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Targeted induction of apoptosis for cancer therapy

current progress and prospects

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Abstract

Important breakthroughs in cancer therapy include clinical application of antibodies, such as Rituximab, and small inhibitory molecules, such as Iressa and Velcade. In addition, recent reports have indicated the therapeutic potential of physiological pro-apoptotic proteins such as TRAIL and Galectin-1. Although unrelated at first glance, each strategy relies on the deliberate and selective induction of apoptosis in malignant cells. Importantly, therapy-resistance in cancer is frequently associated with de-regulation in the mechanisms that control apoptosis. However, cancer cells are often reliant on these molecular aberrations for survival. Therefore, selective induction of apoptosis in cancer cells but not normal cells seems feasible. Here, we review recent progress and prospects of selected novel anti-cancer approaches that specifically target and sensitize cancer cells to apoptosis.

Selective activation of apoptosis in cancer cells

Apoptosis is an elaborate cellular homeostasis mechanism that ensures correct development and function of multicellular organisms. In this respect, the immune system is perfectly equipped to target apoptosis selectively towards cells with potentially dangerous phenotypes. The immune system uses an enormous repertoire of highly selective receptors (e.g. on T and B cells) combined with various potent pro-apoptotic effector mechanisms [e.g. granzymes and fibroblast-associated cell surface (FAS) ligand (FASL)]. However, during tumor progression cancer cells can develop ingenious mechanisms to escape from the immune system – most notably, an increased resistance to apoptosis.

Recent detailed knowledge on molecular aberrations that underlie carcinogenesis has identified various possible targets for therapeutic intervention in cancer. As a result, a plethora of novel targeted approaches has been designed. A common denominator for many of these approaches is the elimination of cancer cells by the preferential induction of apoptosis in these cells while sparing normal cells.

Here, we briefly introduce the normal molecular pathways that underlie apoptosis (Fig.1) and some of the major defects observed in cancer cells. We also highlight recent advances in therapeutic approaches that have been designed to exploit differences between cancer and normal cells to tip the balance of cellular fate towards apoptotic cell death in a selective way (Fig.2). We review some of the most promising approaches that selectively target cancer associated cell-surface molecules. We then deal with strategies that aim to induce apoptosis on the basis of intracellular pathways that might be altered in cancer cells. Subsequently, we review novel approaches that selectively exploit cancer-related alterations in the nucleus to induce apoptosis. In table 1, the respective target, the
Fig. 1. The molecular mechanisms of apoptosis. The mitochondrial pathway of apoptosis is controlled by the BCL-2 family of pro- and anti-apoptotic proteins. When activated, the upstream sensors of intracellular stress – the BH3-only proteins (e.g. BID) – associate with the pro-apoptotic BCL-2 proteins BAX or BAK and translocate to the outer mitochondrial membrane. Subsequently, pores are formed in this membrane, resulting in the release of, among others, the DNases apoptosis inducing factor (AIF) and endonuclease G, both of which translocate to the nucleus and induce DNA fragmentation. In addition, cytochrome c is released from the mitochondria and associates with APAF-1 and pro-caspase-9 into the so-called apoptosome. In the apoptosome, caspase-9 is proteolytically processed into its active form, whereupon effector caspases (e.g. caspase-3) can be activated. BH3-only proteins are inhibited by the anti-apoptotic BCL-2 proteins, such as BCL-2 and BCL-xL, that bind to and inhibit the association of BH3-only proteins with BAX or BAK. The death-receptor pathway of apoptosis is activated upon interaction of a death receptor with its cognate death-inducing ligand, resulting in recruitment of the adaptor protein FADD and pro-caspase-8 to the intracellular death domain of the receptor. Concomitantly, pro-caspase-8 is proteolytically cleaved and activates the effector caspases. In addition, caspase-8 can cleave the BH3-only protein BID, thereby activating a mitochondrial amplification loop. Abbreviations: casp, caspase; cyt c, cytochrome c; pro-casp, pro-caspase.
Fig. 2. Therapeutic strategies for the targeted induction of apoptosis in human cancer cells.

1. Naked monoclonal antibodies (e.g., RituximAb and ApolizumAb) bind to and crosslink their target antigen, which results in activation of the mitochondrial pathway of apoptosis. (2) Galectin-1 binds to carbohydrate moieties on various cell surface-expressed proteins and triggers caspase-independent apoptosis that is characterized by specific release of endonuclease G from the mitochondria. (3) Apoptosis is activated by triggering of death receptors by recombinant forms of the cognate death-inducing ligand. (4) Hypoxia in the tumor microenvironment upregulates the expression of the transcription factor HIF-1α, which protects towards apoptosis. The function of HIF-1α can be specifically inhibited by the small-molecule inhibitor PX-478, whereupon cells undergo apoptosis. (5) The anti-apoptotic proteins BCL-2 and BCL-xL shift the balance of mitochondria towards survival. Inhibition of BCL-2, using the antisense ODN genasense, and inhibition of both BCL-2 and BCL-xL, using small molecule inhibitor ABT-737, blocks the anti-apoptotic function of these proteins and thereby shifts the balance towards induction of mitochondrial apoptosis. (6) Inhibition of the proteasome, using the inhibitor bortezomib, deregulates protein homeostasis leading to cell cycle arrest and activation of the mitochondrial apoptotic pathway. (7) Inhibition of the important chaperone molecule HSP90, using 17-AAG, results in proteasomal degradation of regulatory proteins and subsequent cell-cycle arrest and activation of the mitochondrial apoptotic pathway. (8) Inhibition of the XIAP-mediated block on active caspase-9, using SMAC peptides, sensitizes tumor cells towards apoptosis. Inhibition of the XIAP-mediated block on active caspase-3, using small-molecule inhibitors, results in potent activation of apoptosis. (9) Demethylation of cellular DNA, using decitabine, induces growth arrest and concomitant apoptosis. (10) Restoring the balance between histone acetylation and histone deacetylation with histone deacetylase inhibitors such as valproic acid results in increased acetylation,
whereby transcription of a finite number of genes is up- or downregulated. Subsequently, cancer cells undergo cell-cycle arrest followed by apoptosis. (11) Inhibition of the interaction of wild-type p53 with its negative regulator HDM-2 by blocking the HDM-2–p53 interaction site on HDM-2, using Nutlins, or by blocking the HDM-2–p53 interaction site on p53, using RITA, upregulates p53 target genes and potently induces apoptosis. (12) Re-activation of mutant p53, using PRIMA-1, upregulates p53 target genes and potently induces apoptosis. Abbreviations: casp, caspase; pro-casp, pro-caspase.

