REVIEW

Targeting BCL-2 to enhance vulnerability to therapy in estrogen receptor-positive breast cancer

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ABSTRACT

The last three decades have seen significant progress in our understanding of the role of the pro-survival protein BCL-2 and its family members in apoptosis and cancer. BCL-2 and other pro-survival family members including Mcl-1 and BCL-X_L have been shown to play a key role in keeping pro-apoptotic 'effector' proteins BAK and BAX in check. They also neutralize a group of 'sensor' proteins (such as BIM), which are triggered by cytotoxic stimuli such as chemotherapy. BCL-2 proteins therefore play a central role as guardians against apoptosis, helping cancer cells to evade cell death. More recently, an increasing number of BH3 mimetics, which bind and neutralize BCL-2 and/or its pro-survival relatives, have been developed. The utility of targeting BCL-2 in haematological malignancies has become evident in early phase studies, with remarkable clinical responses seen in heavily pre-treated patients. Since BCL-2 is overexpressed in approximately 75% of breast cancer, there has been growing interest in determining whether this new class of drug could show similar promise in breast cancer. This review summarizes our current understanding of the role of BCL-2 and its family members in mammary gland development and breast cancer, recent progress in the development of new BH3 mimetics, as well as their potential for targeting ER-positive breast cancer.

Introduction

Breast cancer is now recognised to be a complex heterogeneous disease with different intrinsic molecular subtypes.[1,2] In the clinic, these subtypes are largely categorized by the presence or absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression. In general terms, luminal tumors are defined by the expression of ER and/or PR; basal-like tumors are often (but not always) 'triple negative' for ER, PR and HER2 and express CK5/6 and/or EGFR; HER2-enriched tumors overexpress HER2 (usually through amplification). The classification of breast cancer into these distinct subgroups not only provides important prognostic information, but also helps guide clinical therapy through targeting of these predictive biomarkers.

Luminal tumors account for the vast majority of breast cancer. Current standard treatment of ER positive breast cancer involves anti-estrogen therapy using either Selective Estrogen Receptor Modulators (SERMs) such as tamoxifen, or aromatase inhibitors. Recent gene expression studies have highlighted a significant degree of molecular heterogeneity within the ER-positive subtype, which presumably contributes to variable clinical outcomes.[3,4] ER positive breast cancers can be broadly further divided into two categories, based on gene expression profiling: luminal A and luminal B. Luminal B tumors often exhibit higher grade and proliferative activity (as revealed by Ki67 immunostaining) and lower (or absent) progesterone receptor expression. Due to these poor prognostic features, patients with luminal B cancers are more likely to be recommended chemotherapy,[5] albeit in a relatively untargeted manner. Identifying more effective novel agents, employed as either monotherapy or in conjunction with standard therapy, remains an important area of need for managing patients with this common and potentially clinically aggressive breast cancer subtype.

Following the identification of the *Bcl-2* gene in the mid-1980s,[6,7] intense effort was applied to understanding how *Bcl-2* functioned as a proto-oncogene. The discovery that Bcl-2 promotes cell survival[8] and could cooperate with the *c-Myc* oncogene in lymphomagenesis[9,10] focussed attention on its role as a negative regulator of apoptosis in promoting tumorigenesis. The family of pro-survival and pro-apoptotic proteins has expanded over the last 25 years, accompanied by substantial insights into

their mechanisms of action.[11-14] These discoveries have culminated in the development of a new class of drug, BH3 mimetics, which antagonize BCL-2 proteins. These have shown promise in early phase studies in chronic lymphocytic leukaemia and lymphoma, and may ultimately prove to have a broader role in the treatment of certain solid tumors.

BCL-2 has emerged as an important prognostic marker[15] for both ER positive and triple negative breast tumors.[1] *Bcl-2* gene expression is also a component of molecular assays for risk of recurrence such as Oncotype DX[16,17] and PAM50 Prosigna Breast Cancer Assay,[18,19] which are being used increasingly to predict tumor recurrence and guide adjuvant therapy in ER positive, node-negative disease. In spite of this, the precise role of BCL-2 as a therapeutic target in breast cancer is yet to be clarified.[20] Here we review some of the current knowledge on the role of BCL-2 in breast cancer, and discuss the future therapeutic challenges of combining BH3 mimetics with standard treatment in ER-positive breast cancer.

BCL-2 and apoptosis

Apoptosis or programmed cell death is triggered by two pathways: the 'extrinsic pathway' which is mediated by death receptors, and the 'intrinsic pathway', also known as the BCL-2-regulated or mitochondrial pathway.[11] In transformed breast epithelial cells (known as 'type II' cells) the intrinsic pathway also becomes activated in response to the activation of the death receptor pathway. As a result, BCL-2 and its relatives behave as critical arbiters of apoptosis in response to a much broader range of pro-apoptotic stimuli compared to 'Type I' cells such as lymphoid cells.[21]

BCL-2 family members can be broadly divided into three groups (Figure 1): the pro-apoptotic 'effectors' (BAX and BAK), the anti-apoptotic 'guardian' proteins (BCL-2, BCL-X_L, BCL-W, MCL-1, A1 and BCL-B) and the BH3 only 'sensor' proteins (for example BIM, BAD, PUMA, BID, NOXA, BMF). At any moment in a normal cell, the decision between survival and death is dependent on the tightly regulated interactions between these different BCL-2 family members. Anti-apoptotic guardian proteins take center-stage in this process: they can bind and neutralize the BH3-only sensor proteins or bind to BAX and BAK effector proteins (Figure 2a). Binding of

BCL-2 survival proteins to effector proteins BAX and BAK prevents their conformational change and oligomerization, thus blocking the formation of lethal pores in the outer membranes of mitochondria (Mitochondrial Outer Membrane Permeabilization, MOMP). MOMP triggers cytochrome c release and caspase activation, culminating in the destruction of the cell.[13] An excess of BH3-only proteins can overwhelm BCL-2 to indirectly activate BAX and BAK. BH3-only proteins may also directly activate these effectors. Thus, BCL-2 and its relatives block cell death in two ways: (i) they titrate BH3-only proteins to prevent their binding to BAX/BAK, and (ii) protect activated BAX and BAK from further oligomerization.

BCL-2 expression in primary breast cancer

Approximately 75% of primary breast cancers express high levels of BCL-2, with a predominance in ER-positive tumors: BCL-2 is overexpressed in ~85% of ER-positive tumors, 50% of HER2-positive tumors, 41% of triple negative breast cancers (TNBCs) and 19% of basal-like tumors (TNBCs that express EGFR and/or CK5/6).[15,22,23] These findings appear to be consistent with gene expression profiling studies, where *Bcl-2* is predominantly expressed in ER-positive tumors.[4] Interestingly, the frequency of BCL-2-positive tumors is lower (31%) in *BRCA1*-associated cancers, compared to cancers without *BRCA1* mutations,[24] probably attributable to their triple negative status.

Bcl-2 family members in normal mammary gland development and neoplasia

The role of BCL-2 in promoting cancer progression was first described in follicular lymphoma.[6,7] In the Eμ-Myc transgenic and other mouse models of lymphomagenesis, over-expression of BCL-2 (and its relatives) greatly accelerated the onset and the progression of lymphomas,[9] as did the loss of BH3-only proteins[25]. In keeping with these findings, several clinical studies have revealed that BCL-2 expression confers a poor prognosis in hematopoietic malignancies.[26,27] It is likely that improved tumor cell survival facilitates the acquisition of additional molecular changes during tumor development or progression.

The impact of BCL-2 family member overexpression on normal mammary gland development and tumorigenesis has been evaluated using a variety of transgenic and knockout models (Table 1). Enforced expression of *Bcl-2* under the *whey acidic*

promoter (WAP) delayed (but did not prevent) involution following the cessation of lactation.[28] Although Bcl-2 overexpression alone was not tumorigenic, *Bcl-2* was shown to accelerate tumorigenesis by either the c-Myc or SV40T antigen (SV40TAg, which neutralises p53 and Rb) transgene.[28,29] Apoptosis was reduced during the pre-neoplastic period, presumably accounting for the reduced tumor latency observed in the presence of the *Bcl-2* transgene. Conversely, loss of *Bax* delayed involution but did not itself promote tumor formation[30] and *Bax* haploinsufficiency decreased tumor latency in both the C3(1)/SV40TAg and c-Myc transgenic models.[30,31] These results are broadly consistent with murine lymphomagenesis models and BCL-2 overexpression studies in human breast cancer cell lines, where BCL-2 has been generally found to promote tumor growth and metastases *in vivo*.[32,33] As yet, no studies have been reported where BCL-2 family members were conditionally targeted in established mouse mammary tumors. Such genetic studies, like that recently reported for conditional deletion of *Mcl-1* in a Myc-driven lymphoma model,[34] offer a powerful means of anticipating the clinical effect of a pharmacological inhibitor.

The paradox of BCL-2 as a good prognostic marker in breast cancer

The correlation between BCL-2 expression and patient outcome has been extensively studied in primary breast cancer (Table 2). In ER-positive tumors, there appears to be a correlation between high levels of BCL-2 and improved clinical outcome. Although one study suggested that BCL-2 overexpression is associated with worse prognosis,[35] most studies reported favorable outcomes in patients with ER-positive breast cancer who received adjuvant endocrine therapy (Table 2). In a large prospective analysis involving more than 11,000 patients with early breast cancer, BCL-2 was shown as a favorable prognostic marker across molecular subtypes, independent of the adjuvant therapy received.[15] A meta-analysis of 17 studies confirmed the association of BCL-2 with improved disease-free survival and overall survival in breast cancer independent of lymph node status, tumor size and grade as well as a range of other biological variables.[36] More recently, the clinical value of BCL-2 was also explored in early TNBC,[1,37] where there appeared to be disparate findings on BCL-2 as a prognostic marker in this tumor subtype.

