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Displacement of Bim by Bmf and Puma rather than increase in Bim level mediates paclitaxel-induced apoptosis in breast cancer cells

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Abstract

Taxanes exert their antitumor effect via stabilizing microtubule dynamics and initiating G2/M arrest in cancer cells followed by apoptotic cell death. However, the signaling pathways that connect paclitaxel-induced microtubule perturbation to mitochondrial outer membrane permeabilization (MOMP) and cytochrome *c* release are not well characterized. Here we demonstrate that in breast cancer cells paclitaxel induces a novel displacement mechanism: prodeath BH3-only proteins Bmf and Puma competitively displace prodeath BH3-only protein Bim from anti-apoptotic proteins to activate Bax and Bak and commit the cell to apoptotic death. Bim and either Puma or Bmf are required for paclitaxel toxicity. While prior mechanisms of apoptosis induced by taxol have focused on changes in Bim levels, we find that an increase is not required for paclitaxel killing of breast cancer cells. Rather, competitive displacement of Bim from anti-apoptotic proteins is the key step committing the cell to death. This novel mechanism suggests the potential utility of novel therapies targeted at altering BH3-only protein heterodimerization.

Keywords

apoptosis; Bcl-2; Bcl-xL; Bim; Bmf; Puma; BH3; paclitaxel

Introduction

Paclitaxel is a chemotherapeutic drug effective against many human malignancies, including breast cancer. Similar to other taxanes, paclitaxel stabilizes microtubule dynamics, activates the spindle checkpoint and triggers mitotic arrest at the G2/M phase followed by apoptosis.[1] Apoptosis occurs either during mitotic arrest, or following “slippage” out of arrest into the cell cycle. Bax/Bak knockout MEFs are completely insensitive to paclitaxel treatment,

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Conflict of Interest

Dr. Letai is a cofounder of Eutropics Pharmaceuticals. He is an inventor on a patent application for BH3 profiling submitted by Dana-Farber Cancer Institute. Eutropics Pharmaceuticals has licensed this patent from Dana-Farber Cancer Institute.

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indicating the fundamental role of the mitochondrial apoptotic pathway in paclitaxel-induced cell death.[2] Nevertheless, the signaling pathways that connect paclitaxel-induced microtubule perturbation to mitochondrial outer membrane permeabilization (MOMP) and cytochrome *c* release are not well characterized.

The mitochondrial (or intrinsic) apoptotic pathway is utilized by most chemotherapeutic agents to kill cancer cells. The point of commitment to death in the mitochondrial apoptosis pathway is mitochondrial outer membrane permeabilization (MOMP) followed by the release of cytochrome *c* from the mitochondrial intermembrane space into cytosol.[3] Once in the cytosol, cytochrome *c* induces the formation a large multi-protein complex known as the apoptosome.[4] The apoptosome facilitates the activation of caspases, cysteine proteases which are important executors of the apoptotic process. MOMP is strictly controlled by Bcl-2 family proteins. Bax and Bak undergo an activating allosteric change and homo-oligomerize to form pores that cause MOMP. Bax and Bak are essential for mitochondrial apoptosis and prodeath function of BH3-only proteins.[5-7] Bax and Bak are activated by activator proteins that include the BH3-only proteins Bim and Bid, also members of the Bcl-2 family of proteins.[5, 8-11] Prosurvival Bcl-2 proteins (Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and Bfl-1) inhibit MOMP and cytosolic release of cytochrome *c* largely by sequestering activator BH3-only proteins (Bid, Bim and possibly Puma) before they can activate Bax and Bak.[5, 9, 10, 12] Another group of BH3-only proteins called sensitizers, which lack the ability to directly activate Bax or Bak, exert their prodeath function by binding prosurvival Bcl-2 proteins to displace activator BH3-only proteins. Sensitizers include Bad, Noxa, Bik, Puma and Bmf. Additionally; BH3-only proteins may directly displace Bax and Bak from their antiapoptotic binding partners to initiate prodeath signaling and to promote MOMP.[13, 14]

It is clear that a complicated interplay among proapoptotic and antiapoptotic proteins is responsible for determining commitment to cell death from many perturbations. In the present study we sought to determine the components of paclitaxel-triggered mitochondrial proapoptotic signaling in breast cancer cells. We chose breast cancer cells because breast cancer is one of the leading indications for taxane therapy, yet the mechanism of killing is still poorly understood. Here we report that paclitaxel activates the mitochondrial apoptotic pathway in breast cancer cells, in which Bim, Bmf and Puma collaborate in a novel and complex mechanism involving the common final displacement of Bim from antiapoptotic proteins by Bmf and Puma. In contrast with most previously described mechanisms of chemotherapy killing of cancer cells, this mechanism does not rely solely on changes in levels of proapoptotic proteins, but also in changes in their interaction partners.

Results

Paclitaxel kills MCF-7 breast cancer cells via the mitochondrial apoptotic pathway

We first tested whether paclitaxel killed breast cancer cells via the mitochondrial (intrinsic) apoptotic pathway. To evaluate the dose and time dependence of paclitaxel-induced apoptosis in MCF-7 cells, cells were examined after 48 h treatment with increasing doses of paclitaxel (0-100 nM) using Annexin V staining (Figure 1a). Apoptosis induction by paclitaxel (100 nM) was time-dependent, evident by 12 h, 24 h and 48 h after treatment. Release of cytochrome *c* from the mitochondrial intermembrane space to the cytosol is a key

characteristic of mitochondrial apoptosis. Treatment of MCF-7 cells with 100 nM paclitaxel triggered the release of cytochrome *c* from mitochondria into cytosol significantly at 12 h after treatment (Figure 1b). When MOMP is induced via the mitochondrial apoptotic pathway controlled by the Bcl-2 family of proteins, the multidomain proapoptotic Bcl-2 proteins Bax and Bak undergo an allosteric change that exposes an N-terminal epitope, which is accompanied by formation of oligomers on mitochondrial membrane to initiate the release of cytochrome *c*. In paclitaxel-treated MCF-7 cells, activation of Bax and Bak was demonstrated by immunoprecipitation with antibodies recognizing the exposed N-terminal epitope as early as 8 h following paclitaxel treatment (Figure 1c).

Caspase 8 activation is a hallmark of signaling via the extrinsic, or death receptor, apoptotic pathway. Caspase 9 activation, on the other hand, is a hallmark of killing via the intrinsic, or mitochondrial, cell death pathway. We found that cleavage of caspase-9, but not caspase-8 was detected in paclitaxel-treated cells by immunoblotting (Supplementary Figure S1a). Pretreatment of MCF-7 cells with a pancaspase inhibitor and a caspase-9 inhibitor, but not a caspase 8 inhibitor significantly prevented paclitaxel-induced apoptosis. (Supplementary Figure S1a). Selective activation of and dependence on caspase 9 rather than caspase 8 suggests that paclitaxel activates the intrinsic mitochondrial rather than the extrinsic apoptotic pathway.[4, 15, 16] The activation of Bax and Bak, release of cytochrome *c* and dependence on caspase 9 all suggest that death from paclitaxel occurs via the mitochondrial apoptotic pathway.

Since Bcl-2 protein family members are major regulators of the mitochondrial apoptotic pathway, we examined the expression of Bcl-2 family proteins in response to paclitaxel treatment. As shown in Figure 1d, with the exception of an increase in Bax protein, the other proteins examined are relatively stable through 24 hours of treatment. Only at 48 hours, a decrease in Bcl-xL, Bim and Bmf levels is observed. Given how late this occurs, the decreases are likely due to events such as decreased production or increased proteolysis following commitment to apoptosis. There were no substantial alterations of Bcl-2, Mcl-1, Bak, Noxa, Puma protein levels in response to paclitaxel treatment. There are not, therefore, widespread changes in levels of Bcl-2 family proteins observed prior to commitment to apoptosis following treatment with paclitaxel.

Bim is an activator BH3-only protein which has been reported to act as a tumor suppressor and to be essential for apoptosis induction by a number of stimuli such as chemotherapy, irradiation or growth factor withdrawal.[17-19] In particular, paclitaxel was shown to cause increased Bim levels in baby mouse kidney cells.[20] Indeed, in all of these reports, prodeath stimuli were found to kill by increasing Bim levels by either transcriptional or posttranslational mechanisms. However, paclitaxel treatment did not increase Bim protein levels in our experiments suggesting breast cancer cell line MCF-7 was killed via a novel mechanism (Figure 1d).

Paclitaxel treatment induces Bmf/Bcl-2 heterodimerization and disrupts Bim/Bcl-2 interaction

We have found that displacement of Bim from Bcl-2 can cause apoptosis in other systems.[21, 22] Therefore, we tested whether paclitaxel treatment induced a change in Bim binding by

anti-apoptotic proteins that correlated with apoptotic response. As shown in Figure 2a, while a substantial amount of Bim is bound by Bcl-2 prior to treatment; treatment with 100 nM paclitaxel for 12 h causes dissociation of the Bim/Bcl-2 complex. Note that Bim was also bound by Bcl-xL and Mcl-1 in untreated cells, but these interactions were not significantly altered by paclitaxel treatment (Figure 2a). All interactions were verified by reciprocal immunoprecipitation experiments. We also monitored the time course of Bim/Bcl-xL and Bim/Mcl-1 interactions following treatment with paclitaxel and our results did not reveal substantial changes except for slightly increased BimL and BimS interactions with Mcl-1 and BCL-xL(Supplemental Figure S2a). A key question, therefore, is whether treatment with taxol increases the amount of “free” Bim unbound by anti-apoptotic proteins. We found that more than half of cellular Bim was bound to Bcl-2, Bcl-xL and Mcl-1 in MCF-7 cells prtior to treatment, and an increase in unbound Bim levels could be detected following treatment with paclitaxel (Supplemental Figure S2b). Moreover, most of Bim was localized to heavy membrane or light membrane fractions in MCF-7 cells and the subcellular localization of Bim was not altered following paclitaxel treatment (Supplemental Figure S2b).

