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# Bim is reversibly phosphorylated but plays a limited role in paclitaxel cytotoxicity of breast cancer cell lines

Matt Czernick, Aja Rieger, Ing Swie Goping *

*Department of Biochemistry, University of Alberta, 5-73 Medical Sciences Building, Edmonton, Alta., Canada T6G 2H7*

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ABSTRACT

The chemotherapeutic drug, paclitaxel, induces mitotic arrest and then activates the cellular apoptotic program. Although paclitaxel has been in clinical use for over 10 years for the treatment of breast, ovarian, and lung cancer, the molecular mechanisms of paclitaxel-induced cytotoxicity are ill defined. We decided to investigate the regulatory mechanism of the pro-apoptotic BH3-only protein Bim, which is known to play a role in paclitaxel cytotoxicity. We discovered that paclitaxel induces reversible phosphorylation of Bim. Bim initially displays enhanced phosphorylation during paclitaxel-induced mitotic arrest, and then undergoes de-phosphorylation as cells become apoptotic. This dynamic phosphorylation is dependent on mitotic checkpoint signaling. However, while these results suggest that reversible phosphorylation of Bim may contribute to the transmission of a mitotic checkpoint-to-apoptosis signal, we did not observe a strong correlation between Bim protein levels and cellular sensitivity to paclitaxel. Indeed, in contrast to the well-defined role of Bim in paclitaxel-induced cell death in mouse model cells, our depletion studies demonstrate that Bim is not absolutely required for paclitaxel cytotoxicity in breast cancer cell lines. Clearly it is imperative to define the contribution of Bim in paclitaxel-induced apoptosis of clinically relevant targets in order to rationally develop enhanced treatment strategies.

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Taxanes are clinically important anti-cancer drugs that eliminate dividing cells. Paclitaxel, the proto-typic member of this class of agents, is used in the treatment of ovarian, breast, and lung cancers (for review, see [1,2]). This chemotherapeutic drug causes mitotic arrest, although precisely how this mitotic arrest triggers subsequent cell death (apoptosis) is unclear [2,3]. In order to improve anti-cancer therapies, it is imperative to understand how these drugs work at the molecular level in cells that are the therapeutic targets of paclitaxel treatment. Elucidation of paclitaxel-induced cellular signals may reveal novel drug targets and allow rational design for improved combination therapies.

When cells undergo paclitaxel-induced cell death, the mitochondrial apoptotic pathway is activated [4–6]. Presumably, the triggers of this mitochondrial apoptotic pathway are the BH3-only proteins [7,8]. Specifically, the BH3-only protein Bim, has been shown to play a major role in paclitaxel-induced cell death [9–14]. Direct depletion of Bim or indirect depletion of Bim through siRNA repression of Bim transcriptional activators, delays paclitaxel-mediated apoptosis in cell based models [9,11,12,14]. As well, *in vivo* mouse models of *bim−/−* cells confirmed the importance of Bim expression for paclitaxel cytotoxicity [10]. Evidently Bim can transmit a paclitaxel-induced apoptotic signal, but how this is accomplished, and the mechanism of Bim regulation is unknown.

Bim pro-apoptotic activity is controlled by two main mechanisms: transcription (stimulatory) and post-translational modifications (inhibitory and stimulatory) [7,15,16]. Paclitaxel-induced cell death can occur in the absence of protein synthesis, indicating that post-translational mechanisms activate the apoptotic machinery [17]. Phosphorylation is a major regulatory mechanism of Bim activity. Inhibitory phosphorylation of Bim was first described with ERK1/2-mediated phosphorylation of BimEL at S69 in interleukin-dependent B cell lines [18,19]. Of relevance to our studies, during the course of paclitaxel treatment, ERK1/2-mediatedphosphorylation stimulated degradation of BimEL with subsequent attenuation of paclitaxel cytotoxicity [10]. Importantly, these studies underscore the need to further examine Bim phosphorylation patterns in response to paclitaxel treatment and identify how these modifications modulate cellular responses to paclitaxel.