The paradoxically favourable prognostic value of BCL-2 evident in early breast cancer is difficult to reconcile with data based on mouse mammary tumor models and

contrasts with findings for non-Hodgkin lymphoma. One likely explanation for this paradox is that *Bcl-2* is an estrogen-responsive gene.[38] BCL-2 could in part serve as a surrogate biomarker of an intact estrogen signaling pathway, such as in luminal A tumors in which increased response to endocrine therapy is well-recognized. In this setting, potent and effective endocrine therapy would reduce BCL-2 expression and tumor survival. Mitochondrial priming, described below, could also provide another explanation. It is also feasible that BCL-2 may contribute additional, non-apoptotic, functions.[39,40]

Most of the published findings that link BCL-2 expression to favorable outcome refer to patients with *early* breast cancer, rather than those with advanced/metastatic disease that have become refractory to therapy (Table 2). However, even for early stage disease it is important to note that *Bcl-2* is generally expressed at high levels across *both* luminal subtypes, including poorer prognosis luminal B tumors, albeit that slightly higher levels are observed in luminal A tumors.[23] In a study on 205 ER positive metastatic tumors, BCL-2 was shown to be a weakly favourable prognostic factor.[41] In the metastatic setting, previous systemic therapy is likely to have applied selective pressure on tumors, where elevated BCL-2 levels could contribute to drug resistance.

Another issue that has not been evaluated in BCL-2 expression studies is the question of tumor heterogeneity. BCL-2 can be unevenly distributed in tumors. In one study, BCL-2 was found to be upregulated in distinct regions, which were linked to local adaptive resistance and survival of matrix-attached breast cancer cells.[42] Moreover, BCL-2 has also been shown to be expressed in $CD44^{+}/CD24^{-}$ stem cell-like cancer cells.[43] We recently observed increased expression of the pro-apoptotic protein BIM at the tumor border, where *Bim* was shown to be a *bona fide* target of the EMT protein Snai2/Slug.[44] Such biologically relevant information is not generally apparent in studies that evaluate global gene or protein expression in breast tumors.

Approximately 50% of circulating tumor cells (CTC) from patients with different subtypes of breast cancer express BCL-2.[45] Although BCL-2 expression correlated with better outcome, CTCs themselves are linked to poorer prognosis. It is conceivable that CTC assays could be biased by the preferential survival of BCL-2-

positive CTCs. A clinical trial using the CellSearch® technology and immunohistochemistry for BCL-2 on a large scale (NCT01701050) may clarify the role of BCL-2 expression in CTCs for ER-positive metastatic breast cancer including its impact on endocrine sensitivity.

Efforts to standardise BCL-2 reporting as well as the development of prognostic indexes that combine BCL-2 expression with other markers (such as proliferation, apoptosis, differentiation, mitosis or p53)[46-50] will be important to further understand the role of BCL-2 in prognosis. To date, the types of antibodies and scoring methods used to define immunohistochemical positivity have varied between reports. Standardization will be necessary for the development of BH3 mimetics in the clinic, where BCL-2 is likely to be an important predictive (companion) biomarker.

BCL-2 and mitochondrial priming

The precise relationship between BCL-2 levels and resistance to drug therapy has yet to be fully established. The association between BCL-2 and improved clinical outcomes could be explained by the observation that increased BCL-2 levels are often associated with a commensurate increase in the levels of its pro-apoptotic binding partners, due to protection from proteasomal degradation.[51,52] The high levels of pro-apoptotic proteins potentially bring them closer to an 'apoptotic threshold', sensitizing cells to anti-cancer therapy. Exposure of cells to a cytotoxic insult can trigger the sudden release of the pro-apoptotic proteins. Depending on the precise composition of BCL-2 family members in that cell, the acute release of pro-apoptotic proteins could activate the apoptotic cascade. This concept, described as 'mitochondrial priming', could explain why some cancer cells with high levels of BCL-2 are unexpectedly sensitive to conventional therapy.[53]

The growing class of BH3 mimetics

Initial efforts to target BCL-2 proteins involved antisense oligonucleotide technology. Oblimersen sodium (Genasense®), an antisense DNA molecule designed to hybridize with BCL-2 mRNA and to induce hydrolysis, was evaluated in several clinical trials, including neoadjuvant studies in breast cancer. Results were disappointing, with minimal or no tumor response observed.[54,55] This lack of efficacy was probably due to poor tumor penetration and ineffective knockdown of BCL-2 levels *in vivo*.

Subsequent attention turned to the development of agents that disrupt BCL-2 complexes. BH3 mimetics bind to the hydrophobic groove of anti-apoptotic proteins, mimicking the acting of BH3-only proteins in binding to pro-survival proteins, leading to the release of BH3-only proteins from complexes and activation of BAX and BAK. In addition, pro-survival proteins are captured, neutralizing their ability to prevent BAX/BAK oligomerization (Figure 2a).[11] Early efforts to develop BH3 mimetics included the cottonseed extract gossypol and its synthetic derivative AT-101, and the small molecule inhibitor obatoclax (GX15-070). These compounds bind with modest affinity to BCL-2, BCL-X_L and MCL-1. Gossypol and AT-101 has pleiotropic actions that extend beyond 'on target' effects (i.e. cell death mediated by BAX and BAK). These agents have shown limited clinical activity in lymphoid,[56] lung[57-59] and prostate[60] cancer.

Recent efforts to develop potent BH3 mimetics with 'on target' efficacy have been pioneered by AbbVie and Genentech (Table 3). Three BH3 mimetics have been shown to have high anti-tumoral activity with 'on target' effects: ABT-737 (a pre-clinical lead compound), its orally available counterpart ABT-263 (Navitoclax) and GDC-0199/ABT-199 (Venetoclax). *In vitro* and *in vivo* studies have demonstrated good specificity and anti-tumoral activity of these compounds for various cancer types.[14] ABT-737 and navitoclax have been designed to bind BCL-2, BCL-X_L and BCL-W with high affinity, but are unable to antagonise MCL-1 and A1[61,62] (Figure 2b). *In vitro* and *in vivo* studies have demonstrated good specificity and anti-tumoral activity of these compounds on various cancer types, including breast cancer cells. ABT-199 (venetoclax), another 'on target' BH3 mimetic, has recently been developed[63] that binds to BCL-2 with comparable binding affinity to ABT-737 or navitoclax, but does not bind to BCL-X_L, BCL-W, MCL-1 or A1 with high affinity (Figure 2b). Both ABT-263/navitoclax and ABT-199/venetoclax are currently being studied in early phase clinical trials for the treatment of chronic lymphocytic leukaemia (CLL) with promising preliminary results in heavily pretreated patients with chemorefractory disease.[56,64,65] ABT-199 also appears to have the distinct clinical advantage of avoiding dose-dependent thrombocytopenia, which is an acute side-effect of navitoclax due to the dependence of mature platelets on BCL-X_L for survival.[66,67]

A BCL-2/BCL-X_L inhibitor developed by Servier, S44563, has been shown to elicit antitumor activity *in vivo* in pre-clinical models of uveal melanoma.[68] Another BCL-2 specific inhibitor, S55746, is currently undergoing evaluation in a Phase 1 dose escalation study in B cell non-Hodgkin Lymphoma (ISRCTN04804337). There are no reports to date on the effect of these BCL-2 inhibitors on breast cancer cells.

Novel inhibitors of other BCL-2 family members have been developed, although most have yet to be fully assessed in breast cancer models. These include dual BCL-X_L/MCL-1 inhibitors[69] and BCL-X_L-selective inhibitors, WEHI-539,[70,71] A-1155463[72] and A-1331852.[73] The latter two compounds appear to synergize with docetaxel without inducing neutropenia observed with the BCL-2/BCL-X_L inhibitor navitoclax. Finally, MCL-1 inhibitors have recently been reported by several groups.[74-77] Interestingly, monotherapy with the MCL-1 inhibitor A-1210477 appeared to induce death in HCC-1806 breast cancer cells, which have a TNBC phenotype. It will be interesting to determine if MCL-1 inhibitors produce a preferential response in this tumor subtype, where BCL-2 expression is less dominant.

Development of BH3 mimetics in pre-clinical breast cancer models

Various groups have investigated the effect of ABT-737 and ABT-263 on a number of breast cancer cell lines (Table 3). Unlike hematopoietic models, monotherapy with a BH3 mimetic did not appear to induce tumor killing in breast cancer in cell lines.[22,23,42,78] Single agent ABT-199 was similarly shown to have minimal anti-tumor activity in ER-positive or TNBC cells.[23] This lack of vulnerability to single agent therapy could reflect a limited contribution by BCL-2 as an oncogenic driver in breast cancer cells, in contrast to lymphoma. Alternatively, high levels of other BCL-2 family members, such as BCL-X_L (and possibly MCL-1), which appear to be universally expressed across most breast tumors, could contribute to functional redundancy. In contrast to monotherapy, however, combination therapy with a BH3 mimetic and other agents such as chemotherapy or mTOR inhibitors did appear to induce a synergistic tumor response (Table 3). These findings suggest that breast cancer cells may need to be 'primed' and then challenged with a pro-apoptotic insult in order to elicit an anti-tumoral response to BH3 mimetic therapy.

