Identification of a novel Bcl-xL phosphorylation site regulating the sensitivity of taxol- or 2-methoxyestradiol-induced apoptosis

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Abstract Bcl-xL, a close homolog of Bcl2, is an important regulator of apoptosis and is overexpressed in human cancer. Phosphorylation of Bcl-xL can be induced by microtubule-damaging drugs such as taxol or 2-methoxyestradiol (2-ME). By site-directed mutagenesis studies, we have identified that serine 62 is the necessary site for taxol- or 2-ME-induced Bcl-xL phosphorylation in prostate cancer cells. Further studies with the inhibitor of Jun kinase (JNK) and phosphorylation null mutant of Bcl-xL reveal the augmentative role of JNK-mediated Bcl-xL phosphorylation in apoptosis of prostate cancer cells. In summary, our studies suggest that the phosphorylation of Bcl-xL by stress response kinase signaling might oppose the anti-apoptotic function of Bcl-xL to permit prostate cancer cells to die by apoptosis.

Key words: Apoptosis; Bcl-xL; Bcl2; 2-Methoxyestradiol; Taxol; Jun kinase

1. Introduction

The Bcl2 family comprises of two counteracting groups of proteins: the pro-apoptotic and anti-apoptotic [1–3]. Among the anti-apoptotic members, Bcl2, Bcl-xL or Mcl-1 gets phosphorylated by microtubule disarraying agents such as taxol, dolastatin 10, nocodazole or 2-methoxyestradiol (2-ME) [4–25]. The phosphorylation of the Bcl2 family members rendered negative outcome on their biological function [4–32]. Phospho-Bcl2 associates with a member of a peptidyl prolyl isomerase family, Pin1 and transient conformational change induced by Pin1 hinders Bcl2 to execute its normal anti-apoptotic activity against tubulin binding drugs [4,24]. The emerging concept that yielded from these studies is that phosphorylation-induced inactivation of Bcl2 protein on Ser70 residue inside the unstructured 'loop region' (LR) during mitosis might work as a checkpoint to augment apoptosis [11,13,15]. The LR of both Bcl2 and its close homolog Bcl-xL can negatively regulate their functions as evident by enhanced anti-death activity of LR-deficient or phosphorylation-defective mutants [32,33]. The screening of a library of phage-displayed peptides as well as chemical approach identified interaction of taxol with LR of Bcl2 [34,35]. Besides, microtubule-damaging drugs, growth factor-signaling molecules such as insulin-like growth factor binding protein-3 (IGFBP-3) can mediate apoptosis induced by TNF-α through the inactivation of the cell survival protein Bcl2 via serine phosphorylation in prostate cancer cells PC-3 [36].

Unlike Bcl2, the functional significance of tubulin binding drug-induced Bcl-xL phosphorylation remains elusive. We initiated this study on the belief that it is important to determine the precise site of phosphorylation to dissect out the functional consequences of Bcl-xL phosphorylation. Our results indicate that Jun kinase (JNK)-mediated Bcl-xL phosphorylation on serine 62 residue inside LR of Bcl-xL is critical for rendering death advantage against taxol or 2-ME.

2. Materials and methods

2.1. Cell lines and reagents

Human prostate cancer cells DU145 were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 50 μg/ml gentamicin. Mouse monoclonal antibodies against Bcl-xL and Bcl2 were purchased from Pharmingen, USA and Upstate Biotechnology, USA respectively. λ-Protein phosphatase kit was available from New England Biolabs, USA. JNK inhibitor II was purchased from Calbiochem, USA. Phospho-specific Bcl2 antibody was developed in the laboratory as described previously [4]. All other reagents used were of ultrapure grade.

