Sensitization of BCL-2–expressing breast tumors to chemotherapy by the BH3 mimetic ABT-737

Samantha R. Oakes[a,b,1], François Vaillant[a,b,1], Elgene Lim[a,2], Lily Lee[a,b], Kelsey Breslin[a], Frank Feleppa[c], Siddhartha Deb[d], Matthew E. Ritchie[a,b], Elena Takano[d], Teresa Ward[a], Stephen B. Fox[d], Daniele Generali[e], Gordon K. Smyth[a,f], Andreas Strasser[a,b], David C. S. Huang[a,b], Jane E. Visvader[a,b,1,3], and Geoffrey J. Lindeman[a,g,h,1,3]

[a]The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3052, Australia; [b]Department of Medical Biology, University of Melbourne, Parkville, Victoria 3010, Australia; [c]Department of Anatomical Pathology, Royal Melbourne Hospital, Parkville, Victoria 3050, Australia; [d]Peter MacCallum Cancer Centre, East Melbourne, Victoria 3002, Australia; [e]U.O. Multidisciplinare di Patologia Mammaria, Laboratorio di Oncologia Molecolare Senologica, AO. Istituti Ospitalieri di Cremona, Cremona 26100, Italy; [f]Department of Mathematics and Statistics, University of Melbourne, Parkville, Victoria 3010, Australia; [g]Department of Medical Oncology, Royal Melbourne Hospital, Parkville, Victoria 3050, Australia; and [h]Department of Medicine, University of Melbourne, Parkville, Victoria 3010, Australia

Edited by Kornelia Polyak, Dana-Farber Cancer Institute, Boston, MA, and accepted by the Editorial Board June 16, 2011 (received for review March 29, 2011)

Overexpression of the prosurvival protein BCL-2 is common in breast cancer. Here we have explored its role as a potential therapeutic target in this disease. BCL-2, its anti-apoptotic relatives MCL-1 and BCL-XL, and the proapoptotic BH3-only ligand BIM were found to be coexpressed at relatively high levels in a substantial proportion of heterogeneous breast tumors, including clinically aggressive basal-like cancers. To determine whether the BH3 mimetic ABT-737 that neutralizes BCL-2, BCL-XL, and BCL-W had potential efficacy in targeting BCL-2–expressing basal-like triple-negative tumors, we generated a panel of primary breast tumor xenografts in immunocompromised mice and treated recipients with either ABT-737, docetaxel, or a combination. Tumor response and overall survival were significantly improved by combination therapy, but only for tumor xenografts that expressed elevated levels of BCL-2. Treatment with ABT-737 alone was ineffective, suggesting that ABT-737 sensitizes the tumor cells to docetaxel. Combination therapy was accompanied by a marked increase in apoptosis and dissociation of BIM from BCL-2. Notably, BH3 mimetics also appeared effective in BCL-2–expressing xenograft lines that harbored p53 mutations. Our findings provide in vivo evidence that BH3 mimetics can be used to sensitize primary breast tumors to chemotherapy and further suggest that elevated BCL-2 expression constitutes a predictive response marker in breast cancer.

ABT-263 | navitoclax | programmed cell death | mammary | small molecule inhibitor

Basal-like tumors account for ~20% of breast cancers and usually exhibit a triple-negative phenotype, as they lack clinically significant expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2)/ErbB2, and express basal markers such as cytokeratin 5/6 and epidermal growth factor receptor (EGFR) (1, 2). The absence of ER, PR, and HER2 expression precludes endocrine or anti-HER2 therapy. Although a number of cytotoxic drugs (including the taxanes) are efficacious, prognosis remains significantly worse for basal-like triple-negative cancers than for other tumor subtypes (3, 4), highlighting the need for new therapeutic strategies.

Impairment of apoptosis is a hallmark of cancer and can result in resistance to chemotherapy (5). Tumor resistance to apoptosis is frequently acquired through deregulated expression of BCL-2 family members or mutations in the p53 tumor suppressor pathway that ablate the ability of this transcription factor to induce BH3-only proteins (such as PUMA and NOXA), which are critical for the initiation of apoptosis. There are three main classes of BCL-2 family regulators: the prosurvival BCL-2–like proteins; the proapoptotic BH3-only ligands, including BIM that interacts with all prosurvival proteins; and the proapoptotic multi-BH domain effector proteins, which activate caspases and lead to cell demolition (5).

BCL-2 has emerged as an important clinical prognostic marker in breast cancer (6, 7). *BCL-2* gene expression is a component of a 21-gene expression assay (Oncotype DX) that is increasingly being used to predict recurrence of hormone receptor-positive, node-negative breast cancers (8). Although BCL-2 is commonly associated with ER-positive breast tumors (9), it can also be expressed in ER-negative tumors. The frequency of BCL-2 expression in basal-like tumors has not been well-defined. Although BCL-2 is a favorable prognostic marker, it is noteworthy that a significant number of patients with BCL-2–positive disease relapse and die (7). A potential role for BCL-2 as a therapeutic target in breast cancer has not been explored.

The pivotal role of the BCL-2 family as arbiters of the intrinsic apoptotic pathway has stimulated considerable interest in developing anti-cancer agents that specifically act to induce apoptotic cell death. ABT-737, a small molecule that mimics the action of the proapoptotic BH3-only proteins, has been shown to bind and neutralize the prosurvival proteins BCL-2, BCL-XL and BCL-W but not MCL-1 or A1 (10). ABT-737 has demonstrated killing potency in combination settings in cell line-based models (11–17). Furthermore, the potency of ABT-737 with diverse chemotherapeutic agents has been shown in the case of primary leukemia cells and in small-cell lung cancer (SCLC) primary xenografts (18–22).

Here we report elevated expression of BCL-2 among the various subtypes of breast cancer, including basal-like tumors, and investigate the clinical relevance of targeting BCL-2 through the generation of a panel of primary breast tumor xenografts. Primary tumor xenografts can recapitulate the phenotype, biological properties, and drug sensitivity of the original primary tumor, thereby serving as powerful models for preclinical studies (23). These primary tumor xenografts offer many advantages over cell line-based xenografts, which do not consistently predict the clinical potential of drug treatments. Our studies reveal that ABT-737 potentiates the effects of docetaxel chemotherapy in basal-like breast cancers with elevated BCL-2 levels, suggesting that BH3 mimetics in combination therapy have considerable potential for the treatment of this aggressive cancer subtype and other BCL-2–expressing tumors.

