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Forcing Cancer Cells to Commit Suicide

Christel Vangestel,¹ Christophe Van de Wiele,² Gilles Mees,³ and Marc Peeters¹

Abstract

Apoptosis plays a crucial role in the normal development, homeostasis of multicellular organisms, carcinogenic process, and response of cancer cells to anticancer drugs. It is a genetically strictly regulated process, controlled by the balance between pro- and antiapoptotic proteins. Resistance to standard chemotherapeutics also seems to be an apoptosis-related process due to failure to activate the apoptotic machinery. Hence, the molecular pathways (extrinsic and intrinsic) regulating the apoptotic process are attractive targets for potential therapeutic intervention. The goal of proapoptotic drugs is to selectively induce apoptosis in the tumor cell while leaving healthy cells unharmed. Several proapoptotic receptor agonists have recently been developed, activating selectively the extrinsic pathway, and give promising results. Targets for the intrinsic pathway include the Bcl-2 family proteins, the inhibitor of apoptosis proteins, the p53 pathway, and many others. However, several studies have implicated that using monotherapy will probably not be sufficient to sensitize or induce apoptosis in all tumor cells. Most promising results, in terms of killing the tumor cell, will be achieved by the combination of various therapeutic strategies. In this review, promising apoptosis-inducing anticancer therapies are summarized.

Key words: apoptosis, intrinsic and extrinsic pathway, cancer therapy

Introduction

Apoptosis, or programmed cell death, allows the organism to tightly control cell numbers and tissue size, maintaining overall homeostasis. It is involved in many physiologic processes (e.g., development, proliferation/homeostasis, differentiation, regulation, and function of the immune system). Apoptosis is a highly regulated process that allows a cell to self-degrade in order for the body to eliminate unwanted or dysfunctionnal cells. Damaged or unneeded cells are removed by neighboring cells without causing inflammation from leakage of the cell content. Defects in apoptotic cell-death regulation contribute to a variety of pathologic conditions, including cancer.¹ Cancer can occur when the balance between cell proliferation and apoptosis is disturbed, either by an increase in cell proliferation or a decrease in tumor cell apoptosis. The goal of anticancer treatment is to introduce cell death in the tumor cells while leaving healthy cells undamaged. Although ap-

optosis is a key factor influencing therapeutic outcome, tumor cells can also die by nonapoptotic mechanisms, including autophagy, mitotic catastrophe, and necrosis, influencing the overall tumor response to anticancer therapy. Therapeutically targeting apoptotic as well as non-apoptotic cell deaths will enhance the effect of anticancer treatment.²

The morphologic characteristics of the apoptotic cell include chromatin condensation, nuclear fragmentation (i.e., internucleosomal DNA cleavage), plasma membrane blebbing, and cell shrinkage. Ultimately, the cell breaks into small membrane-surrounded structures, called apoptotic bodies. The externalization of phosphatidylserine (PS), a phospholipid normally found on the inner-cell membrane leaflet to the exterior-cell membrane leaflet, is one of the first events of apoptosis and continues until the cell is engulfed. This process is facilitated by a calcium-dependent deactivation of translocase and floppase, as well as the activation of a third enzyme, scramblase.³ PS serves as a signal for the

Departments of ¹Gastroenterology and ²Nuclear Medicine, Experimental Cancerology and Radiotherapy, University Hospital Ghent, Ghent, Belgium.

³Department of Nuclear Medicine and Molecular Imaging, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.

Address correspondence to: Christophe Van de Wiele; Department of Nuclear Medicine, University Hospital Ghent, De Pintelaan 185, 9000 Ghent, Belgium; Tel.: 003293323028; Fax: 003203323807
E-mail: christophe.vandewiele@UGent.be

phagocytes to recognize and engulf the apoptotic bodies, without causing an inflammatory response.⁴ This is in sharp contrast with the necrotic way of cell death, in which cells release their cellular content in the cell's environment, resulting in the damage of neighboring cells and a strong inflammatory response in the surrounding tissue. In comparison to apoptosis, necrotic cells die accidentally in response to an acute injury.

Two main apoptotic pathways have emerged: the mitochondrial pathway (intrinsic pathway) and the death-receptor pathway (extrinsic pathway). The intrinsic pathway is mediated by the Bcl-2 superfamily members that interact with the mitochondrial membrane, while the extrinsic pathway is governed by the tumor necrosis factor (TNF) superfamily of ligands and receptors, interacting at the cytoplasmic membrane.

The Apoptotic Pathways

Apoptosis can be triggered in a cell through either the intrinsic or extrinsic pathway.

The intrinsic pathway

Many death signals originating from cellular stress, DNA damage (i.e., cytotoxic chemotherapy, irradiation), and loss of growth factors (p53-dependent) activate the intrinsic apoptotic pathway mediated by the mitochondria. This pathway is mainly controlled by the balance and interactions between pro- and antiapoptotic members of the Bcl-2 family proteins, first isolated from the t(14; 18) chromosomal breakpoint in follicular B-cell lymphoma, which regulate the permeability of the mitochondrial membrane. In addition to Bcl-2, Bcl-X_L, Bcl-w, A1, and Mcl-1 all belong to the antiapoptotic group and all possess the domains (Bcl-2 homology 1) BH1, BH2, BH3, and BH4. The proapoptotic group is subdivided into the Baxsubfamily (Bax, Bak, and Bok), which all possess the domains BH1, BH2, and BH3, and into the BH3-only proteins, Bid, Bad, Bim, Bmf, Bik, Noxa, and Puma.⁵

In response to apoptotic stimuli, Bax/Bak proteins are activated at the mitochondria, causing mitochondria to release cytochrome (cyt) c and other mitochondrial factors in the cytosol. Also, tBid (activated by caspase-8 in the extrinsic death pathway; see below) can activate Bax/Bak to mediate the release of cyt c from the mitochondria. Cyt c, together with dATP, triggers the assembly of the apoptotic protease-activating factor-1 (Apaf-1) apoptosome ("the wheel of death"), causing the recruitment and activation of caspase-9. Caspase-9, in turn, cleaves and activates the effector caspase-3 or -7. Caspases 3, 7, and 9 are part of a set of cysteine proteases, called the caspases, causing most of the biochemical changes observed in apoptotic cells. These cysteine-dependent aspartate-specific proteases possess a critical active-site cysteine, cleave their substrates after Asp residues, and are present as inactive zymogens (procaspases) in essentially all animal cells.⁶ In mammals, 14 different members of the caspase family have been described. The proapoptotic caspases can be divided into the initiator caspases, including procaspases-2, -8, -9, and -10, and into the executioner (or effector) caspases, including procaspases-3, -6, and -7.^{7,8} The effector caspases subsequently cleave a specific set of death-protein substrates, leading to the morphologic and biochemical features of apoptotic cells.