2.2. Assessment of Bcl2/Bcl-xL phosphorylation

Equal amounts of protein isolated from cells with or without taxol or 2-ME treatment were subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Western blot. The phosphorylated forms of Bcl2/Bcl-xL protein were detected as slower migrating forms on Western blots as described previously [4–9,25]. For detection of phospho-Bcl2, either whole antibody [4–9] or phospho-Bcl2-specific antibody [4] was used. Dephosphorylation reaction by λ-protein phosphatase was performed in accordance with the manufacturer's reaction as described previously [5]. Briefly, freshly prepared lysate was incubated at 30°C for 30 min with 200–400 units of λ-protein phosphatase in a reaction buffer containing 50 mM Tris–HCl, 0.1 mM ethylenediamine tetraacetic acid (EDTA), 5 mM dithiothreitol (DTT), 0.01% Brij 35 (pH 7.5, at 25°C) and 2 mM $MnCl_2$. The reaction was stopped by boiling for 5 min in Laemmli's sample buffer.

2.3. Cell sorting

Cells were stained with Hoechst 33342 (Sigma) at a concentration of 15 μg/ml for 1 h at 37°C. To increase the resolution of DNA distribution, 3,3′-dipentyloxacarbocyanineiodide (Molecular Probes) was added at a concentration of 0.2 μg/ml simultaneously with Hoechst 33342 [4,8,9]. Cells were sorted using a fluorescence-activated cell sorter.

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Abbreviations: 2-ME, 2-methoxyestradiol; JNK, Jun kinase; LR, loop region; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride

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2.4. Construction of Bcl-xL mutants

We started substitution of several serine and threonine residues in Bcl-xL protein by site-directed mutagenesis using overlap extension involving the polymerase chain reaction (PCR) [5,9,37]. The following sets of overlapping primers were designed from the human Bcl-xL cDNA clone sequence.

Site(s) of mutations	Overlapping primers
$Ser^{56} \rightarrow Ala^{56}$	
	288 314
	5' AAT GGC AAC CCA G* CC TGG CAC CTG GCA 3'
	3' TTA CCG TTG GGT CGG ACC GTG GAC CGT 5'
Thr 115→Ala 115	465 491
	5' CAG CTC CAC ATC G *CC CCA GGG ACA GCA 3'
	3' GTC GAG GTG TAG C GG GGT CCC TGT CGT 5'
$Ser^{62} \rightarrow Ala^{62}$	
	306 332
	5' CAC CTG GCA GAC G *C *C CCC GCG GTG AAT 3'
	3' GTG GAC CGT CTG C G G GGG CGC CAC TTA 5'
$Thr^{47} \rightarrow Ala^{47}$	
	261 287
	5' TCG GAG ATG GAG G *CC CCC AGT GCC ATC 3'
	3' AGC CTC TAC CTC C GG GGG TCA CGGTAG 5'

Asterisk indicates mutated nucleotides incorporated in Bcl-xL gene. Numbers indicate the position of nucleotides in Bcl-xL gene. In addition to the above overlapping primers, two external primers (5′ and 3′ ends of Bcl-xL cDNA) were also synthesized with *Eco*RI and *Not*I extensions respectively.

5' end primer (Forward)

102 122

5' GTG GAA TTC AAT GGA CTG GTT GAG CCC ATC 3'

3' end primer (Reverse)

841 818

5' AAT GCG GCC GCT CTG GTC ATT TCC GAC TGA AGA GT 3'

The PCR products were gel purified by Qiagen spin column and digested with *Eco*RI and *Not*I restriction enzymes followed by cloning into pcDNA3 (Invitrogen) at the *Eco*RI and *Not*I sites. Mutations in the clones were verified by automated DNA sequencing. Wild-type Bcl-xL was also constructed in pcDNA3 vector by cloning the PCR amplification product generated by 5′ and 3′ end primers and template Bcl-xL DNA in pBluescript.

2.5. Transfections of wild and mutant Bcl-xL cDNAs

DU145 prostate cancer cell line (expressing low endogenous level of Bcl-xL) was stably transfected with pcDNA3 alone or pcDNA3 plasmids containing either wild-type or various mutant (Ser/Thr to Ala) constructs using the calcium phosphate co-precipitation method and positive clones were selected in G418-containing medium [5,9].

2.6. Determination of apoptosis

Chromatin condensation of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (2 μg/ml)-stained nuclei were scored by epifluorescence microscopy as described previously [8].

