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Original Articles

Combined Bcl-2/Src inhibition synergize to deplete stem-like breast cancer cells

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ABSTRACT

Breast cancer cells with stem cell properties play an important role in tumor progression and thus are key targets for therapy. Here, we show that combined Bcl-2/Src inhibition synergize to deplete stem-like cells. While Src inhibition increases pro-apoptotic PUMA, we find that a significant amount interacts with Bcl-2 and Bcl-xL, promoting resistance to cell death. Consistent with this, the clinically-approved Bcl-2 selective drug venetoclax was sufficient to overcome resistance by preventing PUMA/Bcl-2 binding, enhancing apoptosis. This effect was specific to stem-like breast cancer cells as there was no effect on luminal or basal-like cell types. In contrast, the Mcl-1 inhibitor S63845 potently targeted basal-like, but not stem-like cells, highlighting dependency on distinct sentinel Bcl-2 family members. Our findings reveal Bcl-2/Src inhibition as a superior therapy to target stemness, providing a foundation for a potential personalized strategy to reduce breast cancer progression.

1. Introduction

In breast cancer, a population of tumor cells bearing similarities with normal mammary stem cells has been implicated in aggressive disease [1–5]. These cells have been identified as a sub-population in some patient tumors using cell surface marker profiles such as $CD44^{+}/CD24^{low}$ [1] and $CD49f^{+}/EpCAM^{low}$ [6] as well as gene expression signatures including the claudin-low molecular subtype [4]. Stem-like cells contribute to recurrence [7] and metastasis [8], the leading cause of breast cancer-related deaths [9], and may therefore represent important therapeutic targets in aggressive disease. Unfortunately, since they typically lack cell-cycle genes and instead often display markers of quiescence [8] this may aid stem-like cells in evading traditional chemotherapies, emphasizing the need for more effective targeted approaches to eliminate these cells.

In our prior study, we characterized a unique and particularly aggressive population of stem-like breast cancer cells that express cell surface integrin αvβ3 and the transcriptional repressor Slug [10], similar to activated stem cells we identified in the normal mammary gland during pregnancy [11]. Importantly, we characterized several cell lines with similar properties that could serve as surrogate models for these otherwise rare cells in human disease. These cells also display the claudin-low intrinsic molecular subtype and are therefore distinct from basal-like and luminal breast cancer cells [10,12]. In stem-like cell lines we characterized an αvβ3/Src/Slug signaling pathway that suppressed pro-apoptotic PUMA leading to increased cell survival [10]. We further observed that genetic or pharmacological blockade of c-Src, a non-receptor tyrosine kinase, could increase PUMA levels by reducing Slug, a known PUMA repressor [13]. In fact, Src inhibitors such as dasatinib were the only class of therapy capable of increasing PUMA in stem-like tumor cells, leading to reduced colony-formation, self-renewal and tumor initiation [10]. In contrast, we and others showed that the related pro-apoptotic protein NOXA had minimal effect on these cells [10,14]. Overall, our previous findings indicated that despite the lack of efficacy observed with Src inhibitors in clinical trials [15–17], re-purposing these drugs to target stem-like cells may be a promising new therapeutic approach. However, while effective at depleting anchorage-independent cells, we noted that PUMA had little effect on adherent cells in culture, suggesting the presence of innate resistance mechanisms.

In the present study we sought to overcome resistance to Src inhibition by identifying key factors limiting PUMA-mediated apoptosis in stem-like cells. Pro-apoptotic PUMA is a BH3-only member of the Bcl-2 family. As such, it can bind to pro-survival Bcl-2 proteins, limiting its ability to initiate apoptosis [10]. Therefore, the net effect on apoptosis may not only depend on increasing levels of pro-apoptotic “activators”, like PUMA, but also on inhibiting the appropriate pro-survival Bcl-2 factor. We propose that by identifying the key Bcl-2 family member(s)

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that bind to PUMA in stem-like cells, we may be able to prevent this interaction and overcome resistance to Src inhibition. This approach may lead to discovery of a new combination therapy to more effectively deplete stem-like cells.

2. Materials and methods

2.1. Cell lines

The following breast cancer cell lines were purchased from ATCC: MCF-7, MDA-MB-468, BT549, and MDA-MB-157. LM2-4 cells, a highly metastatic variant of the MDA-MB-231 cell line [18] was a gift from Robert Kerbel. All cell lines were tested and shown to be free of mycoplasma. Cell lines were cultured in complete DMEM medium (DMEM supplemented with 10% fetal bovine serum (FBS) + 1:100 L-glutamine, sodium pyruvate, non-essential amino acids, and antibiotic/antimycotic).

2.2. Cell viability

XTT cell viability assays were performed by first seeding 25,000 LM2-4 or 12,000 BT549, MDA-MB-468, MDA-MB-157 or MCF-7 cells per well into a 96-well tissue culture plate and allowing them to attach overnight. The indicated concentrations of drugs or vehicle alone (DMSO) were then added to the wells in 100 µL phenol-free complete DMEM medium. After 24 h, XTT substrate (Thermo Fisher Scientific) was added to the wells and incubated for 2 h before reading the A450 nm on a plate reader. Cell viability for each of the indicated treatments was expressed as a percent of the vehicle control wells. The following drugs were used to treat cells and compared to DMSO alone (Vehicle): dasatinib (Chemietek), navitoclax, venetoclax and S63845 (Selleckchem).

2.3. Propidium iodide staining

Cell staining with propidium iodide (PI) was performed on live cells plated on 2% Matrigel-coated 8-well chamberslides and treated with the indicated drugs after 24 h. After 48 (LM2-4) or 72 h (BT549) medium was removed and 1 µg/mL PI was added to the cells in 0.1% BSA/PBS for 15 min in the dark. Cells were then washed 2x with 0.1% BSA/PBS and imaged with a Nikon A1R confocal microscope. 6 random 20x fields were captured from each condition and the PI^+ cells per field quantified with ImageJ.

2.4. Generation of stable cells

Stable knockdowns were achieved by transducing LM2-4 cells with lentivirus expressing human-specific shRNA's targeting PUMA (*BBC3*; V3LHS_342433 and V3LHS_342436), c-Src (*SRC*; V2LHS_262793) or a non-silencing control in the pGIPZ vector (Open Biosystems) and pooling puromycin-resistant cells. Successful knockdown of the respective targets was verified by Western blot for each stable cell line as previously described [10].