Author contributions: S.R.O., F.V., E.L., J.E.V., and G.J.L. designed research; S.R.O., F.V., E.L., L.L., K.B., F.F., S.D., E.T., T.W., and S.B.F. performed research; D.G. contributed new reagents/analytic tools; S.R.O., F.V., E.L., L.L., S.D., M.E.R., S.B.F., G.K.S., A.S., D.C.S.H., J.E.V., and G.J.L. analyzed data; and S.R.O., J.E.V., and G.J.L. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. K.P. is a guest editor invited by the Editorial Board.

Data deposition: The sequence data presented in Fig. S2 has been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE28570).

[1]S.R.O., F.V., J.E.V., and G.J.L contributed equally to this work.

[2]Present address: Dana-Farber Cancer Institute, Boston, MA 02115.

[3]To whom correspondence may be addressed. E-mail: visvader@wehi.edu.au or lindeman@wehi.edu.au.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1104778108/-/DCSupplemental.

Results

BCL-2 Is Frequently Expressed at Elevated Levels with BIM, MCL-1, and BCL-XL in Breast Cancer. The expression of BCL-2, BIM, MCL-1, and BCL-XL was evaluated in a panel of 197 primary breast tumors that included 60 luminal, 65 basal-like, 24 HER2-positive, and 29 marker-null tumors, as defined by immunostaining for ER, PR, HER2, CK5/6, and EGFR (24). Luminal tumors were characterized by expression of the steroid hormone receptors ER and/or PR, basal-like tumors by their triple negative status for ER, PR, and HER2 and positivity for CK5/6 and EGFR, whereas HER2-positive tumors displayed intense membrane (3+) staining and/or HER2 amplification as determined by in situ hybridization. Marker-null tumors lacked expression of all markers. BCL-2 was expressed in a large proportion (83.3%) of luminal tumors, consistent with previous reports (7, 25, 26), and in 50.0 and 41.4% of HER2-positive and marker-null breast tumors, respectively. Within the basal-like subtype, 18.5% were found to express BCL-2.

BIM, MCL-1, and BCL-XL were widely expressed among the different breast tumor subtypes: 96.3, 94.4, and 100% in luminal tumors; 57.6, 89.8, and 93.4% in basal-like tumors; 95.7, 100, and 100% in HER2-positive tumors; and 65.5, 65.5, and 85.2% in marker-null tumors, respectively. Concurrent scoring of all four markers was feasible for 159 tumors (Table 1 and Fig. S1). Overall, 93.5, 94.7, and 96.2% of BCL-2–positive tumors expressed BIM, MCL-1, and BCL-XL, respectively: BIM and MCL-1 were almost invariably coexpressed with BCL-2 in luminal and HER2-positive tumors, whereas more than 75% of basal-like or marker-null BCL-2–positive tumors coexpressed these proteins. There were higher numbers of BCL-XL–positive tumors across all subgroups.

Establishment of Human Breast Cancer Xenografts That Recapitulate the Primary Tumor. A bank of human breast tumor xenografts was established by serial passage of primary breast tumor fragments in the cleared mammary fat pads of immunocompromised NOD-SCID-IL2R$\gamma_c^{-/-}$ mice. Approximately 25% (28 of 112) of primary tumors successfully engrafted, a frequency similar to that previously reported (23). The majority of these were derived from tumors designated as "triple negative." A schematic diagram of the strategy used to generate primary tumor xenografts is shown in Fig. 1*A*. In most cases, the xenografted tumors were passaged for two or three rounds.

Five breast-tumor xenograft lines (838T, 24T, 315T, 13T, and 806T) were selected for further analysis. Immunohistochemical analysis of the original patient tumors (Table S1 and Fig. S1) revealed that 315T contained foci of intense ER and PR staining (10% of cells), although 838T, 24T, 315T, and 13T had been reported as triple negative. The primary tumor 806T was HER2-positive, as determined by immunohistochemistry and the presence of HER amplification (11-fold) detected by chromogenic in situ hybridization (CISH), but lacked ER and PR expression.

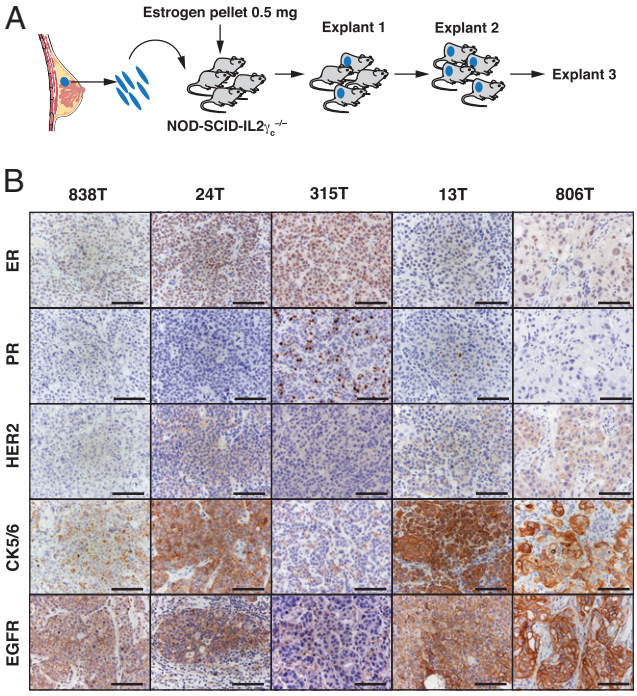

Fig. 1. The establishment of a panel of human breast tumor xenografts. (*A*) Schematic model of the derivation of human breast tumor xenograft models. Human breast tumor tissue was implanted into the mammary fat pads of immunocompromised NOD-SCID-IL2R$\gamma_c^{-/-}$ mice. Upon successful engraftment (explant 1), tumor tissue fragments were prepared and sequentially passaged in mice (explants 2 and 3). (*B*) Immunohistochemistry of ER, PR, HER2, cytokeratin 5/6 (CK5/6), and EGFR expression in breast tumor xenografts denoted 838T, 24T, 315T, 13T, and 806T. Expression of PR, HER2, CK5/6, and EGFR replicated that observed in the original patient tumors (Fig. S1), with the exception of 806T, in which the HER2-amplified fraction failed to passage. Low-level ER expression was detected in xenografts, possibly reflecting the use of estradiol pellets. (Scale bars, 100 μm.)

