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Review

Targeting the Bcl-2-regulated apoptosis pathway by BH3 mimetics: a breakthrough in anticancer therapy?

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Induction of apoptosis in tumor cells by direct activation of the Bcl-2-regulated apoptosis pathway by small molecule drugs carries high hopes to overcome the shortcomings of current anticancer therapies. This novel therapy concept builds on emerging insights into how Bcl-2-like molecules maintain mitochondrial integrity and how pro-apoptotic BH3-only proteins lead to its disruption. Means to unleash the pro-apoptotic potential of BH3-only proteins in tumor cells, or to bypass the need for BH3-only proteins by directly blocking possible interactions of Bcl-2-like pro-survival molecules with Bax and/or Bak, constitute interesting options for the design of novel anticancer therapies. For the optimization and clinical implementation of these novel anticancer strategies, a detailed understanding of the role of individual BH3-only proteins in cell death signaling in healthy cells and during tumor suppression is required. In this review, we will touch on the latest findings on BH3-only protein function and attempts to define the molecular properties of the so-called 'BH3 mimetics,' a novel class of anticancer agents, able to prompt apoptosis in tumor cells, regardless of their p53 or Bcl-2 status.
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Modern anticancer strategies finally move away from the use of crude nonspecific cytotoxic agents toward the application of rationally designed drugs that inhibit well-defined targets in specific cellular signaling pathways involved in tumorigenesis. A key assumption of many of these novel strategies is that the maintenance and progression of cancers are critically dependent on resistance of transformed cells to undergo cell death. During neoplastic transformation, cancer cells are usually selected to gain the ability to survive otherwise deadly cellular and genetic changes. These alterations can be caused by oncogenic stress, impaired cell cycle arrest and/or DNA damage repair functions, genomic instability, telomere erosion, trophic factor deprivation or loss of adhesion.

The link between impaired apoptosis and tumorigenesis became apparent when the antiapoptotic protein Bcl-2 (B-cell lymphoma 2) was initially described as a potential oncogene more than 20 years ago. Bcl-2 was found overexpressed in 90% of human follicular lymphoma and 75% of all cases were harboring the characteristic t(14;18) chromosome translocation that juxtaposes the *bcl-2* gene to the immunoglobulin heavy chain (*IGH*) locus (Adams and Cory[1] and citations therein). Seminal work by Vaux *et al.*[2] identified Bcl-2 as a survival factor shortly thereafter. Genetic analysis in mice overexpressing a *bcl-2* transgene further provided strong evidence that aberrant expression of Bcl-2, although being poorly oncogenic on its own, facilitates onset of malignant disease, once the cell cycle machinery is deregulated by aberrant expression of oncogenes such as *c-myc*.[1] Consistently, Bcl-2 or related pro-survival family members were found frequently overexpressed in many human tumor types identifying Bcl-2 and its subsequently discovered homologs as promising candidates for therapeutic intervention.

Mammals control tissue homeostasis by apoptosis, which allows removal of damaged, infected or otherwise unwanted cells. Therefore, the apoptosis machinery acts as a barrier against cancer but is also rate limiting for the efficacy of anticancer therapy.

Two major pathways can lead to apoptosis in mammalian cells. The stress-induced 'intrinsic' or mitochondrial cell death pathway, regulated by the Bcl-2 family, is more ancient and evolutionarily conserved from worms to mammals than the death receptor (DR)-induced or 'extrinsic' cell death pathway, which co-evolved with the establishment of adaptive immunity in vertebrates. The extrinsic pathway is induced when DRs, members of the TNF (tumor necrosis factor) receptor (R) family (e.g. CD95, TRAIL-R or TNF-R), are engaged by their cognate ligands, belonging to the TNF family (e.g. FasL, TRAIL or TNF). Subsequently, on the cytosolic side of the DR a protein complex called DISC, (death-inducing signaling complex), consisting of the DR, an adapter protein (e.g. FADD in case of CD95) and proteases of the caspase family, that is, pro-caspase-8 or -10 in humans, is formed. Within this

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Abbreviations: Bad, Bcl-2 antagonist of cell death; Bak, Bcl-2 antagonist/killer; Bax, Bcl-2-associated protein X; Bcl-2, B-cell lymphoma 2; Bcl-x, Bcl-2-related protein X; BH, Bcl-2 homology; Bid, Bcl-2 interacting domain death agonist; Bik, Bcl-2 interacting killer-like; Bim, Bcl-2 interacting mediator of cell death; Bmf, Bcl-2-modifying factor; DR, death receptor; Hrk, harakiri; IAP, inhibitor of apoptosis protein; Puma, p53-upregulated modulator of apoptosis; TNF, tumor necrosis factor; XIAP, X-linked inhibitor of apoptosis protein
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complex, pro-caspase-8 or -10 is activated by dimerization leading to their full activation, which, in turn, allows processing of the effector caspase-3, -6 and -7 by proteolytic cleavage. Similarly, apoptosis along the intrinsic pathway, regulated by the Bcl-2 family and mitochondria (discussed in more detail below), also culminates in the activation of these caspases. Once active, effector caspases provoke cellular destruction by cleaving hundreds of proteins within the cell.[3]

Although initially considered as a potent anticancer drug, TNF can only be applied in limited number of clinical settings due to its strong systemic side effects, including endothelial damage and liver cytotoxicity. However, recombinant TRAIL or humanized therapeutic agonistic antibodies targeting TRAIL-RI and/or TRAIL-RII are promising candidates for (combinatorial) anticancer therapy due to their selectivity to trigger apoptosis preferentially in transformed cells and are currently being investigated for their *in vivo* potency in phase II clinical trials. Another example aiming to exploit the extrinsic apoptosis pathway for anticancer therapy is the use of CD95-Ig fusion proteins for its suitability to prevent FasL-mediated graft *versus* host disease while preserving graft *versus* leukemia effects. In addition, a modified version of FasL, which does not cause liver cytotoxicity, is currently under way to enter clinical trials (for recent detailed reviews see Fesik[4] and Kassahn *et al*.[5]).

