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Bcl2 family proteins in carcinogenesis and the treatment of cancer

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Abstract

Deregulation of Bcl2 family members is a frequent feature of human malignant diseases and causal for therapy resistance. A number of studies have recently shed light onto the role of pro- and anti-apoptotic Bcl2 family members in tumour-pathogenesis and in mediating the effects of classical as well as novel front-line anticancer agents, allowing the development of more efficient and more precisely targeted treatment regimens. Most excitingly, recent progress in our understanding of how Bcl2-like proteins maintain or perturb mitochondrial integrity has finally enabled the development of rational-design based anticancer therapies that directly target Bcl2 regulated events at the level of mitochondria. This review aims to give an overview on the most recent findings on the role of the Bcl2 family in tumour development in model systems of cancer, to relate these findings with observations made in human pathologies and drug-action.

Keywords

Apoptosis; Bcl2 family; Cancer

Bcl2 regulated cell death signalling

Bcl2 family proteins regulate apoptosis along the intrinsic mitochondrial apoptosis pathway (Fig. 1) that is activated in response to a number of stress stimuli including growth-factor deprivation, cytokine-withdrawal, Ca^{++} -flux or DNA-damage, caused by UV or gamma-irradiation, but can also be licensed to contribute to cell death triggered by members of the tumour necrosis factor family member such as Fas, TNF or TRAIL [1].

Based on their function, the members of the Bcl2 family can be divided into pro-apoptotic and pro-survival proteins. Pro-survival proteins contain up to four Bcl2 homology (BH) domains. The BH1, 2 and 3 domains are critically involved in the creation of a hydrophobic groove where the α -helix formed by the BH3-domain of a pro-apoptotic partner is binding into [1]. The molecular function of the BH4-domain, although required for the anti-apoptotic function of pro-survival Bcl2-family proteins still remains to be fully elucidated [2]. Pro-apoptotic Bcl2 family members can be subdivided into multi-domain class proteins, harbouring three out of four BH-domains (BH1, 2, 3) i.e. Bak, Bax and Bok and those that only contain the BH3-domain and are referred to as BH3-only proteins, including Bim/Bod, Bid, Bmf, Puma/Bbc3, Noxa/APR, Bad, Bik/Nbk/Blk and Hrk/DP5 [1].

Bcl2 and its pro-survival relatives i.e. Bclx1, Mcl1, Bclw and Bfl1/A1, are known to protect cells from apoptosis until neutralised by BH3-only proteins. Two models are currently discussed how BH3-only proteins promote loss of mitochondrial integrity [3]. The first model is based on the different binding affinities of the BH3-only proteins for the individual Bcl2-like proteins. While Puma and Bim can bind to all the pro-survival molecules with comparable affinities others, such as Bad and Bmf, can only bind to Bcl2, Bclx1 and Bclw, while Noxa binds only to Bfl1/A1 and Mcl1 with significant affinity [4]. According to this model a cell undergoes apoptosis when all pro-survival Bcl2 molecules expressed in that cell are neutralised by BH3-only proteins that have been activated or induced upon a given apoptosis signal. This, subsequently, leads to displacement of Bax or Bak, bound and kept inactive by Bcl2 in the healthy cell, that are now free to oligomerize and perturb the integrity of the mitochondria (Fig. 1). Hence, a cell that depends on Bcl2 and Mcl1 for survival can be killed by Bim alone or, alternatively, a combination of Bmf and Noxa [3].

The competing model instead suggests that the BH3-only proteins can be subdivided into “direct activators” and “de-repressors” or “sensitizers”. The de-repressors can bind only to the pro-survival proteins, while the direct activators, Bim, tBid and possibly Puma, can bind not only to the pro-survival proteins but also to Bax and/or Bak. In this model it is suggested that upon an apoptotic stimulus “de-repressor” BH3-only proteins out-compete “direct activators” for binding to pro-survival molecules, leading to the interaction between “direct activators” and Bak or Bax, triggering their full-blown activation [5–7]. Regardless which model of BH3-only function is correct, both lead to the activation of Bax and/or Bak, enabling mitochondrial outer membrane permeabilization (MOMP) and the release of pro-apoptotic factors such as cytochrome *c* into the cytoplasm, triggering the caspase-cascade, leading to cell destruction.