Since paclitaxel induced displacement of Bim from Bcl-2, we hypothesized that another protein(s) might be binding Bcl-2 to displace Bim. In Bcl-2-overexpressing MCF-7 cells, we tested whether paclitaxel treatment induced binding of BH3-only proteins Puma, Bad, or Bmf, or multi-domain proapoptotic Bax or Bak to Bcl-2. We found that only Bmf increased its interaction with Bcl-2 (Supplemental Figure S2c). Bmf is reported to be regulated through its interaction with myosin V motor complexes and activated and released from its association with dynein light chain 2 (DLC2) in response to anoikis.[23] We next tested whether the pattern of Bmf binding to antiapoptotic proteins was consistent with a potential role as the agent displacing Bim in parental MCF-7 cells. In Figure 2b, we demonstrate that paclitaxel treatment induces a Bmf/Bcl-2 interaction based on immunoprecipitation with a Bcl-2 antibody followed by immunoblotting for Bmf. This coimmunoprecipitation of Bmf with Bcl-2 was confirmed by reciprocal immunoprecipitation by a Bmf antibody and immunoblotting for Bcl-2. In this reciprocal immunoprecipitation Bmf/Bcl-2 interaction was evident as early as 4 h of paclitaxel treatment. Moreover, levels of Bmf complexed to Bcl-xL were significantly decreased following paclitaxel treatment in parallel with increased Bmf/Bcl-2 interaction. Mcl-1 did not interact with Bmf in either untreated or paclitaxel treated cells (results not shown). We also evaluated the interactions between antiapoptotic Bcl-2 proteins and Bax or Bak in response to paclitaxel treatment using coimmunoprecipitation experiments. We could not detect any Bax/Bcl-2, Bak/Bcl-2, Bak/Bcl-xL or Bax/Mcl-1 interaction (Supplemental Figure S2d). In contrast, our results demonstrated Bax/Bcl-xL interaction in MCF-7 cells, which was disrupted following paclitaxel treatment. Bak interacts weakly with Mcl-1 and this interaction was not altered by paclitaxel treatment (Supplemental Figure S2d). Hence, it is plausible that Bax displaced from Bcl-xL can also contribute to paclitaxel-induced cell death in cooperation with Bim displaced from Bcl-2.

Our results suggest that paclitaxel-induced signaling in MCF-7 cells induces Bmf to leave a complex with Bcl-xL to bind to Bcl-2, displacing Bim from Bcl-2 to activate the mitochondrial apoptosis pathway. If this is the case, loss of either Bim or Bmf should cause

reduced killing by paclitaxel. To test our hypothesis, MCF-7 cells were transfected with Bim siRNA, Bmf siRNA or a scrambled sequence siRNA. Reduction of Bim and Bmf levels in siRNA-treated cells was demonstrated by immunoblotting. As seen in Figure 2c, both Bim and Bmf protein levels were efficiently reduced by their corresponding siRNA duplexes and scrambled siRNAs did not have any notable effect. Loss of either Bim or Bmf significantly impaired the ability of paclitaxel to kill. This impairment was particularly prominent for Bim loss. The less complete rescue by Bmf leaves open the possibility that other molecules might also play a role in displacing Bim from Bcl-2. Knockdown of Bim or Bmf also decreased the activation of Bax and Bak in response to paclitaxel treatment in MCF-7 cells, indicating that these BH3-only proteins act upstream of Bax and Bak activation as expected (Figure 2d).

In addition, we also tested whether small-molecule Bcl-2 inhibitor ABT-737 restores the sensitivity of MCF-7 cells to paclitaxel treatment following Bmf and Bim knockdown. ABT-737 treatment effectively rescued the loss of Bmf and to a lesser extent loss of Bim paclitaxel sensitivity, although ABT-737 itself did not show any significant effect when used alone at 100 nM (Supplementary Figure S1b). In addition, ABT-737 did not induce further cell death in untransfected MCF-7 cells when combined with paclitaxel, which could be due to already efficiently engaged prodeath signaling by paclitaxel in these cells.

If displacement of Bim is causing apoptosis, then loss of Bim should reduce sensitivity to peptides which compete for Bim binding to antiapoptotic proteins. We tested this using BH3 profiling, a tool that measures mitochondrial dysfunction after treatment with peptides derived from BH3-only proteins.[21, 24] We compared mitochondrial dysfunction in response to BH3 peptides in MCF-7 cells in with and without knockdown of Bim (Figure 2e). Knockdown of Bim caused decreased sensitivity to peptides, consistent with displacement of pre-existing Bim being the cause of mitochondrial permeabilization.

If Bmf is responsible for Bim displacement, then we would expect that the Bim/Bcl-2 complex would be maintained even after paclitaxel treatment if Bmf levels were reduced. In Figure 2f, we examine levels of the Bim/Bcl-2 complex before and after paclitaxel treatment, in the presence and absence of siRNA for Bmf. As expected, addition of paclitaxel causes loss of the Bim/Bcl-2 complex (compare lanes 1 and 2). However, when Bmf is reduced with siRNA, the Bim/Bcl-2 complex is largely maintained, suggesting that Bmf is necessary for the displacement of Bim from Bcl-2 after paclitaxel treatment (compare lanes 2 and 4). Note that similar results are obtained by reciprocal immunoprecipitation and blotting of Bcl-2 and Bmf, or Bmf and Bcl-2, respectively. On the other hand, depletion of endogenous Bad by siRNA did not have any affect on Bim/Bcl-2 complex and Bim was displaced from Bcl-2 upon paclitaxel treatment, consistent with the ability of Bad to promote MOMP in the absence of Bim in whole cell JC-1 assays (Supplementary Figure S1c). Treatment of cells with scrambled siRNA did not have any effect.

Our findings show that in the MCF-7 breast cancer cell line, paclitaxel treatment causes displacement of Bmf from Bcl-xL which displaces Bim from Bcl-2, followed by Bax and Bak activation and commitment to apoptosis.

Paclitaxel kills other breast cancer cells via the mitochondrial apoptotic pathway

Above we described a novel mechanism of serial displacement of pro-apoptotic proteins from anti-apoptotic proteins that governs commitment to apoptosis in paclitaxel-treated MCF-7 cells. To explore whether paclitaxel activates a similar proapoptotic pathway in other breast cancer cells, we exposed T47D, MDA-MB-468 and BT20 cells to increasing concentrations of paclitaxel for 48 h and 100 nM paclitaxel for 0-48 h. Using identical methods to those in Figures 1 and 2 for MCF-7, we found that paclitaxel induced mitochondrial apoptosis at similar concentrations with similar kinetics in the T47D, MDA-MB-468, and BT20 breast cancer cell lines (Figure 3a). Cytochrome *c* was released from the mitochondria and Bax and Bak were activated with similar kinetics in all cases (Supplementary Figure S3a-S3c, S4a-S4c). Treatment of breast cancer cells with paclitaxel for up to 48 hours also revealed the activation of caspases 3 and 9 in breast cancer cells as assessed by immunoblot analysis of cleaved caspase fragments and fluorogenic caspase assays (Supplementary Figure S1d, e and f). Moreover, paclitaxel-induced apoptosis was blocked by pretreating cells with pancaspase inhibitor Z-VAD-FMK and caspase-9 inhibitor Z-LEHD-FMK, but not the caspase-8 inhibitor Z-IETD-FMK (Supplementary Figure S1d, e and f). In all breast cancer cell lines studied, therefore, paclitaxel kills in a caspase 9-dependent fashion using the mitochondrial apoptotic pathway.

In T47D, MDA-MB-468 and BT20 cells, we also examined the levels of Bcl-2 proteins in response to paclitaxel treatment for 0-48 h. Treatment with paclitaxel led to increased Puma and modestly increased Bim levels at 8-12 h and decreased Bcl-2 levels at 48 h of treatment in T47D cells. Notably, Bmf is undetectable by immunoblot analysis (Figure 3b). In MDA-MB-468 cells, paclitaxel treatment resulted in increased Bcl-xL and Bmf levels, other Bcl-2 proteins were not altered (Figure 3c). Paclitaxel treatment did not induce any significant change in Bcl-2 proteins in BT20 cells (Figure 3d).

Bim displacement from anti-apoptotic proteins is critical for paclitaxel-induced apoptosis

Since Bim displacement was so important in paclitaxel-mediated killing of MCF-7 cells, we also investigated the interaction of Bim with antiapoptotic Bcl-2 proteins Bcl-2, Bcl-xL and Mcl-1 in response to paclitaxel (100 nM) treatment for these other breast cancer cell lines. In Figure 4a, we show that Bim interacts with all three antiapoptotic proteins in untreated T47D cells, but the interaction with Bcl-xL is easiest to detect. Only Bim bound to Bcl-xL decreases following paclitaxel treatment in T47D cells. In MDA-MB-468 and BT20 cells, Bim was also found to be complexed to all three antiapoptotic Bcl-2 proteins. Paclitaxel treatment led to decreased levels of Bcl-2/Bim and Bcl-xL/Bim complexes in these two cell lines (Figure 4b and 4c). Similar to MCF-7 and T47D cells, the Mcl-1/Bim interaction did not change upon paclitaxel treatment in these cell lines (Figure 4b and 4c). In common with MCF-7 cells, displacement of Bim from anti-apoptotic proteins is a consistent event following paclitaxel treatment in all four breast cancer cell lines.