Both the extrinsic and intrinsic apoptosis pathways converge at the level of effector caspase activation, identifying these molecules as 'ideal' drug targets. Caspase activity is regulated by certain members of the inhibitor of apoptosis protein (IAP) family, most potently by XIAP (X-linked IAP), which is able to bind to and inactivate caspase-9, -3 and -7. This interaction is neutralized by IAP antagonists such as SMAC/DIABLO, which is released from mitochondria during apoptosis induction. Although other members of the IAP family including cIAP1, cIAP2 or ML-IAP have also been shown to block cell death, the molecular basis of inhibition is less clear. However, many of these IAP family proteins have been implicated in the pathogenesis and/or drug resistance of a number of malignant diseases, including melanoma, hepatocellular carcinomas or pancreatic cancer.[6] At least three companies developed synthetic compounds targeting IAP–caspase interaction sites. Surprisingly, latest studies revealed that these SMAC mimetics trigger tumor cell death by initiating an autocrine TNF loop rather than by directly activating caspases.[7–9] Since systemic application of TNF is known to cause severe side effects, it will be interesting to see how tolerable and beneficial these compounds are when used to treat cancers in preclinical *in vivo* models. Short-term low-level autocrine TNF production and/or sensitization to TNF signaling, triggered by SMAC mimetics, may be much better tolerated than systemic administration of this cytokine, hopefully paving the way for clinical trials.

Cell Death Signaling Regulated by the Bcl-2 Family: a Simplified View

Whether a cell continues to live in response to diverse forms of stress or undergoes apoptosis along the intrinsic signaling pathway is largely determined by the complex interplay between individual members of the Bcl-2 protein family that can either promote or prevent apoptosis. The five known survival-promoting family members Bcl-2, Bcl-x_L (Bcl-2-related protein x_L), Bcl-w, Mcl-1 and A1 share four Bcl-2 homology (BH) domains (BH1–BH4) among each other, with the exception of Mcl-1 that contains only three of these domains. All these proteins are critical for cell survival, since loss of any of them causes premature cell death of certain cell types. Bcl-2 appears highly critical for the survival of mature lymphocytes and melanocytes, whereas neurons and erythroid progenitors depend on the presence of Bcl-x_L as myeloid progenitors, lymphocytes and hematopoietic stem cells do on Mcl-1 (for a more detailed review see Youle and Strasser[3] and references therein). Consistently, overexpression of Bcl-2 pro-survival molecules is associated with prolonged (tumor) cell survival and drug resistance in a number of model systems, but more importantly, also in tumor patients.

The pro-apoptotic Bcl-2 family members can be divided into two classes: the Bax (Bcl-2-associated protein X)-like proteins (Bax, Bak (Bcl-2 antagonist/killer) and Bok) that contain three BH domains (also called BH123 or multi-domain pro-apoptotic Bcl-2 proteins) and the BH3-only proteins. The latter include Bim (Bcl-2 interacting mediator of cell death), Bid (Bcl-2 interacting domain death agonist), Puma (p53-upregulated modulator of apoptosis), Noxa, Bmf (Bcl-2-modifying factor), Bad (Bcl-2 antagonist of cell death), Hrk (harakiri) and Bik (Bcl-2 interacting killer-like) that are unrelated in their sequence to each other or other Bcl-2 family members with the exception of the BH3 domain. The BH3 domain forms an amphipathic alpha helix of about 24 residues that enables binding to a hydrophobic groove on the surface of pro-survival Bcl-2 molecules, formed by amino acids contained in the BH1, BH2 and BH3 domains, thereby mediating protein–protein interaction.[3] For the pro-apoptotic function of BH3-only proteins, the multi-domain proteins Bax and Bak are absolutely essential.[10] Whereas loss of either Bax or Bak has only little effect on apoptosis induction in most cell types, combined deficiency of both proteins causes perinatal lethality in mice and renders cells highly resistant to overexpression of BH3-only proteins as well as to a broad range of apoptotic stimuli triggering the intrinsic cell death pathway.[11]

Molecular action of BH3-only proteins. It has been anticipated for a long time that all Bcl-2 pro-survival proteins can substitute for each other in inhibiting cell death in response to genotoxic stress and that overexpression of any given BH3-only protein can kill cells in a rather nonspecific manner. However, recent biochemical and genetic evidence suggest that BH3-only proteins act only in a much more specific way to neutralize the pro-survival function of Bcl-2-like molecules. Interestingly, the combination of BH3-only proteins that is required to achieve that differs, depending on the apoptotic stimulus that hits the cell, as well as on the expression pattern of antiapoptotic Bcl-2 molecules.[12]

Bim and Puma, for example, can engage all pro-survival Bcl-2 proteins with comparable binding affinities and are therefore potent killers. In contrast, other BH3-only family members target selectively only subsets of pro-survival Bcl-2 proteins, for example, Bad or Bmf can only counteract the

function of Bcl-2, Bcl-x_L or Bcl-w, whereas Noxa exclusively counteracts Mcl-1 and A1,[13] and therefore possesses only weak or no killing activities on its own (Figure 1a). On the basis of these observations, one can predict that a normal or malignant cell that expresses Bcl-2 and Mcl-1 can only be killed by Bim or Puma but not by activation of Bad or Bmf, which fail to neutralize Mcl-1. Alternatively, combining two 'weak killers' targeting the whole spectrum of Bcl-2 pro-survival molecules, such as Bad and Noxa, should potently induce apoptosis in such a cell and this hypothesis has been confirmed in a number of experimental settings.[13,14]

In an alternative model, called the 'direct activator' model, BH3-only proteins are further split up into two groups (Figure 1b). The 'direct activators' Bim, tBid (the activated truncated form of Bid) and maybe also Puma, are thought to directly bind to Bax/Bak as well as pro-survival family members, whereas all other BH3-only proteins, that is, Bad, Hrk, Bik, Bmf and Noxa, are only able to bind to pro-survival