Weak granular staining for HER2 was also evident in a small percentage of cells within the 13T and 24T tumors, although HER2 was not amplified. Notably, all five primary tumors expressed CK5/6, and all except 315T were strongly positive for EGFR. Furthermore, gene expression profiling of primary tumor xenograft mRNA confirmed the basal-like nature of 838T, 24T, 806T, and 13T and revealed that 315T had a luminal B-like molecular signature (Fig. S2).

Tumor morphology and marker expression were largely maintained in the xenograft models although some differences were apparent (Fig. 1*B* and Fig. S1). The cellular profile of each

Table 1. Coexpression of BCL-2, BIM, MCL-1, and BCL-XL in primary breast cancer subtypes

Breast cancer subtype (no.)	BCL-2 expression	No. of samples (%)	BCL-2 histoscore	% BIM positive (histoscore)	% MCL-1 positive (histoscore)	% BCL-XL positive (histoscore)
Luminal (54)	+	45 (83.3)	6.89	100 (6.73)	97.7 (5.98)	100 (8.74)
	–	9 (16.7)		88.9 (4.78)	88.9 (6.11)	100 (6.89)
HER2 (22)	+	11 (50.0)	5.09	100 (5.09)	100 (5.00)	100 (8.60)
	–	11 (50.0)		90.0 (5.30)	100 (6.55)	100 (8.20)
Basal-like (54)	+	10 (18.5)	3.70	77.8 (4.78)	88.9 (5.00)	100 (6.00)
	–	44 (81.5)		58.1 (2.09)	92.9 (4.38)	89.5 (6.29)
Marker-null (29)	+	12 (41.4)	5.00	75.0 (2.92)	83.3 (3.17)	83.3 (4.17)
	–	17 (58.6)		58.8 (3.65)	52.9 (2.82)	86.7 (4.80)
All tumors (159)	+	78 (49.1)	5.96	93.5 (5.68)	94.7 (5.28)	96.2 (7.49)
	–	81 (50.9)		65.8 (3.14)	84.8 (4.54)	92.1 (6.17)

Of 197 tumor samples in the tissue microarrays, scoring of all four markers was feasible for 159 tumors. For BCL-2 expression: (+) positive, (–) negative. The numbers in parentheses for BIM, MCL-1, and BCL-XL are the mean histoscores. Scoring of samples is described in *SI Materials and Methods*.

xenograft was similar to that of the corresponding primary patient sample, as determined by flow cytometric analysis using antibodies directed against CD49f (α6-integrin) and EpCAM (CD326/epithelial specific antigen) (27) (Figs. S1 and S3). Weak nuclear ER staining, however, was detected in four of the xenografted tumors, with highest levels evident in the 315T xenograft line. These findings may reflect the use of estradiol pellets in the host mice, perhaps leading to the selection of ER-positive tumor cells or slight activation of ER itself. Substantially lower levels of HER2 were observed in the 806T primary tumor xenograft compared with the original tumor. Moreover, CISH for HER2 gene amplification did not reveal any amplification, indicating that selection had occurred against HER2-amplified cells during xeno-transplantation.

Variable BCL-2 and p53 Expression in Breast Tumor Xenografts. We next investigated the expression profile of BCL-2 family members and p53 among the different breast cancer xenografts by Western blot analysis (Fig. 2*A*). BCL-2, BIM_{EL} BIM_L, and BIM_S were found to be differentially expressed, with barely detectable BCL-2 in the 806T tumor xenografts. NOXA and PUMA, the two BH3-only proteins that are transcriptionally up-regulated by p53, were also variably expressed. MCL-1, BCL-XL, and the apoptotic effectors BAX and BAK were present in all of the tumor xenografts. Consistent with the Western blot data and their quantification, immunostaining of the parental primary tumors revealed strong BCL-2 expression in the 838T and 24T tumors, focal staining in 13T and 315T tumors, and undetectable expression in the 806T tumor xenografts (Fig. 2*B*).

High levels of the tumor suppressor p53 were detected in the 838T tumor xenograft by Western blot analysis and moderate levels in the 24T and 315T xenograft lines (Fig. 2*A*). Sequencing of tumor genomic DNA revealed pathogenic mutations in the *p53* gene in tumors 838T (c.581T > G, 194Leu > Arg, exon 6) and 24T (c.404T > G, 135Cys > Phe, exon 5), with loss of heterozygosity for both mutations. The p53 pathway is therefore disabled in the 838T and 24T basal-like tumor xenograft models.

Combination Therapy Induces Stable Regression of Breast Cancer. Because three of the five xenografts displayed high levels of BCL-2, we examined whether the BH3 mimetic ABT-737, either alone or in combination with docetaxel, was efficacious in killing BCL-2–positive breast tumors. A dose titration was first performed on mice bearing 838T and 13T tumors using 50 or 75 mg/kg ABT-737 with 0, 5, 10, or 20 mg/kg docetaxel to establish dose-limiting toxicity. This was determined by >10% weight loss following therapy and the extent of ABT-737–induced thrombocytopenia (28). On the basis of these studies, ABT-737 at 50 mg/kg and docetaxel at 10 mg/kg were selected as suitable doses for tumor response studies. This dosage induced tolerable levels of thrombocytopenia (Table S2 and Fig. S4). Notably, docetaxel alone was efficacious in producing a tumor response in the 838T tumor xenografts (median survival was 76 versus 10 d for control vehicle-treated animals; $P < 0.0001$) but had little effect in other models (Fig. 3).