To test whether Bim was again required for paclitaxel-induced apoptosis in these breast cancer cells, we treated cells with Bim siRNA or a scrambled sequence siRNA followed by paclitaxel treatment for 48 h. We confirmed the efficiency of Bim knockdown by immunoblot analysis. As demonstrated in Figure 5a, knockdown of Bim in T47D, MDA-

MB-468 and BT20 cells efficiently blocked paclitaxel-induced apoptosis. In parallel to Annexin V staining, we also monitored the effect of Bim depletion on paclitaxel-induced activation of Bax and Bak in these cell lines. Our results show that depletion of Bim levels by siRNA-mediated knockdown consistently prevented paclitaxel-induced Bax and Bak activation (Figure 5b). These results revealed the pivotal role of Bim upstream of Bax and Bak activation in paclitaxel-induced apoptosis.

Bmf binds to Bcl-2 and Bcl-xL upon paclitaxel treatment and is required for Bim displacement and cell death

To evaluate the involvement of Bmf in paclitaxel-induced apoptosis in MDA-MB-468 and BT20 cells, we initially monitored whether Bmf interacts with antiapoptotic Bcl-2 family proteins in response to paclitaxel treatment for 0-12 hours using coimmunoprecipitation experiments. As demonstrated in Figure 6a, both Bcl-2 and Bcl-xL interact weakly with Bmf in untreated MDA-MB-468 cells, which was more clearly shown with reciprocal Bmf immunoprecipitation experiments. Dramatically increased Bmf/Bcl-2 and Bmf/Bcl-xL interactions in MDA-MB-468 were detected after 8 h of paclitaxel treatment. Similarly, paclitaxel treatment resulted in induction of Bmf/Bcl-2 and Bmf/Bcl-xL protein complexes in BT20 cells (Figure 6b). In neither case was an interaction between Bmf and Mcl-1 detectable (not shown). Note that this pattern of Bmf binding to Bcl-2 and Bcl-xL matches the displacement of Bim from the same proteins with the same kinetics. Because increased Puma levels and decreased Bim/Bcl-xL interaction were observed in T47D cells, we tested whether Puma can contribute to paclitaxel-induced apoptosis in this cell line. Thereby, we investigated the interaction of Puma with Bcl-xL in response to paclitaxel treatment for 12 h. In Figure 6c, we show that paclitaxel induces increased Bcl-xL-bound Puma levels, which was also evident in reciprocal immunoprecipitation experiments.

We then tested the requirement for Bmf in paclitaxel-induced apoptosis in MDA-MB-468 and BT20 cells by using siRNA-mediated knockdown of Bmf. Pretreatment of MDA-MB-468 and BT20 cells with Bmf siRNA significantly decreased paclitaxel-induced apoptosis after 48 h of paclitaxel treatment (Figure 6d). Of note, this effect was more profound in BT20 cells in which the knockdown was more efficient. As a negative control, Bmf siRNA had no effect on paclitaxel killing of T47D cells which do not express Bmf (data not shown). By contrast, knockdown of Puma in T47D cells significantly decreased paclitaxel-induced apoptosis, suggesting the involvement of Puma in paclitaxel-induced proapoptotic signaling in T47D cells. Treatment of cells with scrambled siRNA did not have any effect on paclitaxel-induced cell death in all three cell lines. Of note, depletion of Puma by RNA interference did not alter the apoptotic response by paclitaxel in MDA-MB-468, BT20 and MCF-7 cells (Supplementary Figure S5a, b and c). In agreement with the inhibitory effect of Bmf knockdown on apoptotic response, activation of Bax and Bak after 12 h of paclitaxel treatment was also suppressed when we depleted Bmf by siRNA in MDA-MB-468 and BT20 cells (Figure 6e). To test whether Bmf is capable of displacing Bim from Bcl-2, we employed fluorescence polarization assays using FITC-labeled Bim BH3 and Bmf BH3 peptides. Unlabeled Bim and Bmf BH3 peptides were titrated to compete with the binding of FITC-labeled BH3 peptides as depicted by loss of fluorescence polarization. Our

data demonstrated that both Bim and Bmf were capable of displacing each other from Bcl-2 with similar efficiencies (Figure 6f).

Since we demonstrated that depletion of Bmf in MCF-7 cells led to decreased displacement of Bim from Bcl-2 (Figure 2f), we tested whether a similar mechanism was also active in MDA-MB-468 and BT20 cells. To address this question, we incubated cells with paclitaxel for 12 h following treatment with Bmf siRNA and performed reciprocal coimmunoprecipitation experiments in total protein lysates with anti-Bcl-2, anti-Bcl-xL and anti-Bim antibodies. As shown in Figure 7a, Bim is displaced from Bcl-2 and Bcl-xL in response to paclitaxel treatment in MDA-MB-468 cells and knockdown of Bmf effectively inhibits this process. Likewise, depletion of Bmf by siRNA treatment greatly diminished paclitaxel-induced displacement of Bim from Bcl-2 and Bcl-xL in BT20 cells (Figure 7b). In both cases, the same effect is observed when reciprocal immunoprecipitations are studied. Reasoning that Puma binds to Bcl-xL and displaces Bim to execute its proapoptotic role in response to paclitaxel treatment in T47D cells, we sought to understand whether a similar mechanism of cooperation exists between Puma and Bim as we have seen between Bmf and Bim. To test this, we treated T47D cells with paclitaxel for 12 h following treatment with Puma siRNA and performed reciprocal coimmunoprecipitation experiments using anti-Bcl-xL and anti-Bim antibodies. Our results show that depletion of Puma by siRNA treatment decreased paclitaxel-induced displacement of Bim from Bcl-xL, consistent with a mechanism whereby increased Puma levels disrupts Bim/Bcl-xL complexes to trigger apoptosis (Figure 7c).

Bim is required for paclitaxel killing in 3-dimensional spheroid culture

Since monolayer cell cultures may fail to recapitulate the *in vivo* milieu of breast tumors, we tested whether Bim and Bmf plays a similar key proapoptotic role in cells grown in 3D culture. Treatment of BT20, MDA-MB-468 and MCF-7 with Bim or Bmf siRNA duplexes did not interfere with the growth and spheroid formation of these breast cancer cells in Algimatrix 3D culture plate (Figure 8a). Notably, BT20 and MCF-7 cells form mass-like spheroids and MDA-MB-468 cells form grape-like spheroids when grown in 3D cell culture conditions. We verified the efficiency and specificity of knockdown of Bim and Bmf in spheroids by immunoblotting of spheroid protein lysates (Figure 8b). Immunoblotting results clearly demonstrated that both Bim and Bmf were efficiently depleted in 3D spheroids. Furthermore, siRNA-mediated depletion of Bim or Bmf protected against paclitaxel-induced cell death as shown viability by alamar Blue assay (Figure 8c), supporting the prodeath role of Bim and Bmf in response to paclitaxel treatment in breast cancer cells.

Discussion

While taxanes have been a mainstay of chemotherapy for breast cancer for two decades, the mechanism by which paclitaxel kills breast cancer cells has been poorly understood. There have been suggestions, however, that Bim is a consistent participant in death signaling from paclitaxel. Observations of loss of paclitaxel killing with loss of Bim have been made in lymphocytes,[17] neuroblastoma,[25] and non-small cell lung cancer.[26] In these studies,

however, it was not clear how Bim was dynamically responding to paclitaxel. In baby mouse kidney epithelial cells, paclitaxel was found to cause an increase in Bim protein levels that could be inhibited by H-ras expression. [20]. A separate study suggested paclitaxel caused a transcriptionally mediated increase in Bim in breast cancer cell lines.[27] Bim levels increased at the relatively late timepoint of 48 hours, however, well after the time we see most breast cancer cells commit to apoptosis, suggesting that this phenomenon may be occurring downstream of the commitment to cell death.

Nonetheless, our initial expectation was that we would observe paclitaxel induce an increase in Bim in our breast cancer cell lines. Instead, we found that Bim levels, with the exception of a modest increase in T47D, stayed constant in breast cancer cells. However, we still found that Bim was required for killing. This apparent paradox was solved by our observation that Bim was displaced from antiapoptotic proteins following paclitaxel treatment in all breast cancer cell lines studied. In three out of the four cell lines, we found that the factor displacing Bim was the BH3-only protein Bmf. In fact, there was remarkable correlation between the temporal and protein-specific binding patterns of Bmf and the displacement of Bim. In MCF-7 cells, following paclitaxel treatment, Bmf bound Bcl-2 but not Bcl-xL or Mcl-1, and Bim was displaced from Bcl-2, but not Bcl-xL or Mcl-1 (Figure 2a, 2b). In MDA-MB-468 and BT20 cells, Bmf bound to Bcl-2 and Bcl-xL, but not Mcl-1 (Figure 6a and 6b), and Bim was displaced from Bcl-2 and Bcl-xL, but not Mcl-1 (Figure 4b and 4c).

In the case of T47D, since no Bmf is detectable, but Bim is nonetheless displaced, it seems likely that a different protein is responsible. Remarkably, we detected increased Puma and Bim levels in T47D cells following paclitaxel treatment (Figure 3d). Puma has been shown to mediate cell death in response to various proapoptotic stimuli[28], [29] and depletion of Puma by siRNA decreased paclitaxel-induced cell death in T47D cells, indicating that the increase in Puma protein levels can functionally complement for the absence of Bmf expression during paclitaxel-induced apoptosis. In fact, Puma was also identified as a direct activator BH3-only protein and it is possible that it may contribute to paclitaxel-induced cell death directly by activating Bax or Bak.[30] Nonetheless, depletion of Puma did not affect paclitaxel-induced cell death response in other breast cancer cell lines (Supplementary Figure S5a, b and c). Specifically, Puma is required for the displacement of Bim from Bcl-xL upon paclitaxel treatment in T47D cells, consistent with a mechanism whereby Puma acts as a sensitizer upstream of mitochondrial cell death pathway. A summary of this model maybe found in Figure 8d.