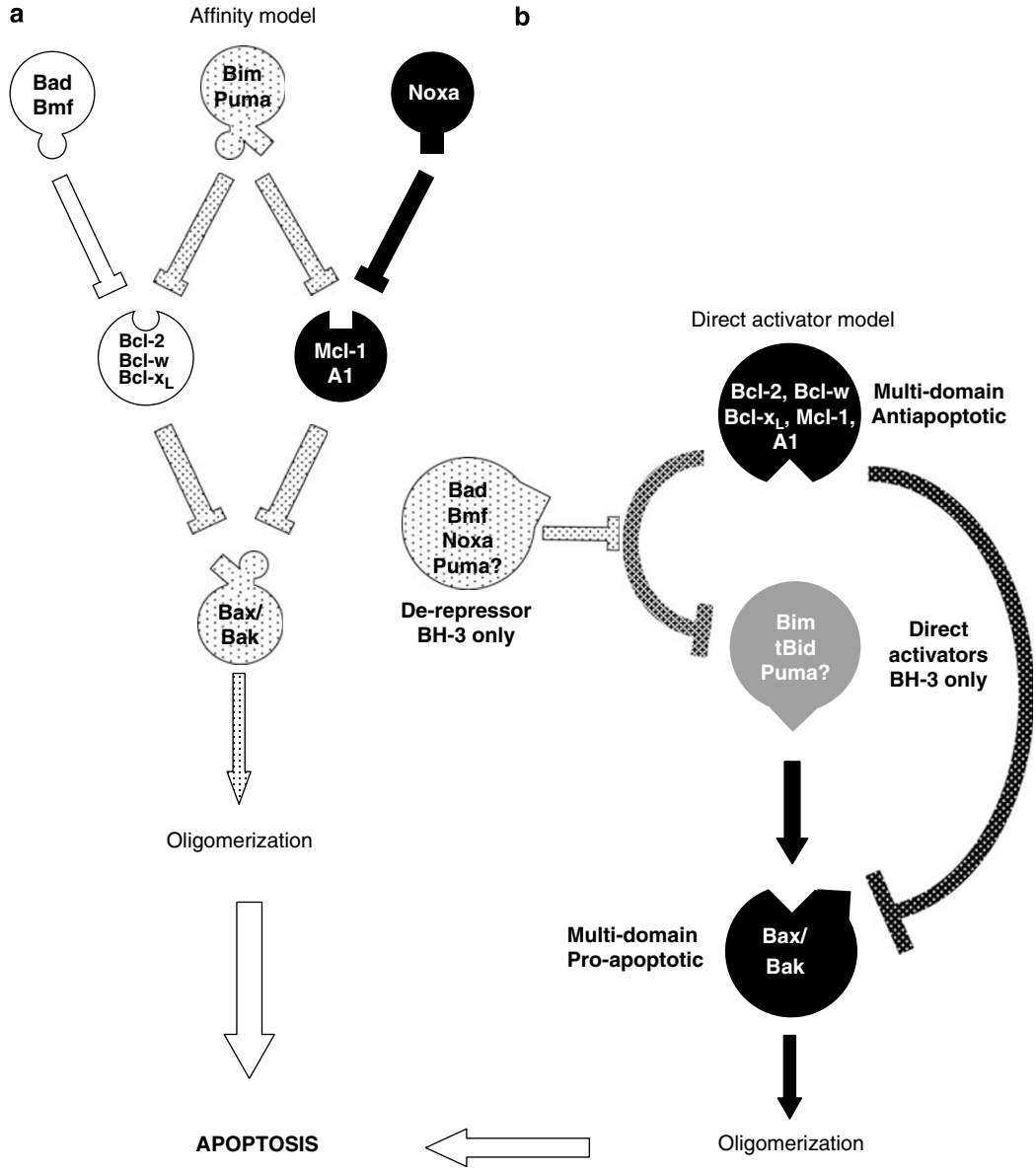

Figure 1 Possible modes of BH3-only protein function. The affinity model (**a**) suggests that BH3-only proteins possess different capacities to interact with Bcl-2 pro-survival homologs. Bim and Puma can engage all pro-survival Bcl-2 proteins with comparable affinities and are therefore potent killers. Other BH3-only family members target selectively only subsets of pro-survival Bcl-2 proteins, for example, Bad or Bmf can counteract the function of Bcl-2, Bcl-x_L or Bcl-w, whereas Noxa can only counteract Mcl-1 and A1. To trigger cell death, all Bcl-2 survival proteins expressed in a given cell must be neutralized to relieve Bak and/or Bax from sequestration by these proteins that promotes their oligomerization triggering apoptosis. In the 'direct activator' model (**b**), BH3-only proteins are split-up into two groups. The 'direct activators' Bim and tBid (and maybe Puma) are thought to directly bind to Bax/Bak as well as pro-survival family members, whereas all other BH3-only proteins, that is, Bad, Hrk, Bik, Bmf and Noxa, are only able to bind to pro-survival Bcl-2 family members and are considered to act as 'sensitizers.' In this model, it is assumed that stress stimuli activate mainly sensitizer BH3-only proteins that subsequently compete with Bim and tBid for binding to Bcl-2 proteins leading to their displacement from antiapoptotic proteins. Free Bim or tBid then in turn directly bind and activate Bax or Bak inducing their oligomerization thus leading to apoptosis

Bcl-2 family members and are considered to act as 'sensitizers.'[15,16] In this model, it is assumed that stress stimuli activate mainly sensitizer BH3-only proteins that subsequently compete with Bim and tBid for binding to Bcl-2 proteins leading to their displacement from antiapoptotic proteins. Free Bim or tBid then in turn directly binds and activates Bax or Bak inducing their oligomerization and subsequent permeabilization of the outer mitochondrial membrane, caspase activation and cell death.[15,16] This model predicts, however, that the sensitizer BH3-only proteins must be highly competitive, either due to higher binding affinities to Bcl-2 and homologs, or vast stoichiometric excess over Bim, Puma or tBid. Direct interaction between Bax and Bim or Bid, however, has so far never been confirmed in response to cell death initiation at the endogenous protein level, leading to the proposition of a '*hit and run*' mode of action.

Recent evidence provided by Willis *et al*.[14] identified a putative Achilles heel of the direct activation model by demonstrating that mice that lack Bim and Bid do not resemble the Bax/Bak double knockout phenotype and that additional knock down of Puma in cells lacking Bim and Bid does not provide protection from BH3-only protein-induced apoptosis. In addition, tBid and Bim_S proteins bearing mutations within their BH3 domains that prevent binding to Bax, but not Bcl-2, killed cells as efficiently as the relevant wild-type proteins.

Taken together, we have to assume that BH3-only proteins kill normal as well as malignant cells by neutralizing the cell-type-specific pool of pro-survival Bcl-2 family members, which usually prevent the assembly of Bax and/or Bak oligomers (Figure 1).