3. Results

3.1. Taxol can trigger Bcl-xL phosphorylation in prostate cancer cells at G2-M phase of cell cycle

Taxol-induced mobility shift of Bcl-xL in mammary cancer cells is known to be the phosphorylated form of Bcl-xL [25]. We also observe similar mobility shift of Bcl-xL (Fig. 1A, lane 3) in DU145-2 cells (prostate cancer cells genetically engineered to overexpress wild-type Bcl-xL) when exposed to taxol. Panel A (Fig. 1, lane 4) indicates the quantitative conversion of modified form to unmodified form of Bcl-xL by λ-phosphatase treatment. λ-Phosphatase is an Mn^{+2}-dependent protein phosphatase with activity towards phosphorylated serine, threonine or tyrosine residues [5,15]. Of note, there is no change of unmodified form of Bcl-xL by λ-phosphatase treatment. The dose–response study reveals that taxol can phosphorylate Bcl-xL even at 10 nM concentration (Fig. 1B).

In order to determine when in the cell cycle Bcl-xL phosphorylation occurs, taxol-treated cells were sorted after 16 h into pools of cells into G1-S or G2-M. As shown in Fig. 1C, lane 1, abundant phosphorylated form of Bcl-xL was present in G2-M-enriched fraction of cells. We were unable to process the G1-S fraction of taxol-treated cells because of the low yield ($< 30\%$ cells). The levels of Bcl-xL from unsorted control and taxol-treated cells were presented in lanes 2 and 3 respectively. These results indicate that anti-cancer drugs that cause G2-M arrest by affecting microtubule integrity induce Bcl-xL phosphorylation predominantly at G2-M phase of the cell cycle.

3.2. Identification of phosphorylation site on Bcl-xL by site-directed mutagenesis

To understand the specific Ser/Thr residue(s) required for taxol-induced Bcl-xL phosphorylation, we started substitution of several serine and threonine residues (Ser56, Ser62, Thr47, and Thr115) in Bcl-xL protein with alanine residues by site-directed mutagenesis [37]. A similar Ser/Thr to Ala substitution approach was undertaken for determining the phosphorylation site(s) of Bcl2 protein [5,9,11,15,38,39]. We chose these amino acid residues because they are located within the flexible loop or in close proximity of this loop. Furthermore, sequences surrounding these amino acids resemble consensus motif for SAPK/JNK substrate. SAPK/JNK is known to phosphorylate Bcl2 in response to taxol [11,15,38].

Cellular extracts from stably transfected wild and mutant clones were analyzed for the status of taxol-induced Bcl-xL phosphorylation. Immunoblot analysis using anti-Bcl-xL monoclonal antibody reveals that only Ser62 to Ala mutation can abrogate taxol-induced Bcl-xL phosphorylation (Fig. 2A, lane 2). Other Ser/Thr to Ala mutants were without any effect (Fig. 2A, lanes 4, 6, 8). Our studies suggest that taxol-induced Bcl-xL phosphorylation does occur on Ser62 residue (Fig. 2A, lane 2). Like taxol, another estrogen metabolite 2-ME can induce Bcl2/Bcl-xL phosphorylation with mitotic arrest in leukemic, breast, prostate or pancreatic cancer cells [18,19,30]. When wild-type Bcl-xL transfected DU145 cells were challenged with this compound, phosphorylation of Bcl-xL was clearly evident (Fig. 2B). But no phosphorylation was visible when S62A mutant overexpressed DU145 cells were exposed