Activity of caspase-3, -7, and -9 is inhibited by inhibitors of apoptosis proteins (IAPs). The IAPs members (XIAP, cIAP1, cIAP2, NAIP, and survivin) are important checkpoints in the execution of apoptosis, because they can bind and inhibit the activated caspases. The IAPs are, in their turn, inhibited by Diablo/Smac and Omi/HtrA2, which are released from the cytosol of damaged mitochondria⁹ (Fig. 1).

The extrinsic pathway

The extrinsic pathway (p53-independent) is mediated by the activation of the death receptors, the cell-surface receptors belonging to the tumor necrosis factor receptor (TNFR) gene superfamily that transmit apoptotic signals after the binding of specific ligands. This family includes TNFR-1, FasR/CD95, and TNF-related apoptosis-inducing ligand receptors (TRAIL)R-1 (DR4) and TRAILR-2 (DR-5). All death receptors are characterized by an intracellular death domain (DD). Upon stimulation, the DD of the death receptors interact with the DD of an adaptor molecule, such as Fas-associated death domain (FADD) or TNFR-associated death domain (TRADD). The adaptor protein contains a second protein motif, the death effector domain (DED), which facilitates the binding to procaspase-8 (or procaspase-10) to form the death-inducing signaling complex (DISC). In this way, caspase-8 (or caspase-10) becomes activated and cleaves and activates, in turn, the effector caspase-3.¹⁰ In type I cells, signals coming from the activated receptors are strong enough to produce a caspase-dependent apoptosis. Type II cells, however, need an amplification signal for apoptotic activation via the mitochondrial pathway, occurring through cleavage of the proapoptotic BH3-only protein, Bid, into a truncated form of Bid (tBid) through caspase-8. In this way, tBid is translocated to the mitochondria, where it activates two other proapoptotic proteins, Bax/Bak, inducing cyt c release and other mitochondrial proapoptotic factors in the cytosol.

Inhibition of the death-receptor pathway occurs by the antiapoptotic FLICE inhibitory protein (Flip), which associates with pro-caspase-8 and competes with pro-caspase-8 and -10 for binding to Fadd¹¹ (Fig. 1).

Apoptosis and Cancer

Cancer can occur when the balance between mitosis and cell death becomes disturbed, either by an increase of cell proliferation or a decrease in apoptosis.¹² Conventional anticancer treatments, such as chemotherapy and radiotherapy, kill tumor cells primarily by the induction of apoptosis through activation of the intrinsic pathway. However, drug resistance in tumor cells, due to failure to activate the apoptotic machinery, is an important clinical problem. Tumor cells can achieve resistance to apoptosis by various mechanisms on multiple levels of the apoptotic pathways.

In general, a tumor cell can become resistant to apoptosis by increased expression or activation of antiapoptotic proteins or by decreased activation or inactivation of proapoptotic proteins.¹³

Increased expression of antiapoptotic proteins

Bcl-2 and Bcl-X_L are frequently overexpressed in a wide variety of tumor cell types, including malignant glioma cells,

FIG. 1. Overview of the two main pathways to apoptosis. The intrinsic (mitochondrial pathway) is mainly controlled by the balance and interactions between pro- and antiapoptotic members of the Bcl-2 family proteins that interact with the mitochondrial membrane. The extrinsic pathway is governed by the tumor necrosis factor (TNF) superfamily of ligands and receptors, interacting at the cytoplasmic membrane. The two pathways converge at the level of caspase-3, and cross-talk is achieved by cleavage of Bid by caspase-8.

myeloid leukemia cells, non-Hodgkin's lymphoma (NHL) cells, and others, shifting the balance toward antiapoptosis.¹⁴ Overexpression of the IAP survivin is highly tumor specific and occurs frequently in most human tumors but not in normal adult tissues.¹⁵ Finally, overexpression of c-FLIP, an inhibitor for pro-caspase-8 activation, has been observed in human melanomas and B-cell-lymphoma cell lines.^{11,16}

Increased activation of antiapoptotic proteins

The Akt/PKB kinases are constitutively active or amplified in many human cancer types. The Akt family proteins are members of the serine-threonine-specific protein kinases and are involved in the regulation of a wide variety of cellular processes, including survival, proliferation, growth, and metabolism.¹⁷ In some tumor cells, the overexpression of Akt/PKB phosphorylates the proapoptotic protein, Bad, resulting in its proteasomal degradation via interaction with the 14-3-3 scaffold protein. Further, Akt/PKB also activates the survival pathway mediated by NF- κ B, and it suppresses the p53 proapoptotic signaling by phosphorylation of the

inhibitor of p53 Hdm-2, which, in its phosphorylated state, is activated for inhibition of p53.¹⁸

Decreased activation of proapoptotic proteins

The tumor-suppressor gene, p53, is a key regulator of apoptosis and carcinogenesis. In normal cells, p53 is inactive, bound to the protein, Hdm-2, an ubiquitin ligase, which causes proteasomal degradation of p53. Oncogenetic stress (i.e., increased expression of oncogenes, such as c-Myc, Ras, and others) leads to the activation of the tumor suppressor, alternative reading frame (ARF), which activates p53 by the sequestration of Hdm-2. This causes an arrest in the cell cycle, to allow for DNA repair, by activation of the transcription factor, p21, or causes apoptosis. If DNA becomes damaged, activation of the kinases, ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR), causes phosphorylation of p53, which disrupts the interaction of Hdm-2-p53, causing a displacement of the p53 protein of the inhibitor, Hdm2, and thus an activation of the p53 transcription factor. Activated p53 translocates to the nucleus, where it activates or suppresses its target genes. p53 activates apoptosis in multiple ways: 1)

transactivation of the death-domain receptors (extrinsic pathway), 2) upregulation of proapoptotic BH-3-only proteins (Puma, Noxa, Bid, Bmf, and Bad; intrinsic pathway), 3) induction of Apaf-1, 4) transrepression of Bcl-2 and survivin, and 5) direct binding to the mitochondria, causing release of cyt c^{19} (Fig. 2).