Targeting the Bcl-2 Family in Cancer Therapy

Antisense-based strategies. Pathologic overexpression of pro-survival Bcl-2 family proteins was frequently observed in many tumor types and identified Bcl-2 and/or Bcl-x_L as possible drug targets in the 1990s. However, since Bcl-2 family members are intracellular proteins that do not show any intrinsic catalytic function, targeting by antibodies or small molecule drugs were no feasible options, fostering attempts to interfere with mRNA expression levels by antisense oligonucleotide (ASO)-based strategies.[17] ASOs are single-stranded chemically modified oligodeoxynucleotides (18–21 mer) that target complementary mRNA for RNaseH-mediated degradation after sequence-specific hybridization and generation of a RNA–DNA heteroduplex, but may exert their effects in part via their immune-modulatory properties (e.g. by TLR activation).[17] Oblimersen (Genasense™) is a 18-bp phosphorothioate oligonucleotide targeting the first six codons of *bcl-2* mRNA and has been evaluated for suitability of the treatment of a number of tumors, including small cell lung cancer (SCLC),[18] prostate cancer,[19] renal cell carcinoma,[20] myeloid leukemia[21] as well as non-Hodgkin lymphomas.[22] This compound is already well advanced in clinical trials for the treatment of refractory CLL,[23] multiple myeloma[24] and melanoma.[25] Knowing the molecular basics of how Bcl-2-like molecules preserve survival and how BH3-only proteins kill cells immediately highlight the limitations of antisense-based strategies that can only target one, or at best, two highly homologous proteins at a time[26] (see above and Figure 1). However, in combination with conventional anticancer treatments Oblimersen may achieve clinical benefits. For example, a recent publication reported promising results regarding the addition of Oblimersen to fludarabine plus cyclophosphamide for the treatment of patients with relapsed or refractory CLL.[23]

Natural and chemical inhibitors of Bcl-2. A number of natural compounds were identified as potential Bcl-2 inhibitors over the past years.[27] Antimycin A, a streptomyces-derived inhibitor of ubiquinone–cytochrome *c* oxidoreductase at the mitochondrial respiration chain, was reported to promote cell death by binding to Bcl-2. A 2-methoxy derivative, no longer inhibiting mitochondrial respiration, was still able to kill Bcl-x_L overexpressing cells.[28] Chelerythrine, a plant alkaloid originally identified in a high-throughput screen for PKC inhibitors, was shown to disrupt Bcl-x/Bax complexes in *in vitro* experiments.[29] In addition, gossypol and apogossypol, derivatives of the cottonseed extract which was first described almost a 100 years ago,[30] were discussed to exert their function by induction of apoptosis due to inhibition of Bcl-2.[31] Another compound, the harmine-derivative compound-6, was suggested to promote cell death by inhibiting transcription of Bcl-2, but may actually do so by influencing the transcription of many genes.[32]

All these compounds, including the non-peptidic chemical compounds HA14-1[33] and BH3I-I[34] that were generated based on computer modeling onto the Bcl-2 structure, were shown subsequently to kill cells largely in a Bax/Bak-independent manner, clearly demonstrating that their cytotoxic effect was not solely due to specific inhibition of, or interaction with Bcl-2 or its homologs.[35] This, however, does not exclude the possibility that these compounds may prove useful for the treatment of certain cancers, since they potently kill tumor cells in various experimental settings. The interaction of these drugs with different Bcl-2 family members may even strongly contribute to their effects, but necrosis,[36] release of AIF,[37] the induction of autophagy[38] or enforced metabolic changes[39] may all contribute to their antineoplastic potential. Therefore, a more detailed analysis of their molecular mode of action is urgently needed.

More selective in this regard appears to be the design and synthesis of the so-called hydrocarbon-stapled BH3 helices, BH3 domain peptides modified to become cell permeable and protease resistant. These stapled peptides called 'stabilized alpha helix of Bcl-2 domains' (SAHBs) showed increased helicity in solution, when compared with unmodified BH3 peptides that appear to exist as random coils, and bind with high affinity to the multi-domain Bcl-2 member binding pocket. A Bid-based stapled BH3 peptide ($SAHB_A$) showed cytotoxicity against a panel of tumor cell lines in the nanomolar range and delayed growth of human leukemia in a xenotransplant model.[40] Very little information about their effects on healthy cells or their molecular mode of action is currently available in the public domain. Direct induction of Bax oligomerization has been proposed to account for the ability of $SAHB_A$ to trigger cell death.[41]

BH3 mimetics. Overexpression of pro-survival Bcl-2 family members is a common feature of many tumors and therefore, drugs mimicking the function of their pro-apoptotic counterparts, the BH3-only proteins, hold large promise as alternative anticancer strategies. The advantage of this therapeutic approach is the possibility to circumvent the need to induce expression of BH3-only proteins that is often compromised in tumor cells either due to gene ablation (e.g. Bim in mantle cell lymphoma), epigenetic silencing of BH3-only protein gene promoters as described for the *bim* gene in renal cell carcinoma and melanoma or, even more frequently, due to inactivation of the p53 pathway that usually triggers activation of Puma and Noxa.[42] Importantly, under all these circumstances, the core executioners of apoptosis, that is, caspases, are still present and could be used to drive apoptosis of tumor cells, once kick started. Therefore, lots of effort was put into defining compounds mimicking the action of BH3-only proteins, and non-peptidic 'BH3 mimetics' were rationally designed based on structural information gathered on protein–protein interactions within the Bcl-2 family.

The first non-peptidic 'BH3 mimetic,' based on a helical peptide-like terphenyl scaffold that showed low nanomolar affinity for the hydrophobic groove of Bcl-x_L, was reported in 2002.[43] Meanwhile additional compounds including the benzenesulfonyl derivative TW-37,[44] a putative BH3 mimetic designed on the structural basis of gossypol, Obatoclax (GX015-070) from GeminX Pharmaceuticals or A-385358 from Abbott, have been tested in preclinical studies. The Abbott compound showed low single-agent efficacy but enhances the cytotoxic activity of a number of DNA-damaging anticancer agents as well as paclitaxel.[45] Obatoclax showed promising *in vitro* efficacy against non-SCLC, mantle cell lymphoma as well as multiple myeloma cells but failed to exhibit efficacy in tumor xenotransplant models *in vivo.*[46–48] TW-37 reportedly induced apoptosis in PC3 prostate cancer cells, diffuse large B-cell lymphoma cells (DLBCLs), acute lymphatic leukemia (ALL) and melanoma cells *in vitro* as well as in DLBCL and melanoma xenotransplant models *in vivo.*[44,49,50] Both compounds appear to increase the efficacy of a range of conventional anticancer agents. Although high binding affinities of these compounds to certain pro-survival Bcl-2 family members have been reported, for example, TW-37 is able to bind Bcl-2, Bcl-x_L and Mcl-1 in the nanomolar or near nanomolar range,[44] little information on their molecular mode of action is currently available. The most studied and certainly promising BH3 mimetic to date is ABT-737 developed by Abbott, now joining forces with Genentech on this project.