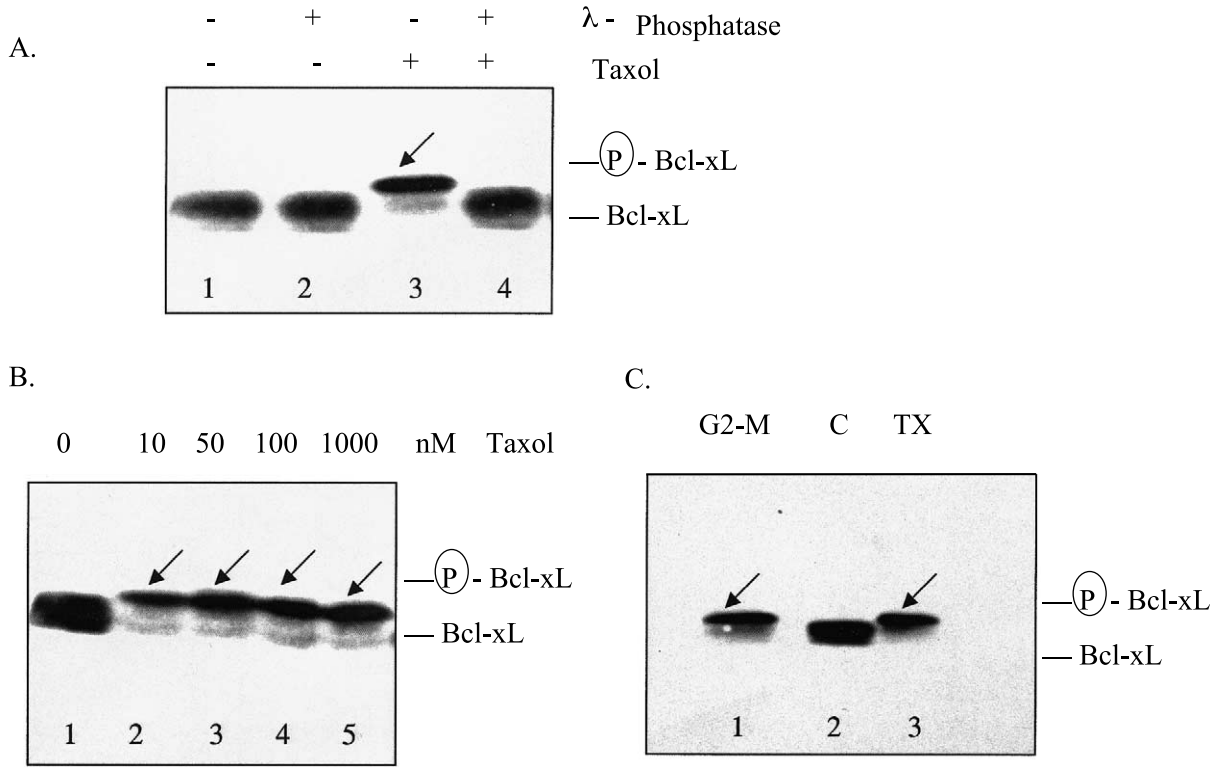

Fig. 1. Tubulin binding agent taxol-induced Bcl-xL phosphorylation at mitotic phase of the cell cycle. A: Taxol induces a modified form of Bcl-xL and λ-phosphatase can abolish this modified form. Lanes 1 and 2, dimethyl sulfoxide (DMSO) control; lanes 3 and 4, taxol; lanes 1 and 3, no λ-phosphatase addition; lanes 2 and 4, 200 units of λ-phosphatase. B: Bcl-xL phosphorylation at different concentrations of taxol. C: Immunoblotting of purified G2-M fraction from taxol-treated DU145-2 clone. Arrow indicates phosphorylated form of Bcl-xL.

to 5 μM 2-ME (Fig. 2B). Thus Ser 62 residue appears to be critical for anti-microtubule agents taxol- and 2-ME-mediated Bcl-xL phosphorylation.