The feasibility of targeting luminal B tumors with combination therapy comprising endocrine therapy (tamoxifen) and a BH3 mimetic (ABT-737 or ABT-199/venetoclax) using patient derived xenograft (PDX) models of primary breast cancer has been explored.[23] These PDX models were derived through orthotopic transplantation of treatment-naïve primary breast tumors into cleared mammary fat pads of immunocompromised NOD/SCID/IL2$\gamma^{-/-}$ (NSG) mice. These represent powerful pre-clinical models in which to test therapeutic targets, as they faithfully recapitulate the behavior of the primary tumor, with a high degree of genomic preservation across primary tumors and matching PDXs over serial passages.[79] Three BCL-2-positive luminal B PDX models were investigated. In all cases ABT-199 alone had little effect, whereas combination therapy with tamoxifen and ABT-199 reduced tumor growth rates and extended animal survival. In one model, complete tumor regression was noted. Notably, ABT-199 appeared to be as effective as ABT-737, suggesting that BCL-2, rather than BCL-X_L is the key target *in vivo*, despite the high levels of BCL-X_L. Whether these models represent the broad spectrum of luminal B breast tumors remains to be determined. Given that PDX models have been shown to reflect poor prognosis disease, it seems likely that these models recapitulate clinically aggressive disease. Of note, two of the responding tumors harbored p53 mutations, consistent with the notion that BH3 mimetics act downstream of p53.[80,81]

One unanticipated finding in these models was that ABT-737 treatment counteracted endometrial hyperplasia normally seen with tamoxifen. This was accompanied by profound endometrial apoptosis, as evidenced by caspase-3 activation. Presumably BCL-X_L (or BCL-2) inhibition induced apoptosis following a proliferative stimulus in the endometrium, where tamoxifen acts as an estrogen agonist. As the effect was more pronounced with ABT-737, it is likely to be at least partially driven by BCL-X_L. Whether this observation is replicated in humans and has clinical utility remains to be determined.

Is BCL-2 a preferred therapeutic target for treatment naïve or refractory disease?

The experiments described above were carried out using PDX models from clinically aggressive, treatment naïve primary tumors. It seems likely, therefore, that combined

therapy with estrogen blockade and a BCL-2 inhibitor should be beneficial for luminal B tumors in the early disease setting. This question would optimally be addressed in the clinic through a neoadjuvant study. Whether BCL-2 inhibition would benefit patients with ER-positive tumors that are highly endocrine responsive (such as luminal A tumors) is not yet known. Although it is plausible that effective endocrine therapy could directly lower BCL-2 levels obviating the need for an inhibitor, the acute disruption of BCL-2 containing complexes by a BH3 mimetic could still potentiate the efficacy of endocrine therapy.

It will also be critical to determine whether a similar tumor response would be elicited in previously treated tumors that have become partially or fully refractory to endocrine therapy. In this setting, maintenance or induction of BCL-2 expression during tumor progression would provide a rationale for specifically targeting BCL-2 to augment tumor vulnerability, best tested in patients with. metastatic breast cancer.

Interestingly, two partially responsive models showed increased expression of phosphorylated AKT. The PI3K/AKT/mTOR pathway is frequently activated in luminal B tumors, where it is associated with driving resistance to endocrine therapy.[82,83] Indeed, improved clinical outcomes have been observed in patients with refractory disease who are treated with combination therapy using exemestane (a steroidal aromatase inhibitor) and the mTOR inhibitor everolimus.[84] Notably, mTOR inhibitors were found to synergize with BH3 mimetic therapy. Treatment of one of the partially responsive PDX models with triple therapy that included tamoxifen, a dual mTOR inhibitor (PKI-587) and ABT-737 significantly improved tumor response and was well-tolerated (Figure 3). The mTOR inhibitors may have contributed to mitochondrial priming by elevating BCL-2 and reducing pBAD levels. These findings suggest that it may be important to explore combination therapy that targets resistance and survival pathways such as PI3K/AKT/mTOR and pro-survival proteins in the clinic.

Since tamoxifen was used in these models, it will also be important to determine whether estrogen deprivation through aromatase inhibitor therapy or estrogen receptor degradation by fulvestrant would be similarly efficacious. Since estradiol pellets were used to propagate the PDX tumors, it was not possible to directly assess aromatase

inhibitor therapy in these models. In one model, however, short-term therapy with tamoxifen appeared to prime the tumors for BCL-2 expression, perhaps augmenting the tumor response. Whether or not this effect is specific to tamoxifen (or other endocrine therapies) is unknown.

Is BCL-2 the primary pro-survival protein to target in ER-positive breast cancer?

Despite the fact that ABT-737 and navitoclax bind to BCL-2, BCL-X_L and BCL-W with similar affinity, there is evidence to suggest that these 'BAD-like mimetics' principally target BCL-2, rather than BCL-X_L and BCL-W in normal lymphocytes and leukaemia.[51,85] Indeed, cells that depend on BCL-2 for survival (such as mature B cells or double positive thymocytes) are more sensitive to ABT-737 than BCL-X_L dependent cells. In addition, over-expression of BCL-2 sensitizes tumor cells to ABT-737, whereas BCL-X_L or BCL-W overexpression helps to protect against ABT-737, indicating that, at least in leukemic cells, BCL-X_L or BCL-W do not prime cell death induced by ABT-737. BIM appears to be the main BH3-only protein in these cell types,[86] where BH3 mimetic induction of cell death is dependent on BCL-2/BIM complex disruption. In contrast, BCL-X_L represents the key target in platelets, where BAK and BAD are likely to be the main pro-apoptotic proteins.[51] These observations underscore the importance of considering the precise nature of the molecular complexes, which is likely to be different in distinct cell types and tumors.

In breast cancer, the nature of the pre-existing complexes remains obscure. Although BCL-X_L is abundantly expressed in all breast tumors, ABT-199 appeared to be as effective as ABT-737 in combination with tamoxifen or mTOR inhibitors, suggesting that the neutralization of BCL-2 is sufficient to induce apoptosis. It is likely that this will differ between individual tumors and across tumor subtypes, where BCL-2 may also serve as a therapeutic target in TNBC[22] and HER2-positive tumors. In the case of the latter, resistance to trastuzumab in HER2/ER-positive tumor cells can be accompanied by elevated BCL-2, which is ameliorated by endocrine therapy.[87] The precise role of targeting BCL-2 and additional BCL-2 family members in breast cancer should become clearer through additional studies that include investigation of newer BH3 mimetics that selectively target BCL-X_L and MCL-1.

Conclusion and future directions

There is now accumulating evidence to indicate that BCL-2, which is overexpressed in the majority of ER-positive tumors, represents a *bona fide* therapeutic target. ABT-199 appeared to elicit a synergistic response when combined with tamoxifen in luminal B PDX models. In contrast to lymphoid malignancies (where BCL-2 is an oncogenic driver), monotherapy with a BCL-2 inhibitor is unlikely to be effective. Targeting two major survival pathways, BCL-2 and the PI3K/AKT/mTOR pathway (the latter associated with endocrine resistance to therapy) could also prove to be effective, although the optimal combination and scheduling of inhibitors remains to be determined. The recent promise shown by the CDK4/6 inhibitor palbociclib in ER-positive breast cancer[88] adds a further complexity on how to best investigate BH3 mimetics in pre-clinical models and in the clinic. The relative merits of combining a BH3 mimetic with tamoxifen versus an aromatase inhibitor or fulvestrant is not known and remains a critical question. Similarly, should BH3 mimetics be first tested in early (treatment naïve) or endocrine refractory disease? The precise mechanisms of tumor response and resistance to BH3 mimetics will also need to be investigated, since they are likely to extend beyond the PI3K/AKT/mTOR signaling pathway and could include MCL-1 upregulation.[89] These important questions are now potentially ripe for testing in clinic trials. The first study on this new class of drug in breast cancer, a phase 1b dose escalation study of the BCL-2 inhibitor venetoclax in combination with tamoxifen in metastatic ER-positive breast cancer ('m-BEP', ISRCTN98335443) will hopefully start to shed light on some of these questions. More broadly, the targeting of other solid tumors with BH3 mimetics represents a promising future challenge.

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Conflict of Interest

DM, JEV and GJL are employees of The Walter and Eliza Hall Institute, which receives commercial income and research funding from Genentech and AbbVie and also collaborates with and receives research funding from Servier. The Royal Melbourne Hospital (Melbourne Health) will receive research funds from AbbVie for an investigator-initiated study on ABT-199 in breast cancer (ISRCTN98335443) where GJL is lead investigator. GJL has served on an Advisory Board for AbbVie and Genentech.

References

1. Abdel-Fatah TM, Perry C, Dickinson P, Ball G, Moseley P, Madhusudan S *et al* (2013). Bcl2 is an independent prognostic marker of triple negative breast cancer (TNBC) and predicts response to anthracycline combination (ATC) chemotherapy (CT) in adjuvant and neoadjuvant settings. *Ann Oncol* **24:** 2801-2807.
2. Curtis C (2015). Genomic profiling of breast cancers. *Curr Opin Obstet Gynecol* **27:** 34-39.
3. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H *et al* (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA* **98:** 10869-10874.
4. Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ *et al* (2012). The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* **486:** 346-352.
5. Goldhirsch A, Winer EP, Coates AS, Gelber RD, Piccart-Gebhart M, Thurlimann B *et al* (2013). Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013. *Ann Oncol* **24:** 2206-2223.
6. Tsujimoto Y, Finger LR, Yunis J, Nowell PC, Croce CM (1984). Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. *Science* **226:** 1097-1099.
7. Bakhshi A, Jensen JP, Goldman P, Wright JJ, McBride OW, Epstein AL *et al* (1985). Cloning the chromosomal breakpoint of t(14;18) human lymphomas:

clustering around J_H on chromosome 14 and near a transcriptional unit on 18. *Cell* **41:** 899-906.