p53 is mutated in more than 50% of human cancers, including lung cancers, breast cancers, and leukemia. In one third of all sarcomas, an amplification of Hdm-2 to inactivate p53 has been observed.²⁰ Overall, tumors with mutated p53 have, in general, a bad prognosis.²¹ In some tumors, such as breast cancer, there is still wt p53 present; however, the p53 is not an active transcription factor anymore. This has been contributed to the lack of the protein apoptosis-stimulating protein of p53 (ASPP), for example, in human breast carcinomas. ASPP binds to a conformational-sensitive region of p53, altering the protein conformation of p53 and enhancing, in this way, the binding activity of p53 to the promoters of the p53 target genes.²² Besides protein-protein interactions,

phosphorylation, and ubiquitination, the transcriptional activity of p53 is also regulated by acetylation and sumoylation.

Inactivation of proapoptotic proteins

Mutations and downregulation of proapoptotic proteins, such as Bax and Bak, are observed in hematopoietic malignancies. In addition, metastatic melanomas do not express APAF-1, thus escaping mitochondria-dependent apoptosis.¹³ Downregulation or inactivation of death receptors, deficient TRAIL receptor transport, and overexpression of decoy receptors has also been observed in many tumor cells.²³

Selective Induction of Apoptosis as Mean of Treatment of Cancer

Understanding the apoptotic pathways, and how tumor cells resist apoptosis at the molecular level, has led to new therapeutic strategies with the goal to selectively induce apoptosis in the tumor cell while leaving healthy cells

FIG. 2. The role of p53 in apoptosis. In normal cells, p53 is inactive, bound to the protein, Hdm-2, a ubiquitine ligase, which causes proteasomal degradation of p53. Oncogenic stress leads to the activation of the tumor suppressor, ARF, which activates p53 by sequestration of Hdm-2. This causes an arrest in the cell cycle, to allow for DNA repair, by activation of the transcription factor, p21, or causes apoptosis. When DNA becomes damaged, activation of the kinases, ATM and ATR, causes phosphorylation of p53, which disrupts the interaction of Hdm-2-p53, causing a displacement of the p53 protein of the inhibitor, Hdm2, and thus an activation of the p53 transcription factor. Activated p53 translocates to the nucleus, where it activates and suppresses its target genes.

unharmful. Some of the most promising strategies will be discussed below.

Immunotoxins and antibodies

Immunotoxins. Immunotoxins are hybrid proteins composed of an antibody fragment linked to a toxin. The immunotoxin binds to a surface antigen on a cancer cell, enters the cancer cell by endocytosis, and kills it. Most success with these agents has been achieved in the treatment of hematologic cancers.

The CD-22-targeted immunoconjugate, inotuzumab-ozogamicin, and the CD-22-targeted exotoxin A are in phase I clinical trials and are used in the treatment of NHL.^{24,25}

The immunoconjugate, gemtuzumab-ozogamicin (GO), used for the treatment of acute myeloid leukemia, contains a humanized monoclonal antibody (mAb) directed against the CD33 surface antigen conjugated to a derivative of the antibiotic calicheamicin, which is 1000-fold more potent than most chemotherapeutics. It induces double-strand DNA breaks, leading to activation of the apoptotic pathways. The most important clinical toxicity associated with this drug is abnormality in hepatic function.²⁶ Several other immunotoxins are currently under investigation for the treatment of solid tumors, including gastrointestinal, breast, mesothelioma, ovarian, and pancreatic cancer.²⁷

Antibodies. For the treatment of NHL, a mAb against the B-cell antigen, CD20, called rituximab, was developed. This antibody inhibits the NF- κ B-signaling pathways and activates the mitochondrial apoptotic pathway.²⁸ It is currently given in combination with the chemotherapy regimen, CHOPC (cyclophosphamide, doxorubicin, vincristine, and prednisolone), in combination with the chemotherapy regimen CVP (cyclophosphamide, vincristine, and prednisolone), as monotherapy to patients with advanced (stages 3 and 4) follicular lymphoma, as part of a clinical trial or as maintenance therapy.

Alemtuzumab, is a humanized therapeutic mAb that recognizes the CD52 antigen expressed on normal and neoplastic lymphoid cells. This humanized antibody was recently approved for the treatment of B-cell chronic lymphocytic leukemia.²⁹

Apoluzimab, a humanized anti-human-leukocyte-antigen-DR (HLA-DR) β -chain-specific antibody, showed proapoptotic activities (although caspase independent) in human chronic lymphocytic leukemia.³⁰

Death-receptor-based cancer therapeutics

Activation of death receptors occurs by binding of one of the three main ligands, FasL (CD95L), TNF- α , or TRAIL, to the death receptors.

Fas-based cancer therapeutics. Agonistic antimurine Fas mAbs were found to be extremely toxic *in vivo*, causing a rapid induction of hepatocyte apoptosis. Also, soluble FasL (sFasL) has been found to be severely toxic in mice, thus limiting the use of Fas-targeted therapy.³¹

TNF-based cancer therapeutics. The severe cardiovascular toxicity of soluble TNF (sTNF) has made it a lesser potential drug candidate. Interestingly, TNF disrupts tumor-

associated blood vessels, while leaving normal vessels unharmed. Therefore, TNF was coupled to the GNGRC peptide (a ligand of CD13), which binds selectively to endothelial cells. In this way, a targeted delivery of TNF was achieved, which proved its benefits in a xenograft-lymphoma mouse model.³²

TRAIL-based cancer therapeutics. Contrary to Fas and TNF, TRAIL binds to tumor target cells while leaving healthy cells unaffected. TRAIL can bind to five receptors, two of which are death receptors (TRAIL-R1 or DR4 and TRAIL-R2 or DR5).