In addition, while the small-molecule Bcl-2 inhibitor ABT-737 did not have any proapoptotic effect when used alone, it restored the sensitivity of Bmf or Bim siRNA-pretreated MCF-7 cells to paclitaxel-induced cell death. Although ABT-737 itself did not induce apoptosis when used at 100 nM concentration, it effectively kills MCF-7 cells with an EC_{50} value of 2.88 μM, at which antiapoptotic reserve is efficiently saturated. Considering this notion, we can argue that coordinated paclitaxel-mediated prodeath signals may be needed for neutralizing antiapoptotic potential, facilitating Bmf to promote apoptosis by displacing Bim. The paclitaxel-sensitizing effect of ABT-737 was more significant in

Bmf depleted cells, supporting the role of Bmf as a sensitizer BH3-only protein which ABT-737 may functionally mimic in MCF-7 cells treated with paclitaxel.

Bim and Bmf share the properties of interacting with dynein motor components, dynein light chains 1 and 2, respectively.[19, 23] JNK-dependent phosphorylation of Bim and Bmf was shown to mediate UV-mediated apoptotic response via releasing these proteins from dynein motor components.[31] It is interesting to note that dynein motors themselves associate with the microtubule network, the target of paclitaxel. It is also possible that Bmf associates preferentially with the actin network via dynein light chain 2 association with the myosin V motor complex there.

This moves the question of how paclitaxel kills breast cancer cells a step further upstream from the mitochondrion. What regulates Bmf's ability to displace the Bim that is prebound to anti-apoptotic proteins? It does not appear to be a matter of increased Bmf levels, as total cellular levels do not generally change in response to paclitaxel in the cells studied here. Our results suggest that there is an obscure mechanism that alters Bmf heterodimerization to cause death from taxol. Candidate mechanisms would include post-translational modification, which we have not yet identified. However, it is intriguing to speculate that identification of the mechanism altering Bmf heterodimerization would provide a novel target for potential chemotherapeutic exploitation, since this is apparently a key feature of paclitaxel's ability to kill breast cancer cells.

Prior examination of the participation of Bcl-2 family proteins participation in death signaling has focused on changes in protein levels, either increase in proapoptotic members, or decrease in antiapoptotic members. In this paper, we show that a novel third mechanism, serial displacement of BH3-only proteins, is responsible for killing of breast cancer cells by paclitaxel. This mechanism has the potential to generate new targets for anticancer therapy by directly targeting cellular components that control BH3-only protein heterodimerization.

Materials and Methods

Cell lines

MCF-7, T47D, BT20 and MDA-MB-468 cells were grown in DMEM/F12 (Invitrogen) supplemented with 2 mM L-Glutamine, 10% heat-inactivated fetal bovine serum (Sigma), 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen) in a humidifed incubator at 37°C and 5% CO_2. 5 μg/ml insulin (Sigma) was added to DMEM/F12 medium used for the culture of T47D and MCF-7 cells.

For 3D cell cultures, cell spheroids were grown in AlgiMatrix 24-well plates (Invitrogen) as recommended by the manufacturer. Spheroids were isolated from matrix using iso-osmolar trisodium citrate for lysis and protein isolation.

Chemicals

Paclitaxel, trehalose, oligomycin, digitonin, succinate and FCCP were purchased from Sigma. JC-1 was purchased from Invitrogen. Caspase-9 inhibitor (z-LEHD-FMK),

pancaspase inhibitor (z-VAD-FMK) and caspase-8 inhibitor (z-IETD-FMK) were obtained from BD Biosciences Pharmingen. ABT-737 was provided by Abbott Laboratories.

Cell viability and apoptotic assays

Apoptosis was evaluated by Annexin V-FITC (BioVision) staining according to the manufacturer's protocols. Apoptosis was quantified by flow cytometry on a FACSCalibur (BD Biosciences), followed by analysis using WinMDI 2.9 software (Scripps Institute, La Jolla, CA). alamarBlue assay (Invitrogen) was used to monitor cell viability in cell spheroids grown in 3D culture as described by the manufacturer and results were expressed as % cell viability.

Coimmunoprecipitation and immunoblotting

Total cell lysates were prepared in 1% Chaps buffer [5 mM $MgCl_2$, 137 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Chaps, 20 mM Tris-HCl (pH 7.5), and protease inhibitors (Complete, Roche)] as described previously.[32] Proteins (800-2400 μg) were immunoprecipitated with anti-Bcl-2 (Bcl-2/100; BD Pharmingen), anti-Bcl-xL (Cell Signaling), anti-Bim (22-40; Calbiochem), anti-Bmf (Cell Signaling), anti-Puma (Cell Signaling), Mcl-1 (S-19; Santa Cruz), anti-Bax (6A7; BD Pharmingen), anti-Bak (Ab-2, Oncogene Research) at 4°C for 2 h. Immunoprecipitates were captured by a 50% slurry of protein G-Sepharose in lysis buffer (GE Healthcare) at 4°C for 2 h or overnight. Immunoprecipitates were then recovered by centrifugation and washed three times in 1% Chaps buffer (0.25% for Bmf coimmunoprecipitations). Immunoprecipitates, total cell extracts (40 μg) and subfractionation lysates (30 μg) were separated on NuPage 10% Bis-Tris gels. Following SDS-PAGE, proteins were transferred onto PVDF membranes (Immobilon, Millipore) and then blocked with 5% dried milk in PBS-Tween20. Membranes were incubated with primary and secondary antibodies (GE Healthcare) in a buffer containing 10% milk diluent blocking concentrate (KPL), detected with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer) and exposed to Biomax MR film (Kodak). For detection of some immunoprecipitates, protein A-HRP (GE Healthcare) was employed as a secondary detection agent. The following antibodies were used for immunoblotting: anti-Bcl-2, anti-Bcl-xL, anti-Bmf, anti-Bad, anti-Puma, anti-Actin, anti-caspase-3, anti-caspase-9, anti-caspase-8, anti-CoxIV (Cell Signaling), anti-Noxa (Calbiochem), anti-Bax (N20; Santa Cruz), anti-Bak (G-23; Santa Cruz), anti-BiP/Grp78 (BD Biosciences) and anti-cytochrome *c* (6H2.B4; BD Biosciences). All critical blots and immunoprecipitation experiments were repeated at least three times.

siRNA transfection

Cells were transfected with Bim siRNA (Hs_BCL2L11_5 HP Validated siRNA; Qiagen), Bmf siRNA (Hs_BMF_5 HP Validated siRNA, Qiagen), Puma siRNA (Hs_BBC3_2 HP Validated siRNA; Qiagen), Bad siRNA (Hs_BAD_3 HP Validated siRNA; Qiagen) and negative control (Scrambled) siRNA (AllStars Negative Control siRNA, Qiagen) by using Hiperfect transfection reagent (Qiagen) according to manufacturer's instructions. Protein knockdown efficiencies by siRNA transfection were verified by immunoblotting after 48 h of transfection. For 3D cell culture experiments, cells were transfected with corresponding

siRNA duplex for 12 h and transferred to 3D culture matrix for the growth of cell spheroids in the presence of siRNA treatment for every 24 hours. Protein knockdown efficiencies in cell spheroids were verified by immunoblotting.

Caspase activation assays

The activity of Caspase-3, -9 and -8 was determined by ApoAlert Caspase Profiling Plate (Clontech) according to manufacturer's protocol. The release of fluorochrome AMC was analyzed at 380 nm excitation and 460 nm emission using a multiplate fluorescence spectrophotometer. Data shown are mean ± SEM of three independent experiments in duplicate and expressed in arbitrary fluorescence units (AFU)/mg of protein.

Whole cell BH3 profiling

Cells were permeabilized in T-EB buffer [300 mM Trehalose, 10 mM Hepes-KOH, pH 7.7, 80 mM KCl, 1 mM EGTA, 1 mM EDTA, 0.1% BSA (w/v), 5 mM succinate] in the presence of 0.005% digitonin, 5 mM β-mercaptoethanol, 10 μg/ml oligomycin, 1 μM JC-1 and treated with BH3 peptides (100 μM). Peptides used in this assay were synthesized by Tufts University Core Facility and purified by HPLC. Stock peptide solutions were prepared in DMSO. Peptide sequences used in this assay were as previously described [21]. Cells were transferred to 364-well plates and JC-1 fluorescence was analyzed at 545 nm excitation and 590 nm emission using a multiplate fluorescence spectrophotometer. Data shown are mean ± SEM of three independent experiments in duplicate and expressed as %$\Delta\psi$m (% mitochondrial membrane potential) loss compared with DMSO-treated cells. FCCP was used as a positive control.

Subcellular fractionation

Subcellular fractionation was performed as described before.[33] Briefly, cells were harvested and washed in ice-cold PBS and then resuspended in an isotonic buffer [250 mM sucrose, 20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulphonyl fluoride, and protease inhibitors (Complete, Roche)] on ice for 20 min. Following incubation, cells were homogenized with Dounce homogenizer and centrifuged at 800*g* for 10 min at 4°C. The resulting supernatant was centrifuged at 8,000*g* for 20 min at 4°C to obtain mitochondria-enriched HM (heavy membrane) pellet and cytosolic supernatant fractions. These fractions were used to monitor cytochrome *c* release from mitochondria. To further obtain LM (light membrane) and S-100 fractions, the remaining supernatant (cytosolic fraction) was further centrifuged at 100,000*g* for 1 h at 4°C. The resulting pellet constituted LM and the supernatant was saved as S-100. HM and LM fractions were lysed in 1% Chaps buffer for immunoblot analysis.