More recently, Bcl2-like proteins have been discussed to protect cells also from non-apoptotic death by preventing exhaustive autophagy. This may be achieved by binding to Beclin-1/ATG6, which contains a BH3-like-domain, leading to inhibition of this master-regulator of autophagy [8]. Although autophagic cell death has been reported to prevent genomic instability and haplo-insufficiency in ATG-genes promotes tumour formation in mice, ample evidence has been provided that autophagy usually facilitates tumour cell survival under unfavourable conditions such as nutrient deprivation [8]. These contradicting observations cause much debate in the cell death field and additional investigations are required to clarify if interactions between Bcl2 or Bclx1 and ATG6 are really another way for pro-survival Bcl2 family members to exert their cell protective and oncogenic function.

Pro-survival Bcl2 family members as oncogenes

A role for Bcl2 in cancer was first identified more than 20 years ago in follicular lymphoma, where it is involved in the t(14;18) chromosome translocation, placing Bcl2 under the control of the immunoglobulin heavy-chain promoter resulting in its deregulated high level of expression [9, 10]. Ever since, a number of mouse models overexpressing or lacking members of the Bcl2 family have been exploited to investigate their contribution to tumour formation, progression and therapy resistance.

Surprisingly, B cell specific transgene expression of Bcl2, controlled by the *E μ* -enhancer in mice, resulted only in a low incidence of lymphomas after long latency, indicating that Bcl2 by itself is only weakly tumourigenic [11]. However, another Bcl2 transgenic strain, where Bcl2 is expressed at higher levels, under the control of the *vav* gene promoter, does display frequent germinal centre cell hyperplasia that eventually develops into follicular lymphoma. The true tumour incidence in these mice, however, is masked by their premature death caused by kidney failure, due to immunoglobulin-triggered nephritis [12]. Strong effects of

high level Bcl2 expression on lymphomagenesis are seen in combination with classical oncogenes. Synergistic effects on lymphoma development have been shown for Bcl2 is together with c-Myc, Pim-1 or v-Abl [13–15]. Transgene expression of Bcl2 in other tissues including myeloid cells and mammary epithelial cells has also documented its role in tumourigenesis. Similarly, overexpression of related Bclxl enhanced B cell lymphomagenesis [16] and tumour formation in pancreatic β -cells of SV40 Large T antigen transgenic mice [17]. Bclxl overexpression also cooperates with c-Myc in pancreatic β -cell tumour formation, preventing c-Myc driven β -cell death and diabetes [18], as well as in skin cancer induced by chemical challenge, when put under the control of the keratinocyte-specific K14 promoter [19]. Transgene expression of Mcl1 in the hematopoietic system under control of the autologous promoter leads to development of lymphomas of various kinds, most frequently follicular lymphoma and diffuse large B cell lymphoma, albeit relatively late in life [20]. A1/Bfl-1 was found to facilitate cellular transformation by *bcr-abl* [21] as well as adenoviral E1A [22]. However, simple expression of A1 as a transgene in the B cell compartment under control of the E μ -enhancer did not result in an increased incidence of lymphomas in mice followed for 18 months [23]. Altogether, these observations are consistent with the fact that the simple overexpression of a pro-survival Bcl2 family member in a selected cell type is usually insufficient to spark malignant disease but leads to an increase in the number of cells “at-risk” for additional transforming events.

Overexpression of Bcl2 as well as other pro-survival Bcl2 family members is common in many types of human cancer and has frequently been correlated with decreased susceptibility to chemotherapeutics and to increased radioresistance. Somewhat contradictory at first sight, in some malignancies such as breast cancer [24–26] and gastric carcinoma [27] high levels of Bcl2 have instead repeatedly been associated with a less malignant phenotype and more favourable prognosis. This conundrum might be related to the observation that enhanced survival of the Bcl2 expressing tumour cells relieves the selection pressure to acquire functions associated with tumour progression, such as genetic instability. Tumour cells acquiring additional mutations would simply not be able to out-compete neighbouring, apoptosis-resistant cells. In contrast, p53 deficiency protects from apoptosis, but also prevents cell cycle arrest and senescence while promoting genetic instability. Therefore, loss of p53 always associates with more aggressive malignancies [28, 29] and still is the major risk factor in human cancers. Consistent with this hypothesis, transgene expression of Bcl2 in the liver of mice was shown to delay TGF-induced as well as c-Myc induced liver carcinogenesis [30, 31]. However, since overexpression of Bclxl accelerated c-Myc induced liver carcinogenesis [31], we have to assume that functions unrelated to cell death inhibition e.g. effects on cell cycle progression exerted by Bcl2 but not Bclxl overexpression, as well as the cellular context, define the oncogenic potential of individual pro-survival molecules of the Bcl2 family.