Direct targeting of TRAIL. Soluble TRAIL (sTRAIL) selectively induces apoptosis in a variety of transformed cell lines, while it seems to be inactive against normal healthy tissue.³³ However, the sTRAIL binds to TRAILR1/R2 and the decoy TRAIL receptors (inactive mutated or truncated receptors), giving it a wider therapeutic spectrum or a narrower and—more important—a more unpredictable therapeutic window.³⁴

Recombinant TRAIL (activates TRAIL-R1 and TRAIL-R2) and agonistic anti-TRAIL-R1 and -TRAIL-R2 antibodies have been developed and entered clinical trials.³⁵ A combination therapy of recombinant TRAIL and the mAb, rituximab, in several cell lines demonstrated a cooperation of these two agents in attenuating or reversing tumor growth.³⁶

The fully human Ab targeting TRAIL-R1/DR4, mapatumumab, is currently being tested in patients with advanced solid malignancies.³⁷ TRAILR-2/DR5-targeting antibodies include lexatumumab, apomab, AMG-655, and the chimeric mAb, LB135. All four Abs are being tested in clinical phase I or II tests.³⁸ Some tumor types, however, express high levels of TRAIL-R1 and not TRAIL-R2, while other tumor types display the opposite expression profile. A patient population should first be screened on the presence of the TRAIL-R1/R2 ratio before treatment is started. However, a direct relationship between receptor expression and activity is not always the case.

Indirect targeting of TRAIL. The TRAIL-expressing adenoviral vector (Ad-TRAIL) was developed to cause better tumor targeting and increased tumoricidal activity. However, because of safety and toxicity issues, an intratumoural injection is needed, limiting its use for the treatment of disseminated tumors.³⁹

Another approach involves CD34⁺ transduced-TRAIL cells migrating from the bloodstream to the tumor tissue because of the expression of adhesion molecules that interact with receptors on endothelial cells. These CD34⁺ transduced-TRAIL cells deliver the TRAIL to the tumor cell. CD34⁺-TRAIL⁺ cells show high killing activity on a variety of tumor-cell types *in vitro* and *in vivo* in NOD/SCID mouse xenografted with sTRAIL-sensitive and TRAIL-resistant tumors.⁴⁰

Bremer et al. and others developed a fusion protein compromising the human TRAIL genetically linked to the antibody fragment, scFvC54, specifically targeting the cell-surface target antigen, EGP2. By fusion of the sTRAIL to a tumor-selective antibody fragment, the binding of the fusion protein to the target-specific antigen converts the soluble death ligand into a membrane-bound molecule and leads to the killing of neighboring cells devoid of the target antigen by the

bystander effect. In this way, tumor cells, devoid of the specific target antigen, which would normally escape the antibody-based therapy, are killed.⁴¹

A major advantage of death-receptor activation, in general, is the induction of apoptosis in malignant cells by direct activation of the caspase cascade, independent of the p53 status and the overexpression of Bcl-2.

Bcl-2 as drug target

Given the prominent role of Bcl-2 members in regulating apoptosis by means of lowering the apoptotic threshold, thereby making the target tumor cell more sensitive to other apoptotic-inducing stimuli or directly shifting the balance toward proapoptosis, Bcl-2 inhibitors and BH3-mimetics are of great interest in anticancer therapy.

Bcl-2 inhibition. Bcl-2 oblimersen (Genasense,[®] Aventis/Genta, Inc. Berkeley Heights, CA) is a synthetic 18-mer phosphorothioate antisense deoxynucleotide to Bcl-2 mRNA that is being tested in a wide range of cancer types (phase III). Several studies show downregulation of Bcl-2 by oblimersen, leading to apoptosis activation.⁴² However, recent studies demonstrate that apoptosis induced by oblimersen happens before, and in the absence of, downregulation of Bcl-2 and, therefore, seems to be Bcl-2 independent.⁴³ The antisense technique has also been developed, and targeting Bcl- X_L and cancer cells treated with these oligonucleotides had a decrease in Bcl- X_L expression and were sensitized for chemotherapy-induced apoptosis.⁴⁴ Importantly, to achieve the greatest therapeutic effect, it will be necessary to downregulate all members of the Bcl-2 family via the antisense strategy, as tumor cells may express more than one antiapoptotic Bcl-2 family member.

BH3-mimetics. Another approach is to develop small molecules and peptides that mimic proapoptotic BH3-only proteins, which bind, and thus inhibit, the antiapoptotic Bcl-2 members. Small molecules, such as HA14-1, antimycin A, tetrocarcin A, and chelerythrine, were shown to induce apoptosis in Bcl-2-overexpressing cells. The BH3-only mimetic gossypol, a soluble polyphenol, binds to and downregulates Bcl- X_L . AT-101 and ABT-263 gossypol derivatives bind to Bcl-2, Bcl-w, and Bcl- X_L with subnanomolar affinity. ABT-737 is specific for Bcl-2 and induces complete tumor regression in 77% of treated mice in xenograft models.⁴⁵

Finally, epigallocatechin-3-gallate (EGCG), a polyphenol in green tea extract, induces apoptosis by various mechanisms, including downregulating expression of Bcl-2 family members and suppression of survivin and the Akt pathway.^{46,47}

Caspase activation

The intrinsic and extrinsic pathway of apoptosis converges at the main effector, caspase-3. Direct or indirect activation of caspase-3 is achieved in multiple ways. Inducible caspase mechanisms have also been developed. More recently, inhibitors of c-Flip, which prevents the activation of procaspase-8, are being tested as potential candidates for anticancer drugs.

