# Anti-tumor Therapeutic Molecules that Target the Programmed Cell Death Machinery

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**Abstract:** Apoptosis is a process that governs the elimination of unwanted, damaged, or infected cells in most organisms. Defects in its execution are associated with several diseases, including cancer. Herein, we discuss novel molecules with potential anti-tumor activity that target components of the apoptotic machinery, specifically Bcl-2 proteins, IAPs and caspases.

**Key Words:** Cancer, apoptosis, IAP, Caspases, Bcl-2.

## INTRODUCTION

Programmed cell death (PCD) or apoptosis is a genetically regulated process that allows for the maintenance of tissue homeostasis and cell numbers, and provides protection against damaged or infected cells that threaten this balance. Initially, the process was thought to occur only in multicellular organisms, but abundant recent evidence suggests that unicellular organisms such as the budding yeast *Saccharomyces cerevisiae* [1-4] and a number of bacterial species [5, 6] can also undergo a form of programmed death. The existence of apoptosis early in evolution speaks to the paramount importance that this process has in defining populations of single-celled organisms as well as in regulating tissue homeostasis within multicellular eukaryotes. In particular, deregulation of the cellular pathways leading to PCD in mammals can cause a number of disease states, including neurodegenerative diseases [7], autoimmunity [8] and**,** most prominently, various cancers [9].

Members of the Bcl-2 family of proteins are the key regulators of PCD. These molecules have evolved to sense diverse forms of intracellular damage, interpret survival/proliferation signals from other cells, and integrate this information in deciding the fate of the cell. The founding member of the family, Bcl-2, was initially identified as a player in the chromosomal translocation t(14,18), which is prevalent in B-cell non-Hodgkin lymphomas [10]. Prior to the discovery of Bcl-2, cancer research was mainly focused on genes whose products directly promote proliferation. The recognition that a gene coding for an anti-death molecule could behave as an oncogene and contribute to tumor development opened an entire new field of research. So far, more than twenty Bcl-2 family members have been identified, some of them encoded by viral genes [10]. These proteins contain at least one of four conserved BH (Bcl-2 Homology) domains, and depending on their function, are further divided into pro- and anti-apoptotic subgroups. Bcl-2, Bcl-xL and Bcl-w contain four BH domains (BH1-4) and function as anti-death proteins that promote cell survival, whereas the structurally similar Bax and Bak, which lack the BH4 domain, are enhancers of the cell death process. Upstream death signals are sensed by yet another group of pro-death proteins, the BH3-only subgroup, whose members Bim, Bid, Bad and others share sequence similarity only in the short BH3 domain [10]. Different members of the Bcl-2 family localize to the cytoplasm or different subcellular compartments in healthy cells. However, upon receiving a death stimulus, most of these proteins are thought to carry out their functions at various intracellular membranes, particularly the ER and mitochondrial membranes.

Under normal conditions, it appears that a carefully orchestrated balance between pro- and anti-death molecules prevents the execution of the apoptotic program. However, exposure to diverse intracellular and extracellular death signals leads to the activation of the upstream BH3-only proteins, which in their active state can bind to and inactivate the anti-apoptotic function of the pro-survival Bcl-2 family members [11]. Once the brake provided by anti-death Bcl-2 members is removed, the pro-death proteins Bax and Bak oligomerize at the mitochondrial membrane leading to the release of apoptogenic factors into the cytosol, an event that initiates a deadly proteolytic cascade (see below) [12]. Although the mechanism of action of Bax and Bak is still poorly understood, it is widely believed that their oligomerization results in the formation of pores in the outer mitochondrial membrane, which directly contributes to the release of mitochondrial proteins such as cytochrome *c* [13].

In many tumor cells, the levels of anti-death Bcl-2 family members are increased, while the levels of Bax and Bak pro-death proteins are decreased and signaling through the BH3-only sensors is impaired. This phenomenon is in part due to inactivating mutations in the tumor suppressor protein p53, whose functions upon activation by stress signals in healthy cells include the transcription of several pro-apoptotic Bcl-2 family genes [14, 15]. The important roles that Bcl-2 proteins play in regulating the apoptotic pathway, together with the fact that tumor cells often alter their levels and manipu-

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late their functions, imply that therapies directed at modulating the activities of Bcl-2 proteins are potentially effective anti-cancer treatments.

During apoptosis, the main consequence of shifting the balance between anti- and pro-survival Bcl-2 proteins in favor of death is the activation of a group of enzymes called caspases. Caspases are a family of cysteine proteases that cleave proteins after aspartate residues (hence the name CASPases) and whose proteolytic activity ultimately leads to the dismantling of the cell [16]. Caspases that play a role in apoptosis are generally divided in two main groups: 1) the initiators, which contain long pro-domains, act early in the death process, and initiate enzymatic cascades by cleaving, and thereby activating, other caspases, and 2) the executioners, which contain short pro-domains, act downstream of, and are activated by the initiators, and directly execute the suicide program by cleaving key cellular proteins that are essential for life. In mammals, activation of caspase cascades can be triggered through two distinct pathways, the intrinsic (mitochondria-mediated) and the extrinsic (death receptor-mediated) pathways (Fig. **1**).

The intrinsic pathway can be initiated by a number of death stimuli, including growth factor withdrawal, UV and γ-irradiation, and DNA damaging chemicals. This pathway is the target of most chemotherapeutic drugs and radiation treatments. All of these stimuli lead to the release of cytochrome *c* and other apoptogenic factors from mitochondria. Once in the cytoplasm, cytochrome *c* binds to the apoptosis protease-activating factor-1 protein (Apaf-1) triggering the assembly of a multiprotein complex called the apoptosome, which contains several molecules of the initiator caspase-9 [17]. Formation of the apoptosome leads to the autocatalytic processing and activation of caspase-9, which in turn cleaves and activates the executioner proteases caspases-3 and –7, thereby propagating the death cascade.