Finally, we discuss directions to integrate these approaches in an attempt to design selective tumoricidal pro-apoptotic strategies with low or non-overlapping toxicity towards normal cells.

**Molecular pathways of apoptosis and cancer-specific defects**

Central to the execution of apoptosis is the coordinated activation of a subset of caspases – executioner caspases – that cleave multiple cellular substrates, ultimately resulting in apoptotic cell death (Fig.1). These executioner caspases (caspase-3, caspase-6 and caspase-7) are themselves activated by so-called initiator caspases. All caspases are produced as inactive pro-enzymes and are activated by proteolytic processing.

In most physiological situations, apoptosis is initiated via the mitochondrial pathway. Central to this pathway is the permeabilization of the outer mitochondrial membrane with subsequent release of several pro-apoptotic factors into the cytosol. One of these factors, cytochrome-c, alters the conformation of the cytosolic protein apoptotic protease activating factor-1 (APAF-1), whereupon this protein oligomerizes with pro-caspase-9 into the so-called apoptosome. Pro-caspase-9 is then autoproteolytically processed and, subsequently, activates effector caspases.

Mitochondrial sensitivity to apoptosis is exquisitely regulated by the B-cell leukemia/lymphoma 2 (BCL-2) family of pro- and anti-apoptotic proteins. These proteins are defined in part by homology shared within four conserved BCL-2 homology (BH) domains. Anti-apoptotic members, such as BCL-2 and BCL-xL, are conserved in all four BH domains, whereas the multidomain pro-apoptotic members BCL-2-associated X protein (BAX) and BCL-2 antagonistic killer (BAK) show conservation in BH1–BH3 domains. The BH3-only proteins of this family are thought to serve as upstream sensors that respond to diverse death signals. BH3-only proteins seem to require BAX or BAK for their pro-apoptotic activity and are sequestered by anti-apoptotic BCL-2 proteins. Interaction of BH3-only proteins with BAX and/or BAK is prevented by anti-apoptotic BCL-2 proteins, which, consequently, restrains initiation of mitochondrial apoptosis.

The second main pathway for apoptosis – the death receptor pathway – has a fundamental role in maintenance of tissue homeostasis, especially in the immune system. This pathway is activated upon interaction of death receptors at the cell surface with their cognate ligands on, for example, T cells, whereupon the adaptor protein FAS-associated death
## Table 1: Cellular target, effector mechanism and (pre)clinical status of selected apoptosis-inducing therapeutic compounds