Direct activation of caspase-3. Soluble RGD peptides bind and activate caspase-3, by binding to the RGD tripeptide

sequence near its active site, and induce apoptosis.⁴⁸ Merck (San Diego, CA) developed a RGD-based candidate drug to inhibit the neoformation of tumor blood vessels. MX-2060, a derivative of gambogic acid, is being tested as a potential caspase-activating anticancer agent and is in its preclinical phase.⁴⁹

Indirect activation of caspase-3. *In vitro*, alpha-fetoprotein penetrates tumor cells, disrupts the association between XIAP and caspase-3, and thus activates caspase-3.⁵⁰ However, more studies are needed to clarify the mechanism of AFP in tumor growth inhibition. Mapmodulin or PHAPI (putative HLA-DR-associated protein) causes an increase in sensitivity to cyt c, enhancing caspase-3 activity in breast cancer cells.⁵¹

Induction of caspase-9/3. Inducible caspases have been engineered by fusing them to chemical dimerization domains. After chemical induction, inducible caspase-9 (icasp9), under the control of an androgen-responsive promoter, is targeted to prostate cancer cells and causes apoptosis in xenografted prostate tumors.⁵² Immunocasp-3 is the protein secreted by Jurkat cells transfected with a plasmid-encoding caspase-3 fused with an antibody against HER2, widely expressed on the membrane of tumor cells. Immunocasp-3 becomes internalized and released in the cytosol of the tumor cell, causing apoptosis in this cell.⁵³

c-FLIP inhibitors. c-FLIP prevents the activation of procaspase-8 in the death-inducing signaling complex (DISC); therefore, inhibitors of this protein are attractive anticancer drugs. c-Flip is overexpressed in various types of tumor cells, including colorectal cancer, and are often resistant to death-receptor-induced apoptosis.⁵⁴ Many of the known anticancer drugs, such as chemotherapy, topoisomerase inhibitors, histone deacetylase inhibitors, and RNA/protein synthesis inhibitors, lower c-Flip expression; however, agents directly targeting c-FLIP mRNA or protein have not yet been developed.⁵⁵

IAP inhibitors

IAPs are a family of caspase-inhibiting proteins characterized by the baculoviral IAP repeat (BIR) domain, including XIAP, cIAP1, cIAP2, NAIP, ML-IAP, livin, survivin, and apollon. Smac/Diablo and Omi/Htra2, which share the IBM (IAP-binding motif), bind to the IAP members and reverse their inhibitory interactions with caspases. A wide variety of IAP-inhibiting strategies are currently under exploration, including antisense strategies and Smac peptides and Smac-mimetic drugs. However, these Smac peptides and Smac mimetics often bind to IAP less efficiently, as compared to wild-type proteins, and tumor cells express different members of the IAP-family. Thus, inhibiting a single IAP member might not be enough to induce significant effects *in vivo*.

XIAP. The X-linked inhibitor of apoptosis protein (XIAP) is upregulated in some cancers and can inhibit caspase-9 as well as caspase-3, causing chemoresistance. Several antisense XIAPs are under investigation and demonstrate a reduction of XIAP levels, which sensitizes tumor cells to chemotherapy.⁵⁶ The 19-mer phosphorothioate antisense oligonucleotide, AEG-35156 (Aegera), targeting XIAP, is currently under investigation in several clinical trials, including four phase I-II

combination clinical trials for the treatment of pancreatic cancer, acute myeloid cancer, advanced breast cancer, and non-small-cell lung carcinoma (NSCLC).⁵⁷ One of the most recent Smac/Diablo peptides is embelin, a small-molecular-weight inhibitor, able to bind XIAP and inhibits cell growth, induces apoptosis, and activates caspase-9 in prostate cancer cells. New, more potent embelin analogs have been designed.⁵⁸

Survivin. Survivin, one of the IAP members, is highly tumor specific. It is expressed in most solid human tumors and hematologic malignancies, while it is only expressed in a few adult tissues. Survivin binds and inhibits caspase-9. Additionally, survivin binds Smac/Diablo, inhibiting its proapoptotic function, and binds XIAP, stabilizing their IAP function. However, survivin is a bifunctional protein that not only suppresses apoptosis, but also regulates cell division.⁵⁹ Recent studies indicate that the nuclear pool of survivin facilitates cell division, and the cytosolic form interacts with Smac/Diablo and other IAP members, and thus enables the IAP function of survivin.⁶⁰ Evasion of apoptosis and ability to proliferate uncontrollably are two of the molecular characteristics found in all human cancers, suggesting a pivotal role for survivin in carcinogenesis. Moreover, survivin appears to be involved in tumor-cell resistance to anticancer drugs and ionizing radiation. Finally, overexpression of survivin in the endothelium of tumors protects against apoptosis during the proliferative and remodeling phase of angiogenesis. This results in the facilitation of angiogenesis. Therefore, survivin is an excellent target for cancer therapy, considering its critical role in cell apoptosis, cell proliferation, and angiogenesis.⁶¹

Antisense survivin oligonucleotides, hammerhead ribozymes, and small interfering RNAs cause a downregulation of survivin expression, accompanied by the induction of caspase-dependent apoptosis, and sensitize tumor cells to other apoptosis-inducing stimuli. Two main gene-therapy approaches targeting survivin have been successfully developed. One uses a plasmid or viral vector to deliver a dominant-negative survivin mutant to tumor cells, causing an increase in the apoptotic index. The other involves the use of a survivin gene promoter to drive the expression of cytotoxic genes in tumor cells, causing a suppression of the tumor growth. The first survivin inhibitors have entered phase I–II clinical trials and may represent novel anticancer drugs, applicable in different types of tumors while leaving healthy tissue unaffected.¹⁵

Restoring the function of p53

The tumor suppressor transcription factor, p53, is inactivated in more than 50% of human tumors and, therefore, cannot carry out its crucial functions, including cell-cycle arrest or apoptosis induction after DNA-damaging stimuli. In addition, some tumors overexpress the p53-negative regulator, Hdm-2, which binds p53, resulting in p53 proteasomal degradation (Fig. 2). Different strategies have been undertaken to target p53, including antagonizing the p53-Hdm-2 interaction, restoration of normal function of mutant p53, and p53-expressing adenoviruses.

Antagonizing p53-Hdm-2 interaction. Several Hdm-2-selective inhibitors have been developed, including Nutlins.