2.5. Immunoblotting and immunoprecipitations

Whole cell lysates were prepared from cell lines with RIPA lysis buffer (100 mM Tris pH 7.5, 150 mM sodium chloride, 0.1% deoxycholate, 0.1% SDS, 50 mM NaF, Protease inhibitor cocktail (Roche), 2 mM PMSF, 2 mM sodium orthovanadate) combined with scraping and the lysates cleared by centrifugation. Additionally, Bcl-2, Mcl-1 and Bcl-xL were pulled-down from LM2-4 cell lysates with 30 µL of 50% protein A/G beads (Pierce) and 2 µg of the respective antibody (S431259; BD Bioscience) overnight at 4 °C. The beads were then washed 3x with lysis buffer prior to eluting proteins with 2x sample buffer and performing Western analysis. Standard Western blotting procedures were performed. The following antibodies were used for immunoblotting: PUMA (D30C10), Bim (C34C5), Bcl-2 (D55G8), Mcl-1 (D2W9E), Bcl-xL (54H6), Cleaved Caspase-3 (Asp175), Cleaved PARP (Asp214) (D64E10), Full-length Caspase-3, and Full-length PARP (Cell Signaling Technology), Hsp90 (Santa Cruz) and β-actin (Sigma). All drug treatments were performed at the specified doses for 24 h prior to harvesting lysates.

2.6. Tumorsphere assays

Primary tumorsphere formation was assessed in cells grown under anchorage-independent conditions in either soft agar or methylcellulose. For soft agar assays, 2000 LM2-4 cells are suspended in 200 µL 0.3% agar/complete DMEM medium and cultured on top of a bottom layer of 200 µL 1% agar in a 48-well dish. An additional 500 µL of complete medium was then added and cells cultured for 14 days prior to fixing and staining colonies with 0.005% crystal violet/20% methanol/PBS and counting colonies consisting of at least 6 cells from 4 fields per well with a 10× objective. Alternatively, 12,000 LM2-4 cells were cultured in 1.2 mL of 1% methylcellulose/complete DMEM medium in a poly-HEMA coated 12-well dish and primary tumorspheres assessed by counting colonies consisting of at least 6 cells from an entire well after 14 days. We measured self-renewal by collecting primary tumorspheres by dilution in at least 3 vol of PBS, dissociating them with trypsin for approximately 10 min, and re-seeding in 1% methylcellulose before evaluating secondary colonies after an additional 14 days. For the drug treatment studies, the indicated concentrations were added only once, immediately after embedding the cells.

2.7. Statistics

Data presentation and statistical tests are indicated in the figure legends. Two-tailed Student's t-tests were used for comparing two means while ANOVA was performed for 3 or more data sets. Post-hoc analysis was performed using an appropriate multiple comparisons test as indicated in the legends. For all analyses, $P < 0.05$ was considered statistically significant. Statistical analysis was performed using GraphPad Prism 5 statistical software.

3. Results

3.1. PUMA synergizes with Bcl-2 inhibition to deplete stem-like breast cancer cells

To enhance the effect of Src inhibition in stem-like cells, we sought to identify the critical pro-survival Bcl-2 family members important for PUMA binding. These studies were conducted in adherent stem-like cell lines similar to those in human disease [10,12] under conditions we previously showed were most resistant to Src inhibition [10]. We began by determining the minimum dose of the Src inhibitor dasatinib needed to robustly increase PUMA levels in the stem-like LM2-4 cell line, a highly-metastatic variant of MDA-MB-231 cells [18]. Consistent with our prior results, as little as 100 nM dasatinib is sufficient to maximally induce PUMA in these cells with no effect on the related pro-apoptotic protein Bim (Fig. 1A). We then used this dose of dasatinib to identify which pro-survival Bcl-2 family members interact with PUMA driven by Src inhibition. PUMA has previously been shown to bind to pro-survival Bcl-2 and Bcl-xL via their BH3 domains [19], suggesting these proteins may be critical mediators of resistance in our cells. In immunoprecipitation assays we found that Bcl-2, but not Mcl-1 could interact with PUMA induced by dasatinib (Fig. 1B). We further showed that Bcl-xL also bound to PUMA (Fig. 1C), indicating that both Bcl-2 and Bcl-xL may be important factors limiting the effect of Src inhibition on stem-like cells.

To examine the relevance of these interactions for Src inhibitor resistance, we sought ways to interrupt complex formation. Several small