<table>
<thead>
<tr>
<th>Therapeutic compound</th>
<th>Cellular target</th>
<th>Mechanism of apoptosis induction</th>
<th>Clinical status</th>
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<th>Refs</th>
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<td><strong>Immunotoxins</strong></td>
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<tr>
<td>Gemtuzumab ozogamicin</td>
<td>CD33</td>
<td>cell death machinery-mediated DNA damage</td>
<td>FDA approved (AML)</td>
<td>CR 56%</td>
<td>7</td>
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<tr>
<td></td>
<td>CD22</td>
<td>cell death machinery-mediated DNA damage</td>
<td>Phase I trial (NHL)</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>CD22</td>
<td>cell death machinery-mediated DNA damage</td>
<td>Phase I trial (NHL)</td>
<td>CR 66%, PR 19%</td>
<td>9</td>
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<tr>
<td><strong>Antibodies</strong></td>
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<tr>
<td>Rituximab</td>
<td>CD20</td>
<td>cross-linking of CD20: mitochondrial apoptosis</td>
<td>FDA approved (NHL)</td>
<td>PR 19%</td>
<td>10</td>
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<td></td>
<td>HLA-DR</td>
<td>cross-linking of HLA-DR: ROS generation, mitochondrial apoptosis</td>
<td>Phase I trial (NHL)</td>
<td>7</td>
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<td>TRAIL-R1</td>
<td>cross-linking of TRAIL-R1: mitochondrial apoptosis</td>
<td>Phase I trial</td>
<td>7</td>
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<td>TRAIL-R2</td>
<td>cross-linking of TRAIL-R2: mitochondrial apoptosis</td>
<td>Phase I trial</td>
<td>7</td>
<td>13</td>
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<td><strong>Death Inducing Ligands</strong></td>
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<td>TNF</td>
<td>TNF receptors</td>
<td>DR-induced apoptosis by TNF-R activation</td>
<td>FDA approved (isolated limb/organ perfusion)</td>
<td>CR 78%</td>
<td>14</td>
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<td></td>
<td>Fas</td>
<td>DR-induced apoptosis by Fas activation</td>
<td>Pre-clinical (preclinical efficacious in animal models)</td>
<td>15</td>
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<td></td>
<td>TRAIL</td>
<td>DR-induced apoptosis by TRAIL-R activation</td>
<td>Pre-clinical (preclinical efficacious in animal models)</td>
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<td>scFv-TRAIL</td>
<td>DR-induced apoptosis by TRAIL-R activation</td>
<td>Pre-clinical (preclinical efficacious in animal models)</td>
<td>18</td>
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<td></td>
<td>TNF receptors</td>
<td>DR-induced apoptosis by TNF-R activation</td>
<td>Pre-clinical (in vitro)</td>
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<tr>
<td><strong>Leucinex</strong></td>
<td>Galcetin-1</td>
<td>Growth arrest followed by caspase-independent apoptosis</td>
<td>Pre-clinical (preclinical efficacious in animal models)</td>
<td>20</td>
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<td></td>
<td>Galcetin-3</td>
<td>Growth arrest followed by caspase-dependent apoptosis</td>
<td>Pre-clinical (in vitro)</td>
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<td><strong>Extra-cellular targets</strong></td>
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<tr>
<td>Bortezomib</td>
<td>Proteasome</td>
<td>Inhibition of proteasome, activating mitochondrial apoptosis</td>
<td>FDA approved (multiple myeloma)</td>
<td>PR 5%, SD 50%</td>
<td>40</td>
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<td></td>
<td>BCL-2</td>
<td>Inhibition of BCL-2, shifting balance to mitochondrial apoptosis</td>
<td>Pre-clinical (preclinical efficacious in animal models)</td>
<td>41</td>
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<td>ABT-737</td>
<td>BCL-2/BCL-XL</td>
<td>Inhibition of BCL-2/BCL-XL, shifting balance to mitochondrial apoptosis</td>
<td>Pre-clinical (preclinical efficacious in animal models)</td>
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<td>IAP</td>
<td>Inhibition of IAP-block on active caspase-3</td>
<td>Pre-clinical (preclinical efficacious in animal models)</td>
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<td>17-AAG</td>
<td>HSP-90</td>
<td>Growth arrest followed by caspase-dependent apoptosis</td>
<td>Phase I trial (advanced cancer)</td>
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<td>Reactivation of hypoxia-inducible factor-1</td>
<td>Pre-clinical (preclinical efficacious in animal models)</td>
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<td>p53</td>
<td>Reactivation of hypoxia-inducible factor-1</td>
<td>Pre-clinical (preclinical efficacious in animal models)</td>
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<td>MDM-2</td>
<td>Reactivation of hypoxia-inducible factor-1</td>
<td>Pre-clinical (preclinical efficacious in animal models)</td>
<td>47</td>
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<td></td>
<td>HIF-1</td>
<td>Inhibition of caspase-3, sensitizes cells to apoptosis</td>
<td>Pre-clinical (preclinical efficacious in animal models)</td>
<td>48</td>
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<td></td>
<td>HIF-1</td>
<td>Inhibition of caspase-3, sensitizes cells to apoptosis</td>
<td>Pre-clinical (preclinical efficacious in animal models)</td>
<td>49</td>
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<td><strong>Epigenetic regulation</strong></td>
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<tr>
<td>Decitabine</td>
<td>DNA-methylation</td>
<td>DNA-methylation; activation of mitochondrial apoptosis</td>
<td>Completed phase II trial</td>
<td>34%</td>
<td>66</td>
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<tr>
<td></td>
<td>Histone deacetylase</td>
<td>DNA-methylation; activation of mitochondrial apoptosis</td>
<td>Completed phase II trial</td>
<td>20%</td>
<td>69</td>
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</table>

Abbreviations: 17-AAG, 17-allylamino-17-demethoxygeldanamycin; AML, acute myeloid leukemia; BCL, B-cell leukemia/lymphoma; CHR, complete hematological response; CLL, chronic lymphocytic leukemia; CR, complete response; DR, death receptor; Fas, fibroblast-associated cell surface; FASL, Fas ligand; FDA, Food and Drug administration; GO, gemtuzumab ozogamicin; HDAC, histone deacetylase; HDACI, HDAC inhibitor; HDM-2, human double minute-2; HIF-1α, hypoxia-inducible factor-1α; HLA-DR, human leukocyte-antigen-DR; HSP90, heat shock protein 90; MDS, myelodysplastic syndrome; OR, objective responders; N.a., not available; NHL, non-Hodgkin’s lymphoma; PHR, partial hematological response; PR, partial response; PRIMA-1, p53-dependent reactivation and induction of massive apoptosis-1; RITA, reactivation of p53 and induction of tumor-cell apoptosis; ROS, reactive oxygen species; SD, stable disease; sFASL, soluble FAS ligand; sTNF, soluble tumor necrosis factor; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; XIAP, X-linked inhibitor of apoptosis protein.
domain (FADD) and initiator caspase-8 or caspase-10 are recruited to the intracellular death domains of these receptors. Assembly of this so-called death-inducing signaling complex (DISC) leads to sequential activation of initiator and effector caspases and, ultimately, results in apoptotic cell death. In some cells, the death-receptor pathway relies on a mitochondrial amplification loop that is activated by caspase-8-mediated cleavage of the BH3-only interacting domain death agonist BID to a truncated form. Truncated BID subsequently activates the mitochondrial pathway. An important regulator of the death-receptor pathway is the caspase-8 homologue cellular FAS-associated death-domain-like interleukin-1b-converting enzyme (FLICE) inhibitory protein (c-FLIP), which competes with and inhibits autocleavage of caspase-8.

Once activated, caspases are subject to regulation by the family of inhibitor of apoptosis proteins (IAPs)\(^2\). The IAP family represents an integral checkpoint in the execution of apoptosis by their ability to bind to and inhibit activated caspases, thereby halting the execution phase of apoptosis.