Fluorescence polarization binding assays

Binding assays were performed using fluorescence polarization as previously described. [21] For Bim BH3 and Bmf BH3 displacement assays, 10 nM fluorescence-labeled Bim BH3 or Bmf BH3 peptide was incubated with 100 μM GST-Bcl-2 in binding buffer for 10 min. Bmf or Bim BH3 peptides were titrated and displacement of Bim BH3 or Bmf BH3 was detected by loss of fluorescence polarization using Safire2 plate reader (Tecan). Non-linear

regression of sigmoidal dose-response curves were fitted by using GraphPad Prism 3.0 software. Data shown are mean ± SEM of four independent experiments in duplicate.

Statistical analysis

Statistical significance of the results was analyzed using Student's t-tail test using GraphPad Prism 3.0 software. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Δψm, MMP	mitochondrial membrane potential
MOMP	mitochondrial outer membrane permeabilization
FCCP	Carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone
FITC	Fluorescein isothiocyanate
BSA	Bovine serum albumin
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide

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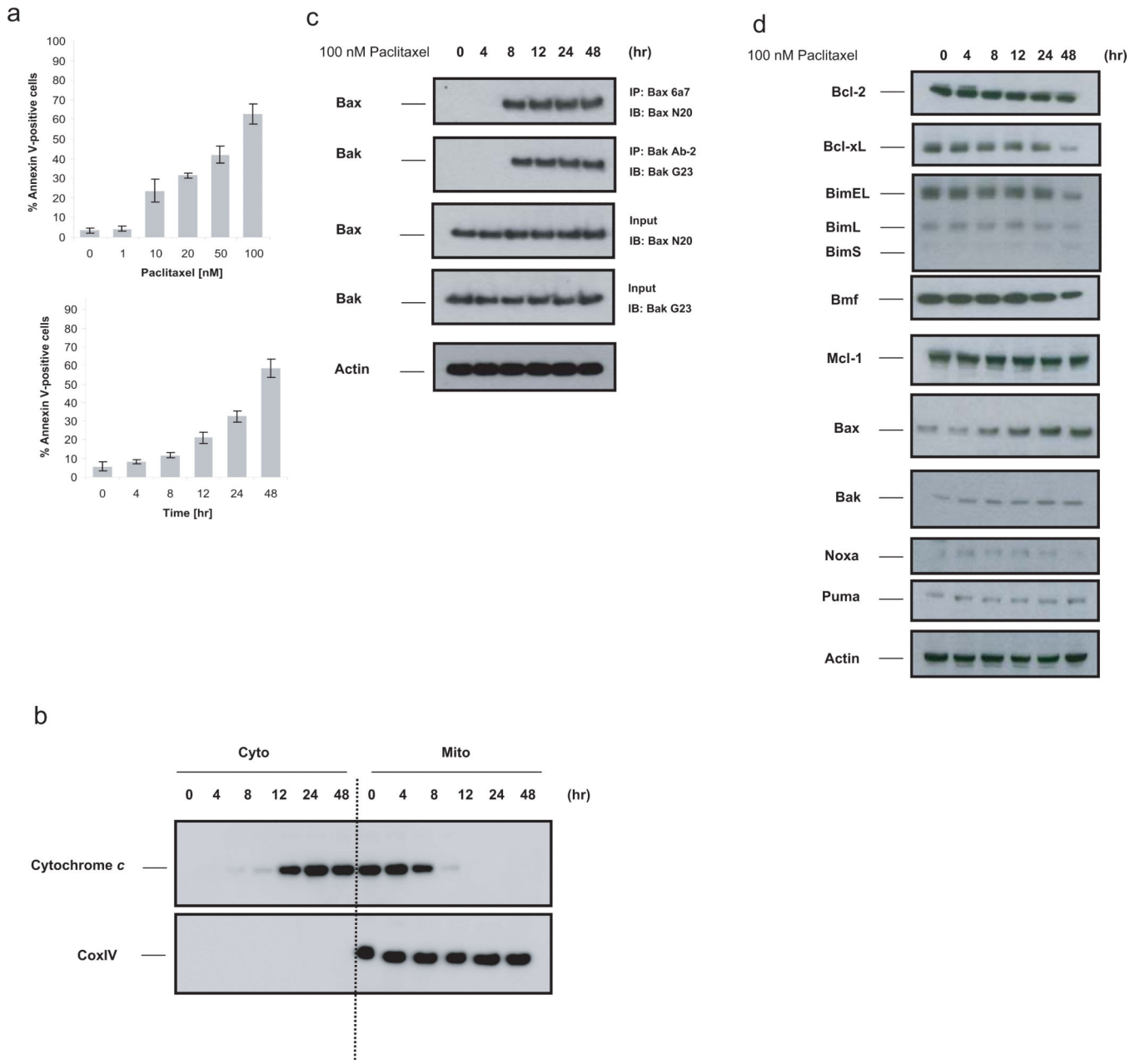

Figure 1.

Paclitaxel activates the mitochondrial apoptotic pathway in MCF-7 cells. **(a)** MCF-7 cells were treated with paclitaxel (0, 1, 10, 20, 50, and 100 nM) for 48 h or 100 nM paclitaxel for 0-48 h and apoptosis was evaluated by Annexin V staining (mean ± SEM, n = 4). **(b)** MCF-7 cells were treated with paclitaxel (100 nM) for 0-48 h. Cytosolic and mitochondrial fractions were immunoblotted for cytochrome *c*. CoxIV was probed as a loading control for mitochondrial fractions. **(c)** Activation of Bax and Bak in MCF-7 cells treated with paclitaxel (100 nM) for 0-48 h was analyzed by immunoprecipitation with active conformation-specific anti Bax (6A7) and anti-Bak (Ab-2) antibodies followed by immunoblot analysis of Bax and Bak. Inputs for immunoprecipitations were also detected

immunoblot analysis. Actin was probed as a loading control. **(d)** Bcl-2 protein levels in MCF-7 cells were detected by immunoblot analysis following treatment with paclitaxel (100 nM) for 0-48 h.

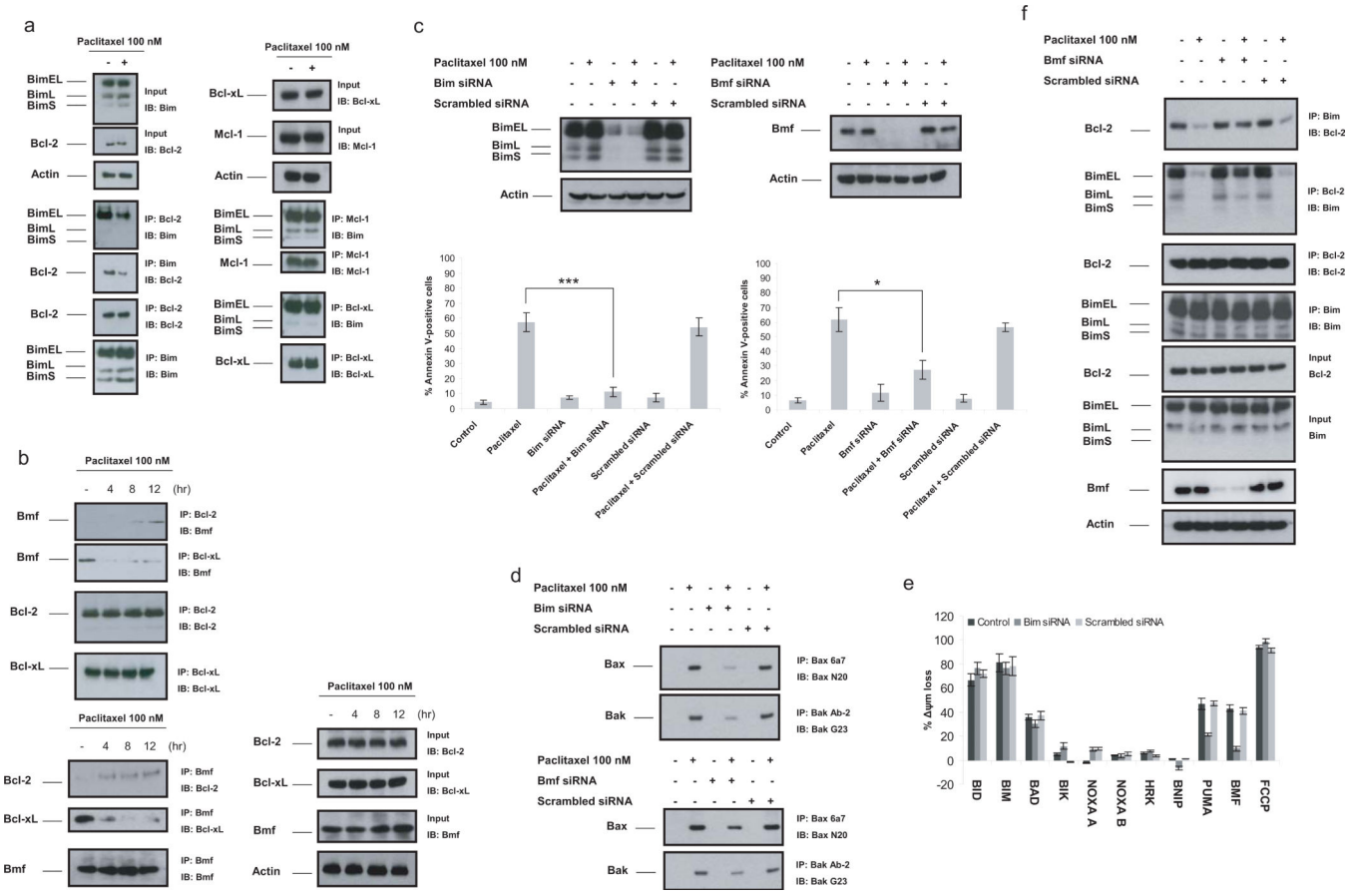

Figure 2.