These inhibitors bind to the p53 pocket of Hdm-2, disturbing the interaction between p53 and Hdm-2 and thereby increase p53 activity. Nutlins activate apoptosis in a tumor-specific way, without being toxic. Chalcone derivatives are antagonists of the Hdm-2-p53 interaction and are in the preclinical phase; however, they seem to have insufficient specificity.⁶² Recently, a potent, highly selective, and orally active small-molecule inhibitor of Hdm-2-p53 interaction, called MI-219 (Ascenta, Ascenta Therapeutics, Malvern, PA), has been developed. It activates the p53 pathway in cells with wild-type p53 and leads to the induction of apoptosis in tumor cells.⁶³

Restoration of normal function of mutant p53. The radio-protector, amifostine, and PRIMA-1 restore the transcriptional activity of some mutant p53 and are in the preclinical phase.⁶⁴ The 9-mer peptide, CDB3, binds and stabilizes the p53 core domain, restoring the activity of mutant p53, and is also in the preclinical phase.⁶⁵

p53-expressing adenoviruses. Wt p53-expressing adenoviruses (Ad-p53) are also being tested in clinical trials; however, low efficiency of the gene-therapy vectors and the hepatotoxicity associated with systemic application has made this approach less successful. Intratumoural injection is, therefore, applied, and some Ad-p53 are being tested in a phase III clinical trial for the treatment of head and neck cancers.

Proteasome inhibitors

Bortezomib, a selective 26S proteasome inhibitor, is the first inhibitor used in cancer therapy, more specific for the treatment of multiple myeloma.⁶⁶ Bortezomib causes a growth arrest in the G2-M phase of the cell cycle in normal and tumor cells; however, only tumor cells are eliminated by apoptosis. In myeloma cells, bortezomib induces Noxa, which causes a displacement of Bim from Mcl-1, leading to the activation of Bax/Bak, release of cyt c out of the mitochondria, and induction of apoptosis.⁶⁷ In mouse carcinomas, a combination of bortezomib and a TRAIL DR5-agonist mAb (MD5-1) was tested. Bortezomib sensitized the tumor cells to TRAIL-mediated apoptosis and increased survival of the mice, in the absence of obvious toxicity.⁶⁸ Several studies are ongoing, in which the proteasome inhibitor, bortezomib, is used in combination with different death-receptor-agonist mAbs or recombinant TRAIL to promote tumor-cell apoptosis.⁶⁹

Galectines

Galectines are a family of β -galactoside-binding lectines (14 members) and are found both in the cell and on the membrane surface. The intracellular galectines-1, -3, and -7 are involved in apoptosis and carcinogenesis. Galectin-1 and -7 are positive regulators of apoptosis. Whether the mechanism of activation of apoptosis is caspase dependent or caspase independent is currently unclear. Galectin-3 can act as an activator or inhibitor of apoptosis by binding to Ras and activating apoptosis, on the one hand, and by binding and inhibiting the antiapoptotic Bcl-2 protein on the other hand. A role in tumor invasion and metastasis for galectine-1 and -3 has also been observed. On binding with integrins, galectin-1 and -3 inhibit the adhesion of tumor cells to one

and another or to the cellular matrix. This may lead to an uncontrolled growth of the tumor cells and the invasion of tumor cells. Metastasis is achieved when galectin-1 and -3 bind to glycoconjugates of endothelial cells, which initiates the penetration of the tumor cells in the bloodstream. Using lactulose amines, the selective inhibition of cancer cell galectin-1 and -3 leads to tumor-selective apoptosis.⁷⁰ Thus, galectines show an opposite working mechanism in carcinogenesis, but might be a promising target for the tumor-specific induction of apoptosis.

*Gene silencing, regulating apoptosis, as target:
Histone deacetylase inhibitors (HDACi)
and DNA-methylation inhibitors*

Histone acetylation works in combination with DNA methylation to regulate gene expression and is required to keep chromatin in an open, transcriptionally active state. The gene silencing of tumor-suppressor genes by DNA methylation and histone deacetylation is an important feature in the development of malignancies. Methylation of promotor regions turns off critical genes that otherwise suppress tumorigenesis. This methylation process is, however, reversible, making DNA-methylation inhibitors attractive anticancer candidate drugs. Moreover, promotor hypermethylation almost never occurs in normal cells, giving tumor-specific therapeutic targets.⁷¹

In cancer, HDACs are often overexpressed or de-/inactivating mutations in histone acetyltransferases (HATs) occur, resulting in gene silencing. In tumor cells, treatment with HDACi and DNA methylation inhibitors can reactivate silenced genes, including tumor-suppressor genes, restoring a normal gene-expression profile. In this way, growth arrest and apoptosis induction can be induced, resulting in the elimination of the tumor cells. HDACi have been shown to have anticancer effects in preclinical studies by several mechanisms, including induction of tumor-cell apoptosis, induction of cellular differentiation, suppression of angiogenesis, and enhancement of host immune surveillance. Induction of apoptosis by HDACi occurs through the upregulation of proapoptotic proteins and downregulation of antiapoptotic proteins.⁷²

DNA methylation inhibitors. Decitabine, a DNA methylation inhibitor, has potent antileukemic actions. In addition, it shows potent antitumor activity against a human tumor xenograft.⁷³ Azacitidine, another DNA-methylation inhibitor, has antitumor effects in hematologic malignancies.⁷⁴

The antineoplastic effects of hydralazine, a DNA-methylation inhibitor, and valproic acid (VA), an HDACi, in a panel of cancer cell lines, has been investigated. VA had strong inhibitory effects in all cell lines, and the effect significantly increased with hydralazine in one of the seven tested cell lines. On the contrary, hydralazine alone showed no inhibitory properties.⁷⁵

HDACi. Examples of HDACi in different ongoing clinical trials include valproic acid (VA), romidepsin, vorinostat, ITF2357, and panobinostat (LBH589). Results of these studies indicate that HDACi are well tolerated. In neuroblastoma cells, HDACi induce cell death via the mitochondrial pathway and activate p53 via hyperacetylation and nuclear re-

localization.⁷⁶ An upregulation of TRAIL and subsequent induction of apoptosis after HDACi treatment in myeloid leukemia cells has been observed, defining another mode of action for HDACi.⁷⁷ Combination therapy of HDACi and bortezomib has been started and demonstrates a synergistic induction of apoptosis in MM cells.⁷⁸

