binding, death receptors, such as CD95 (Apo-1/Fas), aggregate and form membrane-bound signalling complexes. These complexes recruit procaspase-8 molecules via adapter molecules, such as the Fas-associated protein with death domain (FADD) and/or the tumour necrosis factor receptor associated protein with death domain (TRADD). High local concentration of procaspase-8 results in its autoactivation. Under such crowded conditions, the low intrinsic protease activity of procaspase-8 is sufficient to allow the proenzyme molecules to cleave and activate each other and other downstream caspases, leading to the induction of the apoptotic process (9).

The mitochondrial pathway of apoptosis is used extensively in response to diverse forms of cellular stress (DNA damage, growth factor withdrawal, cell-cycle perturbation, exposure to cytotoxic drugs). These stressors promote release of cytochrome c from mitochondria and it seems that pro-apoptotic members of the Bcl-2 family, including Bax, Bad, Bim, and Bid, might have a role in the perturbation of mitochondrial membrane integrity. Once released into the cytosol, cytochrome c associates with the apoptotic protease-activating factor 1 (Apaf 1) and procaspase-9 to form apoptosome. Both, Apaf 1 and cytochrome c are required for caspase-9 activation. It seems that Apaf 1 is not only a transient activator of caspase-9; it is rather an essential regulatory subunit of caspase-9 holoenzyme (24, 25). Therefore, Apaf 1/caspase-9 complex is nowadays thought to represent the true active form of caspase 9 (26).

The death-receptor and mitochondrial pathways converge at the level of caspase-3 activation. The evidence for the cross-talk and integration of these two pathways is provided by Bid, a pro-apoptotic member of the Bcl-2 family. In some cell types, death receptor-associated caspase-8 activation is insufficient to activate downstream caspases. In these cells caspase-8 can propagate the death signal by engaging the mitochondrial pathway through proteolytic processing of the Bid. Truncated Bid translocates to mitochondria where it causes cytochrome c release.

In addition, cytotoxic T lymphocytes (CTL) promote apoptosis by delivering a serine protease granzyme B, which has similar substrate specificity as caspases, into the cell. Granzyme B can promote caspase activation directly or by engaging the mitochondrial pathway by Bid truncation. Activation of caspase-3, either by death-receptor or by mitochondrial pathways, promotes a cascade of events resulting in cellular destruction, as shown in Figure 1.

Caspases differ in their pro-domain structure and this difference is related to the mode of their activation. Caspases with a long pro-domain contain in their pro-domain a protein-protein interaction module, which allows them to bind to and associate with their upstream regulators. Caspases-8 and -10 contain a death effector domain (DED) and they are activated by their interaction with the intracellular domains of death receptors. Caspases with caspase activation and recruitment domains (CARDs), which include caspases-1, -2, -4, -5, -9, -11 and -12, are most probably activated by the Apaf 1/caspase-9 complex. These two domains share little sequence identity, but they fold into a