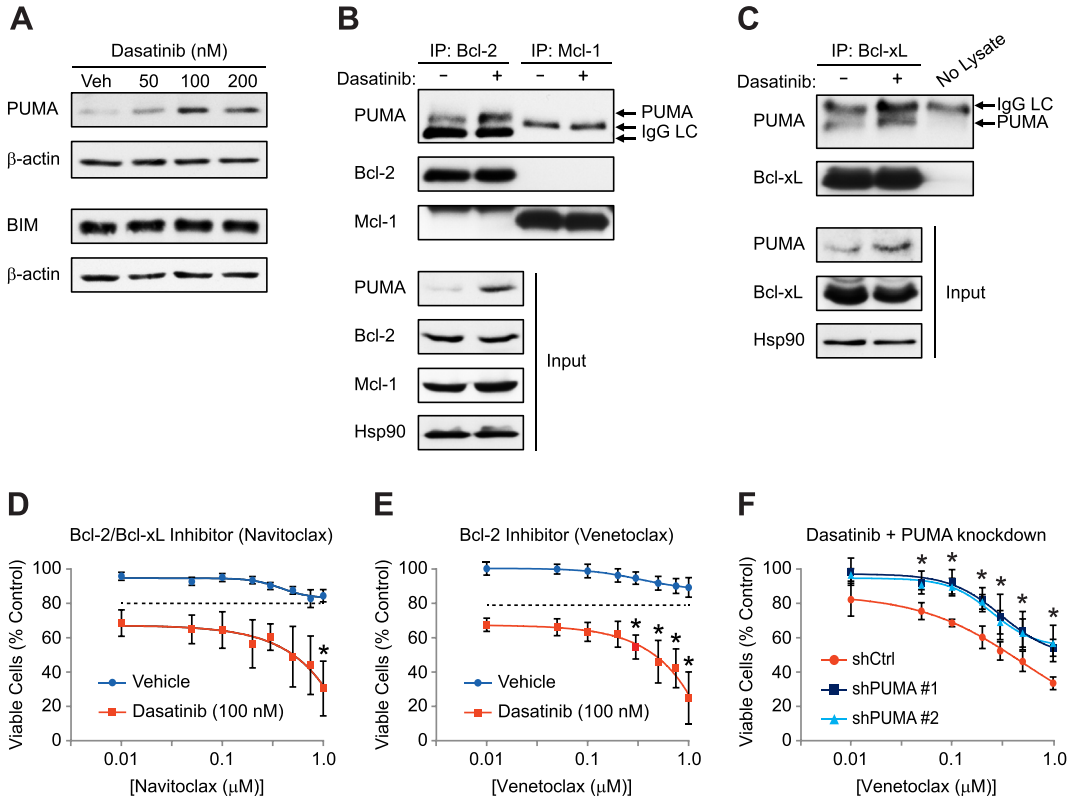

Fig. 1. Bcl-2 inhibition synergizes with PUMA induced by blocking Src. (**A**) Immunoblots of LM2-4 cells after treatment with DMSO vehicle control (Veh) or several concentrations of the Src inhibitor dasatinib for 24 h. β-actin was used as a loading control. (**B** and **C**) Immunoprecipitation experiments to identify the pro-survival Bcl-2 family members that bind PUMA induced by Src inhibition. Arrows indicate the bands for PUMA or the IgG light chains (IgG LC) for each of the antibodies used in these experiments. The correct size for each light-chain band was determined by comparing with a control sample containing only beads and antibody (No Lysate). Input controls show the relative expression level of the indicated proteins in the whole cell lysates for each experiment. Hsp90 is shown as a loading control. (**A-C**) Data shown is representative of 3 independent experiments. (**D-F**) Dose-response XTT experiments comparing the effect of navitoclax or venetoclax in stem-like LM2-4 cells ± 100 nM dasatinib. Curves are plotted relative to vehicle controls for each cell type and fitted by non-linear regression. (**D** and **E**) Statistical analysis performed by two-way ANOVA with Tukey's multiple comparisons test. $^*P < 0.05$ for a navitoclax dose of 1000 nM (vehicle versus dasatinib) (**D**) and venetoclax doses of 300, 500, 750 and 1000 nM (vehicle versus dasatinib) (**E**). Dashed lines indicate the effect of 100 nM dasatinib alone. (**F**) Stable LM2-4 cells expressing non-silencing control shRNA (shCtrl) or either of two different PUMA shRNA's (shPUMA) were dosed with 200 nM dasatinib and cell viability assessed in response to different doses of venetoclax. $^*P < 0.05$ for 50, 100, 200, 300, 500 and 1000 nM venetoclax (shCtrl vs shPUMA #1 and #2). Statistical analysis performed by two-way ANOVA with Sidak's multiple comparisons test. (**D-F**) Data represent the mean ± s. e.m. n = 3 (**D** and **F**) and n = 5 (**E**) independent experiments performed in quadruplicate.

molecule inhibitors that prevent binding of Bcl-2 family members to their respective targets have been characterized and are either clinically-approved or currently being tested in clinical trials [20]. Since PUMA bound to both Bcl-2 and Bcl-xL in our immunoprecipitation experiments, we first examined if the dual specific Bcl-2/Bcl-xL inhibitor navitoclax (ABT-263) could increase the efficacy of Src inhibition. Indeed, treatment with both navitoclax and dasatinib synergized to reduce cell viability, with no effect by navitoclax alone at any dose tested (Fig. 1D). Under these conditions dasatinib alone reduces cell number by only about 20% as indicated by a dashed line in the figure (Fig. 1D), primarily via loss of adhesion [10]. Together, our findings indicate that combined inhibition of Bcl-2/Bcl-xL and Src represent a synergistic therapy to deplete stem-like cells.

Drugs targeting Bcl-xL cause severe thrombocytopenia limiting their use in humans [20]. In an effort to identify an inhibitor that is safe to eventually use in patients, we examined the Bcl-2 selective drug venetoclax (ABT-199) [21] for potential synergy with Src inhibition. Venetoclax lacks the thrombocytopenia associated with Bcl-xL inhibition due to its high binding affinity for Bcl-2 versus Bcl-xL [21] and has the added benefit of being clinically-approved for certain forms of leukemia [20]. In our assays, venetoclax also synergized with Src inhibition to reduce cell viability in LM2-4 cells, in contrast to venetoclax alone, which had no effect at any dose tested (Fig. 1E). Despite the dual specificity of navitoclax for Bcl-2 and Bcl-xL, we noted a similar synergistic response with venetoclax, presumably due to its higher target binding affinity [21]. To examine PUMA's role in this effect, we further asked whether PUMA knockdown could attenuate the response to Bcl-2/Src inhibition. We previously generated LM2-4 stable cells expressing either of two different PUMA shRNA's and showed that they express significantly reduced levels of PUMA relative to a non-silencing control shRNA [10]. Now, we show that PUMA knockdown is indeed capable of decreasing the response to Bcl-2/Src inhibition compared to control cells (Fig. 1F), indicating that PUMA is required for this effect. Overall, our findings show that blocking Bcl-2 alone is sufficient to overcome resistance to Src inhibition by enhancing PUMA-mediated depletion of stem-like cells.

3.2. Selective targeting of stem-like cells with Bcl-2/Src inhibition

Importantly, the synergy we observed between Src and Bcl-2 inhibition was not limited to LM2-4's, as we observed a similar effect in the stem-like BT549 cells (Fig. 2A). In fact, all three stem-like cell lines examined showed synergy between Bcl-2 and Src inhibition while the luminal MCF-7 cells were unaffected (Fig. 2B), supporting a selective effect for this therapy on stem-like cells. Consistent with this, the basal-like MDA-MB-468 cell line failed to respond to combined treatment