8. Vaux DL, Cory S, Adams JM (1988). Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* **335:** 440-442.
9. Strasser A, Harris AW, Bath ML, Cory S (1990). Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. *Nature* **348:** 331-333.
10. Nunez G, Hockenbery D, McDonnell TJ, Sorensen CM, Korsmeyer SJ (1991). Bcl-2 maintains B cell memory. *Nature* **353:** 71-73.
11. Czabotar PE, Lessene G, Strasser A, Adams JM (2014). Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. *Nat Rev Mol Cell Biol* **15:** 49-63.
12. Juin P, Geneste O, Gautier F, Depil S, Campone M (2013). Decoding and unlocking the BCL-2 dependency of cancer cells. *Nat Rev Cancer* **13:** 455-465.
13. Moldoveanu T, Follis AV, Kriwacki RW, Green DR (2014). Many players in BCL-2 family affairs. *Trends Biochem Sci* **39:** 101-111.
14. Hata AN, Engelman JA, Faber AC (2015). The BCL2 Family: Key Mediators of the Apoptotic Response to Targeted Anticancer Therapeutics. *Cancer Discov* **5:** 475-487.
15. Dawson SJ, Makretsov N, Blows FM, Driver KE, Provenzano E, Le Quesne J *et al* (2010). BCL2 in breast cancer: a favourable prognostic marker across molecular subtypes and independent of adjuvant therapy received. *Br J Cancer* **103:** 668-675.
16. Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M *et al* (2004). A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. *N Engl J Med* **351:** 2817-2826.
17. Paik S, Tang G, Shak S, Kim C, Baker J, Kim W *et al* (2006). Gene expression and benefit of chemotherapy in women with node-negative, estrogen receptor-positive breast cancer. *J Clin Oncol* **24:** 3726-3734.
18. Parker JS, Mullins M, Cheang MC, Leung S, Voduc D, Vickery T *et al* (2009). Supervised risk predictor of breast cancer based on intrinsic subtypes. *J Clin Oncol* **27:** 1160-1167.

19. Nielsen TO, Parker JS, Leung S, Voduc D, Ebbert M, Vickery T *et al* (2010). A comparison of PAM50 intrinsic subtyping with immunohistochemistry and clinical prognostic factors in tamoxifen-treated estrogen receptor-positive breast cancer. *Clin Cancer Res* **16:** 5222-5232.
20. Lindeman GJ, Visvader KE (2013). Targeting BCL-2 in breast cancer: Exploiting a tumor lifeline to deliver a mortal blow? *Breast Cancer Management* **2:** 1-4.
21. Roy S, Nicholson DW (2000). Cross-talk in cell death signaling. *J Exp Med* **192:** F21-25.
22. Oakes SR, Vaillant F, Lim E, Lee L, Breslin K, Feleppa F *et al* (2012). Sensitization of BCL-2-expressing breast tumors to chemotherapy by the BH3 mimetic ABT-737. *Proc Natl Acad Sci USA* **109:** 2766-2771.
23. Vaillant F, Merino D, Lee L, Breslin K, Pal B, Ritchie ME *et al* (2013). Targeting BCL-2 with the BH3 mimetic ABT-199 in estrogen receptor-positive breast cancer. *Cancer Cell* **24:** 120-129.
24. Freneaux P, Stoppa-Lyonnet D, Mouret E, Kambouchner M, Nicolas A, Zafrani B *et al* (2000). Low expression of bcl-2 in Brca1-associated breast cancers. *Br J Cancer* **83:** 1318-1322.
25. Adams JM, Cory S (2007). The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* **26:** 1324-1337.
26. Visco C, Tzankov A, Xu-Monette ZY, Miranda RN, Tai YC, Li Y *et al* (2013). Patients with diffuse large B-cell lymphoma of germinal center origin with BCL2 translocations have poor outcome, irrespective of MYC status: a report from an International DLBCL rituximab-CHOP Consortium Program Study. *Haematologica* **98:** 255-263.
27. Perry AM, Alvarado-Bernal Y, Laurini JA, Smith LM, Slack GW, Tan KL *et al* (2014). MYC and BCL2 protein expression predicts survival in patients with diffuse large B-cell lymphoma treated with rituximab. *Br J Haematol* **165:** 382-391.
28. Jager R, Herzer U, Schenkel J, Weiher H (1997). Overexpression of Bcl-2 inhibits alveolar cell apoptosis during involution and accelerates c-myc-induced tumorigenesis of the mammary gland in transgenic mice. *Oncogene* **15:** 1787-1795.

29. Furth PA, Bar-Peled U, Li M, Lewis A, Laucirica R, Jager R *et al* (1999). Loss of anti-mitotic effects of Bcl-2 with retention of anti-apoptotic activity during tumor progression in a mouse model. *Oncogene* **18:** 6589-6596.
30. Shibata MA, Liu ML, Knudson MC, Shibata E, Yoshidome K, Bandey T *et al* (1999). Haploid loss of bax leads to accelerated mammary tumor development in C3(1)/SV40-TAg transgenic mice: reduction in protective apoptotic response at the preneoplastic stage. *EMBO J* **18:** 2692-2701.
31. Jamerson MH, Johnson MD, Korsmeyer SJ, Furth PA, Dickson RB (2004). Bax regulates c-Myc-induced mammary tumour apoptosis but not proliferation in MMTV-c-myc transgenic mice. *Br J Cancer* **91:** 1372-1379.
32. del Bufalo D, Biroccio A, Leonetti C, Zupi G (1997). Bcl-2 overexpression enhances the metastatic potential of a human breast cancer line. *FASEB Journal* **11:** 947-953.
33. Wong CW, Lee A, Shientag L, Yu J, Dong Y, Kao G *et al* (2001). Apoptosis: an early event in metastatic inefficiency. *Cancer Res* **61:** 333-338.
34. Kelly GL, Grabow S, Glaser SP, Fitzsimmons L, Aubrey BJ, Okamoto T *et al* (2014). Targeting of MCL-1 kills MYC-driven mouse and human lymphomas even when they bear mutations in p53. *Genes Dev* **28:** 58-70.
35. Kerr DA, 2nd, Wittliff JL (2011). A five-gene model predicts clinical outcome in ER+/PR+, early-stage breast cancers treated with adjuvant tamoxifen. *Horm Cancer* **2:** 261-271.
36. Callagy GM, Webber MJ, Pharoah PD, Caldas C (2008). Meta-analysis confirms BCL2 is an independent prognostic marker in breast cancer. *BMC Cancer* **8:** 153.
37. Choi JE, Kang SH, Lee SJ, Bae YK (2014). Prognostic significance of Bcl-2 expression in non-basal triple-negative breast cancer patients treated with anthracycline-based chemotherapy. *Tumour Biol* **35:** 12255-12263.
38. Perillo B, Sasso A, Abbondanza C, Palumbo G (2000). 17beta-estradiol inhibits apoptosis in MCF-7 cells, inducing bcl-2 expression via two estrogen-responsive elements present in the coding sequence. *Mol Cell Biol* **20:** 2890-2901.
39. Pietenpol JA, Papadopoulos N, Markowitz S, Willson JK, Kinzler KW, Vogelstein B (1994). Paradoxical inhibition of solid tumor cell growth by bcl2. *Cancer Res* **54:** 3714-3717.

40. O'Reilly LA, Huang DC, Strasser A (1996). The cell death inhibitor Bcl-2 and its homologues influence control of cell cycle entry. *EMBO J* **15:** 6979-6990.
41. Elledge RM, Green S, Howes L, Clark GM, Berardo M, Allred DC *et al* (1997). bcl-2, p53, and response to tamoxifen in estrogen receptor-positive metastatic breast cancer: a Southwest Oncology Group study. *J Clin Oncol* **15:** 1916-1922.
42. Muranen T, Selfors LM, Worster DT, Iwanicki MP, Song L, Morales FC *et al* (2012). Inhibition of PI3K/mTOR leads to adaptive resistance in matrix-attached cancer cells. *Cancer Cell* **21:** 227-239.
43. Madjd Z, Mehrjerdi AZ, Sharifi AM, Molanaei S, Shahzadi SZ, Asadi-Lari M (2009). CD44+ cancer cells express higher levels of the anti-apoptotic protein Bcl-2 in breast tumours. *Cancer Immun* **9:** 4.
44. Merino D, Best SA, Asselin-Labat ML, Vaillant F, Pal B, Dickins RA *et al* (2014). Pro-apoptotic Bim suppresses breast tumor cell metastasis and is a target gene of SNAI2. *Oncogene* **Epub doi:10.1038/onc.2014.313**.
45. Smerage JB, Budd GT, Doyle GV, Brown M, Paoletti C, Muniz M *et al* (2013). Monitoring apoptosis and Bcl-2 on circulating tumor cells in patients with metastatic breast cancer. *Mol Oncol* **7:** 680-692.
46. Silvestrini R, Veneroni S, Daidone MG, Benini E, Boracchi P, Mezzetti M *et al* (1994). The Bcl-2 protein: a prognostic indicator strongly related to p53 protein in lymph node-negative breast cancer patients. *J Natl Cancer Inst* **86:** 499-504.
47. Binder C, Marx D, Overhoff R, Binder L, Schauer A, Hiddemann W (1995). Bcl-2 protein expression in breast cancer in relation to established prognostic factors and other clinicopathological variables. *Ann Oncol* **6:** 1005-1010.
48. Lipponen P (1999). Apoptosis in breast cancer: relationship with other pathological parameters. *Endocr Relat Cancer* **6:** 13-16.
49. Abdel-Fatah TM, Powe DG, Ball G, Lopez-Garcia MA, Habashy HO, Green AR *et al* (2010). Proposal for a modified grading system based on mitotic index and Bcl2 provides objective determination of clinical outcome for patients with breast cancer. *J Pathol* **222:** 388-399.
50. Ali HR, Dawson SJ, Blows FM, Provenzano E, Leung S, Nielsen T *et al* (2012). A Ki67/BCL2 index based on immunohistochemistry is highly prognostic in ER-positive breast cancer. *J Pathol* **226:** 97-107.