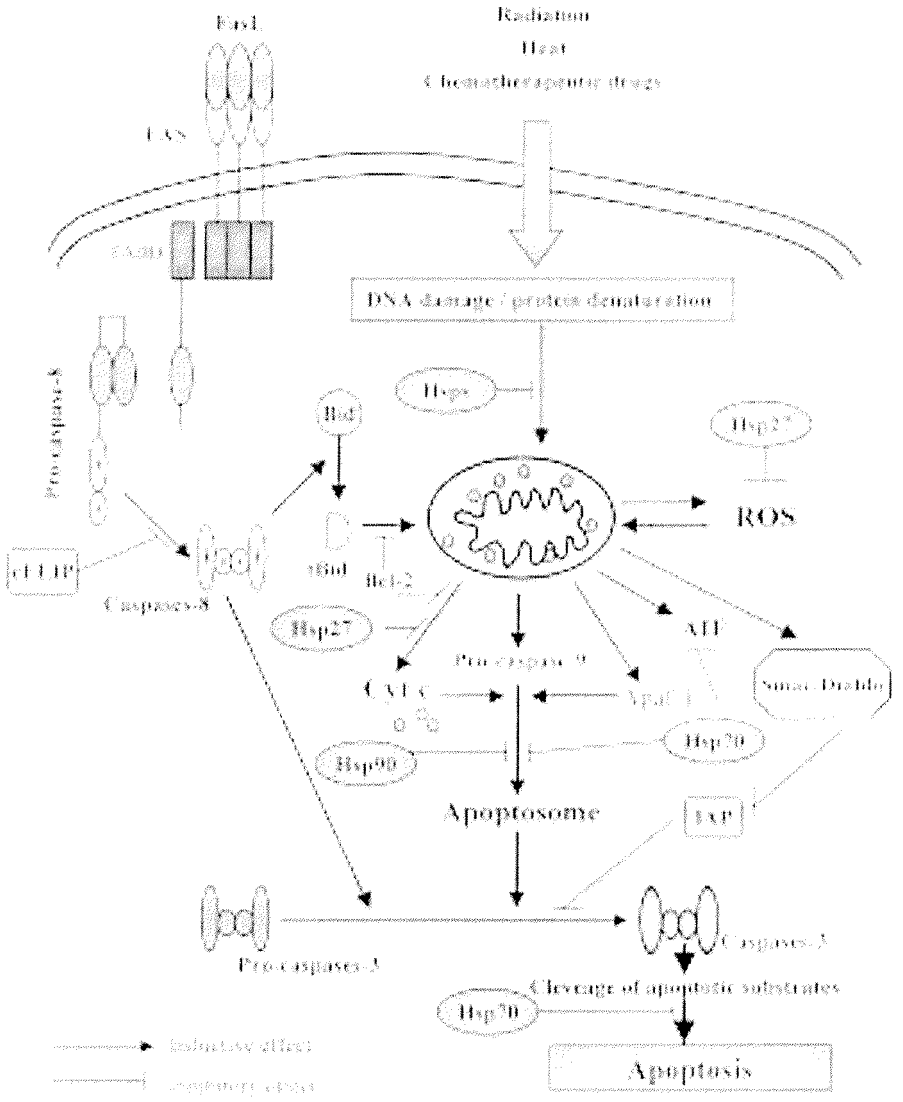

Fig. 1. Apoptotic signalling pathways.

very similar three-dimensional structure consisting of six anti-parallel alpha-helices (27). The same folding pattern is also present in the death domain (DD) of the upstream regulators of apoptosis, such as death receptors and the adapter molecule FADD (28). Caspases with a short prodomain, such as caspase-3, might be activated by most of the known caspase pathways (29).

Neurons, and some other cells, have another way of self-destruction that, unlike the two pathways described above, does not involve caspases. AIF is a flavoprotein normally located in the inner membrane space of mitochondria. Once it is released from mitochondria, it migrates into the nucleus and binds to DNA, leading to DNA destruction and apoptosis (30).

Bcl-2 family

Bcl-2 family members are important regulators of the mitochondrial apoptotic pathway. These members include both anti-apoptotic proteins (Bcl-2, Bcl-x_L, Mcl-1, Bcl-w, A-1) and pro-apoptotic proteins (Bax, Bak, Bid, Bad, Bik).

The family was named after its founding member isolated as a gene involved in B-cell lymphoma (Bcl). Bcl-2 family consists of three functional groups. Members of the first group (Bcl-2, Bcl-x_L, Bcl-w, etc.) are characterised by four conserved Bcl-2 homology (BH) domains (BH1–BH4). They have a C-terminal hydrophobic tail, which localises the proteins to the outer surface of the mitochondrial, ER and nuclear membrane, with the bulk of the protein facing cytosol. They possess anti-apoptotic activity. The second group consists of pro-apoptotic Bcl-2 family members having hydrophobic tails and BH1-BH3 domains (Bax, Bak, Bok, Bcl-x_S). The third group is represented by the Bcl-2 family members (Bik, Blk, Bim_L, Bid, Bad, *etc.*) having pro-apoptotic activity and sharing sequence homology only in the BH3 domain (31–36). Several models have been proposed for the involvement of the Bcl-2 family members in apoptosis regulation. Bcl-2 family members exert their apoptosis-modulating effects at least by controlling the release of cytochrome c from the mitochondrial intermembrane space into cytosol (37, 38). It seems that Bax-like death factors oligomerise and/or interact with voltage-dependent anion channel/adenine nucleotide transporter (VDAC/ANT) to form a protein conducting pore/channel that releases cytochrome c (39–41). Bcl-2 proteins can also interfere with the apoptotic process by heterodimerisation between anti-apoptotic and pro-apoptotic family members (42).

Inhibitors of apoptosis family of proteins (IAP)