ABT-737 administered in combination with docetaxel resulted in significantly improved animal survival in four of the five tumor xenograft models compared with treatment with docetaxel alone. For the models in which little effect was observed with a single cycle of chemotherapy, no further cycles were administered. The most profound response was observed in mice bearing the basal-like tumor xenografts 838T and 24T, in which tumor growth was dramatically inhibited by the combination treatment (Fig. 3*A*) and animal survival was prolonged (Fig. 3*B*). A partial response was evident in mice bearing the basal-like xenograft 13T and the luminal 315T tumor xenograft. Combination therapy had little effect on the 806T tumor xenograft, which expresses virtually undetectable levels of BCL-2 (Figs. 2 and 3). BIM was expressed in all tumor xenografts with high levels present in the most responsive models. ABT-737 as a single agent was ineffective in all five xenograft models. Thus, the combination of ABT-737 with docetaxel proved to be efficacious in eliciting a tumor response and prolonging animal survival in the case of BCL-2–expressing breast cancers.

Given that BCL-2 has been reported to be an estrogen-responsive gene, tumor response was further evaluated in the 838T and 24T xenografts passaged in the presence or absence of estrogen pellets. As noted above, the presence of an estrogen pellet appeared to induce a low level of ER expression in 838T and 24T xenografted tumors. However, BCL-2 levels did not change according to Western blot analysis (Fig. S5), and no significant difference in the rate of tumor growth was observed over several weeks, irrespective of whether an estrogen pellet was present.

To investigate the durability of the therapeutic response, the 838T tumor xenograft model was evaluated for the acquisition of resistance over six cycles. Estrogen pellets were not implanted into mice for these extended experiments as mice succumbed to renal damage, bladder stone formation, and uterine enlargement with chronic estrogen exposure, as reported previously (29). Treatment of mice bearing 838T xenografts with docetaxel alone showed early recurrence, whereas tumors subjected to combination therapy remained undetectable until ~75 d, at which time 8 of 10 tumors resumed growth. Five tumors were re-treated for a further three cycles, and regression was again evident in four cases (Fig. S6*A*). Tumor regression was also evident in mice bearing 24T tumor xenografts over two cycles of combination therapy until the end point at 70 d (Fig. 3).

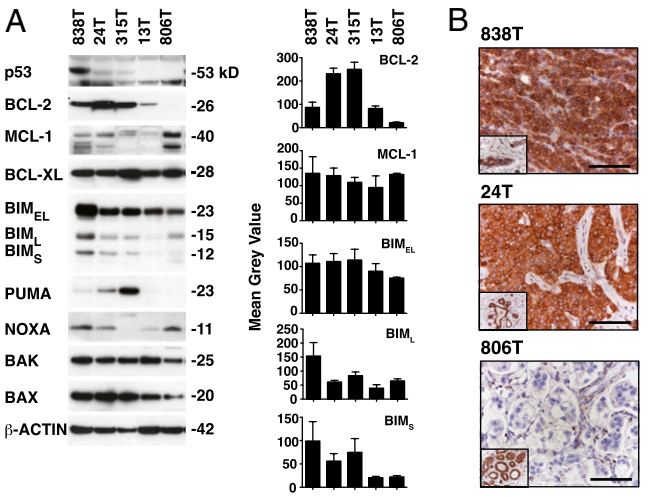

Fig. 2. Variable expression of BCL-2 family members in breast tumor xenografts. (*A*) Western blot analysis of p53, BCL-2, MCL-1, BCL-XL, BIM extra long (EL), long (L) and short (S), PUMA, NOXA, BAK, and BAX in five breast tumor xenografts: 838T, 24T, 315T, 13T, and 806T (*Left*). Probing for β-ACTIN was used as a loading control. Densitometry of three independent tumors for each xenograft line revealed variation in the levels of BCL-2 and BIM expression (*Right*). (*B*) Immunohistochemistry of BCL-2 expression in primary patient breast tumors: 838T, 24T, and 806T. (Scale bar, 100 μm.) (*Insets*) Adjacent normal tissue.

Increased Apoptosis Accompanies the Response to Combination Therapy. To investigate the extent and kinetics of apoptosis during therapy, mice bearing 838T xenografts were treated with ABT-737, docetaxel, or both drugs, and tumors were analyzed at 24 h, 48 h, and 8 d posttreatment (Fig. S6*B*). Immunohistochemistry revealed increased numbers of cleaved (i.e., activated) caspase-3 apoptotic foci in tumors treated with docetaxel alone or combined therapy compared with administration of ABT-737 alone or vehicle at 24 and 48 h postinjection [$P < 0.001$ ($n = 3$) and $P < 0.05$ ($n = 2$), respectively]. Following combination therapy for 8 d, few apoptotic foci were visible, consistent with tumor regression. Similar results were observed upon treatment of mice bearing 24T tumor xenografts with combination therapy (Fig. 4). Docetaxel treatment of 838T and 24T tumor xenografts exerted intermediate effects compared with combination therapy ($P = 0.22$ and $P < 0.01$, respectively). No difference in the numbers of apoptotic cells was apparent for the 315T or 806T tumor xenograft