Bax and Bak in tumour suppression

As apoptosis-promoting proteins, Bax and Bak should potentially work as tumour suppressors. However, their potential should be limited due to their redundancy in apoptosis induction [32], but in some situations Bax and Bak seem to work in a non-redundant manner. There is some evidence that different pro-survival members of the Bcl2 family target Bax and Bak in distinct ways [3, 33]. Loss of Bax, but not of Bak, protects cultured fibroblasts as well as pancreatic β -cells in vivo from apoptosis induced by c-Myc overexpression [34, 35] and certain BH3-only proteins such as Nbk [36] or Puma [37] seem to have a preference for one or the other multi-domain protein for apoptosis induction or may engage Bax directly to kill cells [7].

Until now, there are no reports on the role of Bak in experimental tumour models, while the tumour suppressor potential of Bax has been explored in more detail. Bax deficient mice do not display increased spontaneous tumour incidence, and Bax deficiency has also no effect on tumourigenesis in p53 deficient mice [38]. However, Bax deficiency reduced apoptosis and accelerated tumourigenesis SV40 large T antigen transgenic mice susceptible to brain [39] or mammary tumours [40]. Similarly loss of Bax reduced apoptosis and accelerated c-Myc-driven B cell lymphoma development [41]. These models have in common that haplo-insufficiency of Bax was sufficient to promote tumourigenesis but loss of heterozygosity (LOH) was never observed. Loss of Bax, but not of Bak, was shown to promote c-Myc driven tumourigenesis in pancreatic β -cells. However, Bax loss failed to accelerate tumour development in *ARF*^{-/-} mice but shifted the tumour spectrum away from lymphomas to the development of sarcomas [42]. Furthermore, *Bax/ARF*-double null mice developed uncommon tumours and multiple primary malignancies, which were not normally observed in *ARF*-null mice [42]. Together these observations indicate a tissue specific tumour suppressor potential of Bax. One may also argue that in the absence of a functional oncogenic stress response loss of an apoptosis-regulator acting downstream does no longer influence tumour formation but reveals the tissues and cell types where its pro-apoptotic function is most crucial for normal homeostasis. However, overexpression of Bax in the T cell lineage under the control of the *lck* promoter still enhanced the lymphoma development in p53 deficient mice [38], demonstrating the loss of apoptosis responses independent of the ones mediated by p53 and executed via Bax can contribute to tumour suppression. Mutations in the *bax* gene have been found in human tumours. Frameshift mutations in the *bax* gene were detected in ~50% of colon adenocarcinomas of microsatellite mutator phenotype (MMP) and also in gastric carcinomas of MMP [43, 44]. The presence of *bax* mutation correlated with shorter survival in patients with these cancers [45]. In conclusion, Bax loss promotes tumourigenesis in several experimental settings, and loss of Bax can influence the tumour pathology in human cancer.

BH3-only proteins as barriers against malignant disease

Consistent with their pro-apoptotic role, tumour suppressor potential has been reported for several BH3-only proteins in mouse models of cancer, and, importantly, loss or inactivation of these proteins appears to be a frequent alteration in human cancer [46].

Bim is a crucial regulator of lymphoid tissue homeostasis involved in negative selection of B and T cells as well as cytokine-deprivation, Ca^{++} -flux and corticosteroid-induced cell death of lymphocytes [47]. Aged Bim-deficient mice display a mildly increased incidence of B cell lymphomas [48] and Bim has been shown to suppress c-Myc-driven lymphomagenesis [49]. Loss of a single allele of Bim in *E μ -myc* transgenic mice leads to rapid development of lymphoma compared to *E μ -myc* mice wild type for Bim. In addition, loss of Bim relieved the selection pressure to lose p53 function, which is frequently seen in this tumour model [49]. Consistently, c-Myc was found to induce the expression of Bim in premalignant preB and IgM⁺ B cells, suggesting that Bim is an important mediator of apoptosis induced as a consequence of aberrant *c-myc* activation [49]. Interestingly, mutant versions of *c-myc*, found in patients with Burkitt lymphomas were unable to induce Bim, and as a consequence foetal liver cells transduced with such mutant versions of *c-myc* led to much more rapid development of lymphomas, when transplanted into mice as compared with wt Myc-driven tumours [50].