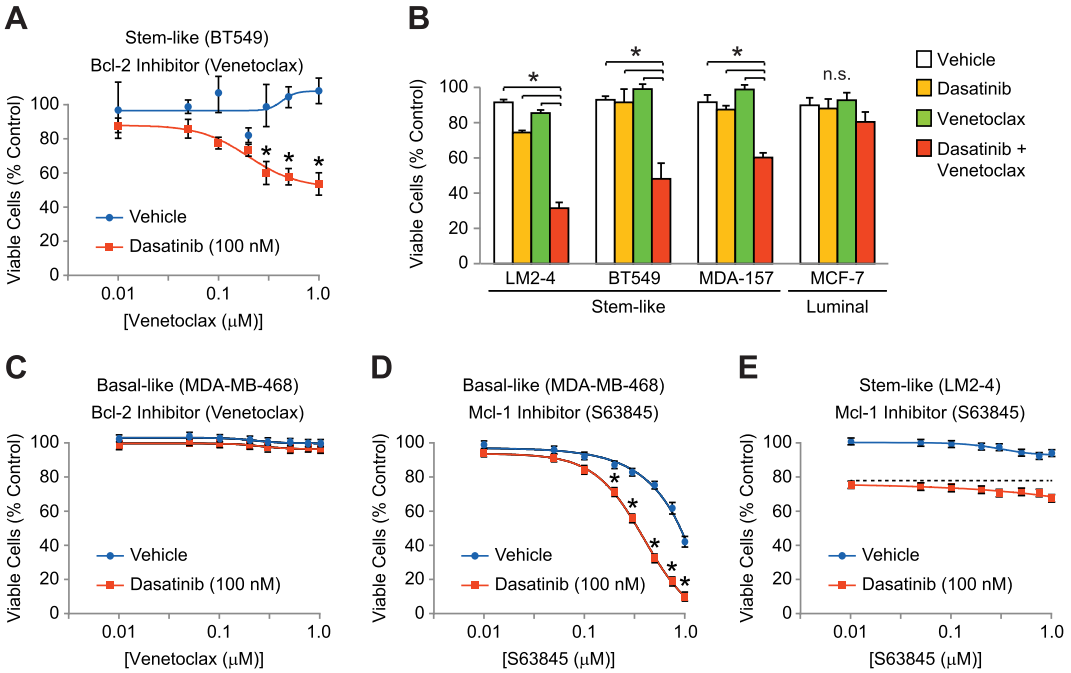

Fig. 2. Combined Bcl-2/Src inhibition specifically depletes stem-like cells. (**A**) Dose-response XTT experiments showing the effect of venetoclax in stem-like BT549 cells ± 100 nM dasatinib. (**B**) Histogram depicts the relative effect of vehicle control (DMSO), dasatinib (200 nM), venetoclax (1 μM) or the two drugs together in multiple stem-like cell lines compared to luminal MCF-7 cells. *$P < 0.05$ for dasatinib + venetoclax vs all other treatments for a given cell type. n. s. = not significant. Statistics performed by one-way ANOVA with Tukey's multiple comparisons test. (**C-E**) XTT cell viability assays comparing venetoclax with the Mcl-1 inhibitor S63845 in basal-like MDA-MB-468 cells (**C** and **D**) and stem-like LM2-4 cells (**E**). (**E**) Dashed line indicates the effect of 100 nM dasatinib alone. (**A** and **C-E**) Curves are plotted relative to vehicle controls for each treatment group (vehicle or dasatinib) and fitted by non-linear regression. Statistical analysis performed by two-way ANOVA with Tukey's multiple comparisons test. (**A**) *$P < 0.05$ for BT549 cells treated with venetoclax doses of 500, 750 and 1000 nM (vehicle versus dasatinib). (**D**) *$P < 0.05$ for MDA-MB-468 cells treated with S63845 (Mcl-1 inhibitor) doses of 200, 300, 500, 750 and 1000 nM (vehicle versus dasatinib). (**A-E**) Data represent the mean ± s. e.m. n = 3 independent experiments performed in quadruplicate.

with venetoclax and dasatinib (Fig. 2C). However, these cells were highly sensitive to the recently developed Mcl-1 inhibitor S63845 [22] (Fig. 2D), consistent with published results achieved by knockdown of Mcl-1 [14]. In contrast, treating LM2-4 cells with S63845 had no effect as a single-agent or when combined with dasatinib (Fig. 2E), consistent with our observation that PUMA induced by Src inhibition did not associate with Mcl-1 (Fig. 1B). These findings support a hierarchy of Bcl-2 family members in different breast cancer cell types, with basal-like cells exhibiting preference for Mcl-1, while stem-like cells are highly-dependent on Bcl-2. Taken together, our results highlight Bcl-2/Src inhibition as a selective therapy to target stem-like breast cancer cells.

3.3. *Bcl-2 inhibition increases "free" PUMA, inducing apoptosis in stem-like cells*

Since venetoclax blocks Bcl-2's ability to bind its targets, we asked if it could prevent the interaction with PUMA we previously observed in response to Src inhibition (Fig. 1B). Indeed, in immunoprecipitation experiments venetoclax prevented PUMA binding to Bcl-2, (Fig. 3A), potentially freeing PUMA to induce intrinsic apoptosis [23]. We examined this possibility by evaluating several apoptotic markers in our stem-like cell lines after Bcl-2/Src inhibition. Staining with propidium iodide (PI) showed very little uptake in cells receiving vehicle or either drug alone (Fig. 3B and C). Only upon treatment with both dasatinib and venetoclax did we observe a 3 and 4-fold increase in PI^+ cells respectively in the stem-like LM2-4 and BT549 cell lines (Fig. 3B and C). Similarly, immunoblotting showed the highest levels of cleaved Caspase-3 (Fig. 3D) and cleaved PARP (Fig. 3E) in cells treated with the combination of Bcl-2 and Src inhibitors. Interestingly, a small amount of cleaved Caspase-3 and PARP were observed in cells treated with dasatinib alone (Fig. 3D and E), consistent with Src inhibition increasing levels of pro-apoptotic PUMA (Fig. 1A). However, this amount of PUMA is apparently unable to reach the threshold required to initiate apoptosis in stem-like cells cultured in adherent conditions (Fig. 3B and C). These results show that combining Src inhibition and venetoclax decreases the amount of PUMA bound to Bcl-2 (Fig. 3A), which is now able to more efficiently drive apoptosis (Fig. 3B–E), providing a mechanism to account for synergy between these two drugs.