Bmf and Bim are required for paclitaxel-induced apoptosis. **(a)** MCF-7 cells were treated with paclitaxel (100 nM) for 12 h and the interaction of Bim with Bcl-2, Bcl-xL and Mcl-1 was detected by coimmunoprecipitation assays. Inputs for coimmunoprecipitations were also subjected to immunoblot analysis and actin was probed as a loading control. **(b)** MCF-7 cells were treated with paclitaxel (100 nM) for 0, 4, 8, and 12 h and the interaction of Bmf with Bcl-2 and Bcl-xL was evaluated by coimmunoprecipitation assays. 5% of the input for coimmunoprecipitation was also subjected to immunoblot analysis and actin was probed as a loading control.

(c) MCF-7 cells were transiently transfected with Bim siRNA, Bmf siRNA or Scrambled siRNA for 48 h. The efficiency of knockdown was monitored by immunoblots. Untransfected and siRNA-transfected cells were treated with paclitaxel (100 nM) for 48 h and apoptosis was evaluated by using Annexin V staining (mean ± SEM, n = 4, *p< 0.05, ***p < 0.001 by two-tailed t test). **(d)** MCF-7 cells were transiently transfected with Bim siRNA, Bmf siRNA or Scrambled siRNA for 48 h. Cells were treated with paclitaxel (100 nM) for 12 h and activation of Bax and Bak was analyzed by immunoprecipitation with active conformation-specific anti Bax (6A7) and anti-Bak (Ab-2) antibodies followed by immunoblot analysis of Bax and Bak. **(e)** MCF-7 cells were transiently transfected with Bim siRNA or Scrambled siRNA for 48 h. Cells were the treated with corresponding BH3 peptides as described in Materials and Methods and loss of Δψm (mitochondrial membrane

potential) was measured using multiplate fluorometer at 100 min. Results were expressed as %Δψm loss and FCCP was used as a positive control. **(f)** MCF-7 cells were transiently transfected with Bmf siRNA or Scrambled siRNA for 48 h. The interaction of Bim with Bcl-2 was evaluated by coimmunoprecipitation assays following 12 h paclitaxel (100 nM) treatment. The efficiency of Bmf knockdown was monitored by immunoblot analysis. Inputs for coimmunoprecipitations were also subjected to immunoblot analysis and actin was probed as a loading control.

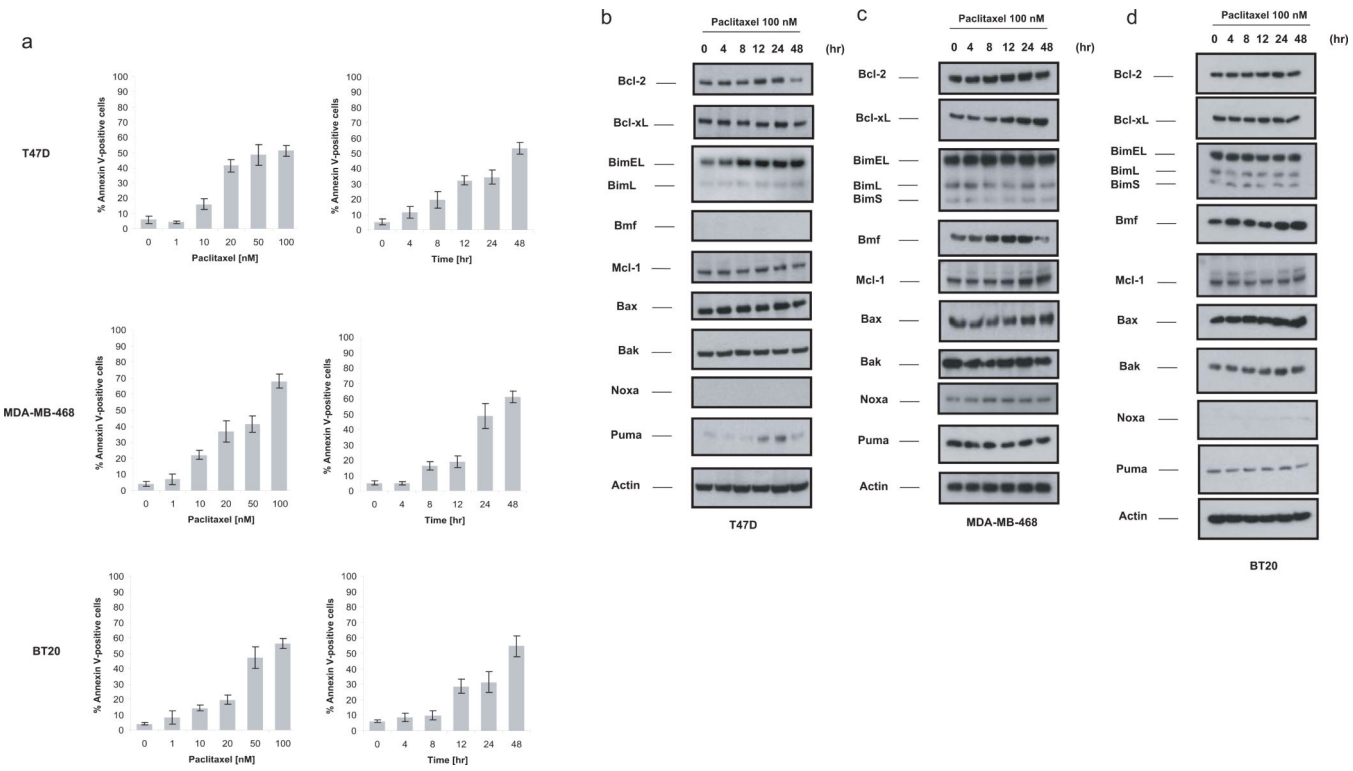

Figure 3.

Paclitaxel activates the mitochondrial apoptotic pathway in breast cancer cells. **(a)** T47D, MDA-MB-468 and BT20 cells were treated with paclitaxel (0, 1, 10, 20, 50, and 100 nM) for 48 h or 100 nM paclitaxel for 0-48 h and apoptosis was evaluated by Annexin V staining (mean ± SEM, n = 4). Bcl-2 family proteins in **(b)** T47D, **(c)** MDA-MB-468 and **(d)** BT20 cells were detected by immunoblot analysis following treatment with paclitaxel (100 nM) for 0-48 h. Actin was probed as a loading control.

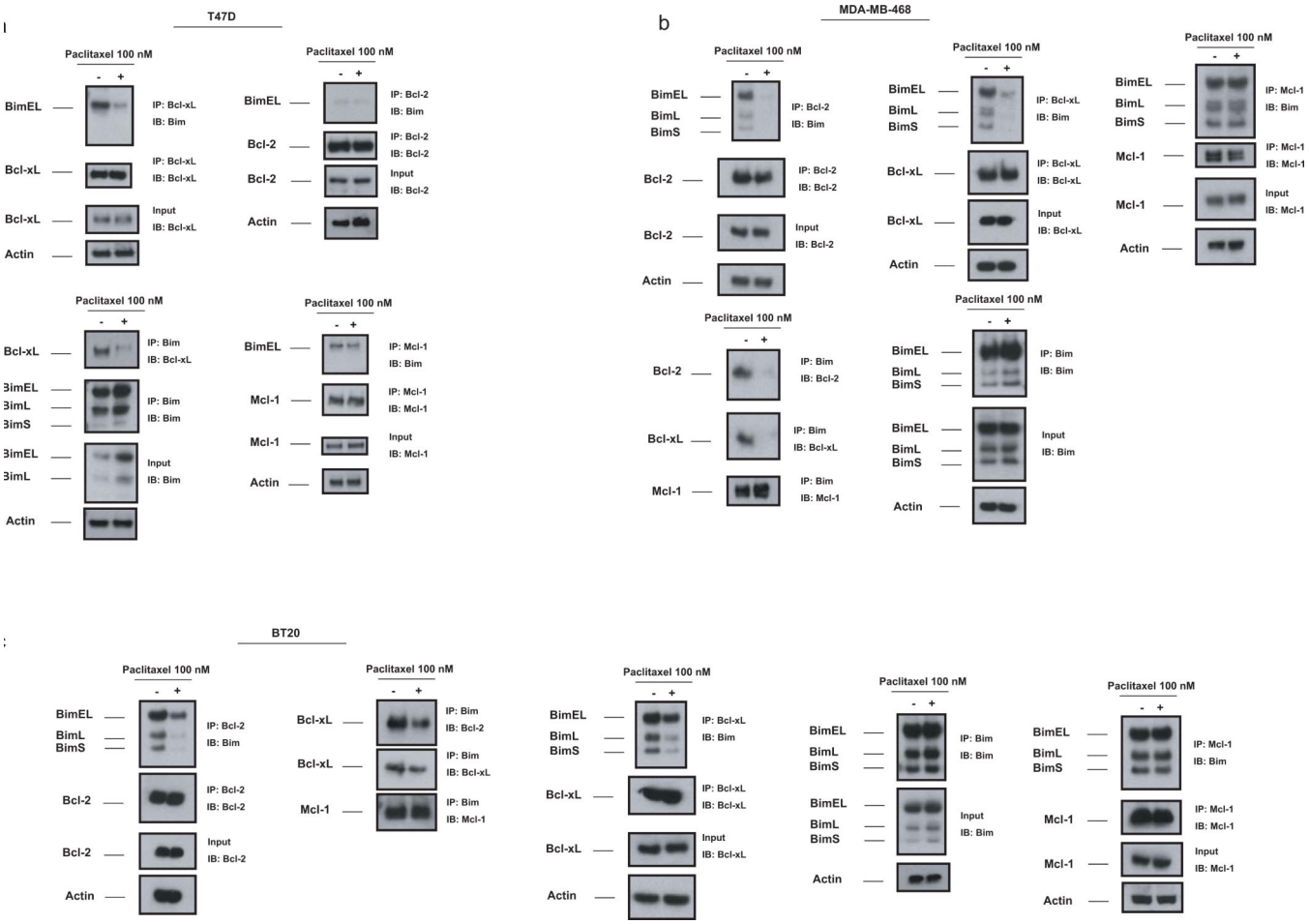

Figure 4.