We have determined that Bim is phosphorylated as cells enter paclitaxel-induced mitotic arrest, and becomes de-phosphorylated as cells exit mitotic arrest and undergo apoptosis. S69 is one of the sites that is subject to this dynamic phosphorylation. Importantly, these changes are absent when mitotic checkpoint signaling is ablated. However, whether these post-translational modifications contribute to paclitaxel cytotoxicity is unclear, because in contrast to the well-defined role of Bim in paclitaxel-induced cell death of mouse model cells, our depletion studies demonstrate that Bim is not absolutely required for paclitaxel cytotoxicity. This was confirmed in breast cancer cells which are targets of paclitaxel chemo-

* Corresponding author. Fax: +1 780 492 0886.
*E-mail address:* igoping@ualberta.ca (Ing Swie Goping).

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therapy. Therefore, other mechanisms must activate the apoptotic program when Bim levels are depleted. Since paclitaxel is a widely used anti-cancer drug, it is important to clarify the contribution of Bim to this form of drug-induced cell death.

## Materials and methods

*Cell lines and reagents.* The human cervical carcinoma cell line HeLa was maintained in DMEM supplemented with 10% fetal calf serum. The human breast carcinoma cell lines, MCF-7, SKBR-3, and MDA-MB-468 kindly provided by Dr. G. Mills (MD Anderson Cancer Center, University of Texas), were maintained in RPMI-1640 supplemented with 10% fetal calf serum. Thymidine and paclitaxel were purchased from Sigma. Tetramethyl rhodamine ethyl ester, perchlorate (TMRE) was purchased from Molecular Probes. Propidium Iodide was from Invitrogen Life Technologies. Antibodies for P-Histone H3 were from Upstate, active caspase 3, Bim, and BubR1 were from BD Biosciences, P-S69-Bim was from Chemicon International, and α-tubulin was from Sigma.

*Cell synchronization to G1/S boundary by double thymidine block.* HeLa cells were plated and the next day, were grown in media containing 2 mM thymidine for 16 h. Cells were thoroughly washed with PBS and incubated in media for 8 h. Thymidine (2 mM) was added to the cells again for 16 h, followed by PBS wash. Cells were then immediately treated with or without 20 nM paclitaxel, which corresponds to a clinically relevant dose [5,20,21].

*Mitochondrial depolarization assay.* Mitochondrial electrochemical potential loss ($\Delta\Psi$ loss) was assessed using the dye tetramethylrhodamine ethyl ester, perchlorate (TMRE, Molecular Probes) which fluoresces in the FL-2 channel in cells with healthy respiring mitochondria. After paclitaxel treatment, cells were labeled with TMRE according to the manufacturer's directions. Mitochondrial depolarization in target cells was assessed by analysis of the FL2-negative population. Percent specific TMRE loss was determined as (% TMRE-negative cells of treated sample − % TMRE-negative cells of untreated control sample). Flow cytometry was performed on a Becton Dickinson FACSCan and analyzed using CellQuest software.

*DNA content analysis by propidium iodide staining.* Samples were fixed at −20 °C in ice-cold 70% ethanol for a minimum of 24 h. After fixation, the samples were stained with 20 μg/ml propidium iodide, 2 mg/ml RNase, 0.1% Triton X-100 in PBS for 30 min at 4 °C. Samples were analyzed in the FL-2 channel on the FACScan as previously described, with the exception that doublet discrimination was achieved by gating on the smaller width G2 cells as determined by analysis of pulse width versus pulse area.

*Western blotting.* Western blotting was performed as previously described [22]. For lysates that were treated with phosphatase, cellular lysates were prepared in CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate) buffer (2% CHAPS, 10% glycerol, 20 mM Tris, pH 7.4, 137 mM NaCl, 2 mM EDTA). Post-nuclear supernatant was collected from a 10 min, 10,000*g* centrifugation. Lysates were treated with or without 20 U of calf intestinal alkaline phosphatase (Sigma) and 11 mM sodium vanadate for 30 min at 30 °C.