Several other oncogenes seem to interfere with apoptosis through downregulation of Bim. H-ras, mutated B-Raf, Bcr-Abl, and mutated, constitutive active EGF-receptor signalling have all been shown to control the levels of BimEL through the activation of ERK protein kinases [51–54]. Phosphorylation of Bim via ERK-mediated phosphorylation at multiple

serines (Ser-55/65/73 in mouse BimEL), leads to its ubiquitination and subsequent degradation by the proteasome [51]. The relevance of these phospho-sites was recently confirmed in vivo in animals that carry amino acid substitutions in Bim replacing Ser-55/65/73 with alanine (Bim^{3SA}). Studies in MEF derived from Bim^{3SA} mice confirmed that these sites are critical for proteasomal degradation upon serum-restimulation and MEF expressing Bim^{3SA} were more susceptible to apoptosis triggered by factor deprivation [55]. It will be interesting to see if the mutant version of Bim also possesses an increased tumour suppressor potential e.g. in the E μ -Myc transgenic mouse model.

All these findings made in mouse models are not only of academic interest, since homozygous loss of the *bim* gene was reported in mantel cell lymphomas, and silencing of the *bim* gene by promoter methylation was reported in 5 out of 10 Burkitt lymphoma samples [56, 57]. As previously mentioned, Burkitt lymphoma patients with poor prognosis express mutant versions of c-Myc that no longer induce expression of Bim. Consistently, Bim expression could not be detected in 6/7 BL samples expressing mutant c-Myc, while it could be easily detected in samples with wild type c-Myc [50].

Furthermore, comparison of the Bim levels in 52 dysplastic nevi, 159 primary melanomas and 52 melanoma metastases revealed that Bim expression decreases as melanoma progresses [58], an observation previously also made for Puma [59], and Bim expression is frequently absent in renal cell carcinoma [60]. A prognostic value of Bim and Puma has recently been demonstrated in human colon carcinoma [61]. The expression levels of Bim in surgically resected primary colon carcinomas from stage II and III patients receiving 5-FU treatment were evaluated and correlated with clinical parameters and disease-free survival (DFS) and overall survival (OS) rates. Patients with elevated expression of Bim had significantly improved DFS and OS, compared with patients that had lost Bim expression, and increased Puma expression also correlated with improved OS [61].

In comparison to the well-established role of Bim in counteracting oncogenesis, the tumour suppressive potential of Bmf is just emerging. Loss of *bmf* has been shown to accelerate the development of thymic lymphoma in a γ -irradiation carcinogenesis protocol in mice [62]. However in the human system, *bmf* has only indirectly been implicated in carcinogenesis. Loss of 15q14/15, which includes the *bmf* gene, has been reported in lung and breast cancer [63, 64]. The observation in breast cancer is intriguing since Bmf has been shown to be involved in mammary morphogenesis and to be repressed by oncogenic Ras [65].

Since p53 is able to induce transcription of the BH3-only proteins Puma and Noxa to trigger apoptosis upon DNA-damage and oncogenic stress it was obvious to study their contribution to p53-mediated tumour suppression. Although the hematopoietic system develops normally in the absence of Puma, thymocytes and myeloid progenitors from Puma deficient mice are protected from apoptosis induced by various p53-dependent as well as p53-stimuli [66, 67]. In addition, MEFs transduced with E1A oncogene normally become sensitised to p53-mediated induction of apoptosis, but this is blocked in the absence of Puma [67].

Importantly, mice deficient for Puma, do not have an increased incidence of tumours, but loss of Puma over Bim appears to triggers spontaneous tumour formation in aged mice [48]. Consistently, loss of Puma has been shown to cooperate with oncogenes such as H-ras or E1A in cellular transformation. Loss of Puma can even substitute for loss of p53 in the transformation of MEF with E1A/Ras, but not with Ras alone [68]. Along that line, loss of Puma can also cooperate with *c-Myc* in the transformation of B cells. E μ -Myc mice deficient for Puma or where Puma was suppressed by retroviral transfection of shRNA in E μ -Myc transgenic stem cells develop lymphomas faster than those containing normal levels of Puma [68, 69] (E. Michalak and A. Strasser, personal communication). In line with

these findings, reduced expression of Puma was also observed in Burkitt lymphoma, which in some of the cases correlated with methylation of the *puma* gene promoter [69].

Loss of 19q13.3 where the *puma* gene is located has been described in glioma [70], neuroblastoma [71] and in some B cell lymphomas [72]. LOH at 19q was reported in 56% of head and neck squamous cell carcinomas and in 27% of primary lung cancers [73]. Similar to Bim, reduced Puma protein expression was observed in primary malignant melanomas compared to non-malignant dysplastic nevi which even further decrease in expression in metastatic melanomas and low expression associated with shorter survival [59]. Of note, increased expression of Puma also predicted increased overall survival in patients suffering from colon carcinoma. The combined variable of Bim and Puma was a strong independent prognostic marker for both DFS and OS in univariate and multivariate analyses, demonstrating for the first time that these two BH3-only proteins significantly impact on treatment success and survival [61].