Many cancers are characterized by inactivating mutations in pro-apoptotic proteins; for example, in the tumor suppressor p53, which is instrumental in the activation of the mitochondrial pathway of apoptosis upon DNA damage by substances such as chemotherapeutics\(^3\). Alternatively, anti-apoptotic proteins such as BCL-2 and IAP family members are frequently upregulated. These cancer-cell specific aberrations enable evasion of apoptosis and also confer resistance to chemotherapeutics that typically work by induction of apoptosis. However, cancer cells are reliant on these aberrations for survival, as evidenced by recent findings that have indicated that these cells paradoxically are more prone to apoptosis than normal cells\(^4\) and can even have ‘ready to go’ active effector caspases\(^5\). However, owing to genetic instability, a population of cancer cells arises in which the apoptosis-prone phenotype is over-ruled by upregulation of various anti-apoptotic mechanisms. It has been proposed that malignant transformation induces intracellular stress signals in an attempt of the cancerous cell to self-terminate. Therefore, selective induction of apoptosis in cancer cells but not in normal cells seems feasible. In recent years, identified molecular aberrations have been the target of novel strategies; some of the most promising will be discussed in this review.

**Targeted induction of apoptosis using antibodies**

Compared to their normal counterparts, cancer cells often display a qualitatively and/or quantitatively different repertoire of cell-surface molecules that can be selectively targeted in cancer therapy. Most established strategies for targeted therapy are based on cancer-cell-selective monoclonal antibodies (MAbs). Often, the tumoricidal effect of antibody-based therapy relies on highly toxic and proapoptotic compounds directly conjugated to antibodies that potently activate apoptosis upon internalization and processing. Although
such toxin-based strategies have often been hampered by toxicity and immunogenicity, a better target validation and the development of strategies to reduce immunogenicity have provided some promising results. For example, the immunoconjugate gemtuzumab ozogamicin (GO) has recently been the first immunotoxin to be clinically validated. GO comprises a humanized anti-CD33 antibody linked to a derivative of calicheamicin. Calicheamicin is an antibiotic isolated from Micromonospora echinospora that is 1000-fold more potent than conventional chemotherapeutics. Calicheamicin induces sequence-specific double-strand DNA breaks, thereby activating apoptosis. GO induces objective responses when used as a single agent in acute myeloid leukemia, but is associated with serious hepatic veno-occlusive disease. A similar CD22-targeted immunoconjugate, inotuzumab ozogamicin, is currently being evaluated in Phase I clinical trials for non-Hodgkin’s lymphoma. Furthermore, in a recent phase I clinical trial with a CD22-targeted immunotoxin that contains Pseudomonas exotoxin A, a complete response rate of 61% was reported.

Naked chimeric or fully human antibodies have also shown remarkable anti-cancer activity predominantly by recruiting the patient immune effector mechanisms such as complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC). However, at least part of the tumoricidal activity of certain antibodies originates from direct activation of apoptosis by crosslinking of the respective target antigen. For example, the chimeric anti-CD20 antibody Rituximab inhibits nuclear factor-κB (NF-κB) signaling and potently activates the mitochondrial pathway of apoptosis by crosslinking of cell-surface-expressed CD20. This finding might also be relevant for the efficacy of Rituximab in vivo. A similar observation has been made for Apolizumab, a humanized anti-human-leukocyte-antigen-DR (HLA-DR) b-chain-specific antibody, which shows potent pro-apoptotic activity in chronic lymphocytic leukemia that depends on cytoskeletal rearrangements and formation of reactive oxygen species (ROS).

Because better strategies for target validation and reduction of immunogenicity have been developed, the promise held by antibody-based approaches for >20 years is slowly turning into reality. However, several issues still remain to be addressed, one of which is the known antigen heterogeneity of tumor cells. Tumor cells that downregulate or lose target-antigen expression can easily escape from antibody-based targeted therapy (e.g. Rituximab). Combinatorial strategies that target multiple antigens is one of the ways to overcome this problem. In addition, approaches have been developed to take advantage of the so-called 'bystander effect', which is based on the principle that targeted tumor cells are not only eliminated but also exploited to convey the therapeutic effect towards neighboring tumor cells devoid of target-antigen expression. Bystander effects have been reported for several antibody-based therapeutic approaches, but in most cases require consecutive processing steps, including drug internalization, enzymatic conversion and
inter-cellular communication (e.g. via gap junctions) between target and bystander cells. These consecutive processing steps can still pose severe limits to the bystander effect because tumor cells in which one or more of these steps are inhibited or absent will be resistant to the bystander effect.

**Apoptosis by activation of members of the tumor necrosis factor (TNF) receptor family**

The direct activation of the apoptotic machinery in cancer cells using recombinant soluble forms of tumor necrosis factor (TNF), FASL and TNF-related apoptosis-inducing ligand (TRAIL) has attracted much attention. TNF, FASL and TRAIL, three major immune effector molecules, all possess high tumoricidal pro-apoptotic activity. However, severe cardiovascular toxicity has limited the therapeutic use of soluble TNF (sTNF) to loco-regional applications, such as isolated limb perfusion, where it has shown impressive clinical responses in combination with conventional chemotherapy.

The therapeutic use of soluble FASL (sFASL) was originally deemed impossible owing to severe toxicity in mice. However, this toxicity has recently been attributed to contaminating multimeric or aggregated forms of sFASL. Indeed, as little as two adjacent trimeric FASLs are sufficient for activating FAS-apoptotic signaling. MegaFASL, a hexameric FASL preparation, showed potent cytotoxic effects on some human malignant hematopoietic cells. Its toxicity towards normal lymphocytes remains to be determined. By contrast, homogeneous trimeric sFASL preparations are devoid of toxicity but are also devoid of tumoricidal activity. We and others have developed promising strategies to restore the activity of sFASL trimers only after tumor-selective delivery as will be discussed later.