Signaling through the extrinsic pathway is initiated by the binding of extracellular ligands (e.g. TNFα, FasL/CD95L or TRAIL) to their cognate cell surface death receptors (e.g. TNFR, Fas/CD95 or TRAILR). Ligand-induced oligomerization of the receptors leads to the formation of a cytoplasmic multiprotein complex known as the death-inducing signaling complex (DISC), which recruits and activates the initiator caspase-8. Once activated, caspase-8 cleaves and activates the executioner caspases-3 and -7. In some cells (type I cells) these events are sufficient to execute the death program while in others (type II cells), which usually contain lower levels of active caspase-8, signaling through the death receptors requires an additional amplification loop. This process involves the caspase-8-mediated cleavage of the BH3-only protein Bid into tBid (truncated Bid), which binds to and activates Bax, triggering the permeabilization of mitochondrial membranes and consequent release of apoptogenic factors. Thus, activation of Bid by caspase-8 allows for crosstalk between the extrinsic and intrinsic cell death pathways [18].

The enzymatic cascades initiated by the intrinsic and extrinsic apoptotic pathways converge at the level of executioner caspases. In healthy cells, executioner caspases such as caspase-3 reside in the cytoplasm as inactive zymogens comprised of three modular regions: a short pro-domain, a large p20 subunit and a small p10 subunit. Activation of caspase 3 is achieved by cleavage mediated by the initiator

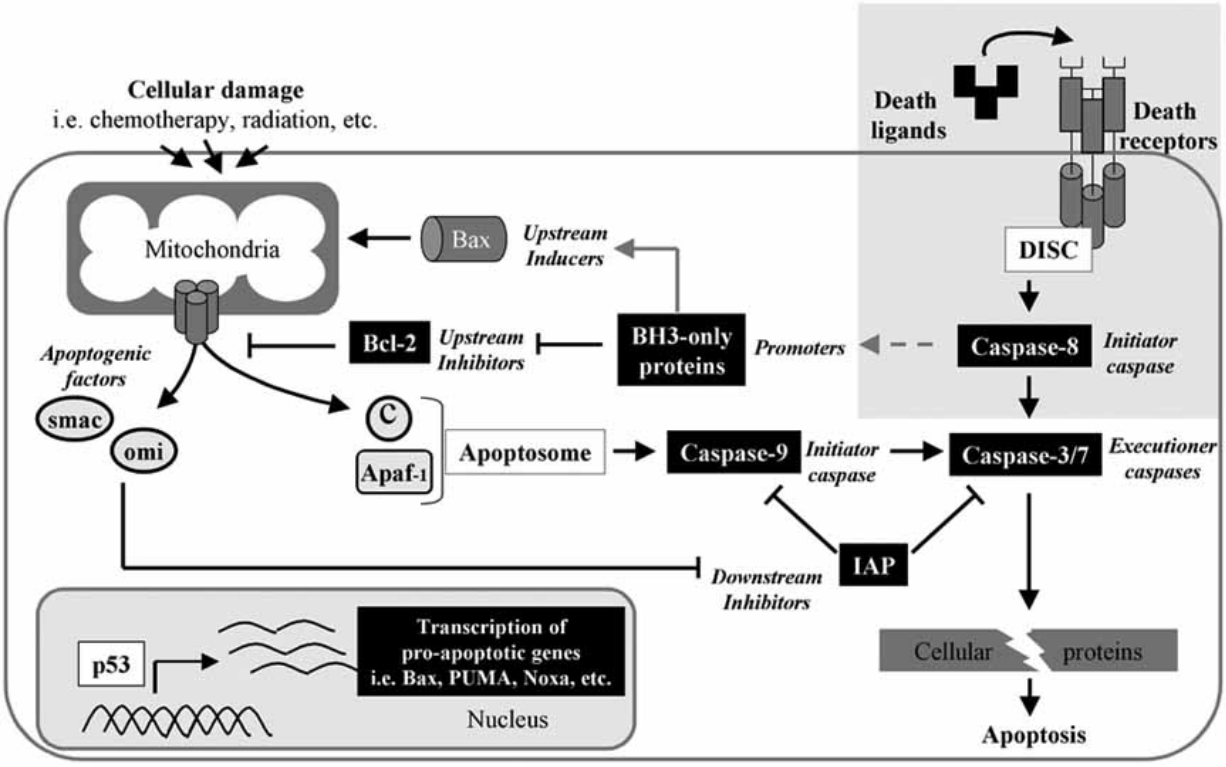



**Fig.** (**1**). Signaling pathways leading to apoptotic cell death. Proteins involved in the execution and regulation of apoptosis are shown. Upper right, the extrinsic pathway of caspase activation is highlighted with a grey box. C, cytochrome c.

caspases at specific aspartate residues within the pro-domain and between the two subunits [19]. The active enzyme is a heterotetramer composed of two p10 and two p20 polypeptides, whose substrates are either inactivated or regulated by cleavage. One of these substrates is ICAD, the inhibitor of a caspase-activated DNAse (CAD). In healthy cells, ICAD remains bound to CAD, effectively sequestering the enzyme in the cytoplasm. In apoptotic cells, caspase-3-mediated cleavage of ICAD results in the release of CAD followed by its translocation into the nucleus where it mediates DNA fragmentation [20]. Other substrates of caspase-3 include proteins involved in the maintenance of normal cellular architecture and proteins involved in the packaging of apoptotic cell corpses for engulfment by neighboring phagocytes [21]. Because of their role as key effector molecules during PCD, caspases are important targets for pharmacological manipulation of apoptotic signaling networks, and the development of drugs that selectively activate these proteases in tumor cells constitutes an attractive avenue for anti-tumor treatments.