3.4. *Combined Bcl-2/Src inhibition is a superior treatment to suppress stemness*

Our prior findings showed that treatment with a Src inhibitor could inhibit stemness properties including colony formation and self-renewal due to higher sensitivity to PUMA expression in 3D culture conditions [10]. To determine if Bcl-2/Src inhibition possessed enhanced stemness blocking properties, we assessed this treatment for its ability to reduce colony-formation in soft agar (Fig. 4A) and self-renewal in methylcellulose (Fig. 4B). While a dose-response with dasatinib alone reduced soft agar colonies as we previously described [10], we observed that adding a single 500 nM dose of venetoclax could substantially improve this response (Fig. 4A). Similarly, while Src inhibition decreased self-renewal in methylcellulose, addition of as little as 200 nM venetoclax helped to significantly deplete self-renewing cells (Fig. 4B). Our results show that combined treatment with Bcl-2/Src inhibitors further reduces stemness properties compared to Src inhibition alone.

Importantly, we also provide genetic evidence for c-Src's role in this response in addition to our use of Src inhibitors (Fig. 4C). We previously showed that stable expression of c-Src shRNA in LM2-4 cells significantly reduced levels of c-Src protein expression and increased levels of PUMA compared to control cells [10]. Now we examined whether silencing c-Src would cooperate with venetoclax to further reduce stemness in the soft agar tumorsphere assay (Fig. 4C). Decreased expression of c-Src alone reduced colonies by roughly 30%, consistent

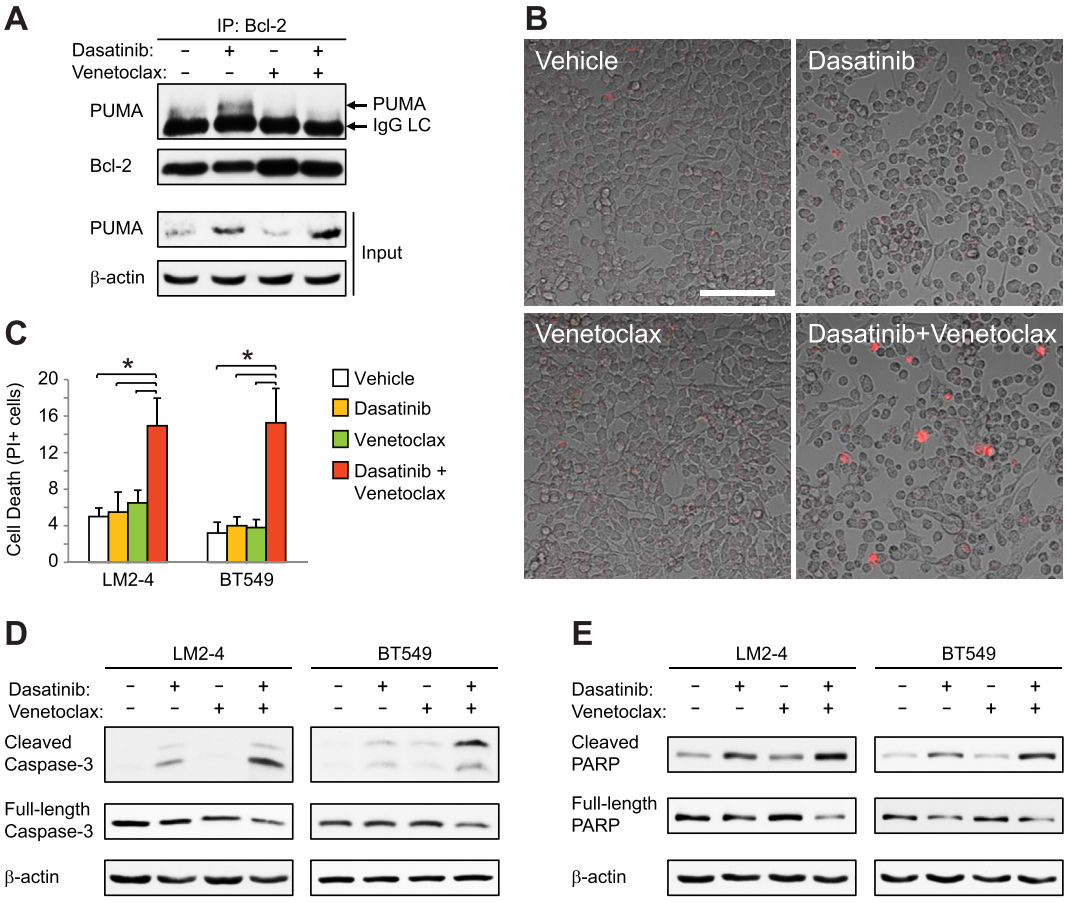

Fig. 3. Bcl-2 inhibition increases "free" PUMA, enhancing apoptosis. (**A**) Immunoprecipitation experiment assessing PUMA's interaction with Bcl-2 ± treatment with 100 nM dasatinib and/or 1 μM venetoclax in LM2-4 cells. Arrows indicate the bands for PUMA or IgG light chain (IgG LC). Input controls show the relative expression level of the indicated proteins in the whole cell lysates. β-actin is shown as a loading control. (**B**) Representative images showing the relative amount of apoptosis after treating LM2-4 cells with vehicle (DMSO), 100 nM dasatinib, 1 μM venetoclax, or the two drugs together. Apoptosis was detected by propidium iodide (PI) fluorescent staining 72 h after drug treatment. Shown for each condition is the PI staining overlayed on the corresponding brightfield image. Scale bar, 100 μm. (**C**) Quantitation of cell death (PI^+ cells per field) for each treatment group in two stem-like cell lines. Statistical analysis performed by one-way ANOVA with Tukey's multiple comparisons test. *$P < 0.05$ for dasatinib + venetoclax vs all other treatments. For each cell type n = 3 independent experiments with data calculated from 6 random fields per experiment. Data represent the mean ± s. e.m. (**D** and **E**) Representative Western blots for markers of apoptosis including cleaved Caspase-3 (**D**) and cleaved PARP (**E**) 48 h (LM2-4) or 72 h (BT549) after treatment with vehicle (DMSO), 100 nM dasatinib, 1 μM venetoclax or both drugs together. (**A,B,D** and **E**) Data shown is representative of 3 independent experiments.

with the effect we observed with Src kinase inhibition (Fig. 4A). However, treatment with venetoclax caused a further 30% reduction in c-Src knockdown colonies at the highest dose tested (Fig. 4C) in contrast to cells expressing control shRNA, which were unaffected at any dose (Fig. 4C). This is surprising since Src inhibitors, such as dasatinib, block kinase activity in all Src family kinases including c-Src, Yes and Fyn, yet we find that c-Src knockdown alone is sufficient to synergize with venetoclax (Fig. 4C). This may be explained by our prior results showing that reduced c-Src was sufficient to increase PUMA levels as well [10]. Together, our findings show that combined Bcl-2/Src inhibition is a superior treatment to block stemness properties over dasatinib alone (Fig. 4D).