TRAIL has generated tremendous enthusiasm because it selectively induces apoptosis in various malignant cell types, but not in normal cells. However, the clinical efficacy of soluble TRAIL (sTRAIL) might be hampered because of the widespread expression of different TRAIL receptors throughout the body. In addition, several tumor types express higher levels of TRAIL-receptor-2 (TRAIL-R2) than of TRAIL-receptor-1 (TRAIL-R1), whereas TRAIL-R2 signaling is only poorly activated by sTRAIL. Intriguingly, in some solid tumor types that express equal levels of TRAIL-R1 and TRAIL-R2, apoptosis was reported to be selectively mediated by TRAIL-receptor-2 signaling. Conversely, apoptotic signaling in chronic myeloid leukemia is mediated exclusively by TRAIL-R1 signaling. This notion has led to the development of TRAIL mutants with selectivity for either TRAIL-R2 or TRAIL-R1.

Recently, we and others have demonstrated that the tumor-selective binding of sTRAIL and sFASL can be strongly enhanced by genetic fusion to a tumor-selective antibody fragment. The binding of such fusion proteins to cell-surface-expressed target antigens
Fig.3. Target-cell-restricted induction of apoptosis by scFv–death-ligand fusion proteins. Specific binding of scFv–death-ligand fusion proteins to the tumor target antigen results in accretion at the cell surface. Subsequently, apoptosis can be induced in an autocrine manner by binding to the cognate death receptor on the same tumor cells. Alternatively, specific binding of scFv–death ligand to the tumor target antigen on one cell can induce crosslinking of cognate death receptors on a neighboring target antigen-positive tumor cell, resulting in paracrine target cell apoptosis. In addition, paracrine crosslinking of cognate death receptors on a neighboring target antigen negative tumor cell results in bystander cell apoptosis. This bystander effect depends only on the presence of functional cognate death receptors. Converts the soluble death ligands into membrane-bound molecules capable of crosslinking agonistic death receptors in an autocrine and paracrine manner (Fig.3). In this way also neighboring tumor cells devoid of target antigen can be effectively eliminated by the so-called bystander effect$^{26,27}$. In this case, the bystander effect solely depends on accretion of fusion proteins to the cell surface of targeted cells and does not require additional
cellular processing other than intact death-receptor signaling pathways. Proof of principle for this approach has been obtained for sTRAIL and sFASL in both solid tumors\textsuperscript{21–24} and leukemia\textsuperscript{25,27}, with no or minimal activity towards normal cells. Activation of TRAIL receptors has also been pursued using agonistic MAbs, of which HGS-ETR-1 and HGS-ETR-2 are currently evaluated in clinical trials\textsuperscript{28,29}. An important difference between these TRAIL-receptor selective MAb and TRAIL is the fact that TRAIL interacts with both its agonistic receptors and its decoy receptors. This might give TRAIL either a wider or a narrower and more unpredictable therapeutic window than that of TRAIL-receptor-selective MAbs. Intriguingly, a recent report has indicated that a mouse agonistic TRAIL-R2 MAb also induced potent tumor-specific T-cell immunity\textsuperscript{30}. As a result, a second challenge with the same tumor cells that were engineered to overexpress c-FLIP could still be eliminated. This strategy might thus be of potential value to overcome acquired TRAIL resistance\textsuperscript{31}. However, epigenetic silencing of TRAIL-receptor expression might pose another daunting challenge to be overcome\textsuperscript{32}.

In conclusion, because much of the molecular pathways of TNF, FASL and TRAIL has been elucidated, the therapeutic potential of these ligands in cancer has been firmly established in pre-clinical studies. However, cancer-cell selective activation remains an issue to be thoroughly addressed to use these molecules for clinical applications.

**Activation of apoptosis by modulating Galectins**

Recently, the physiologically occurring anti-proliferative galectins were shown to have promising anti-tumor activity\textsuperscript{33}. Galectins are a family of lectins with affinity for $\beta$-galactoside residues of cell-surface glycoproteins expressed by both normal and cancer cells. However, upon binding, regulatory functions to which normal and cancer cells respond differently are enforced. For example, galectin-1 blocks the cell cycle in late S-phase by altering the expression of cell-cycle controller proteins such as the transcription factor E2F1\textsuperscript{34}. Intriguingly, in tumor cells this S-phase block is followed by activation of apoptosis due to constitutively high E2F1 levels, whereas in normal cells there is only growth arrest. Several tumor types, including multi-drug resistant tumor cells, are highly sensitive to apoptosis induction by galectin-1\textsuperscript{34–36}. The galectin-1 mediated apoptotic pathway is still largely undefined but is reported to be caspase-independent and to involve the specific release of endonuclease G from the mitochondria\textsuperscript{35}.

Intriguingly, galectins can have contradictory roles in tumor development\textsuperscript{33}. Tumors that ubiquitously express galectin-1 can modulate the anti-tumor immune response by eliminating tumor-infiltrating T cells\textsuperscript{37}. In addition, galectins are involved in tumor metastasis by regulation of tumor-cell adhesion and invasiveness. Selective inhibition of cancer cell-expressed Galectin-1 and Galectin-3 has recently been explored using synthetic lactulose amines. This inhibition results in tumor cell-selective apoptosis induction\textsuperscript{38}. 


Therefore, patient-tailored therapy using either recombinant forms of galectin-1 or, alternatively, inhibitors of endogenous galectin-1 or galectin-3, depending on the tumor, might be a promising approach to induce apoptosis in a cancer-selective way.