*siRNA transfections.* Cells were plated in a 24 well dish the day before transfection. Validated siRNA for Bim (Bim1) and BubR1 were purchased from Qiagen. Smart pools of validated siRNA for Bim (Bim2) were purchased from Dharmacon. Transfections were done according to the manufacturers protocol; using 5 nM siRNA complexed with HiPerFect (Qiagen) for both Bim1 and BubR1 and 100 nM SMARTpool siRNA consisting of a complex of 4 duplex siRNA targeted to Bim complexed with Dharmafect4 (Dharmacon) for experiments with Bim2. The day after transfection, cells were treated with or without 20 nM paclitaxel for 24 and 48 h.

## Results

### *Paclitaxel induces sequential modifications to the BH3-only protein Bim*

Since paclitaxel induces cell death in a cell cycle-dependent manner, we initially synchronized HeLa cells in order to differentiate cell cycle specific events from paclitaxel-specific events. Paclitaxel treatment of HeLa cells resulted in the formation of multipolar mitotic spindles with subsequent apoptosis as judged by cell shrinkage, cell blebbing, and formation of apoptotic bodies. Furthermore, apoptosis was detected through elevated levels of activated forms of the pro-apoptotic multi-domain proteins Bax and Bak, as well as loss of mitochondrial electrochemical potential and increases in caspase activation and phosphatidyl serine externalization (Supplementary Fig. 1). Importantly, these apoptotic features were only measurable after a period of mitotic delay (Fig. 1, observe extended labeling with the M phase marker, P-Histone H3). In fact, caspase activation was detected after loss of M phase marker indicating that cells had exited mitosis prior to undergoing apoptosis (Fig. 1, Lane 48 h with paclitaxel). This confirmed that paclitaxel-induced cell death proceeds through a multi-step mechanism — mitotic arrest, followed by apoptosis [23,24].

Since the apoptotic features of mitochondrial dysfunction (Bax/Bak activation and loss of electrochemical potential), caspase activation and phosphatidylserine externalization are all relatively late stage events, we decided to monitor upstream signaling events that trigger mitochondrial dysfunction. A known molecule that is involved in paclitaxel-induced cell death that can activate the mitochondrial pathway is the BH3-only protein, Bim. Since Bim

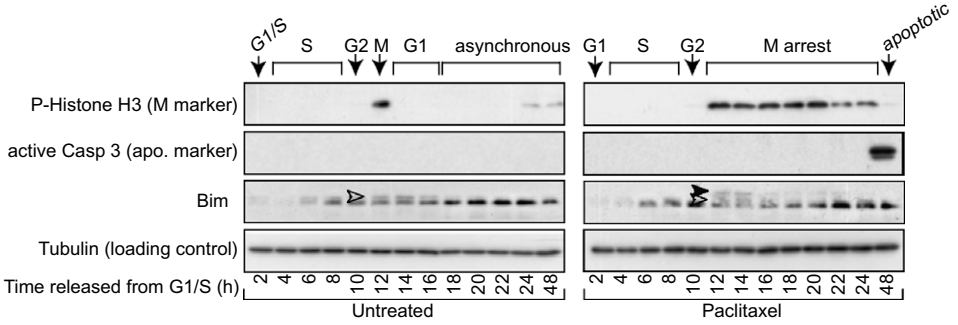


**Fig. 1.** Paclitaxel arrests cells at M phase and induces apoptosis through an ill-defined process. Western blot analysis depicting time line of paclitaxel-induced mitotic arrest and subsequent apoptosis. Whole cell lysates from synchronized cells were harvested at indicated times and analyzed for Mitotic marker (P-Histone H3), apoptotic marker (Caspase 3), Bim, and control (Tubulin).