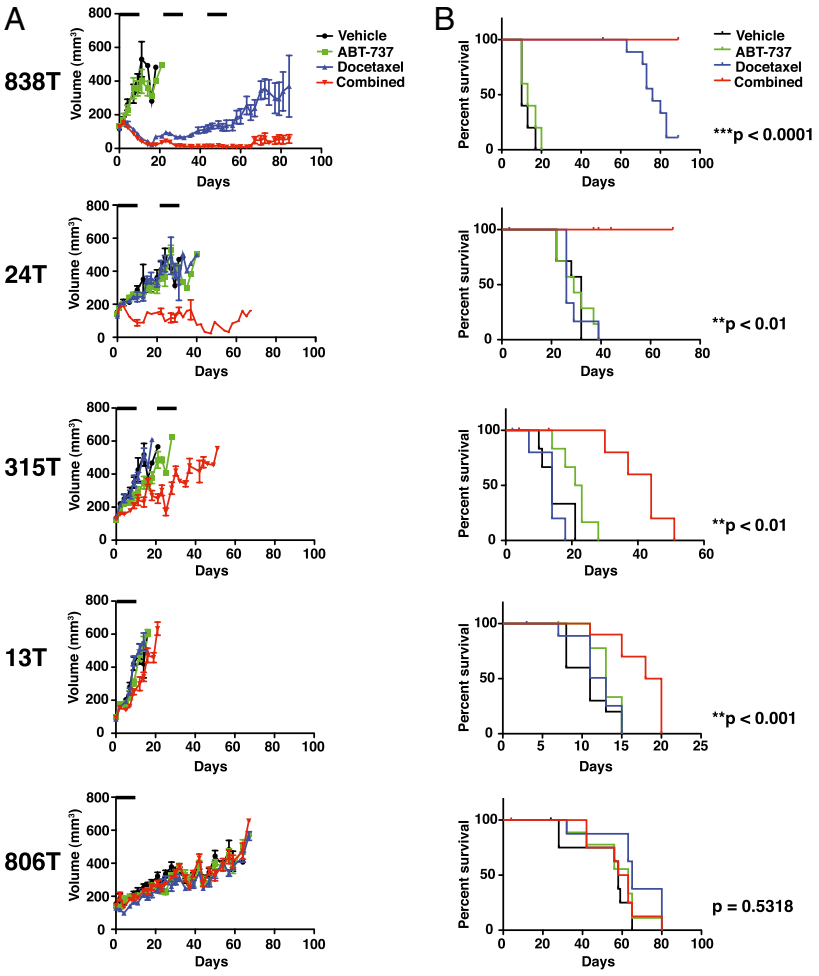

Fig. 3. ABT-737 combined with docetaxel results in decreased tumor growth and increased survival of mice bearing BCL-2–expressing breast tumor xenografts. (*A*) Tumor growth curves and (*B*) Kaplan–Meier survival curves of mice bearing 838T, 24T, 315T, 13T, and 806T human breast tumor xenografts treated with vehicle alone (black line), ABT-737 (50 mg/kg) plus vehicle for docetaxel (green line), docetaxel (10 mg/kg) plus vehicle for ABT-737 (blue line), or combined ABT-737 (50 mg/kg) and docetaxel (10 mg/kg, red line). Estrogen pellets were included for all experiments except those shown for 838T; no difference in tumor growth or animal survival was observed for 838T in the presence or absence of estrogen pellets. Log-rank (Mantel–Cox) *P* values are displayed for combination therapy versus vehicle. Black bars in *A* indicate a treatment cycle of ABT-737 (days 1–10) and docetaxel (day 1).

models in response to combination therapy relative to vehicle ($P = 0.21$ and $P = 0.97$, respectively). Thus, the extent of apoptosis correlated with response efficacy as measured by tumor growth rate and animal survival (Figs. 3 and 4).

Western blot analysis of BCL-2, MCL-1, BCL-XL, BIM, PUMA, NOXA, BAK, and BAX expression in the tumor xenograft models did not reveal any significant differences following the various treatments (Fig. 4*C*). However, coimmunoprecipitation coupled with Western blot analysis revealed a rapid reduction in the amount of BIM bound to BCL-2 following exposure to docetaxel, ABT-737, or combination therapy in the most responsive tumor xenograft line 838T (Fig. 4*D*). A decrease in BCL-2–associated BIM was also observed in 24T tumor xenografts following treatment with ABT-737 or combination therapy, indicating that perturbation of BIM protein complexes in these breast tumors may contribute to activation of the intrinsic apoptotic cascade. Moreover, treatment of the breast cancer cell line MDA-MB-231, which responded synergistically to combination therapy, clearly implicated changes in the BCL-2/BIM complex as a key molecular mechanism underlying the response, whereas no change was observed in BCL-XL/BIM complexes (Fig. 4*E*). Knockdown of either BCL-2 or BIM in MDA-MB-231 cells by siRNAs confirmed the importance of these proteins in mediating responsiveness to combination therapy (Fig. S7).

Discussion

Here we have established primary breast tumor xenograft models, including basal-like cancers, that recapitulate the original patient tumors. Expression analysis revealed that BCL-2 protein expression was highly variable among this panel of breast tumor xenografts, thus providing a platform to test the efficacy of the BH3 mimetic ABT-737 in BCL-2–expressing breast cancers. We demonstrate here that tumors expressing high BCL-2 respond effectively to ABT-737 in combination with docetaxel chemotherapy, whereas the tumor xenograft model expressing very low BCL-2 levels showed no response. BIM also appeared to be more abundant in the responsive tumor xenografts. Conversely, BCL-XL, another reported target of ABT-737, was expressed in both responsive and nonresponsive tumors. Notably, significant inhibition of tumor growth was evident in three basal-like tumor xenograft lines. Of these, a profound response was observed in two tumor xenograft lines (838T and 24T) and partial responses in two further tumor xenograft lines derived from basal-like and luminal cancers. Although ABT-737 has demonstrated single-agent efficacy in some cancers (predominantly cell lines), including follicular lymphoma and chronic lymphocytic leukemia (CLL) (10, 30–32), ABT-737 was not effective as a single agent for any of the primary breast tumor xenograft models tested.