Noxa is the second BH3-only protein that is transcriptionally regulated by p53 after DNA-damage [74], but in contrast to Puma it plays a more tissue restricted and auxiliary role in p53-mediated apoptosis [67, 75]. Nevertheless, although p53-deficient mice rapidly develop tumours, Noxa-deficient, as well as Noxa/Puma double-deficient mice, do not show an increased incidence of spontaneous tumours, demonstrating the inhibition of the pro-apoptotic function of p53 alone is insufficient to foster tumourigenesis [76].

Most recently, the impact of loss of *Noxa* on c-Myc-driven B cell lymphomagenesis has been analysed. Aberrant expression of c-Myc leads to p53 activation and subsequent induction of apoptosis that may engage Noxa and/or Puma to restrain tumour formation. As mentioned above, *Eμ-Myc* transgenic mice lacking *Puma* develop lymphomas significantly earlier than *Eμ-Myc* mice [50, 69], whereas loss of Noxa per se did not (E. Michalak and A. Strasser, personal communication). However, Noxa deficiency did foster *Eμ-Myc* driven tumourigenesis on a background (haplo)-insufficient for *Puma* (E. Michalak and A. Strasser, personal communication), suggesting a modifier, rather than a tumour suppressor role for *Noxa*. Most strikingly, loss of Noxa clearly delayed the onset of c-Myc-driven immature preB cell, but had no impact on the development of mature IgM⁺ B cell tumours. This may be explained by compensatory activation of other more potent BH3-only proteins such as Puma or Bim in the absence of Noxa may be more effective in removing (pre)malignant preB cells. However, this explanation remains to be formally tested but the finding itself suggests that under certain conditions, BH3-only proteins may harbour oncogenic potential (E. Michalak and A. Strasser, personal communication).

A large panel of various human solid tumours were screened for *noxa* mutations. The single mutation found had no impact on the ability of Noxa to induce apoptosis in vitro [77]. Mutations in the Noxa gene promoter were detected in 4 of 106 primary non-Hodgkin lymphomas, but the consequence of these mutations for cell death induction was not investigated [56]. Recently increasing levels of Noxa protein correlated with progression and high expression levels of Noxa protein was reported to associate with shorter time to biochemical recurrence (PSA serum levels >0.3 ng/ml after surgery) in prostate cancer [78].

Mice lacking Bid, a critical link between the extrinsic apoptosis pathway with the mitochondrial pathway in certain cell types, were reported to develop myeloid hyperplasia, which eventually converts into a malignancy resembling chronic myelomonocytic leukaemia in humans [79]. However, so far no other reports are available that support the tumour suppressor function of Bid and its in human cancers is still unclear. A low frequency of inactivating mutations of Bid has been reported in gastric cancers [80]. An extensive study of Bid expression in prostate cancer, colorectal adenocarcinomas, ovarian cancers, brain

tumours and non-Hodgkin B cell lymphomas, revealed that, if anything, Bid expression sometimes increase during disease progression [81]. In the same study, no correlation between Bid expression and the response to a large number of commonly used anticancer was found when tested on the NCI cancer cell line panel, with the exception of two ribonucleotide reductase inhibitors. Also, the level of Bid protein expression in cervical cancers did not correlate with outcome of radiotherapy [82].

The pro-apoptotic potential of Bad is regulated by phosphorylation down-stream of growth factor signalling. Ser-136 phosphorylation is PI3K/AKT dependent, while Ser-112 in Bad is phosphorylated as a result of activation of the ERK-signalling pathway. In both cases phosphorylation leads to sequestration by 14-3-3 proteins suggested resulting in inactivation of Bad [83]. The PI3K/Akt pathway is frequently activated in human cancers due to loss of the negative regulator PTEN. Although it is well known that hyperactivation of Akt facilitates tumourigenesis in part by preventing apoptosis, it is unclear if inactivation of Bad by phosphorylation, one of the many anti-apoptotic effects assigned to Akt, contributes to this effect [84]. Analysis of the tumour suppressor potential of Bad in a defined tumour-model will help to address this question. Noteworthy, Bad-deficient mice appear to develop normally but were reported to develop diffuse large B-cell lymphomas, with an incidence of about 20%, albeit late in life (latency > 15 month) and on a mixed genetic back-ground [85].