51. Merino D, Khaw SL, Glaser SP, Anderson DJ, Belmont LD, Wong C *et al* (2012). Bcl-2, Bcl-xL, and Bcl-w are not equivalent targets of ABT-737 and navitoclax (ABT-263) in lymphoid and leukemic cells. *Blood* **119:** 5807-5816.
52. Del Gaizo Moore V, Schlis KD, Sallan SE, Armstrong SA, Letai A (2008). BCL-2 dependence and ABT-737 sensitivity in acute lymphoblastic leukemia. *Blood* **111:** 2300-2309.
53. Certo M, Moore Vdel G, Nishino M, Wei G, Korsmeyer S, Armstrong SA *et al* (2006). Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell* **9:** 351-365.
54. Moulder SL, Symmans WF, Booser DJ, Madden TL, Lipsanen C, Yuan L *et al* (2008). Phase I/II study of G3139 (Bcl-2 antisense oligonucleotide) in combination with doxorubicin and docetaxel in breast cancer. *Clin Cancer Res* **14:** 7909-7916.
55. Rom J, von Minckwitz G, Marme F, Ataseven B, Kozian D, Sievert M *et al* (2009). Phase I study of apoptosis gene modulation with oblimersen within preoperative chemotherapy in patients with primary breast cancer. *Ann Oncol* **20:** 1829-1835.
56. Anderson MA, Huang D, Roberts A (2014). Targeting BCL2 for the treatment of lymphoid malignancies. *Semin Hematol* **51:** 219-227.
57. Baggstrom MQ, Qi Y, Koczywas M, Argiris A, Johnson EA, Millward MJ *et al* (2011). A phase II study of AT-101 (Gossypol) in chemotherapy-sensitive recurrent extensive-stage small cell lung cancer. *J Thorac Oncol* **6:** 1757-1760.
58. Langer CJ, Albert I, Ross HJ, Kovacs P, Blakely LJ, Pajkos G *et al* (2014). Randomized phase II study of carboplatin and etoposide with or without obatoclax mesylate in extensive-stage small cell lung cancer. *Lung Cancer* **85:** 420-428.
59. Ready N, Karaseva NA, Orlov SV, Luft AV, Popovych O, Holmlund JT *et al* (2011). Double-blind, placebo-controlled, randomized phase 2 study of the proapoptotic agent AT-101 plus docetaxel, in second-line non-small cell lung cancer. *J Thorac Oncol* **6:** 781-785.
60. Sonpavde G, Matveev V, Burke JM, Caton JR, Fleming MT, Hutson TE *et al* (2012). Randomized phase II trial of docetaxel plus prednisone in combination with placebo or AT-101, an oral small molecule Bcl-2 family antagonist, as

first-line therapy for metastatic castration-resistant prostate cancer. *Ann Oncol* **23:** 1803-1808.

61. Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA *et al* (2005). An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* **435:** 677-681.
62. Tse C, Shoemaker AR, Adickes J, Anderson MG, Chen J, Jin S *et al* (2008). ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. *Cancer Res* **68:** 3421-3428.
63. Souers AJ, Leverson JD, Boghaert ER, Ackler SL, Catron ND, Chen J *et al* (2013). ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. *Nat Med* **19:** 202-208.
64. Roberts AW, Seymour JF, Brown JR, Wierda WG, Kipps TJ, Khaw SL *et al* (2012). Substantial susceptibility of chronic lymphocytic leukemia to BCL2 inhibition: results of a phase I study of navitoclax in patients with relapsed or refractory disease. *J Clin Oncol* **30:** 488-496.
65. Seymour JF, Davids MS, Pagel JM, Kahl BS, Wierda WG, Miller TP (2013). Bcl-2 inhibitor ABT-199 (GDC-0199) monotherapy show anti-tumor activity including complete remissions in high-risk relapsed/refractory chornic lymphocytic leukemia and small lymphocytic lymphoma (abstract 7018). *Blood* **122:** 429s.
66. Mason KD, Carpinelli MR, Fletcher JI, Collinge JE, Hilton AA, Ellis S *et al* (2007). Programmed anuclear cell death delimits platelet life span. *Cell* **128:** 1173-1186.
67. Wagner KU, Claudio E, Rucker EB, 3rd, Riedlinger G, Broussard C, Schwartzberg PL *et al* (2000). Conditional deletion of the Bcl-x gene from erythroid cells results in hemolytic anemia and profound splenomegaly. *Development* **127:** 4949-4958.
68. Nemati F, de Montrion C, Lang G, Kraus-Berthier L, Carita G, Sastre-Garau X *et al* (2014). Targeting Bcl-2/Bcl-XL induces antitumor activity in uveal melanoma patient-derived xenografts. *PLoS One* **9:** e80836.
69. Tanaka Y, Aikawa K, Nishida G, Homma M, Sogabe S, Igaki S *et al* (2013). Discovery of potent Mcl-1/Bcl-xL dual inhibitors by using a hybridization strategy based on structural analysis of target proteins. *J Med Chem* **56:** 9635-9645.

70. Lessene G, Czabotar PE, Sleebs BE, Zobel K, Lowes KN, Adams JM *et al* (2013). Structure-guided design of a selective BCL-X(L) inhibitor. *Nat Chem Biol* **9:** 390-397.
71. Sleebs BE, Kersten WJ, Kulasegaram S, Nikolakopoulos G, Hatzis E, Moss RM *et al* (2013). Discovery of potent and selective benzothiazole hydrazone inhibitors of Bcl-XL. *J Med Chem* **56:** 5514-5540.
72. Tao ZF, Hasvold L, Wang L, Wang X, Petros AM, Park CH *et al* (2014). Discovery of a Potent and Selective BCL-XL Inhibitor with in Vivo Activity. *ACS Med Chem Lett* **5:** 1088-1093.
73. Leverson JD, Phillips DC, Mitten MJ, Boghaert ER, Diaz D, Tahir SK *et al* (2015). Exploiting selective BCL-2 family inhibitors to dissect cell survival dependencies and define improved strategies for cancer therapy. *Sci Transl Med* **7:** 279ra240.
74. Leverson JD, Zhang H, Chen J, Tahir SK, Phillips DC, Xue J *et al* (2015). Potent and selective small-molecule MCL-1 inhibitors demonstrate on-target cancer cell killing activity as single agents and in combination with ABT-263 (navitoclax). *Cell Death Dis* **6:** e1590.
75. Bruncko M, Wang L, Sheppard GS, Phillips DC, Tahir SK, Xue J *et al* (2015). Structure-Guided Design of a Series of MCL-1 Inhibitors with High Affinity and Selectivity. *J Med Chem* **58:** 2180-2194.
76. Friberg A, Vigil D, Zhao B, Daniels RN, Burke JP, Garcia-Barrantes PM *et al* (2013). Discovery of potent myeloid cell leukemia 1 (Mcl-1) inhibitors using fragment-based methods and structure-based design. *J Med Chem* **56:** 15-30.
77. Fang C, D'Souza B, Thompson CF, Clifton MC, Fairman JW, Fulroth B *et al* (2014). Single Diastereomer of a Macrolactam Core Binds Specifically to Myeloid Cell Leukemia 1 (MCL1). *ACS Med Chem Lett* **5:** 1308-1312.
78. Chen J, Jin S, Abraham V, Huang X, Liu B, Mitten MJ *et al* (2011). The Bcl-2/Bcl-X(L)/Bcl-w inhibitor, navitoclax, enhances the activity of chemotherapeutic agents in vitro and in vivo. *Mol Cancer Ther* **10:** 2340-2349.
79. Whittle J, Lewis MT, Lindeman GJ, Visvader JE (2015). Patient-derived xenograft models of breast cancer and their predictive power. *Breast Cancer Research* **17:** 17.
80. Cory AH, Edwards CC, Hall JG, Cory JG (2003). Inhibitors of diverse metabolic steps cause increased apoptosis in deoxyadenosine-resistant mouse

leukemia L1210 cells that lack p53 expression: convergence at caspase-3 activation. *Adv Enzyme Regul* **43:** 29-45.