ABT-737: mode of action. ABT-737 is a cell permeating, synthetic BH3 mimetic that was designed by Oltersdorf *et al.*[51] using a NMR structure-based approach to target the BH3-binding groove on Bcl-x_L. It binds with high affinity in the subnanomolar range to Bcl-2, Bcl-x_L and Bcl-w ($K_i < 1$ nM) even in the presence of human serum but only weakly to Mcl-1 and BFL-1/A1 ($K_i > 460$ nM), resembling the binding affinities of the BH3-only protein Bad. Although being unable to trigger cytochrome *c* release on its own, ABT-737 efficiently antagonized the Bcl-2- or Bcl-x_L-mediated inhibition of cytochrome *c* release from isolated mitochondria triggered by application of recombinant myristylated Bid. Subsequently, it was shown that this effect requires the presence of Bax or Bak, suggesting that ABT-737 can engage both multi-domain pro-apoptotic proteins to trigger cell death. Taken together, this indicates that ABT-737 acts as a 'sensitizer' BH3 mimetic that requires the presence of 'direct activator' BH3-only proteins to trigger cytochrome *c* release,[51] according to one model of BH3-only protein function (Figure 1). Consistent with this hypothesis, it was reported that mouse T lymphocytes lacking Bim are less susceptible to the cytotoxic action of ABT-737.[52]

Konopleva *et al.*[53] observed efficient cytochrome *c* release from ABT-737-treated mitochondria isolated from HL-60 acute myeloid leukemia (AML) cells. They report that in HL-60 cells ABT-737 effectively disrupts Bcl-2/Bax heterodimeric complexes and induces a conformational change in Bax, indicative of its activation. Furthermore, knock down of Bak, but not Bim, did abrogate ABT-737-induced apoptosis, suggesting that its direct activator function is not required for killing. They also provide evidence that in MEF, ABT-737 may require the presence of both, Bax and Bak, for efficient killing, since cells lacking either molecule were no longer responsive to the compound.[53] van Delft *et al.*,[35] however, reported in a parallel study that even wild-type MEFs are poor responders and demonstrated that either Bax or Bak can mediate killing by ABT-737, once Mcl-1 is inactivated, for example, by Noxa expression or when its levels are reduced by cytokine deprivation or inhibition of MAPK signaling. Consistent with the study by Konopleva, ABT-737 was found to induce a conformational change in Bax. Taken together, these data suggest that the initially reported observation that ABT-737 can engage Bax and/or Bak to kill cells are correct.

It has been reported that in certain tumor cells that overexpress Bcl-2, Bim is already found associated with Bcl-2 at mitochondria.[54] This may be due to constant oncogenic stress causing BH3-only protein activation that selects for Bcl-2 overexpression to counterbalance BH3-only protein activation (a process often also termed 'Bcl-2-addiction'). It has been discussed that such tumor cells are in a 'primed' state for apoptosis, and it was speculated that 'sensitizer' BH3-only proteins such as Bad or Bmf, or a compound such as ABT-737, should be sufficient to trigger cell death directly by releasing Bim from Bcl-2.[54] In fact, evidence has been presented that in primary B-CLL cells, follicular lymphoma samples and some multiple myeloma cell lines, ABT-737 kills by releasing Bim from sequestration by Bcl-2 triggering subsequent oligomerization of Bax and Bak. In addition, partial knock down of Bim reduced the sensitivity of L363 myeloma cells to ABT-737.[54] All these observations are consistent with a requirement for a direct activator protein to kill tumor cells by ABT-737. However, caution is needed in the interpretation of such results since knock down of Bim in tumor cells or genetic loss of Bim in lymphocytes may simply cause a relative increase in the pool of freely available Bcl-2 pro-survival homologs that can sequester more activated Bax and/or Bak. Hence, ABT-737 will be less efficient in such cells. If correct, this 'resistance' phenotype can simply be overcome by increasing the dose of ABT-737.

Consistent with the idea that BH3-only proteins or *bona fide* mimetics kill cells primarily by neutralizing the total pool of all

Bcl-2 pro-survival molecules expressed in a given cell type, ABT-737 can potently trigger cytochrome *c* release in mouse liver mitochondria or MEF devoid of the direct activator BH3-only proteins Bim and Bid.[14] This indicates that once all Bcl-2 homologs residing at the mitochondria in a particular cell type are neutralized by a stimulus-dependent combination of BH3-only proteins cell death can proceed.

The question whether ABT-737 exclusively kills cells by inducing apoptosis along the mitochondrial pathway to apoptosis (Figure 2), however, cannot be clearly answered yet. Recent observations suggested that ABT-737 also potently disrupts the interaction of Bcl-2 or Bcl-x_L with Beclin-1 under certain experimental conditions, thereby promoting autophagy.[55] Therefore, it is formally possible that autophagy may contribute to the antineoplastic effects of ABT-737, if drug treatment culminates in autophagic tumor cell death. Since the predominant physiological role of autophagy is the promotion of cell survival in response to nutrient deprivation or cellular damage, this phenomenon may also contribute to drug resistance phenotypes, warranting further investigations into this issue (Figure 2).

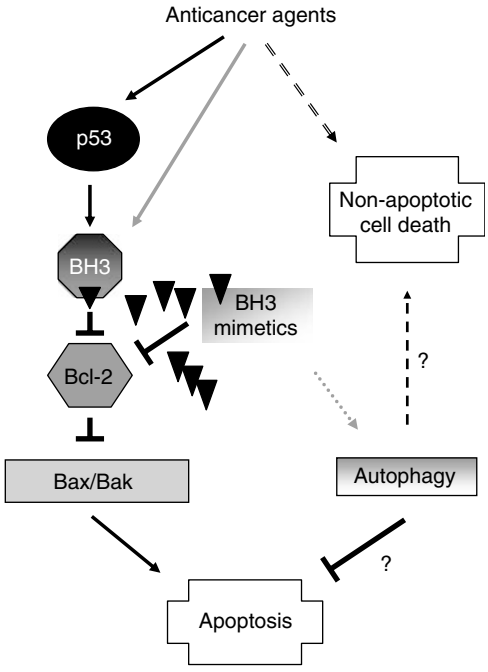

Figure 2 BH3 mimetics trigger tumor cell death by neutralizing Bcl-2 homologs. Many anticancer agents promote apoptosis predominantly by the activation of certain BH3-only proteins. This can be achieved by enforced BH3-only protein gene transcription, for example via activation of p53 such as in the case of Puma or Noxa, or by promoting post-translational modification and/or protein stabilization, as shown for Bim or Bmf. BH3 mimetics circumvent the need for these processes that are often impaired or blocked in tumor cells (e.g. due to inactivation of the p53 pathway, hyperactivation of oncogenes, overexpression of pro-survival Bcl-2 family members) and trigger apoptosis by neutralizing pro-survival Bcl-2 homologs directly. BH3 mimetics may also trigger autophagy in certain cell systems. This phenomenon may contribute or compromise the antineoplastic effects of Bcl-2 inhibitors, depending on whether this leads to autophagic cell death or allows cell survival. Some anticancer agents can also cause non-apoptotic cell death of tumor cells, prompting necrosis-like morphology or trigger expression of death receptors, facilitating apoptosis (not shown or discussed here)