3.3. JNK pathway is responsible for Bcl-xL phosphorylation and apoptosis

Similar to Bcl2, the phosphorylation site for Bcl-xL also conforms to the consensus motif for substrates of MAP kinase and JNK/SAPK [5,11,15]. By employing dominant negative mutants of JNK-signaling pathway, Bcl2 was shown to be phosphorylated by the concerted effort of ASK1/JNK1 in leukemic or breast cancer cells [11,15]. In order to assess whether JNK can phosphorylate Bcl2/Bcl-xL in prostate cancer cells, a potent cell permeable selective inhibitor of c-Jun N-terminal kinase (JNK) was used followed by exposure to taxol. When Bcl2 overexpressing cells, W-34 were treated with taxol, two slower mobility forms were detected by Bcl2 monoclonal antibody (Fig. 3A, lane 3). Of note, both of these slower mobility forms were abolished by λ-phosphatase (Fig. 3A, lane 4) without affecting non-phospho-form (Fig. 3A, lane 2). λ-Phosphatase can even abolish some endogenous phospho-forms of Bcl2 (Fig. 3A, lane 2). These two slower mobility forms of Bcl2 emerged due to taxol treatment were also specifically detected by phospho-specific Bcl2 antibody (Fig. 3B, lane 2) developed in the laboratory [4]. As shown in Fig. 3B, lane 1, the endogenous phospho-form of Bcl2 is also detected by phospho-Bcl2-specific antibody. It is worth mentioning that others and we can detect endogenous phospho-form of Bcl2 in cells when ectopically overexpressed [5,15]. The pretreatment of JNK inhibitor II can successfully abrogate the phospho-forms of Bcl2 in a concentration-dependent manner (Fig. 3B, lanes 3–5). Our inhibitor studies reinstate the involvement of JNK in Bcl2 phosphorylation as observed earlier. Similar to Bcl2, when DU145-2 cells were pretreated with 5–20 μM of JNK inhibitor II, gradual decrease of Bcl-xL phosphorylation was noted (Fig. 3C and E), which might also implicate the JNK-signaling pathway in taxol- or 2-ME-induced Bcl-xL phosphorylation in prostate cancer cells. This inhibitor is known to effectively block the accumulation of phospho-Jun in response to cytokines [40,41]. Moreover, it is noteworthy that the inhibitors of p38 MAP kinase or ERK2 kinase were without any effect on taxol-induced Bcl-xL phosphorylation (Fig. 3D). A similar observation was noted in the case of taxol-induced Bcl2 phosphorylation [15,42].

Next, we examined whether blocking of JNK activity can prevent taxol or 2-ME initiated cell death in DU145-2 cells. As shown in Fig. 3F, co-incubation with 10 μM JNK inhibitor II rendered the cells more resistant to taxol or 2-ME triggered apoptosis.

3.4. Serine 62 residue of Bcl-xL is critical for taxol or 2-ME triggered Bcl-xL phosphorylation and apoptosis

Here we ectopically overexpressed wild as well as Ser62Ala mutant Bcl-xL by stable transfection to test the effects of 2-ME and taxol on the extent of apoptosis. Wild, mutant and vector transfected DU145 cells were challenged with 10 nM taxol or 5 μM 2-ME followed by determination of apoptosis [6]. Previously, it has been shown that human prostate carcinoma cells DU145 lack Bcl2 and are insensitive to 2-ME-induced apoptosis despite the activation of SAPK/JNK [19].