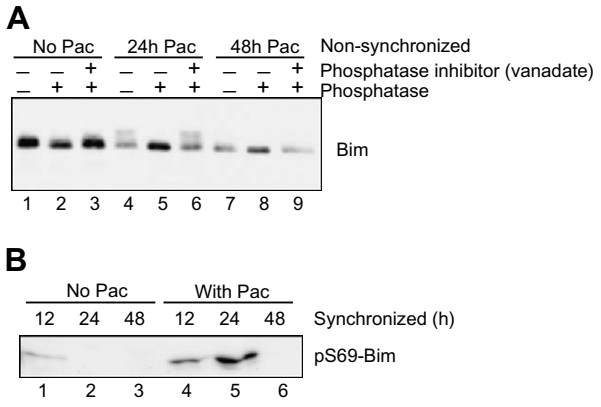


**Fig. 2.** Bim is hyper-phosphorylated at mitotic arrest and undergoes de-phosphorylation during apoptosis. (A) Western blot of non-synchronized cells treated as indicated, demonstrating phosphorylation of BimEL. (B) Bim is phosphorylated at S69 in cells undergoing paclitaxel-induced mitotic arrest, and de-phosphorylated in apoptotic cells, respectively. HeLa cells were synchronized by double thymidine block, then treated with 20 nM paclitaxel and harvested at the indicated time points. Western blot analyses were performed. Blots were probed with anti-Bim antibody specific for phosphorylation on Ser 69.

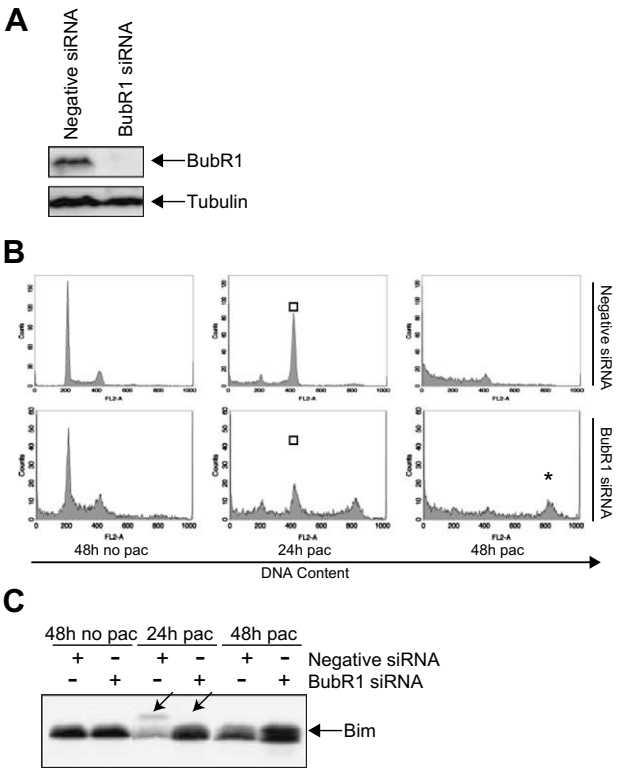


**Fig. 3.** Bim reversible phosphorylation is dependent on mitotic arrest and correlates with cell death. (A) Western blot analysis indicating efficiency of BubR1 knock down (B) DNA content analysis indicates loss of BubR1 abrogates paclitaxel-mediated mitotic arrest. Cells do not die in pseudo-G1, but progress through the cell cycle. * denotes cells with DNA content of 8N. Cells were fixed and stained with PI and analyzed by flow cytometry. (C) Bim hyper- and de-phosphorylated forms only appear in cells that have undergone paclitaxel-induced mitotic arrest. Hyperphosphorylated form is absent (compare arrows) and de-phosphorylated form is diminished in BubR1-deficient cells.

activity can be both negatively and positively regulated by phosphorylation [10,25–27], we wanted to examine paclitaxel-induced phosphorylation of Bim and determine how these modifications regulate Bim pro-apoptotic activity. Therefore, we assessed Bim protein levels and post-translational modifications during a time course of paclitaxel treatment (Fig. 1). In non paclitaxel-treated cells, Bim protein levels increased as cells progressed from G1 into S phase with the newly synthesized Bim migrating as a doublet. As cells exited M phase and became asynchronous over time, Bim migration appeared as a single band. In contrast, during the course of paclitaxel treatment, an additional slower migrating band appeared as cells entered and arrested in M phase (Fig. 1, compare Bim closed vs. open arrowheads). The slower migrating forms of Bim then diminished as cells exited M arrest and were not detectable, as cells became apoptotic (Fig. 1, 48 h).