Pre-clinical Data on ABT-737

A significant number of studies have evaluated the merit of ABT-737 in triggering apoptosis in cancer cells and cell lines *ex vivo* (Table 1). ABT-737 shows cytotoxic effects as a single agent on primary patient-derived follicular lymphoma cells and B-CLL cells. Only weak activity against various solid tumor cell lines was reported but SCLC cells proved to be relatively sensitive in single-agent experiments. ABT-737 potently enhanced the cytotoxicity of paclitaxel against a non-SCLC (NSCLC) cell line by a factor of 4.[51] Primary AML cell lines and patient blasts were reported to be highly sensitive to the mimetic, whereas normal peripheral blood mononuclear cells (PBMCs) proved largely resistant.[53] As effective cancer therapy has to target cancer stem cells for long-term success, it is of special interest that AML stem cells seem to be highly sensitive toward ABT-737 treatment. Two out of six patent-derived AML samples showed complete deletion of cancer stem cells after ABT-737 treatment, one of them being highly resistant to Ara-C treatment. Although the OCI-AML3 cell line is relatively resistant to ABT-737 on its own, combined treatment with Ara-C or doxorubicine revealed synergistic effects on apoptosis induction.[53]

ABT-737 potently synergizes with the Abelson tyrosine kinase inhibitors Imatinib (Gleevec) or INNO-406 in killing human CML cells.[62,63] Imatinib thereby exerts its proapoptotic effects mainly by inducing Bim and Bad.[62] Interestingly, ABT can overcome the drug resistance of *bcr-abl*-transformed mouse leukemia cells lacking these BH3-only proteins as well as in those overexpressing Bcl-2.[62] This is of clinical relevance since Bcl-2 overexpressing Ph^+ CML cells often show reduced sensitivity to Gleevec.[70] The cytotoxic effects caused by inhibition of transforming tyrosine kinase activities by a number of agents including Gefitinib[57] or Erlotinib,[71] both targeting the EGFR kinase activity in epithelial tumors, such as NSCLC or Sunitinib, inhibiting the action of FLT-3 kinase carrying activating internal tandem duplication frequently found in AML patients, are strongly enhanced by the Abbott compound.[60] All these kinase inhibitors trigger apoptosis by inducing transcription of the *bim* gene and presumably also by dephosphorylating Bim and Bad, that are already present in the tumor cells.[57,62,72]

In multiple myeloma, ABT-737 has been extensively tested and was shown to potently act as a single-agent drug on a number of cell lines, including glucocorticoid-resistant ones.[66–68] Additive effects in combination with standard therapeutics such as melphalan, glucocorticoids as well as the recently approved proteasome inhibitor bortezomib (PS341) were demonstrated in cell lines.[68] However, others failed to observe synergy between ABT-737 and bortezomib.[67] In primary patient-derived myeloma cells, however, the BH3 mimetic showed varying efficacy inducing apoptosis in the range from 20 to 90% of cells in one study,[67] but failed to induce apoptosis in a significant portion of primary tumor cells in another report.[66] An important information provided is that not only human PBMCs but also bone marrow-derived progenitors from myeloma patients are not affected in their colony-forming potential by the Bcl-2 inhibitor.[67] Discrepancy exists between the studies whether survival signals provided

Table 1 Preclinical data on ABT-737 efficacy in different tumor types

	ABT-737 efficacy				
	Cell lines		Primary tumor cells		
Tumor type	**Single agent**	**Combinatorial treatment with**	**Single agent**	**Combinatorial treatment with**	**References**
SCLC	+	ND	ND	Carboplatin Etoposide	51,56
NSCLC	–	Gefitinib[a] Erlotinib[a] Paclitaxel[b]	ND	ND	57,58
Breast cancer	–	ND	ND	ND	35
Cervical carcinoma	–	ND	ND	ND	35
Colon cancer	–	Mcl-1 knockdown	ND	ND	59
Bladder cancer	–	Mcl-1 knockdown	ND	ND	59
Glioma	–	Mcl-1 knockdown	ND	ND	59
AML	+	Roscovitine[c] PD98089[d]	+	Ara-C Sunitimib[a]	53,60,61
CML	–	Imatinib[a] INNO-406[a]	ND	ND	60,62,63
CLL	ND	ND	+	ND	51
DLBL/FL	+	ND	ND	ND	64,65
Myeloma	+	ND	+	Melphalan Bortezomib Dexamethasone	54,66–68
ALL	+	L-ASP[e] Vincristine[b] Dexamethasone Roscovitine[c]	ND	ND	61,69

ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; APL, acute promyelocytic leukemia; Ara-C, cytosine arabinoside; CLL, chronic lymphocytic leukemia; CML, chronic myelogenous leukemia; DLBL, diffuse large B-cell lymphoma; FL, follicular lymphoma; ND, not determined; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer [a]Tyrosine kinase inhibitors [b]Microtubule stabilizing agents [c]Inhibitor of cyclin-dependent kinases, e.g. Cdk-2 [d]MAP kinase inhibitor [e]L-Asparaginase amidohydrase

Table 2 Assessing the *in vivo* efficacy of ABT-737

		ABT-737 efficacy		
Tumor type	**Mouse model**	**Single agent**	**Combinatorial**	**References**
SCLC cell lines H146, H1963 and others	Xenograft	Complete regression in 77% of H1963 and 20% in H146	Not tested	51,56
DoHH2 human B-cell lymphoma	Xenograft	Morbidity delayed	Not tested	51
MY5 multiple myeloma cell line	Xenograft	Complete regression	Not tested	66
FDCP1-GFP-ΔRaf-1[a] promyeloid cells	Xenograft	Morbidity delayed	Not tested	53
KG-1 acute myelogenous leukemia line	Xenograft	Morbidity delayed	Not tested	53
Pre B and IgM$^+$ lymphomas	Eμ-myc transgenic and Eμ-myc/bcl-2 double tg mice	Morbidity delayed	Not tested	35
Acute lymphatic leukemia lines	Xenograft	Delayed progression	Combined with VXL[b]	69