The PI3K-Akt pathway as target

The phosphoinositide-3-kinase (PI3K)-Akt pathway regulates many cellular processes, including cell growth, cell proliferation, cell survival, glucose metabolism, and angiogenesis. Activation of PI3K can occur through growth-factor receptors (GFRs), such as insulin-like growth factor-1 receptor (IGF-1R), vascular endothelial growth factor receptor (VEGFR), and G-protein-coupled receptors (GPCRs). PI3K catalyzes the production of the second messenger, phosphatidylinositol 3, 4, 5-triphosphate (PIP₃), at the cell membrane, which binds and activates 3'-phosphoinositide-dependent kinase 1 (PDK1) and the serine-threonine kinase, Akt. The tumor-suppressor gene, phosphatase, tensin homolog deleted on chromosome 10 (PTEN) negatively regulates the activity of Akt by converting PI (3, 4, 5)P₃ back to PI (4, 5)P₂, thus inhibiting the PI3K/Akt pathway. Downstream substrates of Akt control the key cellular processes and include Hdm-2, Bad, the FOXO subfamily of Forkhead family transcription factors, and the serine/threonine kinase mammalian target of rapamycin (mTOR; Fig. 3).

The PI3K/akt pathway is involved in many aspects of carcinogenesis, including antiapoptosis, angiogenesis, and regulation of cell growth.⁷⁹ Activated PI3K/Akt exerts antiapoptotic activities by multiple mechanisms. First, Akt phosphorylates the proapoptotic protein, Bad, causing its sequestration from the mitochondrial membrane by the scaffold 14-3-3 protein, which results in the proteasomal degradation of Bad. Second, Akt phosphorylates the negative regulator of p53, Hdm-2, causing translocation of Hdm-2 to the nucleus, where it downregulates p53, thereby inhibiting p53-induced growth arrest and apoptosis. Third, Akt phosphorylates and inhibits the FOXO transcription factors. These transcription factors regulate the expression of the proapoptotic genes, Bim and FasL (Fig. 3).

The PI3K/Akt/mTOR pathway contributes to tumor angiogenesis, since the hypoxia-inducible transcription factor (HIF) is targeted by mTOR, leading to the activation of vascular endothelial growth factors (VEGFs) and angiogenesis.

In addition, mTor regulates protein synthesis and cell growth by activating the 70-kDa ribosomal, S6 kinase 1 (S6K1), and by inhibiting the elongation factor, 4E binding protein-1 (4E-BP1).⁸⁰

Expression of activated Akt has been observed in many cancers, including multiple myeloma, head and neck cancer, breast cancer, gastric cancer, ovarian cancer, prostate cancer, and melanomas.¹⁸ Loss of the tumor suppressor, PTEN, function, through mutation, deletion, or epigenetic silencing, has also been observed in many human cancers, including bladder, breast, prostate, and endometrial cancers.⁸¹ Further, overexpression or inactivation of Akt renders tumor cells resistant to chemotherapeutic drugs.⁸²

Since the PI3K/Akt pathway is involved in multiple crucial aspects of carcinogenesis—inhibiting apoptosis and, at

FIG. 3. The PI3K/Akt/mTOR signaling pathway involved in cancer. Activation of PI3K can occur through growth-factor receptors (GFRs), such as insuline-like growth-factor-1 receptor (IGF-1R), vascular endothelial growth-factor receptor (VEGFR), and G-protein-coupled receptors (GPCRs). PI3K catalyzes the production of the second messenger, phosphatidylinositol 3, 4, 5,- triphosphate (PIP₃), at the cell membrane, which binds and activates 3'-phosphoinositide-dependent kinase 1 (PDK1) and the serine-threonine kinase, Akt. The tumor-suppressor gene phosphatase tensin homolog deleted on chromosome 10 (PTEN) negatively regulates the activity of Akt by converting PI (3, 4, 5)P₃ back to PI (3, 4, 5)P₂, thus inhibiting the PI3K/Akt pathway. Downstream substrates of Akt control the key cellular processes and include Hdm-2, Bad, the FOXO subfamily of Forkhead family transcription factors, and the serine/threonine kinase mammalian target of rapamycin.

the same time, stimulating cell growth and angiogenesis—blocking this pathway could, therefore, inhibit the growth of the tumor cell and simultaneously induce apoptosis or make tumor cells again sensitive for apoptosis. The components of this pathway are, therefore, promising targets for therapeutic intervention. The molecular targets of the PI3K/Akt pathway can be classified as regulators acting upstream of Akt, Akt as a target itself, and regulators acting downstream of Akt.⁸²

Therapeutic targeting of upstream regulators of Akt. Briefly, the most direct approach to inhibit the PI3K/Akt pathway is by inhibiting PI3K itself. Two pharmacologic PI3K inhibitors, wortmannin and LY294002, have been demonstrated to inhibit cell proliferation, promote apoptosis, and sensitize cancer cells to various types of conventional chemotherapy; however, poor solubility and high toxicity limit their clinical use.⁸¹ Novel inhibitors, such as quinolone,

pyrido-pyrimidine, and IC87114, have been developed, but further studies *in vitro* and *in vivo* are needed. Inhibitors of PDK1 (BX-795, BX-912, and BX-320), serine/threonine protein kinases that activate Akt by phosphorylation, block the activation of Akt and induce apoptosis in tumor cell lines.⁸³

Akt as a target. Inhibition of Akt can be achieved in many ways, such as by Akt antibodies, PI analog inhibitors, ATP-competitive inhibitors, pseudosubstrate inhibitors, Akt pathway inhibitor-2 (API-2), and allosteric Akt kinase inhibitors.⁸² Perifosine, an orally bioavailable alkylphospholipid, is a PI analog inhibitor and sensitizes cancer cells to apoptosis and causes cell-cycle arrest induced by radiation.⁸⁴

Recently, enzastaurin (LY317615), an orally administered ATP-competitive inhibitor, has been shown to dephosphorylate Akt, suppress cell proliferation in a large panel of

human myeloma cell lines, and induce apoptosis by activation of the intrinsic pathway.⁸⁵ Several clinical trials with enzaustaurin in solid tumors and malignant lymphomas are ongoing.