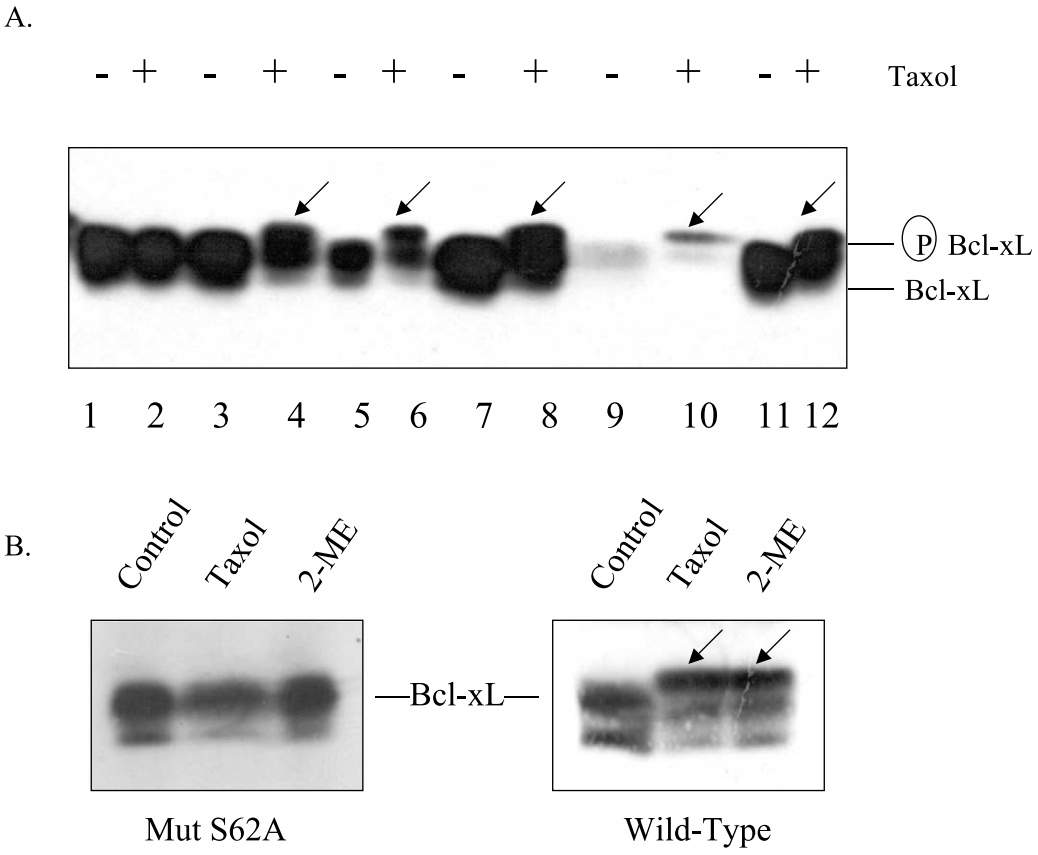

Fig. 2. Effect of microtubule disrupting agents on the phosphorylation status of several Ser/Thr to Ala mutants of Bcl-xL. A: Serine 62 to alanine point mutation abolishes taxol-induced Bcl-xL phosphorylation. Wild and several Ser/Thr to Ala Bcl-xL constructs were stably transfected in DU145 prostate cancer cells. Cells were exposed to 100 nM taxol for 16 h. Cellular extract was subjected to immunoblot analysis with Bcl-xL antibody. Lanes 1, 3, 5, 7, 9 and 11, DMSO treatment; lanes 2, 4, 6, 8, 10 and 12, 100 nM taxol treatment for 16 h. Lanes 1 and 2, Ser62-Ala mutant; lanes 3 and 4, Ser56Ala mutant; lanes 5 and 6, Thr115Ala mutant; lanes 7 and 8, Thr47Ala mutant; lanes 9 and 10, DU145 (neo) cells; lanes 11 and 12, DU145-2 clone. B: S62A Bcl-xL mutant can also abrogate 2-ME triggered Bcl-xL phosphorylation. Arrow indicates phosphorylated form of Bcl-xL.

Parallel to this observation, we have noted negligible amount of Bcl-xL in these cells and very little extent of apoptosis in response to taxol or 2-ME. Due to overexpression of Bcl-xL, both 2-ME- and taxol-induced DNA fragmentations were significantly more enhanced in DU145-2 clone than vector transfected treated cells. A similar response was observed with other wild-type (not shown) or other Ser/Thr to Ala Bcl-xL transfected clones (Fig. 4). In contrast, the overexpression of phosphorylation null mutant of Bcl-xL resulted in no significant increase in the extent of apoptosis triggered by these anticancer agents. Indeed, the insensitivity of the mutant transfected cells to apoptosis indicates the phosphorylation on serine 62 residue to be critical for apoptotic signal transduction cascade triggered by 2-ME or taxol (Fig. 4).