Loss-of-function mutations in Bad, affecting the BH3-domain were reported in colon carcinoma patients [86]. Higher levels of Bad protein expression have been associated with better outcome in androgen dependent prostate cancer and in breast cancer, while the combined levels of Bad and Bid were reported to predict the outcome after 5-FU treatment in colon cancer patients [61, 87, 88].

Expression Bik/Nbk (Blk in mice) is frequently lost, as a consequence of allelic loss and/or silencing by methylation in renal cancer, suggesting that loss of Bik might be responsible for apoptosis resistance and that this protein might act as a tumour suppressor in this cancer [89]. Bik mutations have also been found in B cell lymphomas, although the role of these mutations is not clear [90].

Last but not least, silencing of *Hrk* by promoter methylation has been described in colorectal and gastric cancer, in astrocytomas and glioblastomas, primary central nervous system lymphomas as well as in prostate cancer [91–94]. Tumour promoting effects of loss of either *Blk* or *Hrk* in mouse models have not been described so far.

BH3-only proteins as mediators of anticancer therapy-mediated apoptosis

Investigations into the molecular mechanisms of apoptosis induced by conventional and new front-line anticancer agents have revealed the critical role of BH3-only proteins in the process of drug-responsiveness and resistance (Fig. 2). Bim, for example, has been found to be an important mediator of cell death induced by tyrosine kinase inhibitors (TKI's) targeting Bcr-Abl tyrosine kinase as well as mutant EGF receptor (EGFR). Imatinib, also known as Gleevec, targets the Bcr-abl fusion protein product found in Philadelphia chromosome positive [Ph⁺ t(9;22)] leukaemias. Treatment of the Ph⁺ K562 cell line with Imatinib led to transcriptional upregulation as well as decreased phosphorylation and proteasomal degradation of Bim, resulting in strong accumulation of Bim and tumour cell death [53, 95]. Bim together with Bad, which is also dephosphorylated and activated upon Imatinib treatment, seem to be responsible for most, if not all cell killing by Imatinib, as *bim*^{-/-} *bad*^{-/-} foetal liver cells transformed with *bcr-abl* were almost completely protected from drug-induced apoptosis [53]. Two other TKI's Gefitinib (Iressa) and Erlotinib (Tarceva), which target mutated and constitutively active versions of the EGFR, have also been found to induce apoptosis in lung and colon cancer cells predominantly through

activation of Bim, mainly by antagonizing ERK-mediated proteasomal degradation [52, 96, 97].

Dephosphorylation and activation of Bad was also observed in a breast cancer cell lines and a xenotransplant model by the EGFR tyrosine kinase inhibitor Gefitinib. Consistent with a rate-limiting role of Bad in this type of tumour cell apoptosis, siRNAs targeting *bad* attenuated cell death induction by this treatment [98]. Besides Bim and Bad activation by tyrosine kinase inhibitors, Bmf was also shown to be upregulated in response to TKI's in CML, however its role was not significant, as knockdown of Bmf in this setting did not affect the apoptosis [99]. Bmf and Bim have been shown to mediate apoptosis induction in juvenile acute lymphoblastic leukaemia in response to treatment with glucocorticoids [100], arsenic trioxide in multiple myeloma [101], and in TGF-beta induced cell death in breast epithelial cell lines [102] and B cell chronic lymphatic leukaemia (CLL) [103]. Bmf appears to be preferentially upregulated following application of histone deacetylase inhibitors (HDACi) in numerous tumour entities, at least in vitro [104] but the relevance of its induced expression is not completely clear. For example, the upregulation of Bmf in response to HDACi appears not to translate into apoptosis in CLL cells, as RNAi of Bmf did not abrogate apoptosis. Instead, Bim and Noxa, both upregulated following HDACi treatment in CLL cells, mediated apoptosis in response to HDACi [99]. In squamous cells carcinoma, however, pre-treatment with low nanomolar amounts of HDACi could increase Bmf expression and thus sensitise these cells also to radiation-induced death [105].

Puma and Noxa are clearly relevant for the effects by classical DNA damaging agents as well as radiotherapy, but somewhat surprisingly, Noxa seem to be a relevant mediator of apoptosis in response to inhibition of the proteasome. In malignant melanoma, but not primary melanocytes, as well as multiple myeloma, but not peripheral blood lymphocytes non-selective inhibition of the proteasome by Bortezomib/Velcade causes an increase in the expression of Noxa preceding tumour cell apoptosis. In contrast, Bim is induced by this treatment in normal as well in cancer cells, but the former are highly resistant to this drug [106, 107]. Noteworthy, induction of Noxa is not simply a consequence of impaired proteasomal degradation of the pro-survival member Mcl1, which complexes with Noxa for co-degradation in the proteasome, but occurs also at the transcriptional level in a c-Myc-dependent manner. Notably, these changes are independent from the p53 status of the tumour cell [108]. γ -secretase inhibitors (GSI) which inhibit Notch receptor processing and activation was reported to induce apoptosis in melanoma and multiple myeloma cell lines in a Noxa dependent manner. In both cases GSI treatment led to induction of Noxa, and knockdown of Noxa reduced apoptosis induced by GSI [109, 110].