[a]A constitutively active version of Raf-1 [b]In combination with vincristine, dexamethasone and L-asparaginase amidohydrase

by the interaction of tumor cells with bone marrow stroma cells, IGF-1 or IL-6 interfere with the effects of ABT-737.[66,68]

In childhood ALL, the BH3 mimetic efficiently synergized with L-asparaginase amidohydrolase (L-ASP) that depletes asparagines and glutamine in leukemic cells and vincristine in a panel of seven ALL cell lines tested. Interestingly, synergy with dexamethasone was only observed in 2/7 cell lines.[69] Presumably, this reflects the status of a functional glucocorticoid receptor in this panel of cell lines or their ability to upregulate the GC receptor that has been shown to be rate limiting in GC-induced leukemia cell apoptosis.[73] Importantly, ABT-737 was able to induce significant cell death in GC refractory cell lines and clinical studies including this BH3 mimetic in the initial GC monotherapy are warranted. Normal PBMCs were not affected in their viability using the BH3 mimetic in combination with either L-ASP or vincristine.[69]

Taken together, these studies indicate that ABT-737 shows strong potency as a single agent against a variety of different lymphatic tumor types and that it may help to overcome drug resistance phenotypes when used in combination therapy. The latter observation also extends to epithelial tumors that, with few exceptions such as SCLC, proved relatively resistant to ABT-737 monotherapy.

ABT-737 activity *in vivo*. An important question was whether ABT-737 shows tumor cell toxicity *in vivo* without grossly affecting normal physiology (Table 2). Oltersdorf *et al*.[51] tested ABT-737 in a mouse xenograft model using

H146 and H1963 SCLC cell lines. Single-agent therapy was started 30 days post-inoculation and ABT-737 caused complete regression of the established tumor xenografts within 20 days. Furthermore, tumors did only grow back in a low percentage of animals over the duration of the study. Tumor regression was due to apoptotic cell death, as indicated by caspase activity found in histological tumor sections as early as 2 h after the first treatment. Importantly, toxicity to normal mouse tissue was limited to short-term lymphopenia and thrombocytopenia that can be easily managed in the clinic.[74,75] Morbidity caused by dissemination of DoHH2 human leukemia cells in SCID mice was also significantly delayed by ABT-737 treatment, but not by its enantiomer.[51] In a xenotransplant model using the MY5 multiple myeloma line, application of the BH3 mimetic induced complete regression of established tumors as a daily-applied single agent.[66] *In vivo* efficacy of ABT-737 was also tested in SCID mice that received FDC-P1 cells, transformed by a constitutively active from of the Raf-1 proto-oncogene, or human KG-1 leukemia cells. Treatment of SCID mice carrying transplanted tumors resulted in a 50% decrease of tumor burden and in about twofold extended survival time.[53] Similarly, ABT-737 delayed the progression of *c-myc*-driven mouse B-cell lymphomas and even those overexpressing Bcl-2.[35] However, disease progression was only delayed but not prevented in these two models. Another convincing example of the putative *in vivo* efficacy of ABT-737 comes from a study investigating its synergy with standard VXL (vincristine, dexamethasone and L-ASP) treatment in killing off pediatric ALL samples derived from treatment-refractory relapsed patients that were transplanted into NOD/SCID mice. The event-free survival was increased more than sixfold when compared to untreated control animals and this effect was further increased about twofold by ABT-737 when compared with mice treated with VXL alone.[69]

Taken together, ABT-737 is able to act potently as an anticancer agent against lymphoid as well as certain epithelial tumors *in vivo*, but will most likely not be sufficient to cure established neoplasias when used as a single agent. However, its potential to increase the efficacy of standard treatment protocols appears to be enormous and should help to reduce therapy-associated side effects significantly.

Resistance mechanisms: is it all just about Mcl-1?. As mentioned before, in certain tumor cell types the efficacy of ABT-737 appears to be limited (Table 1). A number of studies have investigated possible resistance mechanisms and all of them come to the same conclusion. Tumor cells that do not respond to the BH3 mimetic appear to display high levels of the Bcl-2 pro-survival homolog Mcl-1.[35,53,56,58,64] In particular, solid tumors such as NSCLCs are responding very poorly to the ABT-737 compound *in vitro*. The basis of this phenomenon is not well investigated but tissue microarray analysis of tumor samples from chemotherapy-naïve NSCLC patients revealed a correlation with high levels of Mcl-1.[58] Since enforced expression of Noxa, which selectively targets Mcl-1, restored sensitivity of NSCLC cells to ABT-737 *in vitro*, one may speculate that ABT-737 may kill solid tumors most effectively when combined with radiation therapy, which can trigger induction of Noxa, at least in the context of wild-type p53.[76] Consistent with these observations, Lin *et al*.[59] identified Mcl-1 in an RNAi-based screen as the primary resistance factor in ABT-737-insensitive H196 SCLC cells. Knock down of Mcl-1 restored sensitivity of these cells but also proved effective in conferring sensitivity onto otherwise resistant colon or bladder cancer cell lines.

Mcl-1 expression also appears to protect non-malignant cells from ABT-737-mediated cytotoxicity. This is consistent with biochemical data demonstrating that ABT-737 does not bind to Mcl-1 (and A1).[13] HeLa and MCF-7 cells express high levels of Mcl-1 and are highly resistant to ABT-737 treatment. Reduction of Mcl-1 protein levels by various means such as shRNA, factor deprivation, the CDK2 inhibitor roscovitine or even DNA-damaging drugs increased apoptosis after ABT-737 treatment.[35] These results were interpreted in a manner that all Bcl-2 pro-survival proteins present in a given cell type, here Bcl-2 and Mcl-1, the latter not targeted by ABT-737, need to be restrained to free Bax and Bak to induce apoptosis. The indirect activation model (see Figure 1) suggests that Bak is activated only when it is displaced from both Mcl-1 and Bcl-x_L by BH3-only proteins. As ABT-737 has only low binding affinity to Mcl-1, it cannot displace Mcl-1 from Bak. In fact, this is not important in cells expressing only low levels of Mcl-1 or when a potent Mcl-1 antagonist like Noxa is present, which facilitates ABT-737-induced apoptosis in MEFs.[35] In line with these experiments, it was argued that expression levels of *mcl-1* and also *a1* mRNA are low in most follicular lymphomas, CLL and SCLC tumor cells where ABT-737 shows single-agent efficacy.[35,51] Interestingly, those SCLC lines that are less sensitive to the compound also display increased levels of Mcl-1 expression.[45] It has also been argued that high amounts of Mcl-1 could suffice to sequester enough 'direct activators' to protect the cells from the apoptotic faith and that this phenomenon could also be accommodated in the 'direct activator' model[77] (Figure 1). However, no experimental evidence supporting this hypothesis has been presented to date.