Bim is displaced from Bcl-2 and Bcl-xL following paclitaxel treatment. **(a)** T47D, **(b)** MDA-MB-468 and **(c)** BT20 cells were treated with paclitaxel (100 nM) for 12 h and the interaction of Bim with Bcl-2, Bcl-xL and Mcl-1 was detected by coimmunoprecipitation assays. Inputs for coimmunoprecipitations were also subjected to immunoblot analysis and actin was probed as a loading control.

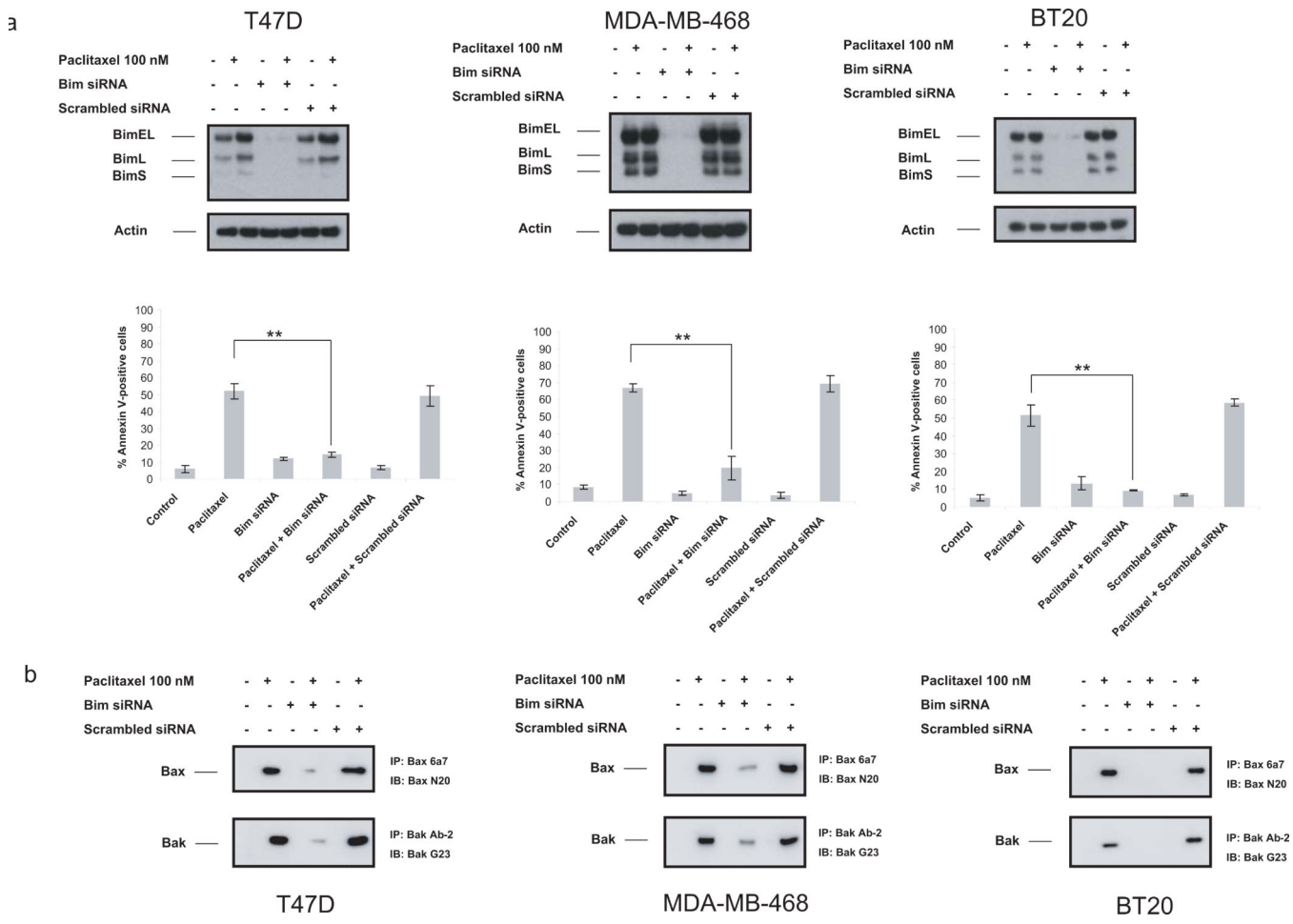

Figure 5.

Bim is required for paclitaxel-induced apoptosis in breast cancer cells. **(a)** T47D, MDA-MB-468 and BT20 cells were transiently transfected with Bim siRNA or Scrambled siRNA for 48 h. The efficiency of knockdown was monitored by immunoblots. Untransfected and siRNA-transfected cells were treated with paclitaxel (100 nM) for 48 h and apoptosis was evaluated by using Annexin V staining (mean ± SEM, n = 4, **p< 0.01 by two-tailed t test). **(b)** T47D, MDA-MB-468 and BT20 cells were transiently transfected with Bim siRNA or Scrambled siRNA for 48 h. Cells were treated with paclitaxel (100 nM) for 12 h and activation of Bax and Bak was analyzed by immunoprecipitation with active conformation-specific anti Bax (6A7) and anti-Bak (Ab-2) antibodies followed by immunoblot analysis of Bax and Bak.

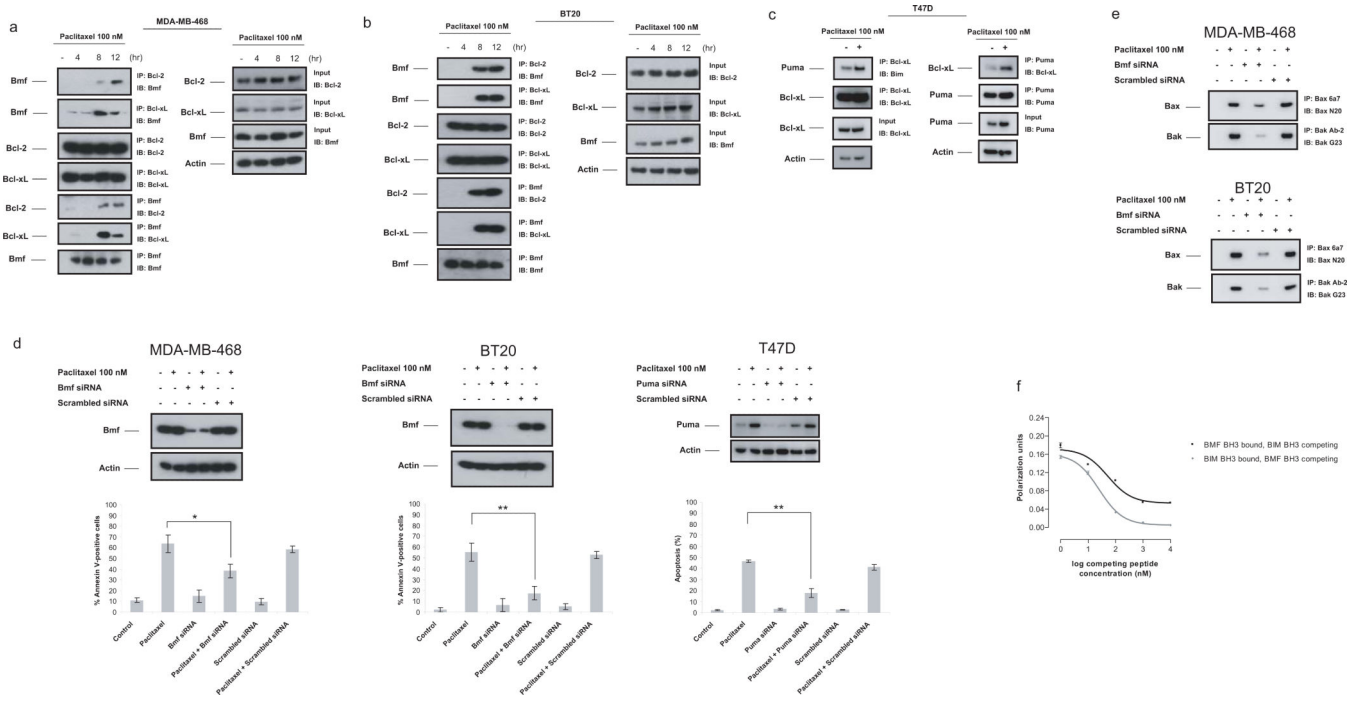

Figure 6.