The deadly effects of caspase action on cellular substrates are irreversible. Thus, their enzymatic activity in healthy cells is tightly regulated. Caspase function is controlled on various levels by several cellular factors, including upstream inhibitory components acting on initiator multiprotein complexes, as well as further downstream inhibitory molecules that block the activity of enzymatically active caspases. The latter is partly a function of the IAP (inhibitor of apoptosis) family of proteins, which in mammals include c-IAP-1, c-IAP-2, XIAP, NAIP and Survivin among others [22]. A structural hallmark of the IAP proteins is the baculoviral IAP repeat (BIR) domain, of which up to three tandem copies can be present in a single molecule (domains BIR1-3). IAPs such as XIAP, selectively bind to activated caspase-9 through their BIR3 domains, and to active caspases–3 and –7 through a linker region located between BIR domains 1 and 2 [16]. Association between the specific IAP domains and the caspases leads to the inhibition of enzymatic activity. The action of IAPs is in turn regulated by Smac/DIABLO and Omi/HtrA2, pro-apoptotic factors which**,** like cytochrome *c*, are released from mitochondria in dying cells (Fig. **1**). Smac/DIABLO and Omi/HtrA2 contain a short amino-terminal peptide sequence that disrupts binding between caspases and IAPs, efficiently releasing the active proteases and blocking the action of IAPs.

Tumor cells can only subsist if they ignore the requirement for suicide that is imposed on all cells of multicellular organisms. Failure to execute PCD is usually a reflection of defective or absent molecular components of the cell death machinery**.** In principle, each of these components represents a potential target for clinical intervention. In the following sections, recent developments in the generation of anti-tumor molecules that target the apoptotic pathway will be reviewed.

## 1. BCL-2 FAMILY MEMBERS AS ANTI-CANCER TARGETS

As discussed above, due to their central role in the intrinsic mitochondrial pathway of cell death, members of the Bcl-2 family of proteins are important potential targets in anti-cancer therapy. Therapeutic agents that act on these proteins do so by either enhancing the activities of pro-death proteins such as Bax, or by blocking the function of anti-death Bcl-2-like proteins. A number of molecules that target this arm of the intrinsic pathway are currently being developed or used in cancer treatment.

### 1.1. Agents that Target Anti-Apoptotic Bcl-2 Family Members

Although *bcl-2* was initially found to be overexpressed in cells of follicular B-cell lymphoma origin, subsequent studies revealed that elevated levels of Bcl-2 protein are common in a number of other malignancies, including lung cancer, prostate cancer, breast cancer, melanoma, and a plethora of hematological malignancies [23]. In all these cases, Bcl-2 overexpression generally correlates with poor responses to therapy and prognosis. A variety of experimental approaches have been explored in the development of anti-cancer compounds that block Bcl-2 function. These include the use of antisense oligonucleotides to *bcl-2* that target gene expression at the mRNA level, peptides that mimic BH3 domains and bind to the pocket of Bcl-2 where pro-apoptotic BH3-only proteins would normally bind, and small molecule inhibitors of Bcl-2 function. Since the Bcl-xL protein shares high sequence homology with Bcl-2 and many tumors that overexpress Bcl-2 also have high levels of Bcl-xL, many of the treatments being developed use a combined therapeutic agent to target both anti-death factors. Several of these approaches have shown promise at the experimental stage and are currently being tested in preclinical and clinical trials.

#### *1.1.1. Antisense Oligonucleotides Targeting bcl-2 and bcl-xL*

The initial observation that antisense oligonucleotides to *bcl-2* could modulate the survival of malignant cells came from studies by Reed and coworkers who reported that an 18-nucleotide-long antisense oligomer could efficiently knockdown Bcl-2 protein levels in leukemic cells, resulting in direct inhibition of growth and survival [24, 25]. Subsequent studies showed that killing of acute myeloid leukemia (AML) cells with this *bcl-2* oligonucleotide could act synergistically with commonly used chemotherapeutic drugs [26, 27].

Currently, new-generation antisense oligonucleotides targeting the *bcl-2* mRNA are undergoing phase-3 clinical trials for multiple myeloma, chronic lymphocytic leukemia and non-small cell lung cancer, and phase-2 for hormone refractory prostate cancer [28]. These therapies utilize a synthetic stabilized phosphothioate oligonucleotide called Genasense/G3139, developed by Genta Inc. (Berkeley Heights, NJ). Genasense/G3139 is a single stranded DNA molecule which hybridizes to the first 18 nucleotides within the *bcl-2* open reading frame and upon complex formation, induces mRNA degradation by RNase H-like endonucleases [29]. The drug has been delivered in combination with a number of cytotoxic and immunotherapeutic agents in an attempt to subside a variety of hematologic malignancies and solid tumors. The first phase-1 trial reported great potential for Genasense as a therapeutic agent in patients with non-Hodgkin's lymphoma [30]. This antisense oligonucleotide also had promising effects on other B-cell malignancies, adult AML [31] and

small-cell lung carcinoma [32]. In preclinical trials, Genasense could strongly enhance the efficacies of dacarbazine and gimatecan in a xenograft melanoma mouse model [33]. Synergistic effects with dacarbazine were also noted in a phase-3 clinical trial for patients with advanced melanoma. However, the drug has not been granted FDA approval for treatment of malignant melanoma because it failed to prolong survival despite slowing down disease progression. The lack of correlation between clinical responses and antisense-mediated down-regulation of Bcl-2 expression in melanoma patients has raised some concerns about the efficacy of this novel anti-tumor drug. However, studies involving patients with other malignant diseases are currently being evaluated, and Genasense may still prove to be an important anti-cancer therapeutic.