**Intracellular activation of apoptosis**

*Apoptosis by proteasome inhibition*

Protein homeostasis is pivotal to cell survival and is mainly regulated by the ubiquitin–proteasome pathway (UPP)\(^39\), which controls the half-life of the majority of cellular proteins. Inhibition of the UPP in cancer cells has yielded promising results. This has been highlighted by the recent approval of the proteasome inhibitor bortezomib (Velcade) for the treatment of multiple myeloma\(^40\). An important feature of bortezomib is the differential response of normal and cancer cells\(^41\), the basis of which is still a mystery. It has been shown that after bortezomib treatment both normal and cancer cells are growth arrested in the G2–M phase of the cell cycle\(^41\). However, cancer cells are eliminated by an as yet incompletely characterized apoptotic pathway that converges on mitochondria, whereas normal cells resume division after treatment. Apoptosis by bortezomib is characterized by stabilization of p53\(^42\) and upregulation of the BH3-only protein NOXA\(^41\). Bortezomib potently augments the apoptotic activity of other therapeutics (e.g. TRAIL\(^43\)) that does not depend on p53 status or BAX expression.

*Apoptosis by inhibition of anti-apoptotic BCL-2 family members*

Central to the control of the mitochondrial pathway of apoptosis is the balance between pro- and anti-apoptotic members of the BCL-2 family\(^1\). In cancer, anti-apoptotic proteins such as BCL-2 and BCL-xL are frequently overexpressed, thus shifting the balance towards cell survival. Therefore, therapeutic inhibition of these endogenous inhibitors of apoptosis is an attractive approach that has recently been explored using the antisense oligonucleotide genasense directed against BCL-2\(^44\).

Additionally, the interaction of BCL-2 with BAX and/or BAK has been targeted using small-molecule mimetics that fit the groove on BCL-2 where these pro-apoptotic members bind\(^45\). More recently, a small-molecule mimetic for BCL-2 and BCL-xL, ABT-737, has been reported to have high-affinity inhibition for this anti-apoptotic protein\(^46\). ABT-737 induced complete tumor regression in 77% of treated mice with no secondary tumor outgrowth in xenograft models. However, some cell lines were resistant to ABT-737, possibly because of overexpression of other anti-apoptotic BCL-2 family members, such as myeloid-cell leukaemia factor (MCL-1)\(^47\). Taken together, therapeutic inhibition of these endogenous inhibitors of apoptosis holds great promise for tipping the balance towards apoptosis in cancer. Because various anti-apoptotic BCL-2 members are known to be upregulated, the future of this approach is likely to involve combination strategies that inhibit multiple anti-
apoptotic BCL-2 family members, such as the use of bi-specific BCL-2–BCL-xL antisense oligonucleotide.\textsuperscript{48}

**Apoptosis by inhibition of the X-linked inhibitor of apoptosis protein (XIAP)**

Inhibition of activated caspases by IAPs is an integral checkpoint during apoptosis.\textsuperscript{2} The first identified member, the X-linked inhibitor of apoptosis protein (XIAP), is upregulated in various cancers and can inhibit both initiator caspase-9 and effector caspase-3. The so-called Baculovirus IAP repeat (BIR)3 domain of XIAP inhibits caspase-9, whereas the BIR2 domain together with the N-terminal linker inhibits caspase-3. Relieving the XIAP-mediated block on caspase-9 was pursued using peptides that contain a BIR3-domain binding motif derived from the XIAP-neutralizing protein second mitochondrial activator of caspases (SMAC). These peptides did not induce apoptosis as single agents, but synergized the apoptotic activity of chemotherapeutic agents and TRAIL. More recently, one of these peptides that specifically relieved the block on not only caspase-9 but also caspase-3 was shown to activate apoptosis as a single agent depending on the expression level of IAPs.\textsuperscript{49}

Intriguingly, inhibition of the XIAP-caspase-3 interaction using selected polyphenylurea compounds potently and selectively induced apoptosis in cancer cells but not in normal cells.\textsuperscript{5} These results strongly indicated that cancer cells are intrinsically more prone to apoptosis than normal cells probably owing to the presence of ‘ready to go’ processed effector caspases. Indeed, processed active caspase-3 has been detected in tumor cell lines and tumor tissue.\textsuperscript{5} Therefore, the inhibition of XIAP and other IAP family members such as survivin can help selectively restore sensitivity to apoptosis in cancer cells, which might be especially useful in combination with other cancer-selective pro-apoptotic approaches, such as TRAIL and p53 reactivation as described below.

**Apoptosis by inhibitors of heat shock protein 90 (HSP90)**

The activity of many proteins that are involved in carcinogenesis depends on heat shock protein 90 (HSP90) for their maturation and stability.\textsuperscript{50} HSP90 is part of a large multichaperone complex that exists in two configurations. In the first configuration, known as open or intermediate, client proteins are loaded. This complex switches to a closed or mature state upon ATP-binding and hydrolysis, resulting in substitution of original co-chaperones with other co-chaperones that help maintain the protein in an active configuration and direct protein maturation. Several HSP-90 inhibitors can lock this multichaperone complex in the open state, leading to proteasomal degradation of client proteins. Most prominent of these is 17-allylamino-17-demethoxygeldanamycin (17-AAG), which has shown promising activity in Phase I clinical trials.\textsuperscript{51} 17-AAG induces growth arrest and apoptosis (e.g. by degradation of B-RAF or 70 kDa zeta-associated
protein ZAP-70). Importantly, it has recently been shown that inhibition of HSP90 abrogates the TNF-induced NF-κB activation by preventing formation of the normally occurring IkB-kinase (IKK) complex. Therefore, combination of HSP90 inhibition and TNF inhibition might be a promising therapeutic approach, as evidenced by the recently described synergistic activation of apoptosis by 17-AAG and TNF.