Bmf is required for paclitaxel-induced apoptosis in breast cancer cells. **(a)** MDA-MB-468 and **(b)** BT20 cells were treated with paclitaxel (100 nM) for 0, 4, 8, and 12 h and the interaction of Bmf with Bcl-2 and Bcl-xL was evaluated by coimmunoprecipitation assays. Inputs for coimmunoprecipitations were also subjected to immunoblot analysis and actin was probed as a loading control. **(c)** T47D cells were treated with paclitaxel (100 nM) 12 h and the interaction of Puma with Bcl-xL was evaluated by coimmunoprecipitation assays. Inputs for coimmunoprecipitations were also subjected to immunoblot analysis and actin was probed as a loading control. **(d)** MDA-MB-468 and BT20 cells were transiently transfected with Bmf siRNA or Scrambled siRNA for 48 h. T47D cells were transiently transfected with Puma siRNA or Scrambled siRNA for 48 h. The efficiency of knockdowns was monitored by immunoblots. Untransfected and siRNA-transfected cells were treated with paclitaxel (100 nM) for 48 h and apoptosis was evaluated by using Annexin V staining (mean ± SEM, n = 4, *p< 0.05, **p< 0.01 by two-tailed t test). **(e)** MDA-MB-468 and BT20 cells were transiently transfected with Bmf siRNA or Scrambled siRNA for 48 h. Cells were treated with paclitaxel (100 nM) for 12 h and activation of Bax and Bak was analyzed by immunoprecipitation with active conformation-specific anti Bax (6A7) and anti-Bak (Ab-2) antibodies followed by immunoblot analysis of Bax and Bak. **(e)** Fluorescence polarization assays were performed using recombinant GST-Bcl-2 (100 μM) and FITC-labeled Bim and Bmf BH3 peptides (10 nM). Unlabeled Bim and Bmf BH3 peptides were used to compete with binding of FITC-labeled Bim and Bmf BH3 peptides to test the ability of Bim and Bmf to displace each other from Bcl-2. Data shown are mean ± SEM of four independent experiments in duplicate.

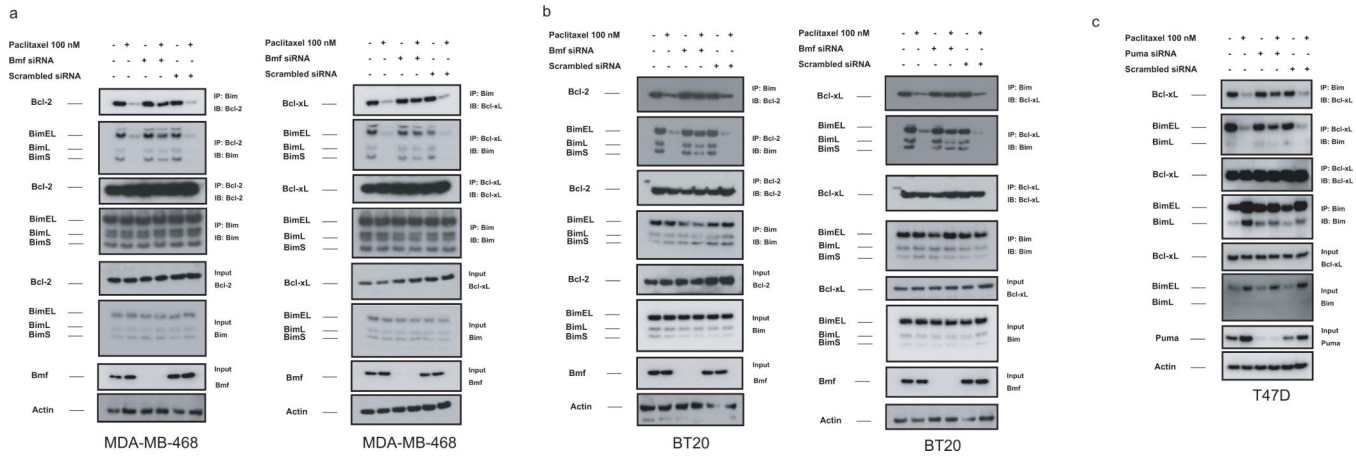

Figure 7.

Bmf displaces Bim from Bcl-2 and Bcl-xL following paclitaxel treatment. **(a)** MDA-MB-468 and **(b)** BT20 cells were transiently transfected with Bmf siRNA or Scrambled siRNA for 48 h. The interaction of Bim with Bcl-2 and Bcl-xL following paclitaxel treatment (100 nM, 12 h) was evaluated by coimmunoprecipitation assays. The efficiency of Bmf knockdown was monitored by immunoblot analysis. Inputs for coimmunoprecipitations were also subjected to immunoblot analysis and actin was probed as a loading control. **(c)** T47D cells were transiently transfected with Puma siRNA or Scrambled siRNA for 48 h. The interaction of Bim with Bcl-xL following paclitaxel treatment (100 nM, 12 h) was evaluated by coimmunoprecipitation assays. The efficiency of Puma knockdown was monitored by immunoblot analysis. Inputs for coimmunoprecipitations were also subjected to immunoblot analysis and actin was probed as a loading control.

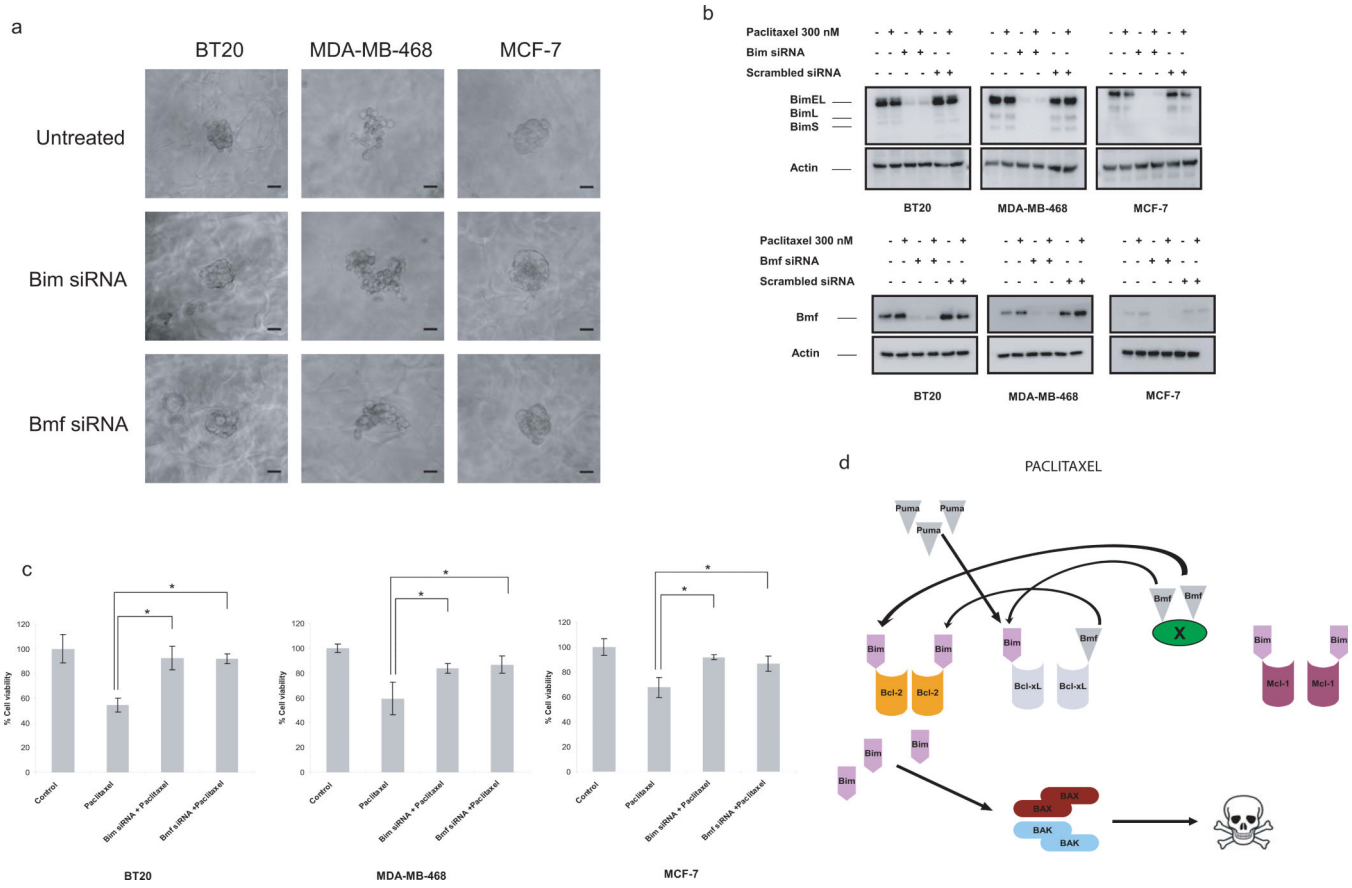

Figure 8.

Bim and Bmf mediate paclitaxel-induced apoptosis in breast cancer cellular spheroids. **(a)** MCF-7, MDA-MB-468 and BT20 cells were transiently transfected with Bim siRNA, Bmf siRNA or Scrambled siRNA for 12 h. Cells were subsequently grown in 3D Algimatrix culture plates with the presence of corresponding siRNAs. Microscopic evaluation of spheroids was done to verify that siRNA treatment did not interfere with 3D growth of breast cancer cells. Scale bars, 24 μm. **(b)** Spheroids were isolated and lysed in 1% CHAPS buffer. The efficiency of Bim and Bmf knockdown in 3D cultures was monitored by immunoblot analysis. Actin was probed as a loading control. **(c)** Following formation of spheroids in 3D culture, cells were treated with paclitaxel (300 nM) for 48 h. Cell viability was assessed by alamarBlue assay (mean ± SEM, n = 4, *p< 0.05 by two-tailed t test). **(d)** Schematic of model: In untreated cells, Bim is sequestered by prosurvival Bcl-2 proteins, Bcl-2, Bcl-xL and Mcl-1. Bmf is either bound to Bcl-xL or other protein(s) (designated as protein X). Following paclitaxel treatment; Bmf is displaced from Bcl-xL or protein X and binds to Bcl-2 and Bcl-xL to displace Bim, which in turn activates Bax and Bak to promote apoptosis. Alternatively, increased Puma protein levels and increased binding of Puma to Bcl-xL in response to paclitaxel treatment displaces Bim from Bcl-xL, triggering apoptosis.