The effectiveness of antisense-based therapies directed against *bcl-2* could potentially be improved by simultaneous down-regulation of other pro-survival proteins such as Bcl-xL. Due to alternative splicing of the *bcl-xL* mRNA, the *bcl-xL* gene has several protein products, which differ in their apoptotic activities. Bcl-xL is an inhibitor of apoptosis, whereas Bcl-xS enhances the pro-death phenotype. To take full advantage of these opposing functions of the splice variants, Taylor and colleagues have developed antisense oligonucleotides, which hybridize to a region of the gene proximal to the splice donor site necessary for the production of Bcl-xL [34]. This approach ensures that only the short pro-death form of the gene is produced. Cancer cells of various origins treated with these oligonucleotides are sensitized to chemotherapy-induced apoptosis underscoring the potential benefits of antisense *bcl-xL* therapy [35-37]. Antisense oligonucleotides that down-regulate both Bcl-2 and Bcl-xL by targeting the mRNA homology regions shared between the two genes have also been developed [38]. Such bispecific oligonucleotides effectively kill different cancer cells, [39-42] and are currently being tested in preclinical trials.

### *1.1.2. Peptides that Target the BH3 Binding Pocket of Bcl-2-Like Anti-Apoptotic Proteins*

The BH3 domain of pro-death proteins is a small 9 amino acid sequence which forms an amphipathic α-helix that binds to a hydrophobic surface pocket of Bcl-2-like anti-death proteins. This binding pocket represents a regulatory site where BH3-containing endogenous antagonists bind to and inhibit the Bcl-2-like cytoprotective activity. Therefore, peptides and small molecules mimicking the BH3 domain could in principle dock at this groove and effectively promote apoptosis by blocking anti-death activities and inducing cytochrome *c* release. Indeed, such Bcl-2-binding peptides have been shown to promote apoptosis of leukemia and lymphoma cells *in vitro* as well as in a mouse model of myeloid leukemia [43]. However, a major downfall of many of these synthetic compounds has been their limited solubility and permeability as well as susceptibility to proteolytic cleavage. Several strategies have been employed by different groups to bypass this limitation. Coupling of a synthetic peptide corresponding to the BH3 domain of Bak to the *Drosophila* Antennapedia homeoprotein internalization domain allowed for rapid peptide internalization and induction of apoptosis in HeLa cells [44]. Moreover, treatment with BH3 peptides resensitized HeLa cells overexpressing Bcl-xL to Fas-mediated apoptosis. In another study, a cell-permeable Bcl-2-binding peptide (cpm-1285) was designed by chemically conjugating decanoic acid to a BH3 peptide derived from the pro-apoptotic protein Bad [45]. While cpm-1285 had no effect on normal human peripheral blood lymphocytes, it effectively killed HL-60 tumor cells. The peptide was also effective in slowing down human myeloid leukemia growth in an *in vivo* SCID mouse model, demonstrating a proof of concept for the use of BH3 peptides as anticancer therapeutics. Delivery of Bax- and Bad-derived BH3 peptides by cationic transfer into Jurkat cells that overexpress Bcl-2 or Bcl-xL has also shown selective inhibition by these peptides [46]. Bad-BH3 peptides exhibit greater affinity for Bcl-xL, while Bax-BH3 peptides are much more potent inhibitors of Bcl-2 function. This selectivity displayed by BH3 peptides implies that there might be possible limitations to their use as therapeutics since they will only inhibit certain target proteins in cells that express more than one anti-apoptotic Bcl-2 member.

Recently, an additional chemical strategy called hydrocarbon stapling was used to create BH3 peptides with improved pharmacological properties [47]. This approach resulted in the generation of SAHBs (stabilized α-helix of Bcl-2 domains) peptides that contain non-natural amino acids on the surface opposite the region of interaction with Bcl-2-like proteins. SAHBs were shown to specifically kill leukemia cells and inhibit the growth of human leukemia xenografts *in vivo* [47].

Despite abundant recent evidence for the application of BH3 peptides in cancer therapy, the exact mechanism of action of these peptides remains unclear. It has been suggested that BH3 peptides can kill cells in a Bcl-2-independent fashion due to the fact that they are α-helical in structure. Theoretically, α-helixes can, independently of their sequence, insert into and disrupt the integrity of the mitochondrial membrane thereby releasing pro-apoptotic mitochondrial factors. This notion is supported by experiments showing that a mutant α-helical Bad BH3 peptide that was no longer able to bind to Bcl-2 could induce cell death as effectively as the wild type peptide [48]. Regardless of the ambiguity in their modes of action, the use of BH3 peptides may provide a useful strategy for experimental and therapeutic modulation of protein-protein interactions in the apoptotic pathway

### *1.1.3. Small Molecule Inhibitors of Anti-Apoptotic Bcl-2 Proteins*

Several interesting small molecules that interact with and block the actions of Bcl-2, Bcl-xL and Mcl-1 have been recently reported (Fig. **2**). One of them, Gossypol (**1**), is a natural polyphenolic aldehyde-containing compound isolated from cottonseeds. In a recent study, this small molecule, which is a dual direct inhibitor of Bcl-2 and Bcl-xL, was shown to induce cell death in a BxPC-3 pancreatic cell line with no effect on normal peripheral blood lymphocytes [49]. Similar to this study, tissue culture studies in other cancer cell lines have shown that the (-) enantiomer of Gossypol has a more potent toxic effect than the (+) enantiomer [50, 51]. While it has been reported that racemic gossypol is active