The mechanism by which ABT-737 synergizes with docetaxel to cause tumor regression is yet to be established but is likely to involve destabilization of interactions between BIM, BCL-2 and MCL-1. Combined treatment with docetaxel and ABT-737, which competitively binds the hydrophobic groove of BCL-2, BCL-XL, and BCL-W (but not MCL-1 or A1), led to release of BIM from BCL-2. These findings are consistent with the recent observation that paclitaxel-induced killing of breast cancer cells in vitro is associated with competitive displacement of BIM from anti-apoptotic proteins by BH3-only proteins or ABT-737 (33). In lymphoma cell lines, ABT-737 responsiveness and BCL-2 dependence correlated with high levels of BIM sequestered by BCL-2 (34), and in CLL cells from patients, BCL-2 complexed to BIM proved to be the pivotal target for ABT-737 (31). Fur-

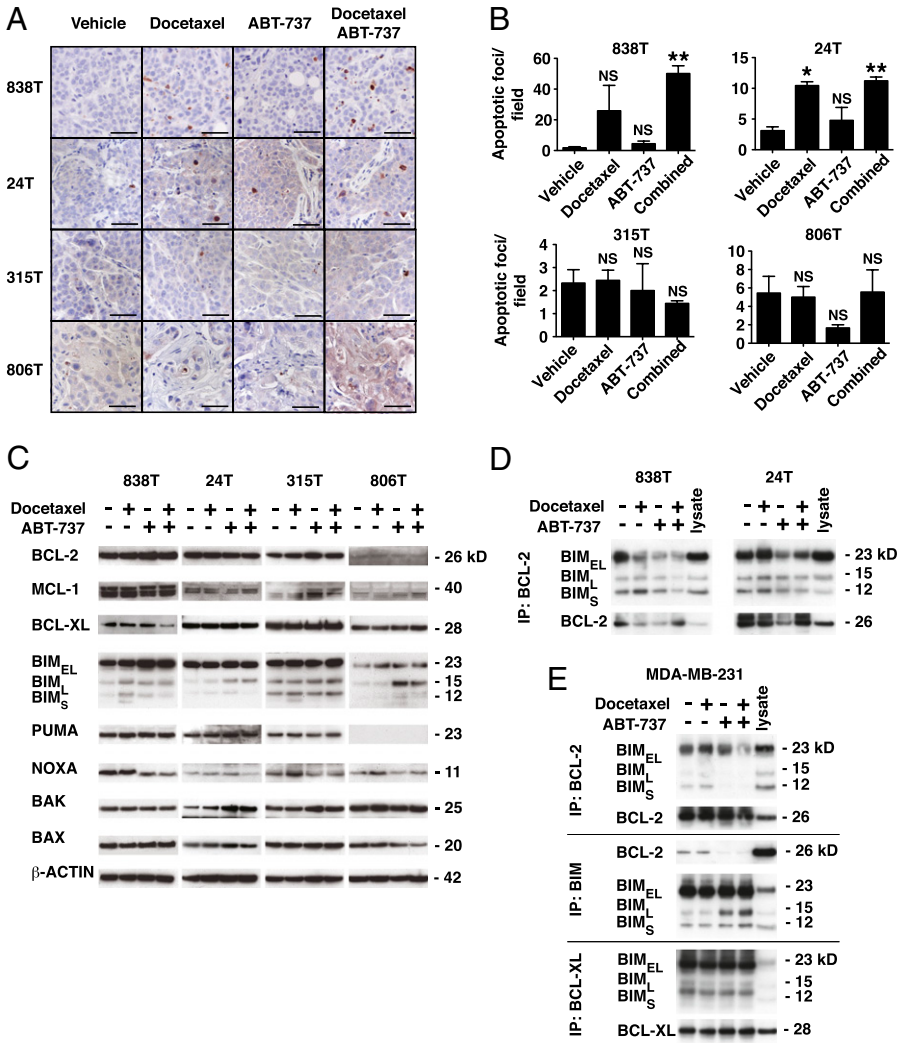

Fig. 4. Combination therapy results in increased apoptosis and dissociation of BIM from BCL-2. (*A*) Immunohistochemical staining for cleaved caspase-3 of 838T, 24T, 315T, and 806T xenografts treated with vehicle alone, ABT-737 (50 mg/kg) plus vehicle for docetaxel, docetaxel (10 mg/kg) plus vehicle for ABT-737, or combined ABT-737 (50 mg/kg) and docetaxel (10 mg/kg) collected at 24 h. (Scale bar, 50 μm.) (*B*) Analysis of the number of cleaved caspase-3–positive apoptotic foci [$*P < 0.01$, $**P < 0.001$, and NS (not significant) of each column mean compared with vehicle]. (*C*) Western blot analysis of BCL-2, MCL-1, BCL-XL, BIM extra long (EL), long (L) and short (S), PUMA, NOXA, BAK, and BAX in 838T, 24T, 315T, and 806T tumor xenografts collected at 24 h after combined treatment with ABT-737 and docetaxel. Probing for β-ACTIN was used as a loading control. (*D*) BCL-2 immunoprecipitates from 838T and 24T tumor lysates collected 24 h posttreatment with ABT-737 and/or docetaxel were analyzed by Western blotting using antibodies against BIM and BCL-2. (*E*) BCL-2, BIM, and BCL-XL immunoprecipitates from MDA-MB-231 cells collected 24 h posttreatment with ABT-737 and/or docetaxel and analyzed by Western blotting using antibodies against BIM, BCL-2, or BCL-XL.

thermore, only cells dependent on BH3-activator proteins bound to BCL-2 but not MCL-1 were found to be sensitive to ABT-737–induced killing. Thus, in primary breast cancers that express elevated BCL-2 levels, chemo-sensitization by ABT-737 may require BIM occupation of BCL-2 to prime cancer cells for death. Interestingly, acquired resistance in SCLC xenografts was linked to decreased expression of BCL-2, BAX, and BIM, and a perturbation of BCL-2–BIM complexes (18). Although MCL-1 expression in the breast cancer xenograft models did not correlate with responsiveness, a role for MCL-1 cannot be excluded.

The p53 status of basal-like breast cancers may also impact on sensitivity to a BH3 mimetic and chemotherapy, as the most responsive of the human breast tumor xenograft lines harbored p53 mutations. Indeed, p53 mutations in breast tumors have been linked with responsiveness to paclitaxel (35), although an in silico analysis comparing changes in gene expression with clinical drug response did not find an association between p53 status and chemoresponsiveness (36). Notably, it has been speculated that ABT-737 is more effective in tumors harboring p53 mutations, because ABT-737 acts downstream of p53 where an intact apoptotic cascade is present (37). A key point to emerge from this study is that combination therapy using BH3 mimetics can be very effective in tumors harboring p53 mutations.