In summary, all these observations confirm that activation of one or more BH3-only proteins mediates the effects of all anticancer agents currently used or developed. The detailed knowledge about their molecular mode of action will certainly help us to increase the drug-panel available for the treatment of malignant diseases and will equally importantly help to properly target these novel treatment strategies.

Targeting Bcl2 pro-survival molecules by mimicking the function of BH3-only proteins as a concept of antitumor therapy

Although interfering with Bcl2 pro-survival function has long been considered a suitable means to kill tumour cells, Bcl2 proteins were simply deemed undruggable by pharmacological means. Therefore, early trials were aiming to exploit antisense-based strategies to knockdown expression of Bcl2 or Bclxl. Chemically modified antisense-oligode-oxynucleotides (ASOs), targeting the mRNA of Bcl2 or Bclxl for RNaseH-mediated degradation are still considered to harbour significant potential as anticancer agents. For

example, Genasense (G3139/Oblimersen), an 18-mer phosphorothioate oligonucleotide has been developed to target the first six codons of Bcl2 mRNA. The drug effectively inhibited the proliferation of human lymphoma cells carrying the t(14;18) translocation [111, 112]. A number of clinical trials investigated the efficacy of the drug in the clinic, but until now it did not obtain FDA approval. Notably, ASOs may not only act via downregulation of target mRNA but were also reported to trigger inflammatory responses, caused by CpG motive-mediated activation of toll-like receptor-9, especially when administered at high doses [113]. This additional bioactivity may even be beneficial. However, knowing the molecular basics of how Bcl2 like molecules preserve survival and how BH3-only proteins kill cells makes the limitation of this strategies, that can only target one, or at best, two highly homologues proteins at a time, immanent. Over the past years a number of small-molecule drugs aiming to target the Bcl2 pathway have been developed or identified in compound-screens (Fig. 3). Among these are natural compounds, such as Antimycin A, a mitochondrial respiratory chain inhibitor, Chelerythrine, a plant alkaloid and Gossypol, a polyphenol found in cotton seeds already used in Chinese herbal medicine [114]. All these substances seem to promote cell death by inhibiting Bcl2 (Antimycin A and Gossypol) or by disrupting interaction between anti- and pro-apoptotic proteins (Chelerythrine) [115–117]. Since phase I clinical trials revealed high toxicities of Gossypol a semi-synthetic variant of Gossypol, Apogossypol was designed to be investigated further [116]. Other natural compounds were identified within tea polyphenols. In particular, certain green tea catechins and black tea theaflavins were reported to be potent inhibitors of Bclxl and Bcl2 [118].

A number of chemical compounds designed to inhibit Bcl2 action are currently evaluated in pre-clinical models and early clinical trials. For example, HA14-1 is a chromene-derivate that was identified by computer modelling and proposed to inhibit Bcl2 [119]. Using a similar approach, a 2-methoxy derivative of Antimycin, A3, was identified. A3 is inactive as an inhibitor of cellular respiration, but still retains its properties of antagonizing Bclxl [117]. Additional compounds were identified in a Bak BH3-peptide displacement assay, such as BH3I-1, a thiazolidin derivative, and BH3I-2, a benzene sulfonyl derivative both proposed to antagonize Bclxl [120]. However, more detailed analysis revealed that none of these substances killed cells in a Bax or Bak-dependent manner and, although of potential therapeutic use, cannot be considered *bona-fide* Bcl2 inhibitors [121].

True Bcl2 inhibitors, also called “BH3-mimetics”, are non-peptidic compounds that have been rationally designed on the basis of protein-protein interaction between anti- and pro-apoptotic proteins of the Bcl2 family. TW-37, a putative BH3-mimetic was designed based on the structure of gossypol and a Bim BH3-peptide binding to Bcl2 [122]. This molecule can displace Bid from its binding to Bcl2, Bclxl and Mcl1 at low micromolar concentrations and is proposed to kill in a Bak/Bax dependent manner. However, since it was reported to induce p53 in melanoma cell lines this may not be its only bioactivity [123].