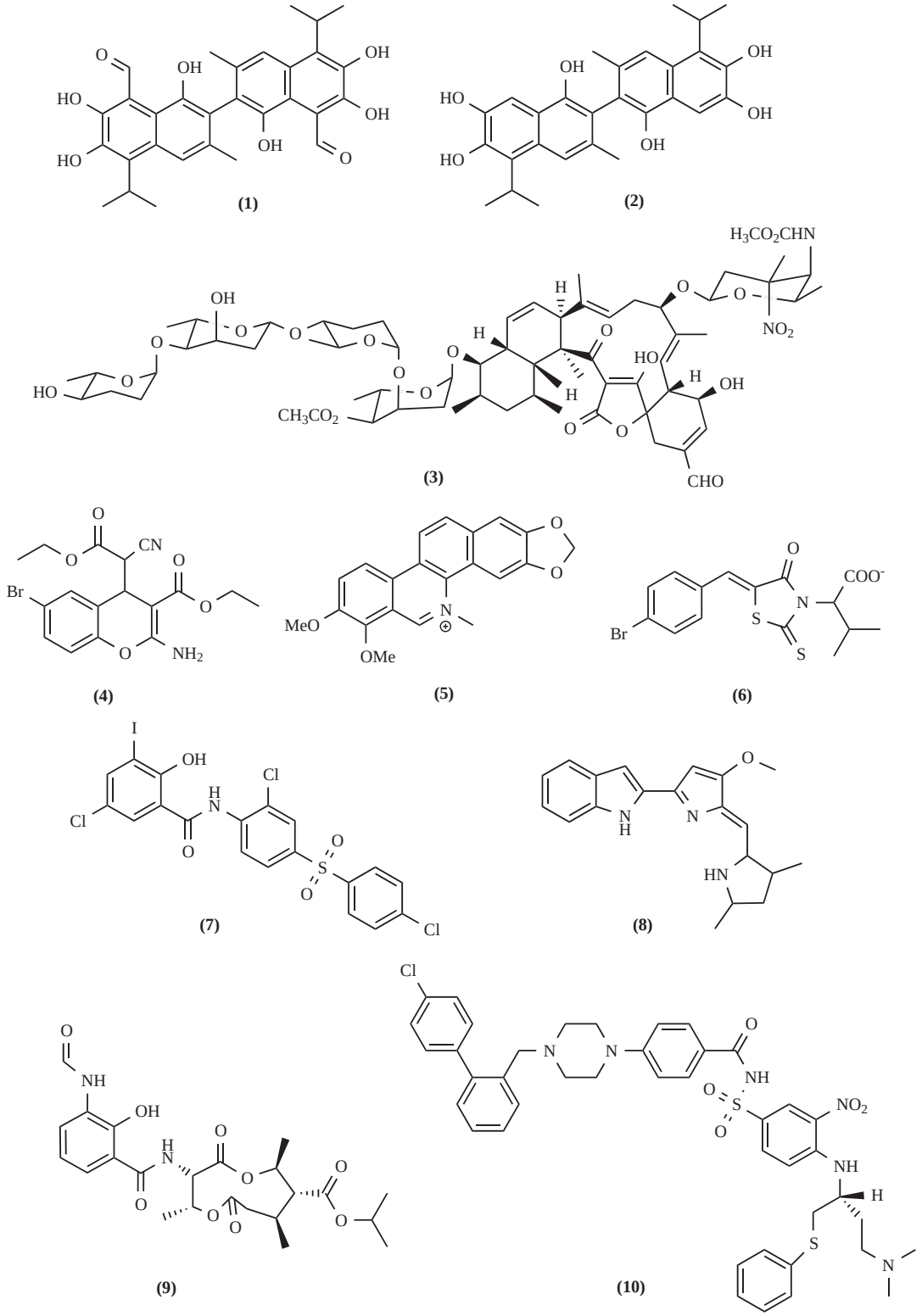



**Fig. (2).** Inhibitors of anti-apoptotic Bcl-2 family members.

against many cancer cell lines both *in vitro* and *in vivo*, it also generated toxic side effects in phase-1 clinical trials possibly due to the fact that it contains two aldehydes [52, 53]. Therefore, attempts to produce less toxic derivatives of Gossypol that still retain the Bcl-2 inhibitory function have resulted in the synthesis of Apogossypol (**2**), which lacks the two aldehyde groups [54]. Apogossypol binds to and inhibits Bcl-2 and Bcl-xL and is capable of inducing apoptosis in tumor cell lines as well as in cells isolated from patients with chronic lymphocytic leukemia.

A number of other chemical inhibitors of Bcl-2-like function have been identified and most of these are currently in preclinical trials for anti-cancer therapy. These include: (i) Tetrocarcin A (TC-A) (**3**), an antibiotic from *Actinomyces* isolated by virtue of its Bcl-2-opposing activity in a high throughput screen (HTS) of a natural library [55]; (ii) HA14-1 (**4**), a chromene derivative identified in a computational screen of a chemical library based on the structure of the Bcl-2 molecule [43]; (iii) chelerythrine (**5**), a natural benzophenanthridine alkaloid, identified by HTS of extracts derived from natural products [56]; (iv) BH3I-1 (**6**) and –2 (**7**) (BH3 inhibitors-1 and –2), isolated by their ability to displace a fluorescent Bak BH3 peptide from Bcl-xL in a fluorescent polarization assay [57]; v) GX15-070 (**8**), a broad spectrum inhibitor of Bcl-2 proteins (Gemin X, Montreal, Canada) [29]; vi) Antimycin A (**9**), an inhibitor of mitochondrial electron transfer that binds Bcl-xL [58]; vii) ABT-737 (**10**), a small molecule BH3 mimetic designed by using a structure-based approach to target the groove of Bcl-xL [59], and many more.

In preclinical trials, all of these compounds have been reported to selectively kill cancer cells of various origins. While their exact specificities for the different anti-death Bcl-2 proteins are not clear, they are all potent inhibitors of at least Bcl-2 and Bcl-xL function. As studies progress into the later stages of clinical trials, it remains to be determined whether any of these compounds will have cytotoxic side effects or issues with stability and susceptibility to proteases that might make them less marketable as anti-cancer drugs. Nevertheless, abundant research so far has proven that targeting protein-protein interactions among Bcl-2 family members is a *bona fide* and promising approach to developing novel anti-cancer therapies.