**Activation of apoptosis in the nucleus**

*Apoptosis by restoring p53 activity*

The tumor-suppressor p53 is instrumental in the cellular response to stress signals and is crucial in the prevention of tumor development and the success of various anti-cancer strategies. Over 50% of tumors possess inactivating mutations in p53, whereas in tumors that retain wild-type p53 its function is often impaired as a result of overexpression of the negative regulator human double minute-2 (HDM-2). HDM-2 binds to p53 and, consequently, p53 is subject to rapid proteasomal degradation. Therefore, the restoration of p53 activity is a potentially promising therapeutic approach. Several HDM-2-selective inhibitors have been designed to treat tumor cells that express wildtype p53. A recently developed class of HDM-2 inhibitors, the so-called Nutlins, disrupt the interaction between wild-type p53 and HDM-2 by binding to the HDM-2–p53 binding pocket on HDM-2. Nutlins potently and tumor-specifically activate apoptosis without toxicity. Conversely, the reactivation of p53 and induction of tumor-cell apoptosis (RITA) inhibits the HDM-2–p53 interaction by binding to p53 and, similarly, induces massive apoptosis in p53 wild-type tumor types.

In tumors that express p53 mutant, reactivation of p53 has been attempted using activating compounds such as p53-dependent reactivation and induction of massive apoptosis-1 (PRIMA-1). These compounds restored transcriptional activity of mutant p53, resulting in potent induction of apoptosis.

In conclusion, the central role of p53 in therapeutic apoptosis induction highlights the rationale to restore its full functionality in cancer cells to overcome therapy resistance. Rational combinatorial strategies of p53 reactivation with, for example, XIAP inhibition might also help to lower the threshold for apoptosis induction specifically in cancer cells.

*Apoptosis by inhibition of hypoxia inducible factor-1α (HIF-1α)*

Hypoxia in the tumor microenvironment contributes to malignant progression and protects cancer cells from drug-induced apoptosis. Hypoxia-inducible factor-1α (HIF-1α) is a pivotal component in the hypoxia response during tumor development. The effect of HIF-1α upregulation on solid-tumor growth is a balance between reduced cell proliferation and enhanced survival, the latter being proportionally greater. Recently, a small molecule inhibitor of HIF-1α, named PX-478, has been described to have promising tumoricidal
effects in xenograft tumor models. The anti-tumor effect of PX-478 positively correlated with tumoral HIF-1α levels and was accompanied by massive apoptosis. Additionally, antisense DNA strategies directed against HIF-1α have been designed, which revealed potent synergistic activity in combination with chemotherapy. These data clearly indicate that modulation of molecules or pathways involved in tumor hypoxia can restore sensitivity to therapeutic apoptosis induction.

### Epigenetic regulation of apoptosis

#### Apoptosis by DNA-methylase inhibitors

DNA methylation has a regulatory role in gene expression during normal development but can also mediate epigenetic silencing of genes in cancer. Many individual genes including tumor suppressors have been shown to undergo de novo methylation in specific tumor types. Specific DNA methyltransferases methylate DNA at the carbon-5 position of cytosine. An important example is the methylation of the E-cadherin promoter, which has an essential role in metastasis and invasiveness of breast cancer. Reversal of cancer-specific DNA methylation has been pursued using nucleoside-based inhibitors, such as 5-aza-deoxycytidine (also known as decitabine), which is currently evaluated in Phase II clinical trials for myeloid malignancies. Decitabine directly incorporates into the DNA and traps methyl-binding proteins (MBPs) on the DNA template, thereby depleting the cellular store of MBPs. Concomitantly, genomic DNA is demethylated during continuous rounds of DNA replication. Decitabine shows single-agent activity by induction of growth arrest that is followed by mitochondrial apoptosis, but is associated with substantial toxicity.

#### Apoptosis by histone deacetylase inhibitors (HDACi)

Histone acetylation is another important epigenetic regulator of transcription, with histone acetylation being associated with active genes and deacetylation with transcriptionally inactive genes. In cancer, the balance between histone acetylation and deacetylation is often disturbed by inactivating mutations in histone acetyltransferases (HATs) or by overexpression of histone deacetylases (HDACs). HDAC inhibitors (HDACi) such as valproic acid (VPA) showed promising results in clinical trials. In vitro, HDACi induce growth arrest, differentiation and apoptosis in various cancer cells. Recent insight into the mode of action of HDACi has revealed that the anti-leukemia activity relies on upregulation of TRAIL and subsequent induction of apoptosis. These findings provide an additional rationale for the observed synergistic pro-apoptotic effect of combined treatment with HDACi and TRAIL. Notably, it has become clear that HDACi also modulate acetylation of various non-histone proteins. An interesting example is HSP90, which is destabilized by acetylation, resulting in enhanced proteasomal degradation of several oncogenic client proteins including RAF, ERBB1 and ERBB2. Taken together, as more insight is gained into
the molecular mechanisms of the activity of HDACi, rational combination strategies have been and will be identified. In this respect, combination with inhibition of DNA methylation might further help to reactivate a set of genes that resensitize cells to apoptosis.

**Conclusions and perspectives**

Targeted therapies that are designed to induce apoptosis selectively in cancer cells are currently the most promising anti-cancer strategies. These strategies aim to target and specifically kill tumor cells with no or minimal collateral damage. However, a fundamental problem is still that ‘primitive’ targeting is often simply not specific enough to enable the delivery of highly toxic agents. Therefore, the problem of cancer selectivity remains an important issue. As a consequence, the toxicity of targeted agents has to be reduced often to the point of not being of sufficient therapeutic benefit. It is therefore useful to integrate the concept of how the immune system deals with the targeted delivery of its potentially highly dangerous effector mechanisms. In the immune system, effector mechanisms are tightly controlled both spatially and temporally. An elaborate system of consecutive proof-reading steps and highly selective receptors on specialized effector cells enables the delivery of a highly cytotoxic freight that is activated only at the site of the lesion. In many cases, the immune system eradicates cancerous cells by targeted apoptosis induction with cell-surface-expressed FASL and TRAIL as important effector molecules.