IAPs have a protective role during the apoptotic process (43). The prototype of IAPs was first discovered in baculovirus (44). Two motifs were identified in baculovirus IAPs: the baculovirus IAP repeat (BIR) and the so-called RING domain. The BIR is a ~70 residue zinc-binding domain, which seems to be essential for the anti-apoptotic properties of IAPs. In several cases, this domain has been directly involved in the binding and inhibition of caspases (45). The RING domain is also a Zn-binding motif present in baculoviral IAPs and several mammalian IAPs. RING-containing proteins can catalyse both their own degradation and select target proteins through ubiquitylation (46). It has been shown that IAPs participate in the ubiquitylation of some apoptotic substrates (47–49). All human IAPs, except NAIP, function as direct inhibitors of activated effector caspases, caspases-3 and –7. cIAP1 and cIAP2 may inhibit death receptor and mitochondrial apoptotic pathways (50). The level of IAPs expression is regulated transcriptionally, post-transcriptionally or by regulatory proteins. For example, survivin, a member of the IAP family, is regulated in a cell cycle-dependent manner (51), while cIAP2 and XIAP are regulated by the nuclear factor NF-κB (52). Post-transcriptional control includes ubiquitylation of IAPs, directing them either to degradation by proteasomes or modifying their biological activity and/or subcellular localisation (47, 53). IAP regulatory protein known as Smac/DIABLO (second mitochondria-derived activator of caspases/ direct IAP-binding protein with low pI) has been identified (54, 55). Smac/DIABLO is localised in the mitochondrial inter-membrane space and can be released into cytosol in re-

sponse to apoptotic stimuli. This protein binds to XIAP and probably several other IAPs, thus neutralising their anti-apoptotic activity (56).

Heat shock proteins (Hsps)

Hsp70 and hsp27 protect cells from death-inducing stimuli. Hsp70 renders cells highly resistant to death induced by tumor necrosis factor (TNF), monocyte, oxidative stress, ceramide, UV-radiation, caspase-3 overexpression and several hemotherapeutic drugs (57–60).

It has been reported that hsp70 can rescue cells from apoptosis induced by TNF after activation of effector caspases, and that it can delay the death process induced by cytochrome c (61). Hsp70 binds to Bcl-2-associated athanogene 1 (BAG-1), the anti-apoptotic protein that inhibits its chaperon activity (62). Hsp70 could also antagonise apoptosis by inhibition of AIF (63).

Hsp27 possesses survival-enhancing activity and blocks apoptotic pathways induced by death receptors, monocytes, hydrogen peroxide and anticancer drugs (58, 64, 65). Hsp27 protects cells from death caused by ROS indirectly by increasing the cellular glutathione levels (64), and directly by neutralising the toxic effects of oxidised proteins by its chaperone-like activity (66).

In some scenarios, anti-apoptotic activity is related to the prevention of activation of stress kinases. These kinases, namely c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 MAPK, belong to the superfamily of mitogen-activated protein kinases (MAPKs). Stress kinases are activated in many stressful conditions through a signalling pathway that involves the small GTP-binding proteins, MAP3Ks and MAP2Ks, which in turn phosphorylate and activate JNK/SAPK and p38 MAPK (67). It has been shown that elevated levels of hsp70 expression inhibit activation of JNK and p38 (68).

Pro-engulfment signal

Cells undergoing apoptosis can display a number of »eat me« signs. Some of them are relatively well characterised, such as exposure of phosphatidylserine, which is normally restricted to the inner membrane leaflet, to the outer face (69), or changed surface sugars, which can be detected by phagocyte lectins (70). Studies of the ingestion of apoptotic cells *in vitro* by phagocytes have revealed a variety of candidate molecules involved in phagocyte recognition of apoptotic cells and their clearance. They include integrins, scavenger receptors, CD14, C1q receptor, β_2-GPI receptors on phagocyte cells and TSP-binding sites, oxidised LDL-like sites, ICAM-3 and C1q-binding sites on apoptotic cells. Molecules localised on the phagocyte cell surface can interact with their partner molecules on the apoptotic cell surface directly or via serum-derived bridging molecules, such as TSP, iC3b, and β_2-GPI (71–74).

Engulfment of apoptotic cells was previously considered only as a protective waste disposal. However, recent data indicate that the removal of apoptotic cells by phagocytes modulates inflammation, controls tissue remodelling by phagocyte-directed cell killing, and regulates the immune response (75).

APOPTOSIS AND DISEASES

Nervous system

Neurotrophins regulate neural survival through the action of protein kinase pathways, such as the phosphatidylinositol-3 kinase (PI-3K)/Akt and MAPK pathways (76, 77). Trophic factor withdrawal in developing brain induces apoptosis through JNK activation and subsequent phosphorylation of transcriptional factor c-Jun, which in turn induces expression of DP5/Hrk, a known activator of pro-apoptotic protein Bax (78).

Pathological apoptosis in adult brain and physiological apoptosis in developing brain share a similar mechanism in the effector phase of the process, but the key difference lies in the mechanism of apoptosis induction. Toxic insults resulting from biochemical or genetical accidents might trigger neurodegenerative diseases by co-opting apoptotic signalling pathways, either through free-radical generation or caspase activation. Amyloid-β protein, which is implicated in the pathogenesis of Alzheimer's disease, might induce apoptosis by interacting with neuronal receptors, p75 neurotrophin receptor or amyloid precursor protein (79). Different mutations of presenilin, the other protein playing an important role in Alzheimer's disease, increase neuronal vulnerability to apoptosis (80).