### 1.2. Drugs that Target Pro-Death Proteins of the Bcl-2 Family

Most of the research in understanding how to utilize the function of Bcl-2 family members in cancer therapies has been focused on designing inhibitors of the anti-death proteins of this family. Albeit limited and not as diverse, some studies have also designed gene therapy approaches to enhance the activities of pro-death Bcl-2 proteins. Adenoviral vectors expressing these genes have recently been constructed with the goal of delivering them into cancer cells. Adenovirus-mediated overexpression of Bax, Bcl-xS, Bik and others has been a successful strategy for killing various cancer cell lines [60-62], therefore opening new possibilities for cancer treatment. However, their use in clinical settings has been limited by their intrinsic cytotoxicity and the myriad of difficulties typically associated with any form of gene therapy.

## 2. CASPASES AS ANTI-CANCER TARGETS

The selective activation of caspases might be a valuable strategy for combating cancer and other diseases where insufficient cell death leads to pathogenesis. Several strategies that trigger caspase activation have been developed and several drugs are currently being tested in preclinical trials.

### 2.1. Small Molecule Activators of Caspases

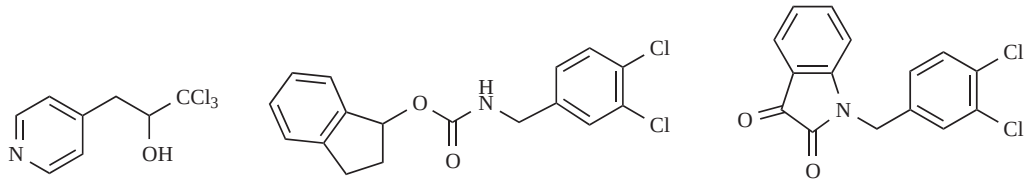
The pharmacological activation of caspases by small cell-permeable drugs may prove to be an effective avenue in the treatment of cancer cells. Caspase-3 is kept in an inactive state in healthy cells by an intramolecular electrostatic interaction called the "safety catch" [61]. The latter consists of a triplet of aspartate residues contained within a flexible loop near the large and small subunit junction. As mentioned above, activation of caspase-3 is achieved through the cleavage of the junction between its large and small subunits. Roy and colleagues showed that removal of the safety catch results in autolytic maturation of caspase-3 and susceptibility to upstream proteases such as caspase-9 and granzyme B [61]. This study suggested that the Asp tripeptide of the safety catch plays an important regulatory role in modulating the sensitivity of cells to apoptosis. Therefore, efforts are being directed at screening for caspase activators that disrupt the safety catch and lower the threshold of caspase activation.

Short peptides containing an arginine-glycine-aspartate (RGD) motif can also activate caspase-3. Normally, intracellular matrix proteins containing an RGD motif can block integrin-dependent signaling and cell adhesion, which was thought to be the cause for the ensuing cell death. However, it has been shown that RGD peptides can directly activate caspase-3 independent of integrins and therefore promote apoptosis [63]. Interestingly, caspase-3 contains an RGD sequence near its active site, which keeps the protease in an inactive state. Therefore, it is plausible that binding of an RGD activator molecule displaces intramolecular bonding leading to caspase-3 activation and apoptosis. The identification of small molecule activators of caspases that exploit this mechanism may lead to a new line of potential anti-cancer therapeutics.

Several research groups have used an HTS approach to identify caspase-3 activating drugs in cell-based assays. The obtained drugs do not seem to activate caspases directly, but rather indirectly by modulating the formation of initiator multiprotein complexes that trigger apical caspase activation. One example is PETCM (**11**), a small compound identified by Jiang *et al.*, which accelerates caspase activation by promoting apoptosome formation *via* its interaction with the cellular inhibitor prothymosin [64] (Fig. **3**). Similarly, Nguyen and Wells identified carbamate- and indolone-based compounds (**12**, **13**) which promote apoptosome formation *via* induction of Apaf-1 oligomerization [65]. Other molecules such as the MX-2060 compounds developed by Maxim Pharmaceuticals, appear to modulate the activation of caspase-8 *via* the transferring receptor [66]. Specifically, the compound MX-2167 has been shown to induce apoptosis in a number of cancer cell lines and reduce tumor growth up to 90% in an animal model of prostate cancer.

### 2.2. Gene Therapy and Chimeric Protein Approaches

Several gene therapy approaches have been developed to replace defective caspases in tumor cells with their wild type counterparts. These have included adenovirus-mediated expression of caspases-3, -6, -8 and –9, which have resulted in both *in vivo* and *in vitro* anti-tumorogenic activities [67]. In addition, adenoviral gene therapy approaches have also been used in the delivery of caspase constructs that can be activated "on demand" by the addition of a cell-permeable compound. The strategy relies on the fusion of caspases to one or



**(11)** **(12)** **(13)**

**Fig. (3).** Activators of caspases.

more chemically inducible dimerization domains forming chimeric "death switches", which can be activated by chemical inducers of dimerization specific for a given construct [68]. These drugs induce intracellular protein aggregation leading to caspase activation. Specifically, adenovirus-mediated activation of inducible caspases-1 and –3 was shown to inhibit the growth of prostate cancer both *in vitro* and *in vivo* [68]. Additionally, inducible caspase-9 under the control of a prostate-specific androgen-responsive promoter was able to suppress prostate tumor growth in nude mice [69], and inducible caspases-3 and -6, but not caspase-8, could activate apoptosis in glioma cell lines [70, 71]. Chemically inducible caspases have also been reported to have an effect as antiangiogenic tumor therapy [72].