81. Anderson MA, Tam C, Seymour JF, Bell A, Westerman DA, Juneja S *et al* (2013). Selective Bcl-2 Inhibition with ABT-199 Is Highly Active Against Chronic Lymphocytic Leukemia (CLL) Irrespective of TP53 Mutation or Dysfunction. *Presented at 55th American Society of Hematology Conference*.
82. Kirkegaard T, Witton CJ, McGlynn LM, Tovey SM, Dunne B, Lyon A *et al* (2005). AKT activation predicts outcome in breast cancer patients treated with tamoxifen. *J Pathol* **207:** 139-146.
83. Perez-Tenorio G, Stal O, Southeast Sweden Breast Cancer G (2002). Activation of AKT/PKB in breast cancer predicts a worse outcome among endocrine treated patients. *Br J Cancer* **86:** 540-545.
84. Baselga J, Campone M, Piccart M, Burris HA, 3rd, Rugo HS, Sahmoud T *et al* (2012). Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. *N Engl J Med* **366:** 520-529.
85. Rooswinkel RW, van de Kooij B, Verheij M, Borst J (2012). Bcl-2 is a better ABT-737 target than Bcl-xL or Bcl-w and only Noxa overcomes resistance mediated by Mcl-1, Bfl-1, or Bcl-B. *Cell Death Dis* **3:** e366.
86. Bouillet P, Metcalf D, Huang DC, Tarlinton DM, Kay TW, Kontgen F *et al* (1999). Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science* **286:** 1735-1738.
87. Wang YC, Morrison G, Gillihan R, Guo J, Ward RM, Fu X *et al* (2011). Different mechanisms for resistance to trastuzumab versus lapatinib in HER2-positive breast cancers--role of estrogen receptor and HER2 reactivation. *Breast Cancer Res* **13:** R121.
88. Finn RS, Crown JP, Lang I, Boer K, Bondarenko IM, Kulyk SO *et al* (2015). The cyclin-dependent kinase 4/6 inhibitor palbociclib in combination with letrozole versus letrozole alone as first-line treatment of oestrogen receptor-positive, HER2-negative, advanced breast cancer (PALOMA-1/TRIO-18): a randomised phase 2 study. *Lancet Oncol* **16:** 25-35.
89. Williams MM, Cook RS (2015). Bcl-2 family proteins in breast development and cancer: could Mcl-1 targeting overcome therapeutic resistance? *Oncotarget* **6:** 3519-3530.

90. Veis DJ, Sorenson CM, Shutter JR, Korsmeyer SJ (1993). Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell* **75:** 229-240.
91. Humphreys RC, Krajewska M, Krnacik S, Jaeger R, Weiher H, Krajewski S *et al* (1996). Apoptosis in the terminal endbud of the murine mammary gland: a mechanism of ductal morphogenesis. *Development* **122:** 4013-4022.
92. Murphy KL, Kittrell FS, Gay JP, Jager R, Medina D, Rosen JM (1999). Bcl-2 expression delays mammary tumor development in dimethylbenz(a)anthracene-treated transgenic mice. *Oncogene* **18:** 6597-6604.
93. Walton KD, Wagner KU, Rucker EB, 3rd, Shillingford JM, Miyoshi K, Hennighausen L (2001). Conditional deletion of the bcl-x gene from mouse mammary epithelium results in accelerated apoptosis during involution but does not compromise cell function during lactation. *Mech Dev* **109:** 281-293.
94. Print CG, Loveland KL, Gibson L, Meehan T, Stylianou A, Wreford N *et al* (1998). Apoptosis regulator bcl-w is essential for spermatogenesis but appears otherwise redundant. *Proc Natl Acad Sci USA* **95:** 12424-12431.
95. Fu NY, Rios AC, Pal B, Soetanto R, Lun AT, Liu K *et al* (2015). EGF-mediated induction of Mcl-1 at the switch to lactation is essential for alveolar cell survival. *Nat Cell Biol* **17:** 365-375.
96. Schorr K, Li M, Bar-Peled U, Lewis A, Heredia A, Lewis B *et al* (1999). Gain of Bcl-2 is more potent than bax loss in regulating mammary epithelial cell survival in vivo. *Cancer Res* **59:** 2541-2545.
97. Rucker EB, 3rd, Hale AN, Durtschi DC, Sakamoto K, Wagner KU (2011). Forced involution of the functionally differentiated mammary gland by overexpression of the pro-apoptotic protein bax. *Genesis* **49:** 24-35.
98. Mailleux AA, Overholtzer M, Schmelzle T, Bouillet P, Strasser A, Brugge JS (2007). BIM regulates apoptosis during mammary ductal morphogenesis, and its absence reveals alternative cell death mechanisms. *Dev Cell* **12:** 221-234.
99. Joensuu H, Pylkkanen L, Toikkanen S (1994). Bcl-2 protein expression and long-term survival in breast cancer. *Am J Pathol* **145:** 1191-1198.
100. Chang J, Clark GM, Allred DC, Mohsin S, Chamness G, Elledge RM (2003). Survival of patients with metastatic breast carcinoma: importance of prognostic markers of the primary tumor. *Cancer* **97:** 545-553.

101. Rolland P, Spendlove I, Madjd Z, Rakha EA, Patel P, Ellis IO *et al* (2007). The p53 positive Bcl-2 negative phenotype is an independent marker of prognosis in breast cancer. *Int J Cancer* **120:** 1311-1317.
102. Trere D, Montanaro L, Ceccarelli C, Barbieri S, Cavrini G, Santini D *et al* (2007). Prognostic relevance of a novel semiquantitative classification of Bcl2 immunohistochemical expression in human infiltrating ductal carcinomas of the breast. *Ann Oncol* **18:** 1004-1014.
103. Tawfik K, Kimler BF, Davis MK, Fan F, Tawfik O (2012). Prognostic significance of Bcl-2 in invasive mammary carcinomas: a comparative clinicopathologic study between "triple-negative" and non-"triple-negative" tumors. *Hum Pathol* **43:** 23-30.
104. Hwang KT, Woo JW, Shin HC, Kim HS, Ahn SK, Moon HG *et al* (2012). Prognostic influence of BCL2 expression in breast cancer. *Int J Cancer* **131:** E1109-1119.
105. Larsen MS, Bjerre K, Giobbie-Hurder A, Laenkholm AV, Henriksen KL, Ejlertsen B *et al* (2012). Prognostic value of Bcl-2 in two independent populations of estrogen receptor positive breast cancer patients treated with adjuvant endocrine therapy. *Acta Oncol* **51:** 781-789.
106. Kim HS, Park I, Cho HJ, Gwak G, Yang K, Bae BN *et al* (2012). Analysis of the potent prognostic factors in luminal-type breast cancer. *J Breast Cancer* **15:** 401-406.
107. Ermiah E, Buhmeida A, Khaled BR, Abdalla F, Salem N, Pyrhonen S *et al* (2013). Prognostic value of bcl-2 expression among women with breast cancer in Libya. *Tumour Biol* **34:** 1569-1578.
108. Bozovic-Spasojevic I, Ameye L, Paesmans M, Larsimont D, Di Leo A, Dolci S *et al* (2014). Prognostic, predictive abilities and concordance of BCL2 and TP53 protein expression in primary breast cancers and axillary lymph-nodes: a retrospective analysis of the Belgian three arm study evaluating anthracycline vs CMF adjuvant chemotherapy. *Breast* **23:** 473-481.
109. Kutuk O, Letai A (2008). Alteration of the mitochondrial apoptotic pathway is key to acquired paclitaxel resistance and can be reversed by ABT-737. *Cancer Res* **68:** 7985-7994.
110. Crawford A, Nahta R (2011). Targeting Bcl-2 in Herceptin-Resistant Breast Cancer Cell Lines. *Curr Pharmacogenomics Person Med* **9:** 184-190.

111. Zheng L, Yang W, Zhang C, Ding WJ, Zhu H, Lin NM *et al* (2011). GDC-0941 sensitizes breast cancer to ABT-737 in vitro and in vivo through promoting the degradation of Mcl-1. *Cancer Lett* **309:** 27-36.
112. Seveno C, Loussouarn D, Brechet S, Campone M, Juin P, Barille-Nion S (2012). gamma-Secretase inhibition promotes cell death, Noxa upregulation, and sensitization to BH3 mimetic ABT-737 in human breast cancer cells. *Breast Cancer Res* **14:** R96.
113. Li JY, Li YY, Jin W, Yang Q, Shao ZM, Tian XS (2012). ABT-737 reverses the acquired radioresistance of breast cancer cells by targeting Bcl-2 and Bcl-xL. *J Exp Clin Cancer Res* **31:** 102.
114. Yin S, Dong Y, Li J, Fan L, Wang L, Lu J *et al* (2012). Methylseleninic acid potentiates multiple types of cancer cells to ABT-737-induced apoptosis by targeting Mcl-1 and Bad. *Apoptosis* **17:** 388-399.