4. Discussion

Stem-like cells contribute to breast cancer progression and metastasis, therefore discovering ways to target these cells holds promise for identifying new treatments for patients. We previously showed that increasing PUMA levels via Src inhibition was an effective therapy for depleting stem-like cells in anchorage-independent conditions [10]. However, Src inhibition was relatively ineffective against adherent cells [10] suggesting the presence of innate resistance factors. Here, we sought to identify mechanisms of PUMA-resistance as a way to enhance depletion of stem-like cells by Src inhibition. We now describe Bcl-2 as an important resistance factor that binds to PUMA, limiting cell death. We further show that the Bcl-2 inhibitor venetoclax is sufficient to overcome this resistance by synergizing with Src inhibition to enhance PUMA-mediated apoptosis. In fact, combining these drugs represents a superior precision therapy for targeting stemness compared to either treatment alone, laying the groundwork for possible effects on reducing metastasis or recurrence after chemotherapy.

Early enthusiasm for use of Src inhibitors in Triple-Negative or metastatic breast cancer was tempered by a lack of efficacy in patients during multiple clinical trials [15–17]. In prior work we showed that these drugs may be more effective if re-purposed to target stem-like breast cancer cells to prevent new metastases from forming [10]. Our current study improves upon this work by providing the initial evidence that combining the clinically-approved Src inhibitor dasatinib with the Bcl-2 selective inhibitor venetoclax produces synergistic cell death in stem-like cells. In addition to enhancing the effects of Src inhibition, another potential advantage of our approach is that it may target stem-like cells that are quiescent, a hallmark of these cells in human disease [8]. This is in contrast to commonly used chemotherapies in breast cancer, such as docetaxel, which preferentially target rapidly-dividing tumor cells. Therefore, our combined Bcl-2/Src inhibitor therapy may not only overcome resistance to Src inhibitors, but may also produce

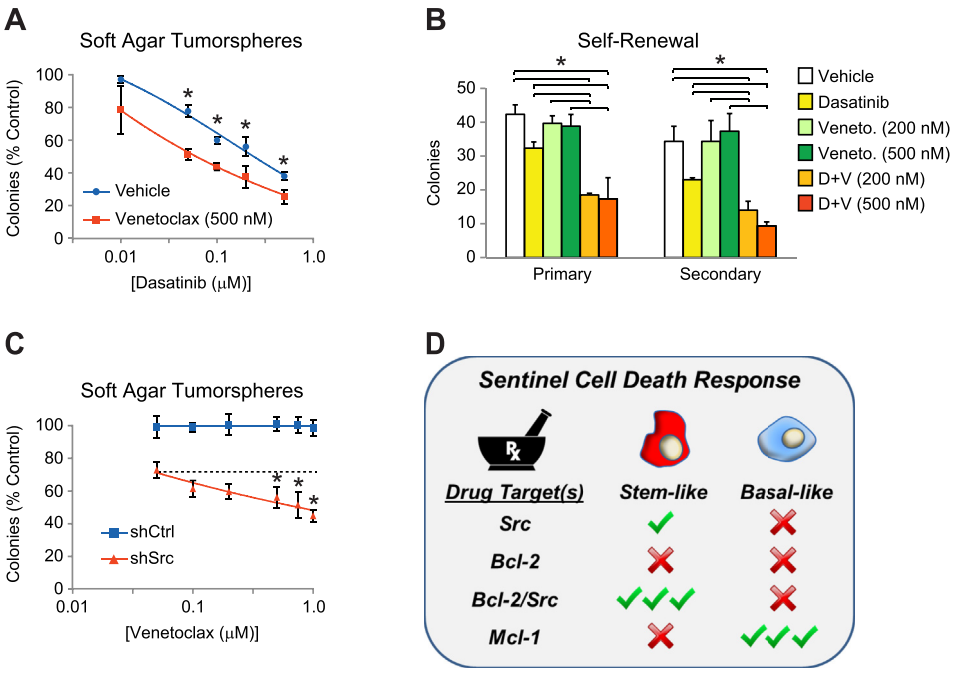

Fig. 4. Combined Src/Bcl-2 inhibition is a superior therapy to reduce stemness properties. (**A–C**) Anchorage-independent tumorsphere assays to assess the effect of dual Src and Bcl-2 inhibition on stemness relative to Src inhibition alone. (**A**) Soft agar tumorsphere assays showing colony number after treatment with dasatinib alone or when combined with venetoclax (500 nM). Curves are plotted relative to vehicle controls for each treatment group (vehicle or venetoclax) and fitted by non-linear regression. $^*P < 0.05$ for 50, 100, 200, and 500 nM dasatinib (vehicle vs venetoclax). (**B**) Self-renewal assays in methylcellulose after treatment with vehicle (DMSO), dasatinib (100 nM), venetoclax (200 or 500 nM) or the two drugs together (D + V). Shown are the total colony counts per well. Statistical analysis performed by one-way ANOVA with Tukey's multiple comparisons test. Primary and secondary tumorspheres; $^*P < 0.05$ for dasatinib + venetoclax (200 and 500 nM) vs all other treatments. n = 4 independent experiments. (**C**) Soft agar colony assays with stable LM2-4 cells expressing non-silencing control shRNA (shCtrl) or c-Src shRNA (shSrc) treated with venetoclax at the indicated doses. The effect of Src knockdown alone (vehicle) is indicated by the dashed line. Colonies per field are shown as a percent of the shCtrl vehicle control and curves fitted by non-linear regression. $^*P < 0.05$ for Src knockdown +500, 750 and 1000 nM venetoclax (each dose versus vehicle). (**A** and **C**) Statistical analysis performed by two-way ANOVA with Sidak's multiple comparisons test. n = 4 independent experiments performed in triplicate. (**A–C**) Data represent the mean ± s. e.m. (**D**) Schematic summarizing our discovery that Src/Bcl-2 inhibition selectively targets stem-like breast cancer cells. This represents a significant improvement over the effect we previously described with Src inhibition alone. We contrast this with data showing that basal-like cell types are highly sensitive to Mcl-1 inhibition.

improved outcomes for breast cancer patients by providing a more effective treatment for stem-like cells.