Our data reveal that BCL-2 expression in breast cancer may serve as a predictive biomarker for responsiveness to ABT-737 combined with docetaxel chemotherapy. Interestingly, BCL-2 was found to be highly expressed in ∼20% of basal-like primary breast cancers and was often associated with abundant BIM. Expression of these proteins may identify a subset of breast cancer patients who are likely to benefit most from treatment with the orally bioavailable BH3 mimetic ABT-263 (Navitoclax). BCL-XL and MCL-1 levels may also contribute to modulating the response to ABT-737, as exemplified by their roles in other tumor types. Similar to ABT-737, ABT-263 can induce complete tumor regression in certain xenograft models, either as a single agent or combined with clinically relevant drugs (38), and is currently undergoing several phase I/II clinical trials.

In summary, we have developed preclinical models of primary breast cancer, including basal-like and luminal tumor xenografts, and provide in vivo evidence that ABT-737 can be used to sensitize primary breast tumors to taxane therapy. Combination therapy resulted in more durable responses and improved overall survival. Our results provide a rationale for the development of clinical protocols evaluating ABT-263 as an adjunct to conventional chemotherapy in BCL-2–expressing basal-like and luminal breast cancers.

Materials and Methods

Detailed methods are described in *SI Materials and Methods*.

Human Xenograft Establishment. Mice were anesthetized with ketamine/xylazine (200 mg/kg)/(20 mg/kg) i.p., and analgesia was administered with carprofen (5 mg/kg) subcutaneously. Human breast tumor fragments (0.5–1 mm × 0.5–1 mm × 5–8 mm) were inserted into inguinal mammary fat pads of 3- to 4-wk-old NOD-SCID-IL2Rγc$^{-/-}$ female mice. Silastic estrogen pellets (0.5 mg) were prepared as previously described (39) and implanted subcutaneously at the time of surgery. Following engraftment, tumors were

minced and digested in 150 U/mL collagenase (Sigma) and 50 U/mL hyaluronidase (Sigma) for 1–1.5 h at 37 °C. The resulting organoid suspension was sequentially digested with 0.25% trypsin 1 mM EGTA and 5 mg/mL dispase (Roche Diagnostics) for 1 min at 37 °C. A single-cell suspension was obtained by filtration (40 μm), and, where required, red blood cells were removed by lysis. Cell sorting is described in *SI Materials and Methods*.

Immunohistochemistry. Human breast cancer xenografts were collected and fixed in 4% paraformaldehyde before embedding in paraffin. Sections were subjected to antigen retrieval and then incubated with antibodies against ER (Novocastra), PR (Novocastra), HER2 (Dako), cytokeratin 5/6 (Dako), EGFR (EGFR.25, Novocastra), BCL-2 (BCL-2–100; Alexis/Enzo), BIM (3C5; Alexis/Enzo), MCL-1 (Alexis/Enzo), or BCL-XL (54H6; Cell Signaling) for 30 min at room temperature, followed by biotinylated anti-IgG secondary antibodies (Vector Labs). Signal detection was performed using ABC Elite (Vector Labs) for 20 min and 3,3′-diaminobenzidine (Dako) for 5 min at room temperature.

Tumor Monitoring and Chemotherapy Administration. Cohorts of 24–32 female mice were seeded with single-cell suspensions of human breast tumors. Treatment was initiated when the tumor volume reached 100–150 mm^3. For survival studies, mice were randomized into four groups: vehicle for both docetaxel and ABT-737, docetaxel (10 mg/kg) plus vehicle for ABT-737, ABT-737 (50 mg/kg) plus vehicle for docetaxel, and ABT-737 (50 mg/kg) plus docetaxel (10 mg/kg). ABT-737 (or vehicle) was injected i.p. daily for 10 d, and a single dose of docetaxel was injected i.p. 4 h after the initial injection of ABT-737. A cycle of treatment refers to 21 d after the initiation of treatment. For short-term experiments, mice were collected at 24 h after injection of docetaxel. Eye bleeds were performed at 2, 8, or 11 d after initiation of treatment, and whole-blood count analysis was performed using an ADVIA 120 hematological analyzer (Bayer). Tumors were collected at the ethical end-point volume of 500 mm^3.

Western Blot Analysis. Tumors were homogenized in lysis buffer (20 mM Tris·HCL, 135 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, and 10% glycerol). Western blot analysis was performed using 30 μg protein lysate per lane; membranes were probed with antibodies (listed in *SI Materials and Methods*), and detection was performed using HRP-conjugated anti-IgG secondary antibodies and ECL (GE Healthcare Life Sciences). Densitometry was performed using the mean gray value of inverted scanned images of Western blots in triplicate.

ACKNOWLEDGMENTS. We are grateful to K. Lowes, B. Pal, J. Corbin, M. Chapman, and the Royal Melbourne Hospital Tissue Bank staff for expert technical assistance; K. Stoev and A. Morcom for animal care; and S. Mihajlovic and E. Tsui for histology support. We thank P. Bouillet for discussions and Abbott Laboratories (S. Rosenberg, S. Elmore, and colleagues) and Genentech (W. Fairbrother) for providing ABT-737 and discussions. Primary tumor samples were provided by the Victorian Cancer Biobank (VCB). This work was supported by the Victorian Government through the Victorian Cancer Agency/Victorian Breast Cancer Research Consortium (J.E.V., G.J.L., and S.B.F.) and an Operational Infrastructure Support grant, the National Health and Medical Research Council Australia (NHMRC; to J.E.V., G.J.L., A.S., and D.C.S.H.) and the Australian Cancer Research Foundation. S.R.O. was supported by the NHMRC and the National Breast Cancer Foundation (NBCF); E.L. by the NHMRC and NBCF; L.L. by the NHMRC and the Cancer Therapeutics CRC; S.D. by the VCB; and A.S., D.C.S.H., J.E.V., and G.J.L. by NHMRC Research Fellowships.