### *Bim undergoes reversible phosphorylation in response to paclitaxel treatment*

Since Bim activity is regulated by phosphorylation, we assessed whether phosphorylation contributed to the altered migration of Bim in response to paclitaxel treatment. Non-synchronized HeLa cells are in mitotic arrest 24 h post paclitaxel treatment and this correlated with the appearance of the slower migrating form of Bim (Fig. 2A, compare lanes 1 and 4). Treatment with calf intestinal phosphatase demonstrated that these alterations in Bim migration were due to phosphorylation and subsequent de-phosphorylation of Bim. Bim becomes hyper-phosphorylated during mitotic arrest (Fig. 2A, compare lanes 4 and 5) and undergoes de-phosphorylation as cells become apoptotic (Fig. 2A, compare lanes 4 and 7). Furthermore, using a phospho-specific antibody we showed that Bim is phosphorylated at S69 as cells enter mitosis (Fig. 2B, lane 1). This modification is independent of paclitaxel addition although paclitaxel treatment resulted in an enhanced staining with the pS69 antibody (Fig. 2B, lanes 4 and 5). In either case, phosphorylation at S69 is diminished as cells exit mitosis and either proliferate in the non-treated state, or undergo apoptosis in the paclitaxel-treated state (Fig. 2B, lanes 2 and 6). Importantly, these results demonstrate that paclitaxel induces transient phosphorylation of Bim at S69 as well as other uncharacterized sites, with de-phosphorylation correlating with the onset of apoptosis.

### *Hyper-phosphorylation of Bim is dependent on paclitaxel-induced mitotic arrest*

Hyper-phosphorylation of Bim occurs at mitotic arrest and we wanted to functionally determine whether these modifications were dependent on mitotic arrest. Therefore we performed siRNA-mediated depletion of the mitotic checkpoint protein, BubR1. Depletion of BubR1 severely inhibits the mitotic checkpoint machinery such that cells do not arrest in M phase after treatment with paclitaxel. While paclitaxel-treated control cells arrested at M phase after 24 h treatment with paclitaxel, paclitaxel-treated BubR1 depleted cells did not arrest and continued through the cell cycle (see Fig. 3B). DNA content PI analysis showed paclitaxel-dependent accumulation of cells at M phase with a DNA content of 4N, whereas similarly treated BubR1 depleted cells had a diminished number of cells in 4N but an increased number of cells with DNA content of 8N (Fig. 3B, asterisk) indicative of continued cell cycle progression. Accumulation of hyper-phosphorylated Bim only occurred in checkpoint competent control cells (Fig. 3C, compare arrows) indicating that Bim hyper phosphorylation is dependent not on paclitaxel treatment alone, but upon the acquisition of paclitaxel-induced mitotic arrest. However, the functional relevance of the progression of hyper-phosphorylated forms of Bim in mitotic arrest to the hypo-phosphorylated forms of Bim in apoptotic cells, was not clear, so we decided to conduct mutagenesis structure/function studies of Bim.

*Bim depletion is not sufficient to ablate paclitaxel cytotoxicity*

In order to conduct these functional studies of Bim, we initially depleted Bim levels and assessed the level of diminished paclitaxel cytotoxicity. Surprisingly, we found that transient knock down of Bim had minimal effects on paclitaxel-induced cell death of HeLa cells (Fig. 4A, Bim1). To rule out that this was not an anomaly of the siRNA target site, we confirmed this lack of effect with an independent pool of Bim targeted siRNA sequences (Fig. 4A, Bim2). Clearly, although these treatments resulted in robust depletion of Bim as determined by Western blot analysis, there was no significant diminishment in sensitivity to paclitaxel. Furthermore, we saw no difference in paclitaxel cytotoxicity of depleted Bim cells in time course and paclitaxel concentration course analyses (data not shown). Given that the role of Bim in paclitaxel-induced apoptosis is well established in different cell types, these results demonstrated that paclitaxel responsiveness may be cell specific. Therefore, we decided to analyze the contribution of Bim to paclitaxel-induced apoptosis of cell lines that would be clinically relevant targets of this chemotherapeutic drug. We used siRNA to deplete Bim levels in the breast carcinoma cell lines MCF-7, SKBR-3, and MDA-MB-468. Similar to HeLa cells, depletion of Bim in all 3 breast cancer cell lines (Fig. 4B, Western blots), had minimal effect on cytotoxicity imparted by paclitaxel (Fig. 4B, bar graphs). Clearly, although paclitaxel can induce dynamic modification of Bim phosphorylation that correlates with acquisition of mitotic arrest and subsequent apoptosis, high levels of Bim protein are not necessary for paclitaxel-mediated apoptosis in the cell lines