Table 1. Mouse models evaluating BCL-2 family members in mammary gland development and neoplasia

BCL-2 family member	Mouse model	Role in normal mammary gland development	Contribution to mammary tumorigenesis	Reference
Pro-survival genes:				
Bcl-2	Bcl-2$^{-/-}$	No mammary gland phenotype reported	-	Veis et al 1993[90]
	WAP-Bcl-2 transgene	Possible contribution to regulation of TEB apoptosis and structure.	-	Humphreys et al 1996[91]
	WAP-Bcl-2 transgene	Possible role in involution.	Insufficient to induce mammary tumors alone. Accelerated *c-myc* induced tumorigenesis.	Jäger et al 1997[28]
	WAP-Bcl-2 transgene and DMBA tumor induction	-	Protection following carcinogen treatment, possibly through anti-proliferative effect.	Murphy et al 1999[92]
	WAP-Bcl-2 x WAP-TAg transgene	-	Accelerated tumor onset.	Furth et al 1999[29]
Bcl-X_L	MMTV-cre;Bcl-x$^{f/f}$, WAP-cre;Bcl-x$^{f/f}$	Role in controlling apoptosis in the first stage of involution.	-	Walton et al 2001[93]
Bcl-w	Bcl-w$^{-/-}$	No mammary gland phenotype	-	Print et al 1998[94]
Mcl-1	MMTV-cre;Mcl-1$^{f/f}$, K5-cre;Mcl-1$^{f/f}$, WAPi-cre;Mcl-1$^{f/f}$, ROSA26-cre;Mcl-1$^{f/f}$	Required for morphogenesis during puberty and pregnancy and essential for sustaining lactation. Role in stem/progenitor cell function.	-	Fu et al 2015[95]
Pro-apoptotic effectors:				
Bax	Bax$^{-/-}$ x WAP-Bcl-2 transgene	Role during the first stage of involution, with delayed involution.	-	Schorr et al 1999[96]
	Bax$^{-/-}$, Bax$^{+/-}$ x C3(1)/SV40TAg transgene	Bax loss may play a role in branching, although a primary ovarian defect not excluded.	Accelerated tumor development.	Shibata et al 1999[30]
	Bax$^{+/-}$ x MMTV-Myc transgene	-	No significant acceleration in tumor onset but modest increase in tumor multiplicity.	Jamerson et al 2004[31]
	WAP-Bax transgene	Lactational defect.	-	Rucker et al 2011[97]
Pro-apoptotic sensors:				
Bim	Bim$^{-/-}$	Required for timely clearing of lumen in TEBs in puberty.	-	Mailleux et al 2007[98]
	Bim$^{-/-}$ x MMTV-PyMT transgene	-	Role in suppressing metastasis	Merino et al 2014[44]

Abbreviations: TEB, terminal endbud.

Table 2. Correlation of BCL-2 expression with clinical outcome in breast cancer

Tumor Stage	Sample Size	Histology	Outcome Measure	Bcl-2 expression an independent prognostic factor?	Bcl-2 predictive for treatment?	Reference
Early	174	Mixed	Overall survival	No	-	Joensuu et al, 1994[99]
Metastatic	205	ER+	Overall survival	Favorable; p=0.07	Bcl-2 predicted longer TTF with tamoxifen	Elledge et al, 1997[41]
Early	346	Mixed	Survival from first recurrence	Favorable; p=0.06	-	Chang et al, 2003[100]
Early	819	Mixed	Overall survival	No	-	Rolland et al, 2007[101]
Early	442	Mixed	Overall survival Disease free Survival	Favorable; p<0.001 Favorable; p<0.001	-	Trere et al, 2007[102]
Early	5,892	Mixed	Overall survival Disease free survival	Favorable; p=0.05 Favorable; p=0.07	-	Callagy et al, 2008[36]
Early	11,212	Mixed	Overall survival	Favorable; p<0.001	-	Dawson et al, 2010[15]
Early Early	124 458	TNBC Non-TNBC	Overall survival Overall survival	No Favorable; p=0.008	- -	Tawfik et al, 2012[103]
Early	7,230	Mixed	Overall survival Disease free survival	Favorable; p=0.001 Favorable; p=0.001	-	Hwang et al, 2012[104]
Early Early	257 1,191	ER+ ER+	Disease free survival Disease free survival	Favorable; p=0.001 Favorable; p=0.028	- -	Larsen et al, 2012[105]
Early	159	Luminal A & luminal B	Disease free survival	Favorable; p=0.034	-	Kim et al, 2012[106]
Early	170	Mixed	Overall survival	Favorable; p<0.0001	-	Ermiah et al, 2013[107]
Early and locally advanced	736	TNBC	Progression free survival Breast cancer specific survival	Favorable; p=0.0004 Favorable; p=0.006	Bcl-2 predicted benefit from anthracycline chemotherapy	Abdel-Fatah et al, 2013[1]
Early	428	Mixed	Overall survival	Favorable; p<0.001	Bcl-2 associated with benefit from CMF over high dose EC	Bozovic-Spasojevic et al, 2014[108]
Early Early Early	492 315 177	TNBC Basal TNBC Non-basal TNBC	Overall survival	No No Unfavorable; p=0.003	Bcl-2 predicted lack of benefit from adjuvant anthracycline chemotherapy	Choi et al, 2014[37]

Abbreviations: TNBC, Triple negative breast cancer; TTF, Time to treatment failure; CMF, Cyclophosphamide, Methotrexate and 5-Fluorouracil; EC, Epirubicin and Cisplatin.

Table 3. BH3 mimetic targeting of breast cancer cells

BH3 mimetic* (Target)	Breast cancer cell line	Combination therapy	Effect of combined therapy on BCL-2 proteins	Reference
ABT-737 (BCL-2, BCL-W and BCL-X_L)	MCF-7, MDA-MB-468	Paclitaxel	Increased BCL-2 (priming)	Kutuk and Letai, 2008[109]
ABT-737	BT474 (HER2$^+$) and BT474-trastuzumab resistant clones	Trastuzumab	Increased BCL-2	Crawford and Nahta, 2011[110]
ABT-737	MDA-MB-231	PI3 kinase inhibitor GDC-0941	Decreased MCL-1	Zheng et al, 2011[111]
ABT-737	MCF-7, BT549, MDA-MB-231, ZR75.1, T47D	γ-secretase inhibitor GSIXII	Noxa upregulation	Seveno et al, 2012[112]
ABT-737	MDA-MB-231, MDA-MB-231R	Radiation		Li et al, 2012[113]
ABT-737	MDA-MB-231	methylseleninic acid (MSeA)	Decreased MCL-1 and pBAD	Yin et al, 2012[114]
ABT-737	MCF-7.10A, MDA-MB-468, HCC-1569, T47-D and PDX models	mTOR inhibitors BEZ-235	Increased BCL-2 and BIM	Muranen et al, 2012[42]
ABT-737	MDA-MB-231 and PDX models	Docetaxel		Oakes et al, 2012[22]
ABT-263 Navitoclax (BCL-2, BCL-W and BCL-X_L)	MCF-7, MDA-MB-231	Camptothecin, Docetaxel, Etoposide, Rapamycin, Gemcitabine, Doxorubicin	Decreased MCL-1 and increased BIM	Chen et al, 2011[78]
GDC-0199/ ABT-199 Venetoclax (BCL-2)	MCF-7 and ER-positive PDX models	Tamoxifen and mTOR inhibitors	Increased BCL-2 and BIM (for tamoxifen) or pBAD (for mTOR inhibitors)	Vaillant et al, 2013[23]
A-1155463, A-1331852 (BCL-X_L)	28 breast cancer cell lines	Docetaxel		Leverson et al, 2015[73]
A-1210477 (MCL-1)	HCC-1806	-		Leverson et al, 2015[74]

* Does not include Obatoclax and Gossypol/AT-101. Abbreviation: PDX, patient-derived xenograft

Figure Legends

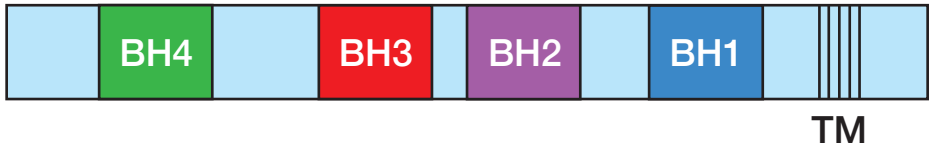
Figure 1. The BCL-2 family. BCL-2 family members are generally stratified in 3 groups according to their structure and function. The anti-apoptotic proteins (or pro-survival guardians) are characterised by four BCL-2 Homology (BH) domains. Pro-apoptotic proteins comprise effector and sensor proteins. The latter are also called 'BH3-only proteins', since they contain only the BH3 domain. TM, Transmembrane domain.

Figure 2. BCL-2 proteins are at the centre of the apoptotic cascade. (**a**) Apoptosis is regulated through a tripartite signaling cascade. The pro-survival guardians can bind to pro-apoptotic sensors, as well as the effectors BAX and BAK, preventing their activation. The pro-apoptotic sensors lie downstream of many stress-activated pathways, including p53. Once activated, they are able to activate BAX and BAK, either directly, or indirectly by sequestering the pro-survival proteins. BAX and BAK undergo conformational change and oligomerization forming pores in the mitochondria, resulting in caspase activation and cell death. BH3 mimetics mimic the BH3 of pro-apoptotic proteins. They bind the hydrophobic groove of the pro-survival proteins, preventing their binding to both apoptotic sensors and effectors. (**b**) Differential binding affinity of BH3 only proteins and BH3 mimetics. The BH3 domains of pro-apoptotic proteins bind to specific pro-survival proteins, shown here for BIM, BAD and NOXA. This specificity has been exploited in the development of BH3 mimetics. Navitoclax (ABT-263) is a 'BAD-like' mimetic that can bind BCL-2, BCL-X_L and BCL-W. Venetoclax (GDC-0199/ABT-199) is a specific and potent inhibitor of BCL-2. MOMP, mitochondrial outer membrane permeabilization; Cyt C, cytochrome C.

Figure 3. The combined inhibition of the BCL-2 and PI3K/AKT/mTOR survival pathways may offer a therapeutic benefit with endocrine therapy in ER-positive breast cancer. Both mTOR inhibitors and tamoxifen have been shown to increase the priming of cancer cells by modulating the levels of BCL-2 family members. PI3K/mTOR inhibitors and tamoxifen synergize with BH3 mimetics in the induction of apoptosis.