Bcl-2 family inhibitors are currently being investigated in multiple clinical trials, with the Bcl-2 selective inhibitor venetoclax representing the first to be clinically-approved [20]. While effective as single agents in certain leukemias, these drugs are less successful in solid tumors [20], suggesting they may work best in combination with other treatments. This may be due to a requirement for induction of pro-apoptotic activators downstream of p53, which is often deficient in solid tumors. In fact, a recent study showed that p53 activation with an MDM2 inhibitor could confer sensitivity to venetoclax in leukemias that were previously resistant [24]. However, p53 is mutated in most aggressive breast tumors and cell lines, so this strategy would be ineffective. We now show that the clinically-approved Src inhibitor dasatinib increases levels of PUMA, an important p53 effector, and this synergizes with venetoclax, representing a superior therapy for depleting stem-like breast cancer cells. This suggest that re-activating critical p53 response genes, such as PUMA, may be an effective strategy to enhance sensitivity to Bcl-2 family inhibitors in solid tumors.

Central to this effort will be the elucidation of the most effective pro- or anti-apoptotic proteins to target in different breast cancer cell types. Observations from our lab and others suggest that different breast cancer cell types are dependent on distinct Bcl-2 family members. We previously found that stem-like cells are highly-sensitive to PUMA, consistent with publications showing PUMA-mediated cell death is the preferred response in normal adult stem cell populations [25]. We now show that PUMA synergizes with Bcl-2 inhibition to eliminate stem-like cells with no effect on luminal (Fig. 2B) or basal-like (Fig. 2C) cell types. In contrast, we also provide the first evidence that the Mcl-1 selective inhibitor S63845 [22] targets basal-like (Fig. 2D), but not stem-like cells (Fig. 2E). Our work is supported by observations from other laboratories showing that increased pro-apoptotic Bim synergizes with venetoclax in ER^+ luminal tumors [26] and, in a different study, reduced expression of pro-survival Mcl-1 preferentially killed basal-like cells [14]. Future studies defining the sentinel cell death/survival pathways in particular breast cancer cell types will help define the most effective personalized treatment for an individual patient's breast cancer.

Conflicts of interest statement

None.

CRediT authorship contribution statement

Qi Sun: Conceptualization, Methodology, Investigation. **Yufen Wang:** Methodology, Investigation. **Jay S. Desgrosellier:** Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Supervision, Funding acquisition.

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References

[1] M. Al-Hajj, M.S. Wicha, A. Benito-Hernandez, S.J. Morrison, M.F. Clarke, Prospective identification of tumorigenic breast cancer cells, Proc. Natl. Acad. Sci. U. S. A 100 (2003) 3983–3988.

[2] E. Lim, D. Wu, B. Pal, T. Bouras, M.L. Asselin-Labat, F. Vaillant, H. Yagita, G.J. Lindeman, G.K. Smyth, J.E. Visvader, Transcriptome analyses of mouse and human mammary cell subpopulations reveal multiple conserved genes and pathways, Breast Cancer Res. 12 (2010) R21.