One of the main challenges to be addressed in contemporary targeted therapy is to create technologically more advanced pro-apoptotic molecules. We and others have provided proof of principle for target-cell-restricted apoptosis induction using recombinant fusion proteins in which a tumor-selective antibody fragment is fused to either sTRAIL or sFASL. Only upon selective binding to the tumor cell surface the otherwise inactive fusion protein is activated and, subsequently, tumor cell apoptosis is induced in an autocrine or paracrine manner.

Recently, this strategy has been greatly advanced by the construction of a so-called TNF prodrug. The TNF prodrug is a tripartite fusion between a tumor-selective antibody fragment, soluble TNF and a TNF receptor-derived inhibitor module. Additionally, recognition motifs of the matrix metalloproteinase (MMP)-2 were engineered between the TNF and the TNF-receptor-1 domain. After tumor selective binding of the TNF prodrug, the inhibitor module is removed by target cell-expressed MMPs, ensuring strictly antigen-dependent activation of apoptosis.

However, even advanced concepts such as the TNF-prodrug strategy will fail when the targeted tumor cells are resistant to apoptosis due to one or more defects in death receptor or caspase apoptosis pathways. Therefore, reagents that show caspase-independent pro-apoptotic activity, such as Galectin-1, are of particular interest. Alternatively, seemingly
apoptosis-resistant tumor cells can have elevated apoptotic thresholds that cannot be reached without first sensitizing tumor cells using one or more activating drugs. Indeed, the combinatorial use of various pro-apoptotic agents is also of interest. In this respect, the specific targeting of cancer-related anti-apoptotic aberrations, such as silencing of p53 and upregulation of BCL-2 and IAPs, are promising targets for intervention. Because it has recently become clear that cancer cells rely heavily on these aberrations for survival and are in fact more prone to undergo apoptosis than normal cells, modulation of these specific aberrations should provide cancer specificity to some extent. Integration of various concepts can be used to design combinatorial-treatment strategies that enhance or restore the sensitivity of tumor cells to (targeted) apoptosis induction with minimal effects on normal cells. In this respect, the most-promising combinations might involve those drugs that work along different or complementary apoptotic signaling routes with non-overlapping toxicities towards normal cells.

However, an important issue to be addressed is the question of how ‘selective’ cancer-cell selective is and ultimately can be. Because both normal and cancer cells crucially rely on apoptosis, it is important to consider whether specific modulation of apoptosis in cancer cells is feasible. In other words, is there a large enough therapeutic window between sensitivity to apoptosis in normal and cancer cells? Single-agent therapy is likely to prove not selective enough in most cases. The best way forward seems the combined treatment of cancer cells with therapeutics that are designed to exploit several cancer related aberrations whereby the therapeutic window is increased.

However, the application of such rational combinatorial strategies will rely heavily on the identification of specific cancer-related aberrancies in each patient. Therefore, further development of reliable, cost-effective and high throughput diagnostic tools will be required to enable the successful application of such patient-tailored therapeutic approaches.

Another important issue to address is the occurrence of drug-resistance upon highly selective cancer therapy, such as for the inhibition of the constitutively active breakpointcluster-region (BCR)–ableson-proto-oncogene (ABL) tyrosine kinase in certain forms of leukemia. These findings indicate that even ‘magic bullets’ seem to lose their magic as single agents.

In addition, all of the approaches discussed here still have to deal with some inherent problems of the respective strategy. For example, the technology for antisense strategies is still fraught with technical limitations, whereas for protein-based therapies immunogenicity is an important issue to address. Humanizing strategies and epitope remodeling are some of the possible ways to reduce immunogenicity. An additional fundamental problem for protein-based therapies in solid tumors is the limited tumor penetration. In such cases, initial tumor-debulking by surgical resection followed by protein-based therapy might prove the best way forward. Taken together, several important questions remain to be
Chapter 2

Targeted induction of apoptosis for cancer research: current progress and prospects

Box 1. Outstanding questions

Despite the elucidation of many of the molecular aberrations that underlie carcinogenesis and the resultant development of a plethora of therapeutic approaches targeting these aberrations, several issues remain to be addressed to determine the feasibility of selective activation of apoptosis in cancer.

- Can apoptosis be selectively activated in cancer cells, being apoptosis a key process for both normal and cancer cells? Although many of the approaches discussed in this article to some extent show selectivity for cancer cells, the ‘holy grail’ of selective elimination of cancer cells has yet to be uncovered.

- How can adverse effects on other normal tissues be avoided upon application of targeted cancer-cell selective therapeutics?

- How can rational combinatorial strategies be designed to activate divergent or complementary pathways of apoptosis? Can rational combinatorial strategies be identified in order to raise the therapeutic window for cancer-selective activation of apoptosis.

- How can patient-tailored combinatorial strategies be designed efficiently? In other words, how can patients be rapidly and reliably diagnosed?

- How can immunogenicity of protein-based therapeutics be avoided or minimized?

- How can poor tumor-penetration of protein-based therapeutics be overcome?

addressed (Box 1).

Thanks to laser-capture microscopy and DNA-microarray technology, it is now possible to obtain large quantities of gene-expression data from individual cancer cells. However, currently it is still difficult to extract meaningful information from such large quantities of data and to connect them to tumor-specific phenomena or drug information. Nevertheless, further improvements in this field are anticipated that might make it possible to identify hitherto unknown routes for tumor-specific apoptosis induction, which, in turn, can contribute to new discoveries in medical, pharmaceutical and life sciences.

Taken together, as molecular aberrations in apoptosis regulation in cancer cells are elucidated, the rational design of combinatorial approaches paves the way towards enhanced and tumor-selective apoptosis induction that in the future will help fight cancer in a clinical setting.
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Chapter 2


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