Pro-survival 'Guardians':

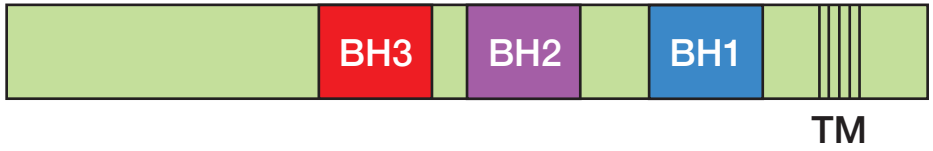
BCL-2, BCL-X_L, BCL-W,
A1, MCL-1, BCL-B



Pro-apoptotic proteins:

Apoptosis 'Effectors'

BAX, BAK



Pro-apoptotic 'Sensors'

BIM, BID, PUMA, NOXA,
BAD, BMF, HRK, BIK

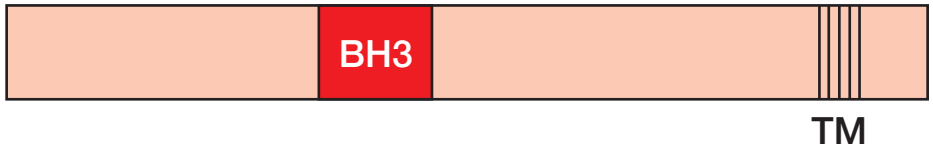


Figure 1

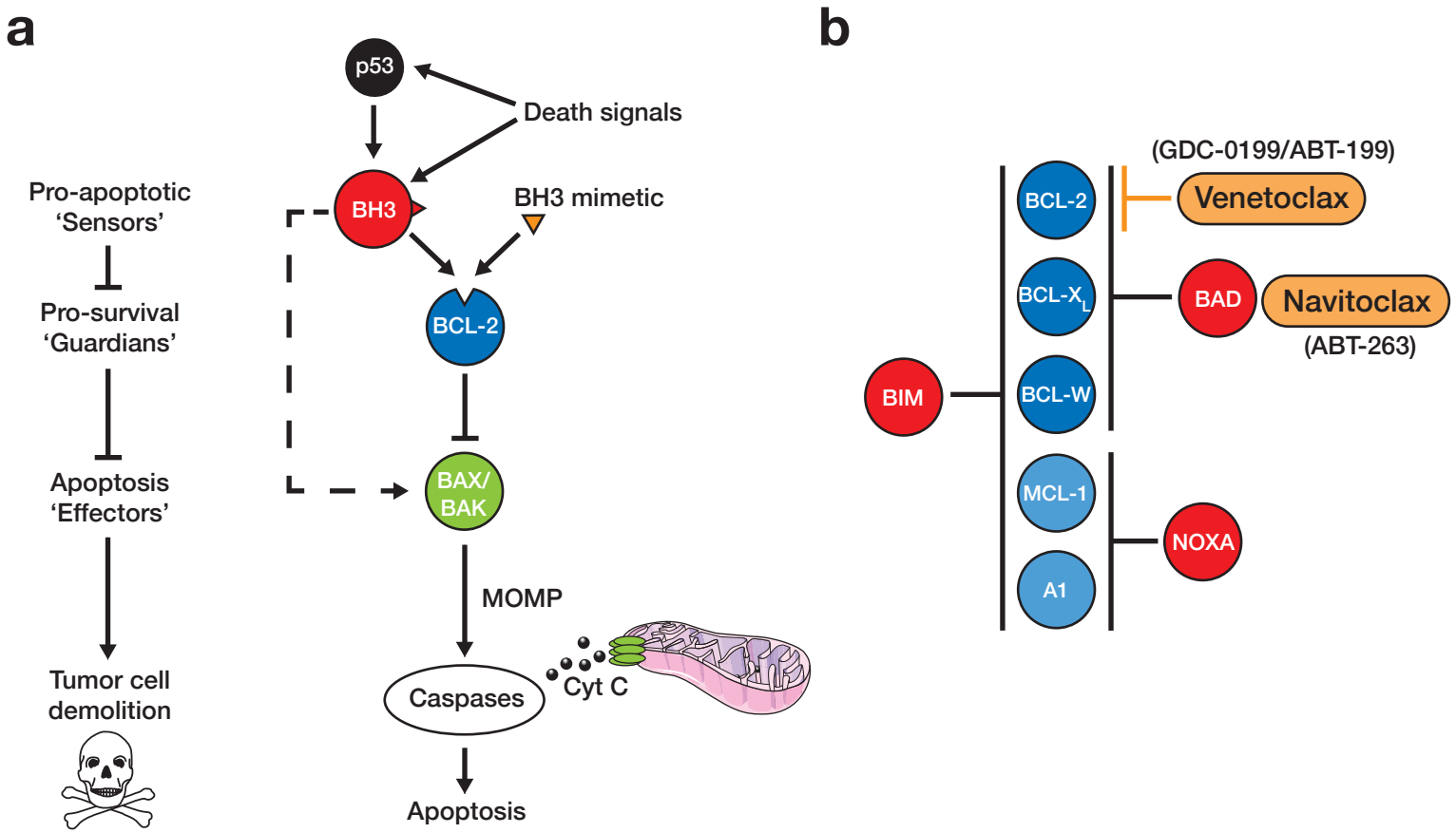

a
p53
Death signals
Pro-apoptotic
'Sensors'
BH3
BH3 mimetic
Pro-survival
'Guardians'
BCL-2
Apoptosis
'Effectors'
BAX/
BAK
MOMP
Tumor cell
demolition
Caspases
Cyt C
Apoptosis
b
(GDC-0199/ABT-199)
BCL-2
Venetoclax
BCL-X$_L$
BAD
Navitoclax
(ABT-263)
BIM
BCL-W
MCL-1
NOXA
A1

Figure 2

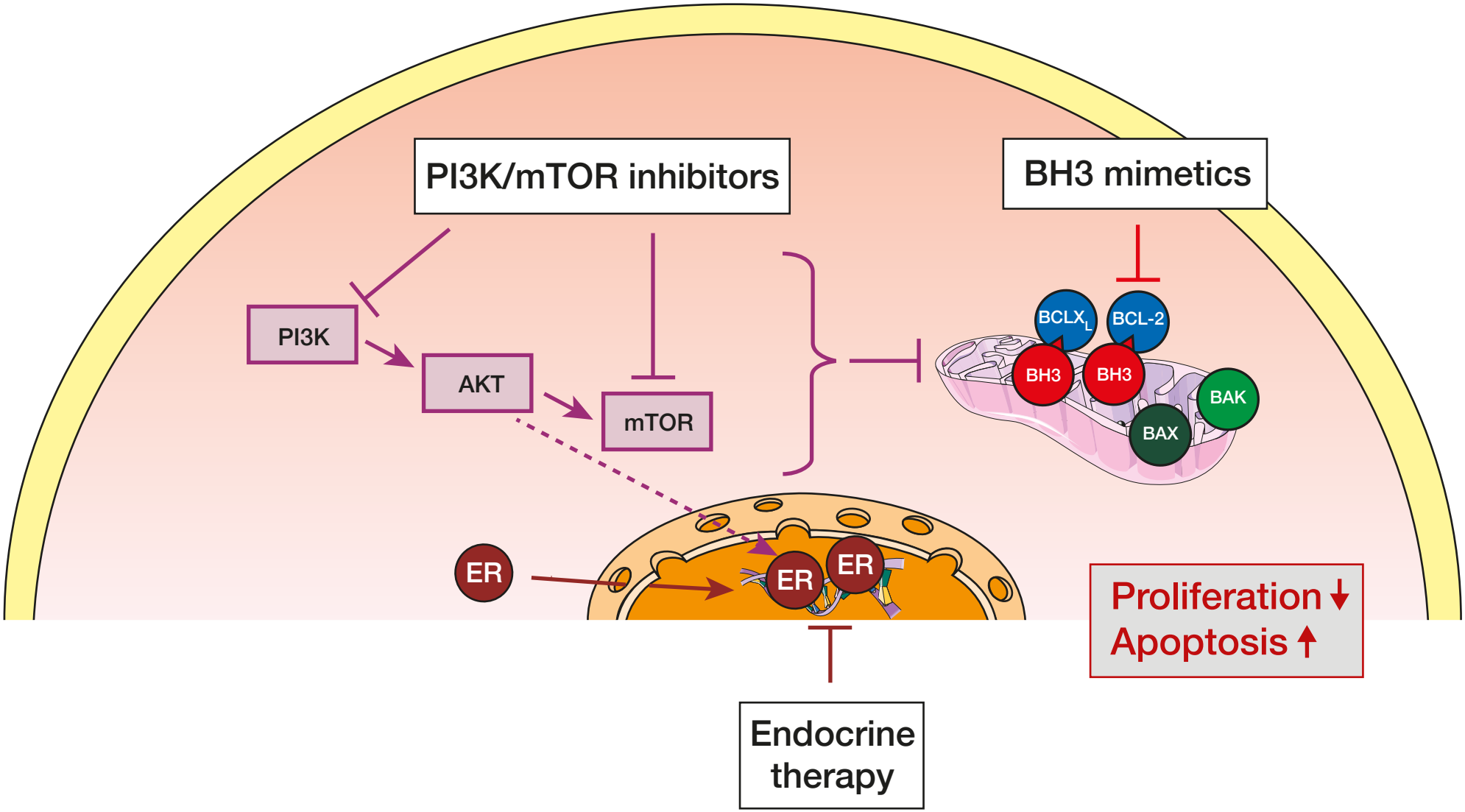

PI3K/mTOR inhibitors
BH3 mimetics
PI3K
AKT
mTOR
BCLX$_L$
BCL-2
BH3
BH3
BAK
BAX
ER
ER
ER
Proliferation ↓
Apoptosis ↑
Endocrine therapy

Figure 3