1. Reis-Filho JS, Tutt AN (2008) Triple negative tumours: A critical review. *Histopathology* 52:108–118.
2. Cheang MC, et al. (2008) Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype. *Clin Cancer Res* 14: 1368–1376.
3. Carey LA, et al. (2007) The triple negative paradox: Primary tumor chemosensitivity of breast cancer subtypes. *Clin Cancer Res* 13:2329–2334.
4. Dent R, et al. (2007) Triple-negative breast cancer: Clinical features and patterns of recurrence. *Clin Cancer Res* 13:4429–4434.
5. Adams JM, Cory S (2007) The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* 26:1324–1337.
6. Callagy GM, et al. (2006) Bcl-2 is a prognostic marker in breast cancer independently of the Nottingham Prognostic Index. *Clin Cancer Res* 12:2468–2475.
7. Dawson SJ, 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.
8. Paik S, et al. (2004) A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. *N Engl J Med* 351:2817–2826.
9. Bhargava V, Kell DL, van de Rijn M, Warnke RA (1994) Bcl-2 immunoreactivity in breast carcinoma correlates with hormone receptor positivity. *Am J Pathol* 145: 535–540.
10. Oltersdorf T, et al. (2005) An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* 435:677–681.
11. Chipuk JE, et al. (2008) Mechanism of apoptosis induction by inhibition of the anti-apoptotic BCL-2 proteins. *Proc Natl Acad Sci USA* 105:20327–20332.
12. Cragg MS, et al. (2008) Treatment of B-RAF mutant human tumor cells with a MEK inhibitor requires Bim and is enhanced by a BH3 mimetic. *J Clin Invest* 118:3651–3659.
13. Kim KW, Moretti L, Mitchell LR, Jung DK, Lu B (2009) Combined Bcl-2/mammalian target of rapamycin inhibition leads to enhanced radiosensitization via induction of apoptosis and autophagy in non-small cell lung tumor xenograft model. *Clin Cancer Res* 15:6096–6105.
14. Li M, et al. (2010) Combined inhibition of Notch signaling and Bcl-2/Bcl-xL results in synergistic antimyeloma effect. *Mol Cancer Ther* 9:3200–3209.
15. Okumura K, Huang S, Sinicrope FA (2008) Induction of Noxa sensitizes human colorectal cancer cells expressing Mcl-1 to the small-molecule Bcl-2/Bcl-xL inhibitor, ABT-737. *Clin Cancer Res* 14:8132–8142.
16. Paoluzzi L, et al. (2008) The BH3-only mimetic ABT-737 synergizes the antineoplastic activity of proteasome inhibitors in lymphoid malignancies. *Blood* 112:2906–2916.
17. Witham J, et al. (2007) The Bcl-2/Bcl-XL family inhibitor ABT-737 sensitizes ovarian cancer cells to carboplatin. *Clin Cancer Res* 13:7191–7198.
18. Hann CL, et al. (2008) Therapeutic efficacy of ABT-737, a selective inhibitor of BCL-2, in small cell lung cancer. *Cancer Res* 68:2321–2328.
19. Kang MH, et al. (2007) Activity of vincristine, L-ASP, and dexamethasone against acute lymphoblastic leukemia is enhanced by the BH3-mimetic ABT-737 in vitro and in vivo. *Blood* 110:2057–2066.
20. Kohl TM, et al. (2007) BH3 mimetic ABT-737 neutralizes resistance to FLT3 inhibitor treatment mediated by FLT3-independent expression of BCL2 in primary AML blasts. *Leukemia* 21:1763–1772.
21. Kuroda J, et al. (2007) Apoptosis-based dual molecular targeting by INNO-406, a second-generation Bcr-Abl inhibitor, and ABT-737, an inhibitor of antiapoptotic Bcl-2 proteins, against Bcr-Abl-positive leukemia. *Cell Death Differ* 14:1667–1677.
22. Mason KD, et al. (2009) The BH3 mimetic compound, ABT-737, synergizes with a range of cytotoxic chemotherapy agents in chronic lymphocytic leukemia. *Leukemia* 23:2034–2041.
23. Marangoni E, et al. (2007) A new model of patient tumor-derived breast cancer xenografts for preclinical assays. *Clin Cancer Res* 13:3989–3998.
24. Nielsen TO, et al. (2004) Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. *Clin Cancer Res* 10:5367–5374.
25. Bertolo C, et al. (2008) Differences and molecular immunohistochemical parameters in the subtypes of infiltrating ductal breast cancer. *Am J Clin Pathol* 130:414–424.
26. 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.
27. Lim E, et al. (2009) Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. *Nat Med* 15:907–913.
28. Mason KD, et al. (2007) Programmed anuclear cell death delimits platelet life span. *Cell* 128:1173–1186.
29. Kang JS, et al. (2009) Low dose estrogen supplementation reduces mortality of mice in estrogen-dependent human tumor xenograft model. *Biol Pharm Bull* 32:150–152.
30. Certo M, et al. (2006) Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell* 9:351–365.
31. Del Gaizo Moore V, et al. (2007) Chronic lymphocytic leukemia requires BCL2 to sequester prodeath BIM, explaining sensitivity to BCL2 antagonist ABT-737. *J Clin Invest* 117:112–121.
32. Konopleva M, et al. (2006) Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. *Cancer Cell* 10:375–388.
33. 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.
34. Deng J, et al. (2007) BH3 profiling identifies three distinct classes of apoptotic blocks to predict response to ABT-737 and conventional chemotherapeutic agents. *Cancer Cell* 12:171–185.
35. Kandioler-Eckersberger D, et al. (2000) TP53 mutation and p53 overexpression for prediction of response to neoadjuvant treatment in breast cancer patients. *Clin Cancer Res* 6:50–56.
36. Sekine I, Shimizu C, Nishio K, Saijo N, Tamura T (2009) A literature review of molecular markers predictive of clinical response to cytotoxic chemotherapy in patients with breast cancer. *Int J Clin Oncol* 14:112–119.
37. Cory S, Huang DC, Adams JM (2003) The Bcl-2 family: Roles in cell survival and oncogenesis. *Oncogene* 22:8590–8607.
38. Tse C, et al. (2008) ABT-263: A potent and orally bioavailable Bcl-2 family inhibitor. *Cancer Res* 68:3421–3428.
39. Laidlaw IJ, et al. (1995) The proliferation of normal human breast tissue implanted into athymic nude mice is stimulated by estrogen but not progesterone. *Endocrinology* 136:164–171.