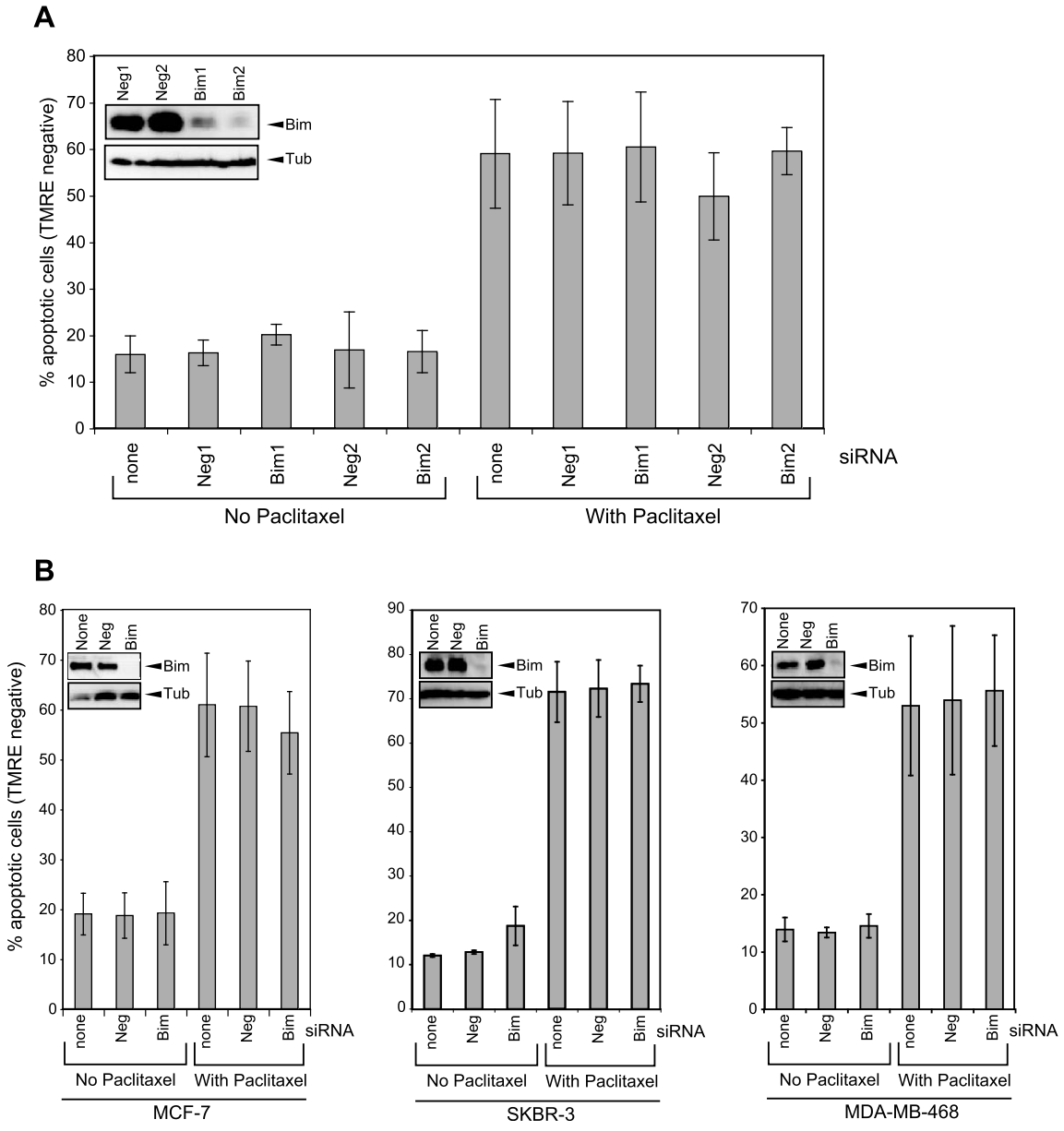


**Fig. 4.** Depletion of pro-apoptotic Bim does not decrease cellular sensitivity to Paclitaxel. (A) HeLa cells were transiently transfected with siRNA as indicated and knock-down efficiency was monitored by Western blots, with none indicating non-treated cells, Neg indicating cells transfected with a negative control siRNA and Bim indicating cells transfected with a Bim-specific siRNA(inset). Cells were treated with paclitaxel for 48 h and apoptosis was analyzed relative to loss of mitochondrial electrochemical potential as assessed by FACS quantitation of TMRE-negative cells (lower). (B) MCF-7, SKBR-3, and MDA-MB-468 breast carcinoma cells were transiently transfected with siRNA as indicated and knock-down efficiency was monitored by Western blot (inset) as compared to a tubulin (Tub) loading control. Cells were treated with paclitaxel for 48 h and apoptosis was analyzed relative to loss of mitochondrial electrochemical potential as assessed by FACS quantitation of TMRE-negative cells.

tested. Possibly, other pro-apoptotic mechanisms can compensate for decreased Bim expression.

## Discussion

Taxanes, including paclitaxel, cause cells to arrest in mitosis and undergo apoptosis through a multi-step pathway. The pro-apoptotic protein Bim, has been shown to contribute to paclitaxel-mediated apoptosis in numerous cellular model systems. We observed that Bim undergoes reversible phosphorylation dependent on paclitaxel-induced mitotic arrest. Possibly the mitosis-specific phosphorylation of Bim at S69 antagonizes Bim by promoting Bim proteasomal degradation [10,28]. Indeed phosphorylation appeared to reduce Bim protein levels and this may in part contribute to cell survival during paclitaxel-induced mitotic arrest. Although it is tempting to speculate that Bim subsequently may couple mitotic arrest signals to the downstream apoptotic machinery, importantly, we found that transient depletion of Bim does not impart paclitaxel resistance to HeLa cells or the breast carcinoma cell lines MCF-7, SKBR-3, and MDA-MB-468. Therefore, more studies need to be undertaken in order to reconcile the previously well-characterized role of Bim in paclitaxel-induced cell death in mouse model cells with its role in clinically relevant cells such as breast cancer cells.