[3] S. Pece, D. Tosoni, S. Confalonieri, G. Mazzarol, M. Vecchi, S. Ronzoni, L. Bernard, G. Viale, P.G. Pelicci, P.P. Di Fiore, Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content, Cell 140 (2010) 62–73.
[4] A. Prat, J.S. Parker, O. Karginova, C. Fan, C. Livasy, J.I. Herschkowitz, X. He, C.M. Perou, Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer, Breast Cancer Res. 12 (2010) R68.
[5] B.T. Spike, D.D. Engle, J.C. Lin, S.K. Cheung, J. La, G.M. Wahl, A mammary stem cell population identified and characterized in late embryogenesis reveals similarities to human breast cancer, Cell Stem Cell 10 (2012) 183–197.
[6] E. Lim, F. Vaillant, D. Wu, N.C. Forrest, B. Pal, A.H. Hart, M.L. Asselin-Labat, D.E. Gyorki, T. Ward, A. Partanen, F. Feleppa, L.I. Huschtscha, H.J. Thorne, S.B. Fox, M. Yan, J.D. French, M.A. Brown, G.K. Smyth, J.E. Visvader, G.J. Lindeman, Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers, Nat. Med. 15 (2009) 907–913.
[7] C.J. Creighton, X. Li, M. Landis, J.M. Dixon, V.M. Neumeister, A. Sjolund, D.L. Rimm, H. Wong, A. Rodriguez, J.I. Herschkowitz, C. Fan, X. Zhang, X. He, A. Pavlick, M.C. Gutierrez, L. Renshaw, A.A. Larionov, D. Faratian, S.G. Hilsenbeck, C.M. Perou, M.T. Lewis, J.M. Rosen, J.C. Chang, Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features, Proc. Natl. Acad. Sci. U. S. A 106 (2009) 13820–13825.
[8] D.A. Lawson, N.R. Bhakta, K. Kessenbrock, K.D. Prummel, Y. Yu, K. Takai, A. Zhou, H. Eyob, S. Balakrishnan, C.Y. Wang, P. Yaswen, A. Goga, Z. Werb, Single-cell analysis reveals a stem-cell program in human metastatic breast cancer cells, Nature 526 (2015) 131–135.
[9] American Cancer Society, Breast Cancer Facts & Figures 2015-2016, American Cancer Society, Inc, Atlanta, 2015.
[10] Q. Sun, J. Lesperance, H. Wettersten, E. Luterstein, Y.S. DeRose, A. Welm, D.A. Cheresh, J.S. Desgrosellier, Proapoptotic PUMA targets stem-like breast cancer cells to suppress metastasis, J. Clin. Investig. 128 (2018) 531–544.
[11] J.S. Desgrosellier, J. Lesperance, L. Seguin, M. Gozo, S. Kato, A. Franovic, M. Yebra, S.J. Shattil, D.A. Cheresh, Integrin alphavbeta3 drives slug activation and stemness in the pregnant and neoplastic mammary gland, Dev. Cell 30 (2014) 295–308.
[12] A. Prat, O. Karginova, J.S. Parker, C. Fan, X. He, L. Bixby, J.C. Harrell, E. Roman, B. Adamo, M. Troester, C.M. Perou, Characterization of cell lines derived from breast cancers and normal mammary tissues for the study of the intrinsic molecular subtypes, Breast Canc. Res. Treat. 142 (2013) 237–255.
[13] W.S. Wu, S. Heinrichs, D. Xu, S.P. Garrison, G.P. Zambetti, J.M. Adams, A.T. Look, Slug antagonizes p53-mediated apoptosis of hematopoietic progenitors by repressing puma, Cell 123 (2005) 641–653.
[14] F. Petrocca, G. Altschuler, S.M. Tan, M.L. Mendillo, H. Yan, D.J. Jerry, A.L. Kung, W. Hide, T.A. Ince, J. Lieberman, A genome-wide siRNA screen identifies proteasome addiction as a vulnerability of basal-like triple-negative breast cancer cells, Cancer Cell 24 (2013) 182–196.
[15] R.S. Finn, C. Bengala, N. Ibrahim, H. Roche, J. Sparano, L.C. Strauss, J. Fairchild, O. Sy, L.J. Goldstein, Dasatinib as a single agent in triple-negative breast cancer: results of an open-label phase 2 study, Clin. Cancer Res. 17 (2011) 6905–6913.
[16] A. Gucalp, J.A. Sparano, J. Caravelli, J. Santamauro, S. Patil, A. Abbruzzi, C. Pellegrino, J. Bromberg, C. Dang, M. Theodoulou, J. Massague, L. Norton, C. Hudis, T.A. Traina, Phase II trial of saracatinib (AZD0530), an oral SRC-inhibitor for the treatment of patients with hormone receptor-negative metastatic breast cancer, Clin. Breast Canc. 11 (2011) 306–311.
[17] C.I. Herold, V. Chadaram, B.L. Peterson, P.K. Marcom, J. Hopkins, G.G. Kimmick, J. Favaro, E. Hamilton, R.A. Welch, S. Bacus, K.L. Blackwell, Phase II trial of dasatinib in patients with metastatic breast cancer using real-time pharmacodynamic tissue biomarkers of Src inhibition to escalate dosing, Clin. Cancer Res. 17 (2011) 6061–6070.
[18] R. Munoz, S. Man, Y. Shaked, C.R. Lee, J. Wong, G. Francia, R.S. Kerbel, Highly efficacious nontoxic preclinical treatment for advanced metastatic breast cancer using combination oral UFT-cyclophosphamide metronomic chemotherapy, Cancer Res. 66 (2006) 3386–3391.
[19] H.C. Chen, M. Kanai, A. Inoue-Yamauchi, H.C. Tu, Y. Huang, D. Ren, H. Kim, S. Takeda, D.E. Reyna, P.M. Chan, Y.T. Ganesan, C.P. Liao, E. Gavathiotis, J.J. Hsieh, E.H. Cheng, An interconnected hierarchical model of cell death regulation by the BCL-2 family, Nat. Cell Biol. 17 (2015) 1270–1281.
[20] D. Merino, G.L. Kelly, G. Lessene, A.H. Wei, A.W. Roberts, A. Strasser, BH3-Mimetic drugs: blazing the trail for new cancer medicines, Cancer Cell 34 (2018) 879–891.
[21] A.J. Souers, J.D. Leverson, E.R. Boghaert, S.L. Ackler, N.D. Catron, J. Chen, B.D. Dayton, H. Ding, S.H. Enschede, W.J. Fairbrother, D.C. Huang, S.G. Hymowitz, S. Jin, S.L. Khaw, P.J. Kovar, L.T. Lam, J. Lee, H.L. Maecker, K.C. Marsh, K.D. Mason, M.J. Mitten, P.M. Nimmer, A. Oleksijew, C.H. Park, C.M. Park, D.C. Phillips, A.W. Roberts, D. Sampath, J.F. Seymour, M.L. Smith, G.M. Sullivan, S.K. Tahir, C. Tse, M.D. Wendt, Y. Xiao, J.C. Xue, H. Zhang, R.A. Humerickhouse, S.H. Rosenberg, S.W. Elmore, ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets, Nat. Med. 19 (2013) 202–208.
[22] A. Kotschy, Z. Szlavik, J. Murray, J. Davidson, A.L. Maragno, G. Le Toumelin-Braizat, M. Chanrion, G.L. Kelly, J.N. Gong, D.M. Moujalled, A. Bruno, M. Csekei, A. Paczal, Z.B. Szabo, S. Sipos, G. Radics, A. Proszenyak, B. Balint, L. Ondi, G. Blasko, A. Robertson, A. Surgenor, P. Dokurno, I. Chen, N. Matassova, J. Smith, C. Pedder, C. Graham, A. Studeny, G. Lysiak-Auvity, A.M. Girard, F. Grave, D. Segal, C.D. Riffkin, G. Pomilio, L.C. Galbraith, B.J. Aubrey, M.S. Brennan, M.J. Herold, C. Chang, G. Guasconi, N. Cauquil, F. Melchiore, N. Guigal-Stephan, B. Lockhart, F. Colland, J.A. Hickman, A.W. Roberts, D.C. Huang, A.H. Wei, A. Strasser, G. Lessene, O. Geneste, The MCL1 inhibitor S63845 is tolerable and effective in diverse cancer models, Nature 538 (2016) 477–482.
[23] J. Yu, L. Zhang, P.M. Hwang, K.W. Kinzler, B. Vogelstein, PUMA induces the rapid apoptosis of colorectal cancer cells, Mol. Cell 7 (2001) 673–682.
[24] R. Pan, V. Ruvolo, H. Mu, J.D. Leverson, G. Nichols, J.C. Reed, M. Konopleva, M. Andreeff, Synthetic lethality of combined bcl-2 inhibition and p53 activation in AML: mechanisms and superior Antileukemic efficacy, Cancer Cell 32 (2017) 748–760 e746.
[25] D. Liu, L. Ou, G.D. Clemenson Jr., C. Chao, M.E. Lutske, G.P. Zambetti, F.H. Gage, Y. Xu, Puma is required for p53-induced depletion of adult stem cells, Nat. Cell Biol. 12 (2010) 993–998.
[26] F. Vaillant, D. Merino, L. Lee, K. Breslin, B. Pal, M.E. Ritchie, G.K. Smyth, M. Christie, L.J. Phillipson, C.J. Burns, G.B. Mann, J.E. Visvader, G.J. Lindeman, Targeting BCL-2 with the BH3 mimetic ABT-199 in estrogen receptor-positive breast cancer, Cancer Cell 24 (2013) 120–129.