Other chimeric proteins containing effector caspases have also been used to activate apoptosis in tumor specific cell lines. These chimeras, called immunocasp-3 and immunocasp-6, are composed of a single chain anti-erbB2/HER2 antibody and the translocation domain of *Pseudomonas* exotoxin-A fused to an active caspase-3 or –6 molecule [73, 74]. The specificity for tumor cells is provided by erbB2/HER2, which is normally not expressed in adult tissue, but is overexpressed in a large number of human cancers including breast, gastric, lung, prostate, bladder and others. Upon binding to the extracellular domain of HER2, the antibody-containing chimera is internalized and the translocation domain of exotoxin-A releases the chimera into the cytoplasm leading to caspase activation. Prolonged mouse survival and significant tumor regression in mouse xenografts of HER2-positive cells have been observed upon exposure to immunocasp-3 or immunocasp-6 regardless of the delivery injection method. The caspase-3 chimera is currently being evaluated in preclinical trials.

Another genetic approach was developed by Tse and Rabbitts, who constructed a single-chain antibody fused to caspase-3 [75]. Specific binding between these chimeras and a respective multivalent antigen results in increased concentration of the fusion protein and autoactivation of caspase-3 due to the close proximity of caspase-3 molecules. The construct has conferred toxicity in transfected CHO cells, suggesting that caspase-3 expression combined with antibodies against tumor-specific antigens could provide a promising strategy for the selective induction of apoptosis in tumor cells.

## 3. IAPs AS ANTI-CANCER TARGETS

Caspase activity is tightly controlled in cells by the endogenous IAP family of anti-apoptotic proteins and, not surprisingly, pathologic overexpression of IAPs has been reported in many cancers. Survivin, for example, which plays a dual role in suppressing apoptosis and regulating cell division, is not expressed in normal cells but is overexpressed in cancer cells [76]. Therefore, part of the current research efforts in the development of anti-cancer therapies is aimed at inhibiting IAP function.

### 3.1. Antisense Oligonucleotides Targeting IAPs

Antisense technology against Survivin and XIAP is presently being evaluated in preclinical trials by Isis Pharmaceuticals (Carlsbad, CA), Eli Lilly (Indianapolis,IN) and Aegera (Montreal, Quebec, Canada). Antisense inhibition of XIAP function has been reported to sensitize a variety of cell lines to chemotherapy [77, 78]. In animal models of non-small cell lung cancer, antisense knockdown of XIAP combined with vinorelbine delays tumor establishment [79]. Recently, AEG35156/GEM640, a second-generation phosphorothioate-based antisense oligonucleotide targeting XIAP has entered phase-1 clinical trials. It remains to be determined whether antisense therapy that targets caspase inhibitors is an effective cancer treatment strategy.

### 3.2. Drugs that Disrupt the Association Between IAPs and Caspases

To target the IAP and caspase-3/-7 interaction, two independent groups employed an enzyme depression assay in which XIAP-mediated suppression of caspase-3 is overcome by chemical compounds [80, 81]. They identified two classes of XIAP antagonists**,** including polyphenylurea derivatives such as the TPI1396 compounds (**14**) and benzenesulfonamide derivatives such as the TWX compounds (**15**), both of which release caspase-3 by targeting areas adjacent to the second BIR domain (Fig. **4**). The phenylurea-based compounds were shown to induce apoptosis in cultured tumor cell lines and primary leukemias, as well as suppress the growth of tumor xenografts in mice [74]. Moreover, the drugs induce apoptosis through a Bcl-2- and Bcl-xL-independent mechanism, suggesting that they might be useful therapeutic agents against cancers that overexpress both of these proteins.

Mimicking endogenous antagonists of IAPs is another strategy for inhibiting IAP function. SMAC and Omi/HtrA2 are mitochondrial proteins, which upon release from the organelle during apoptosis bind to and inactivate cytoplasmic IAPs thereby releasing caspases. Currently, several companies are developing small molecule SMAC mimetics and SMAC peptides to inhibit IAP function. SMAC-like peptides have been shown to sensitize cancer cells and mouse xenograft models of human glioma and non-small cell lung can-