The synthetic API-2 molecule, also named tricibine, selectively inhibits Akt and causes suppression of cell growth and induction of apoptosis in xenografts with constitutively activated Akt in PTEN mutations.⁸⁶

The allosteric Akt kinase inhibitors, Akti, cause caspase-3 activity in multiple cell lines and sensitize tumor cells, but not normal cells, to apoptotic stimuli. However, maximum activity is achieved only in combination with other chemotherapeutics or biologics, such as TRAIL.⁸⁷

Therapeutic targeting of downstream regulators of Akt. The best-studied downstream substrate of Akt is the threonine kinase, mTOR. The mTOR inhibitor, rapamycin, and its analogs (CCI-779 and RAD-001) are well-known anticancer drugs. Inhibition of mTOR decreases phosphorylation of two downstream targets, 4E-BP1 and S6K, resulting in the inhibition of protein synthesis. Combination treatment of mTOR inhibitors and other standard chemotherapeutics demonstrate enhanced levels of apoptosis, inhibition of tumor growth, and angiogenesis.⁸¹ A key role for mTOR in the apoptotic resistance of mesothelioma cells in a three-dimensional culture has recently been demonstrated.⁸⁸

Vascular endothelial growth-factor inhibitors (VEGFi)

VEGF-dependent actions include angiogenesis, increased vascular permeability, paracrine/autocrine growth-factor release, and inhibition of apoptosis. The antiapoptotic VEGF actions are mediated by the induction of PI3K and Akt, which, in turn, phosphorylates Bad, resulting in proteasomal degradation of this proapoptotic protein, enhancing cell survival.⁸⁹ VEGFi not only cause proapoptotic effects by inhibiting the PI3K-Akt pathway, but also increase the probability of reverting the disease to a dormant status. Many VEGFi are being developed and tested in clinical trials.⁹⁰ Bevacizumab (Avastin, Genentech/Roche, San Francisco, CA) is a recombinant humanized mAb directed against VEGFR and was used for the treatment of various metastatic cancers. Bevacizumab, in combination with chemotherapy (gemcitabine or oxaliplatin), yielded encouraging results in phase I and II studies.⁹¹ In 2004, it was approved for combination with chemotherapy for colon cancer, and in 2008, it was approved for use in breast cancer and advanced NSCLC.^{92,93}

Sorafenib is an oral small-molecule tyrosine kinase inhibitor of several types of receptors, including VEGFR, c-Raf, B-Raf, and platelet-derived growth-factor receptor (PDGFR), and is used for the treatment of renal-cell carcinoma. A current study demonstrates an enhanced antitumor activity in patients with solid tumors, when treated with the combination of bevacizumab and sorafenib.⁹⁴

Sunitinib is another oral tyrosine kinase inhibitor that targets the VEGFR, PDGFR, c-Kit receptor, and FLT-3 receptor. Inhibition of these receptor tyrosine kinases blocks signal transduction and thereby affects tumor growth, progression, metastasis, and angiogenesis. It is approved by the U.S. Food and Drug Administration for treatment of gastrointestinal stromal tumor and renal-cell carcinoma.

A recent, orally VEGFR2-tyrosine kinase inhibitor, called YM359445, exhibits greater antitumor activity, compared with other VEGFR2 tyrosine kinase inhibitors.⁹⁵

Some of the trials testing VEGFi (semaxanib, SU668) are being ended because of toxicity reasons. Although some VEGFi drugs are related with toxicity, VEGFi show promising clinical results, especially in combination with different anticancer drugs.

Hypoxia-inducible factor-1 α (HIF-1 α)

Once tumors begin to grow, a lack of oxygen (hypoxia) suppresses their further growth; however, in this condition, tumor cells begin to express the transcription factor, HIF-1 α , which, in turn, activates a set of target genes, helping cells to survive in hypoxic conditions. The role of HIF-1 α in hypoxia-induced apoptosis is not entirely clear. On the one hand, HIF-1 α can initiate apoptosis by increasing the expression of proapoptotic Bcl-2 proteins and by stabilizing wild-type p53. On the other hand, hypoxia itself can inhibit apoptosis by inducing the expression of the antiapoptotic protein, IAP-2. The antiapoptotic function of HIF-1 α becomes evident, since cells with high amounts of HIF-1 α are more resistant to hypoxia-induced apoptosis. Despite the opposite functions of HIF-1 α on the apoptotic machinery, HIF-1 α inhibitors have been successfully developed as cancer drug.⁹⁶ PX-479, a HIF-1 α inhibitor, causes massive apoptosis in xenograft tumor models.⁹⁷ Also, an antisense DNA strategy against HIF-1 α , in combination with chemotherapy, has been developed, with promising results.⁹⁸ To enhance the efficacy of cancer treatment, a liposomal drug-delivery system, consisting of liposomes as nanocarriers, was designed to efficiently introduce antisense HIF-1 α and the anticancer drug, doxorubicin, into the tumor cells. In this way, the development of resistance in cancer cells is prevented, and the efficacy of chemotherapy is increased.⁹⁹

Conclusions

Clarifying the apoptotic signal-transduction pathways has helped to discover new anticancer apoptosis-based therapies, with the goal to destroy harmful tumor cells while leaving normal cells unaffected. The process of apoptosis is controlled at multiple molecular levels, each of which is influenced by different pro- and antiapoptotic proteins. However, since apoptosis is a common feature for tumor as well as for normal cells, selective induction of apoptosis in the cancer cells might be difficult. A tumor cell also represents a mixture of heterogeneous cells that may have acquired different apoptosis-resistance mechanisms. Moreover, one single tumor cell might have developed multiple apoptosis-resistance mechanisms. Overexpression of more than one member of the IAP families has already been observed.¹⁰⁰ Therefore, monotherapy will probably not be sufficient to sensitize or induce apoptosis in all tumor cells. The combination of various therapeutic strategies will likely be more effective. Indeed, proapoptotic receptor agonists, such as TRAIL, have demonstrated a synergistic effect in combination with several other conventional therapies and has been shown to overcome chemoresistance.³⁸ In addition, several studies are ongoing, in which the proteasome inhibitor, bortezomib, is used in combination with different death-receptor-agonist mAbs or recombinant TRAIL to promote tumor-cell apoptosis.

The novel agents at all stages in clinical trial phases, and the rapid progress in revealing the apoptotic process at the molecular level, grounds for optimism.

Disclosure Statement

No financial conflicts of interest exist.

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