Immune system

In the immune system, apoptosis regulates lymphocyte maturation, receptor repertoire selection and homeostasis. At the initiation of the immune response, it is important for the cells of the immune system to be resistant to apoptosis, which enables them to exert their function. On the other hand, in order to prevent autoimmunity, it is important to switch off the activated cells to down regulate the immune response. Two members of the TNF receptor superfamily, CD40 and CD95, have adverse roles in this context: CD40-CD40L stimulate survival, and the CD95-CD95L system induces cell death (81).

Cancer

Tumour cells express several proteins that render them resistant to apoptosis: anti-apoptotic members of the Bcl-2 family, AIPs, hsp70 and hsp27. Increased activity of such protective proteins might result in aggressively growing and therapy-resistant tumours (65, 82–84).

Apoptosis modulating therapy

Targets of apoptosis-modulating therapy are molecular components of the cell death machinery, such as Bcl-2, TNF-related apoptosis-inducing ligand (TRAIL), survivin, caspases, hsp70 and hsp27. Bcl-2, survivin, hsp70 and hsp27 are targets for disruption of gene function with anti-sense oligonucleotides, caspases are targets for regulation of their activity using specific inhibitors, and TNF-related apoptosis can be regulated by recombinant TRAIL. Apoptosis-directed therapeutic agents are expected to selectively influence the apoptotic process in disorders where insufficient apoptosis occurs

(for example cancer) or in those diseases where excessive apoptosis occurs and needs to be attenuated (*e.g.*, in neurodegenerative diseases) (85).

CONCLUSION

In this review, we have focused on some areas of research investigating the mechanism, molecular components, regulators and disregulators of the apoptotic process.

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SAŽETAK

Biokemija apoptotične stanične smrti

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Apoptoza je fiziološki proces programiranog staničnog samoubojstva. Taj je proces važan za razvoj i održavanje tkiva. Reguliranje programirane stanične smrti omogućava organizmu da kontrolira broj stanica i veličinu tkiva, te da se zaštiti od stanica koje ugrožavaju homeostazu. Promijenjena aktivnost mnogih gena utječe na aktiviranje programa samouništenja u stanicama. Apoptoza zahtjeva koordinirano djelovanje i fino usklađivanje mnogih proteina, koji su ili regulatori ili izvršitelji procesa. Rak, autoimune i imunodeficitne bolesti, reperfuzija te neurodegenerativne bolesti povezane su s poremećenom regulacijom apoptoze. Moduliranje ekspresije i aktivacije ključnih molekularnih čimbenika apoptotičkog procesa predstavlja atraktivan terapeutski pristup u liječenju mnogih bolesti.

Ključne riječi: apoptoza, kaspaze, Bcl-2 proteinska obitelj, obitelj čimbenika koji induciraju apoptozu (AIF), obitelj inhibitora apoptoze (IAP), proteinske kinaze aktivirane mitogenima (MAPKs), proteini toplinskog šoka (Hsps)

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ABBREVIATIONS

AIF – apoptosis-inducing factor
Apaf 1 – apoptotic protease-activating factor 1
BAG-1 – Bcl-2-associated athanogene 1
BH – Bcl-2 homology
BIR – baculovirus inhibitor of apoptosis repeat
CAD/DFF 40 – caspase-activated DNase/DNA fragmentation factor 40
CARD – caspase activation and recruitment domain
CTL – cytotoxic T lymphocyte
DD – death domain
DED – death effector domain
ER – endoplasmic reticulum
FADD – Fas-associated protein with death domain
FAK – focal adhesion kinase
Hsp – heat shock protein
IAP – inhibitor of apoptosis
ICAD/DFF 45 – inhibitor of CAD/DNA fragmentation factor 45
JNK/SAPK – c-Jun N-terminal kinase/stress-activated protein kinase
MAPK – mitogen-activated protein kinase
MAP2K – MAPK kinase
MAP3K – MAPK kinase kinase
MLCK – myosin light chain kinase
NF-κB – nuclear factor kappa B
PAK2 – p21-activated kinase 2
PI-3K – phosphatidylinositol-3 kinase
ROS – reactive oxygen species
Smac/DIABLO – second mitochondria-derived activator of caspases/direct IAP-binding protein with low pI
TNF – tumour necrosis factor
TRADD – TNF receptor associated protein with death domain
TRAIL – TNF-related apoptosis-inducing ligand
VDAC/ANT – voltage-dependent anion channel/adenine nucleotide transporter