Konopleva *et al*. describe an alternative mechanism on how ABT-737 efficiency may be compromised. In OCI-AML3 acute leukemia cells, application of the MEK-1 inhibitor PD98059 showed strong synergistic effects with the Bcl-2 inhibitor and this correlated with decreased Mcl-1 protein levels, as previously reported, and also with decreased phosphorylation of Bcl-2 in its unstructured loop. Employing site-directed mutagenesis they show that cells expressing Bcl-2 carrying phospho-site-inactivating alanine mutations are more sensitive to ABT-737 (and less Bax was found associated with Bcl-2 in co-IP experiments), whereas replacement of the relevant serine/threonine residues by charged amino-acid residues rendered these cells more resistant to apoptosis.[53]

Since Bfl-1/A1 is also not bound efficiently by ABT-737 it will be interesting to investigate whether a correlation between the mimetic and A1 expression exists in B-CLL, the only model where high A1 levels was reported to contribute to drug resistance so far.[78] However, since Bfl-1/A1 expression appears to be highly restricted to few lymphocyte subsets its contribution to drug resistance phenotypes may be of limited clinical impact.

In conclusion, these findings implicate that expression of Mcl-1 is rate limiting for the efficacy of ABT-737 in tumor tissues, and BH3 mimetics or other compounds that target specifically Mcl-1 may help overcome this limitation. However, since healthy cells also appear to depend on Mcl-1 for survival, combinatorial targeting of a broader range of pro-survival molecules also poses a significant threat to non-malignant tissue. Therefore, the development of novel BH3 mimetics with distinct inhibition spectra targeting different combinations of Bcl-2 pro-survival family members may be a goal for the near future. Alternatively, selective inhibitors of individual Bcl-2 pro-survival family members may even be more useful, since they could be freely combined to treat a broad range of tumors that differ strongly in their dependence on distinct Bcl-2 homologs for survival.

ABT-263: the next generation. To provide more dosing flexibility, a Bcl-2 family inhibitor that is orally bioavailable was discovered at Abbott. This compound, ABT-263, displays subnanomolar binding to Bcl-2, Bcl-x_L and Bcl-w like ABT-737. Also, similar to ABT-737, the new compound shows single-agent efficacy on lymphomas, CLL and SCLC cell lines and potently synergizes with γ-irradiation and other cancer agents. As a single agent, ABT-263 regresses tumors (e.g. SCLC) *in vivo* and yields cures in some of the animal models.[79] When combined with anticancer agents such as the proteasome inhibitor Velcade, the anti-CD20 antibody rituximab, or standard chemotherapy, ABT-263 is broadly active against a wide variety of tumors. Recently, ABT-263 has entered three phase I/IIA clinical trials for the treatment of patients suffering from lymphoma, CLL and SCLC. Although it is still early, responders have been identified in CLL patients.[80] The side effects are those predicted by the preclinical studies and are likely mechanism based. For example, thrombocytopenia has been observed which is likely related to the inhibition of Bcl-x_L.[69]

Prognostic Potential of BH3 Mimetics

On the basis of the findings discussed above, expression levels of antiapoptotic Bcl-2 family members in cancer cells provide useful prognostic markers for the efficacy of ABT-737 and also provide a powerful tool to predict sensitivity to various chemotherapeutic agents.

Letai and co-workers[64,65] introduced a strategy called BH3 profiling that aims to predict dependence of cancer cells on individual antiapoptotic proteins for survival and, therefore, indirectly also sensitivity to ABT-737. The principle of this assay is to isolate mitochondria from tumor cells and incubate them with a panel of BH3 peptides to determine subsequent cytochrome *c* release. This method has the potential to predict sensitivity of tumor cells to the Abbott compound as a single agent since ABT-737 only triggers efficient cytochrome *c* release in cells that depend on Bcl-2 for survival but not in those that depend on Mcl-1. This assay system may have some merit in predicting the intrinsic responsiveness of tumor cells to different BH3 mimetics in the near future, and may help to decide which mimetic should be used in putative future combination treatment regimens. Whether it will allow a prognosis on the *in vivo* responsiveness of certain tumors to conventional anticancer agents, as proposed by the authors, remains questionable. The authors suggested that tumor cells 'primed for death,' for example, those that show Bcl-2 in complex with Bim and therefore are sensitive to 'sensitizer' BH3 peptides (e.g. a Bad BH3 peptide) in the BH3 profiling assay, would be more responsive to anticancer agents as 'unprimed' tumor cells.[65] In this study, 'unprimed' tumor cells did not show detectable levels of Bim associated with Bcl-2 and were only susceptible to 'direct activator' Bim- or Bid-based BH3 peptides. The severe *caveat* of this study is, however, that only one 'unprimed' model cell line was investigated and it responded very similar to adriamycin, etoposide or vincristine, as at least one out of the five 'primed' cell lines tested.[65] So the prediction that tumor cells need to be in a 'primed state' to undergo drug-induced apoptosis appears to be premature. More experimental evidence and investigations of additional tumor model systems, besides follicular lymphoma and DLBL, are needed to prove or disprove the usefulness and general validity of the BH3-profiling concept.

Conclusions

On the basis of the detailed knowledge on how apoptosis is regulated at the molecular level, much progress has been made over the last couple of years to target specific components of the apoptosis machinery for anticancer therapy. Three major concepts emerge and are currently translated into clinical application. Activation of DRs such as TRAIL-R by recombinant ligand or agonistic antibodies and suppression of IAP mediated caspase inactivation as well as direct activation of the proapototic Bcl-2 family members Bax and Bak by small molecule drugs. It is time now to evaluate their suitability for the treatment of distinct malignant disease in humans as well as their long-term clinical applicability and merits.

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