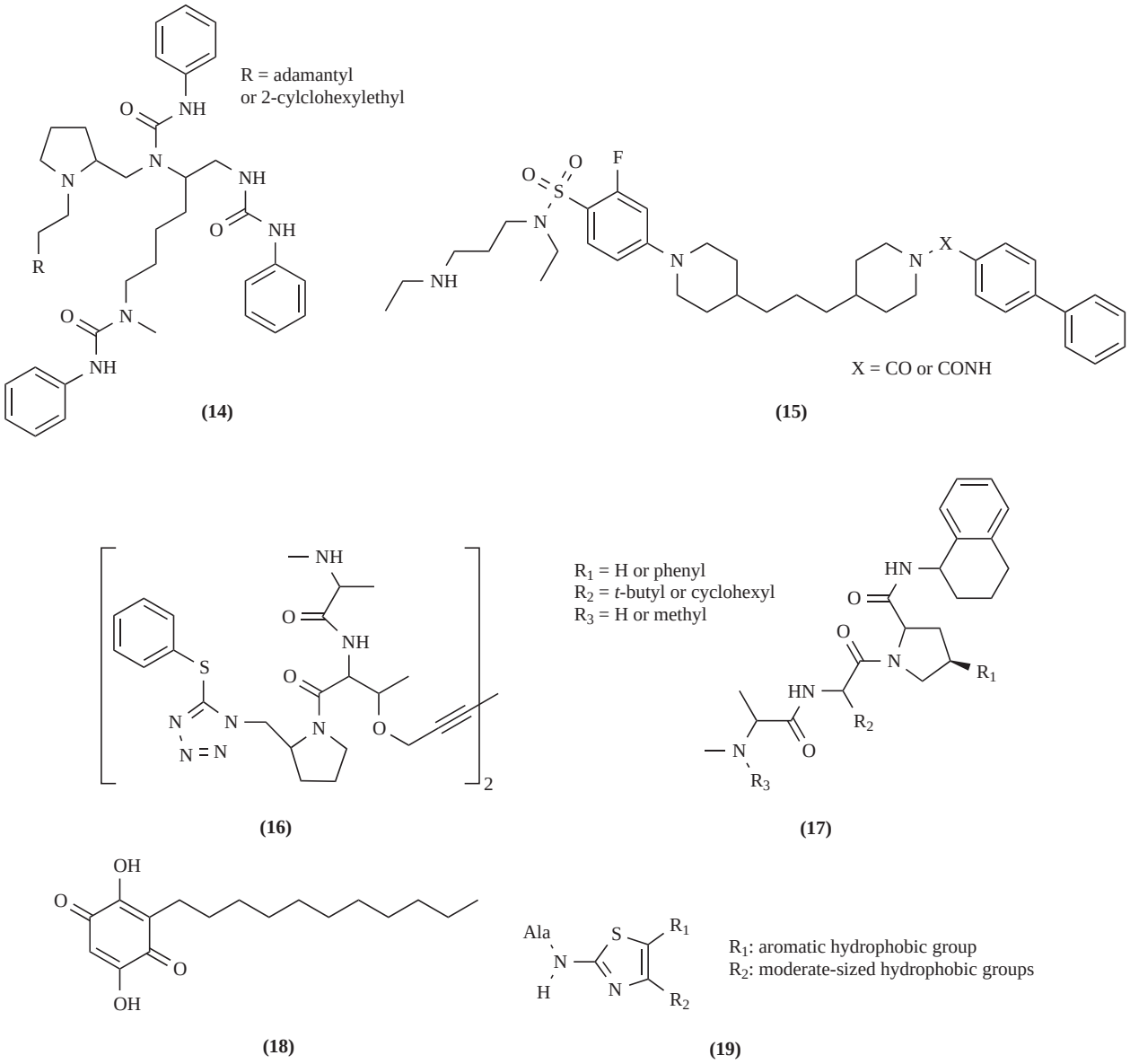



**Fig. (4).** Inhibitors of IAPs.

cer to chemotherapy and TRAIL treatment [82-84]. Recently, an *in silico* analysis of the SMAC-IAP-binding motif conformation aided in the development of a SMAC mimetic called compound 3 (**16**), which targets XIAP, cIAP1 and cIAP2, and synergizes with TRAIL and TNF to kill cultured tumor cells [85] (Fig. **4**). These data provide proof of concept evidence that compounds mimicking the effects of IAP inhibitors can potentially be used as drugs for anti-cancer treatment.

The three-dimensional structure of SMAC bound to the BIR3 domain of XIAP revealed that the N-terminal four amino acids of SMAC bind to the same pocket of XIAP which is normally occupied by the small subunit of caspase-9, suggesting competition for binding [86]. Therefore, peptides that mimic the N-terminus of SMAC should dislodge XIAP from caspase-9 thereby activating the caspase cascade. Indeed, capped tripeptides (**17**) have been developed which bind to the BIR3 domain of XIAP with high affinity. These peptides were effective in killing a number of human tumor cell lines [87]. Other antagonists targeting the SMAC-binding site include embelin (**18**), a cell-permeable compound of the Japanese Ardisia herb [15] and thiazole- and imidazole-based non-peptidic small molecule inhibitors (**19**) [88] (Fig. **4**).

Certain tumors simultaneously overexpress several IAP family members [89]. Therefore, inhibition of a single IAP protein might not yield significant effects *in vivo*. Further complexity is added to drug design when interactions with other proteins and post-translational modifications of IAPs are taken into account. For example, XIAP activates NF-κB, c-Jun $NH_2$-terminal kinase and SMAD signaling through interaction with the type I transforming growth factor-β receptor. Even though IAP-based therapies still encounter some unresolved issues, they remain a very promising avenue for cancer treatment.

## CONCLUDING REMARKS

In the past few years, an enhanced understanding of the molecular basis of the apoptotic cell death program has emerged leading to the identification of potential targets for cancer treatment. In particular, the elucidation of three-dimensional structures and mechanism of action of key players of the cellular suicide machine has opened new avenues for pharmacological intervention aimed at modulating levels of expression of these proteins or impairing their ability to associate with other cellular factors. Although several anti-cancer compounds that target the apoptotic signaling pathway are currently being tested in preclinical and clinical trials, it is clear that much work is needed to develop efficient treatments. The rapid advances in high throughput screening assays for drugs that disrupt protein-protein interactions should greatly aid this process. One of the greatest challenges that remains for any potential new treatment is to manage the redundancy of the cell death network, in which entire families of proteins operate at any given stage in the suicide process. Therefore, compounds that simultaneously target several members of these families at conserved sites will most likely offer the highest therapeutic efficacy.

## ABBREVIATIONS

AML = Acute Myeloid Leukemia

Apaf-1 = Apoptosis Protease-Activating Factor-1

Bcl-2 = B-cell Leukemia 2

BH = Bcl-2 Homology

BIR = Baculoviral IAP Repeat

HTS = High Throughput Screen

IAP = Inhibitor of Apoptosis Proteins

ICAD = Inhibitor of Caspase-Activated DNAse (CAD)

PCD = Programmed Cell Death

RGD = Arginine-glycine-aspartate

SAHBs = Stabilized Alpha-Helix of Bcl-2 domains

SMAC = Second Mitochondria-derived Activator of Caspases

TNF = Tumor Necrosis Factor

TRAIL = Tumor necrosis factor-Related Apoptosis-Inducing Ligand

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