Obatoclax (or GX15-070) can bind to Bcl2, Bclxl, Bclw and Mcl1 in the nanomolar range and kills cells in a Bax/Bak dependent manner [124, 125]. Consistently, it showed single agent activity against CLL, where it also synergizes potently with chlorambucil and fludarabine [126]. In cholangiocarcinoma cells, Obatoclax showed additive effects with TRAIL [127], Velcade [128], Lapatinib and GW2974 (two human epidermal growth factor receptor-2 kinase inhibitors) [129]. In a subset of lung cancer cell lines, Obatoclax induced apoptosis and showed synergistic action together with Cisplatin in non-small cell lung cancer (NSCLC) cells [130]. The antimyeloma activity of Velcade, Dexamethasone or Melphalan was also enhanced by co-treatment with Obatoclax [130], as were the effects of AraC in leukemic cell lines and in primary AML samples [125].

Using nuclear magnetic resonance (NMR)-based methods to target the BH3-binding groove of Bclxl Abbott Laboratories developed the first rationally designed BH3-mimetic, ABT-737 [131]. Unlike Obatoclast, ABT-737 is a Bad-like BH3-mimetic, neutralising Bcl2, Bclw and Bclxl but not Mcl1 or A1, leading to Bax/Bak-dependent apoptosis [121, 132]. As a single agent it was extremely efficient to induce cell death in patient-derived primary B-CLL [132, 133]. Its efficiency has been confirmed in samples from patients affected with follicular lymphoma, mantle cell lymphoma and marginal cell lymphoma. Furthermore, this drug is able to induce cell death alone in multiple myeloma and in SCLC cell lines [131]. The biggest limitation of ABT-737 is its inability to inhibit Mcl1. In fact resistance to treatment with this drug has been linked exclusively to Mcl1 overexpression [131]. However, this problem can be overcome by using ABT-737 in combination with other drugs targeting Mcl1 for degradation, such as the CDK-inhibitor seliciclib [121], or, as mentioned above, a BH3-mimetic that neutralises it, such as Obatoclast [125]. ABT-737 acts in a synergistic manner with doxorubicin or AraC on AML cells [134], as well as with TKI's such as Gleevec or INNO-406 on CML cells or with Gefitinib on NSCLC cells [52, 53, 95].

The only noted side effects of ABT-737 so far are short-term lymphopenia and thrombocytopenia, which can be dealt with effectively in the clinic [131]. To overcome the limited bioavailability of ABT-737, Abbot Laboratories designed ABT-236, which has the same binding capacity for Bcl2 family members and killing-potency as ABT-737, now tested in Phase I/II clinical trials. Oral administration of this drug alone induced complete regression of tumour xenograft model of SCLC and ALL, and showed synergistic drug-action in xenograft models of aggressive B-cell lymphoma and multiple myeloma [135].

Conclusions

Finally, 20 years after the discovery of Bcl2 and its role in apoptosis we have reached a stage where the gathered knowledge about this signalling pathway translates into clinical application. Although this pathway has been considered undruggable for a long time the development of BH3-mimetics has shown that continuous world wide basic and translational research can overcome almost all obstacles. It is now time to evaluate the suitability of different BH3-mimetics for the (combinatorial) treatment of distinct malignant disease in humans and evaluate their long-term clinical applicability and merits. An exciting time for all researchers involved, and hopefully a fruitful one for those waiting for treatment and cure.

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Fig. 1.

Cell death signalling regulated by the Bcl2 family. Prosurvival-members of the Bcl2 family preserve mitochondrial integrity by preventing activation of Bax and/or Bak until neutralised by BH3-only proteins. This leads to mitochondrial outer membrane permabilization, release of apoptogenic factors, including cytochrome *c*, required for apoptosome formation and activation of the caspase cascade. Activation of death receptors can licence the mitochondrial apoptosis pathway by caspase-8 mediated cleavage of Bid into tBid, connecting the extrinsic with the intrinsic apoptosis pathway in an amplification loop

Fig. 2.

Tumour suppressing drugs acting via BH3-only proteins. Classical and novel anticancer agents trigger apoptosis by engaging one or more BH3-only proteins, by one or more different molecular mechanisms. *Solid arrows* indicate transcriptional activation; *dashed arrows* posttranslational stabilization; *dotted arrows* transcriptional and posttranslational activation/stabilization of BH3-only proteins in response to drug-treatment

Fig. 3. Summary of available BH3-mimetics and Bcl2 inhibitors. Chemical, non-peptidic and natural compounds currently tested for their ability to promote cell death by antagonizing the expression or pro-survival function of Bcl2-family members