We were puzzled by our observation that depletion of Bim did not protect cells from paclitaxel-mediated apoptosis. This is in contrast to numerous reports that demonstrate a paclitaxel-mediated dependence on Bim [9–14]. In fact, in one of these cases, similar to our studies, transient siRNA knock-down was employed in breast cancer MCF-7 cells and Bim depletion reduced annexin V positivity [12]. This apparent discrepancy may be due to differences that have been reported in MCF-7 cell lines from different sources [29]. In our studies we used validated fingerprinted MCF-7 cell lines (courtesy of Dr. G. Mills, MD Anderson Cancer Center, University of Texas). These MCF-7 cells did not display annexin V positivity during paclitaxel treatment, nor did the cells display caspase activation. That is to be expected given that MCF-7 harbor a deletion in the caspase 3 gene and do not express caspase 3 [30]. We therefore analyzed mitochondrial dysfunction through loss of electrochemical potential as assessed by staining with the potentiometric dye, TMRE. Using these assays, we observed a lack of dependence on Bim in not only MCF-7 breast carcinoma cell lines, but also SKBR-3, MDA-MB-468 and HeLa cell lines, using multiple RNAi-target sequences. Since siRNA studies do not mimic genetic knock out systems, it remains a possibility that minimal levels of Bim are required to induce paclitaxel cytotoxicity. Alternatively, other pro-apoptotic mechanisms compensate for decreased Bim expression.

We report these results in order to establish that in some cases, Bim signaling may not be absolutely necessary for paclitaxel-induced cell death. This is extremely relevant in the analysis of human breast carcinoma cells, since paclitaxel is widely used in anti-breast cancer therapy. Ultimately, understanding the molecular mechanisms that control how paclitaxel-induced mitotic arrest activates the apoptotic machinery of specific cell types has the potential to contribute to the development of improved chemotherapeutic treatment options.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.12.025.

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