4. Discussion

The investigation reported here demonstrates for the first time that Ser62 is the critical site for taxol- or 2-ME-induced Bcl-xL phosphorylation. In contrary to Bcl2 or Mcl-1, which can be phosphorylated on multiple Ser/Thr residues [5,15,21,27,38], Bcl-xL is phosphorylated on single serine residue following taxol exposure and G2-M arrest. Taxol or 2-ME can cause rapid activation of SAPK/JNK in tumor cell lines previously tested by others [19,38]. A very recent report [19] clearly indicated the involvement of SAPK/JNK in 2-ME-induced Bcl2 phosphorylation and apoptosis in several common epithelial carcinoma cells. The inhibitory effect of JNK inhibitor on taxol or 2-ME triggered Bcl-xL phosphorylation

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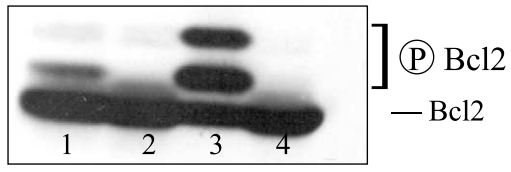
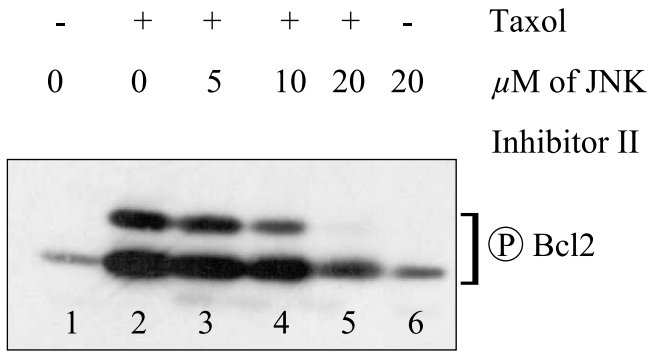
Fig. 3. JNK inhibitor can diminish taxol- or 2-ME-induced Bcl2/Bcl-xL phosphorylation as well as apoptosis. A: λ-Phosphatase (λ-PPase) can abolish only taxol-induced slower mobility forms of Bcl2 in W-34 cells. Western blot was carried out with monoclonal antibody against Bcl2 that recognizes both non-phospho- and phospho-forms of Bcl2. In vitro λ-phosphatase assay was performed using cellular extract isolated from Bcl2 overexpressing prostate cancer cells. Reduction of taxol-induced phosphorylation of Bcl2 (B) and Bcl-xL (C) in cells pretreated with JNK inhibitor II. D: Lack of inhibitory effect of PD98059 (ERK2 inhibitor) or SB202190 (p38 MAP kinase inhibitor) on taxol-induced Bcl-xL phosphorylation. W-34 cells (PC-3 cells engineered to overexpress Bcl2) or DU145-2 cells were pretreated with either JNK inhibitor II (5–20 μM) or PD98059 (100 μM) or SB202190 (1–10 μM) followed by treatment with taxol. For analyzing Bcl2 phosphorylation (B), phosphorylation site-specific Bcl2 antibody [4] was used. For Bcl-xL we have used Bcl-xL monoclonal antibody. E: Effect of JNK inhibitor II on 2-ME triggered Bcl-xL phosphorylation. DU145-2 cells were either pretreated or not pretreated with 10 μM JNK inhibitor II for 8 h followed by treatment with 5 μM 2-ME for 16 h. F: Effect of JNK inhibitor II on taxol- or 2-ME-induced apoptosis. Apoptotic nuclei were visualized by DAPI staining. Approximately 500 cells were scored in each category. Results are means ± S.D. of three independent experiments.

A.



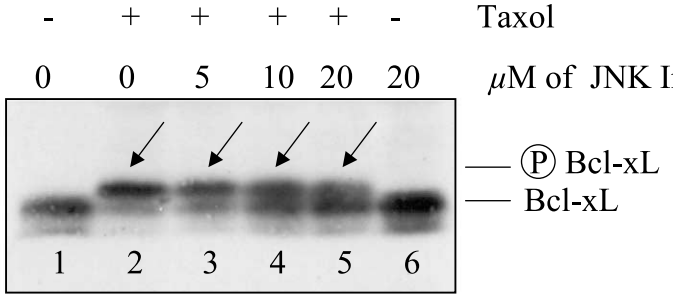
W-34 Cells

B.



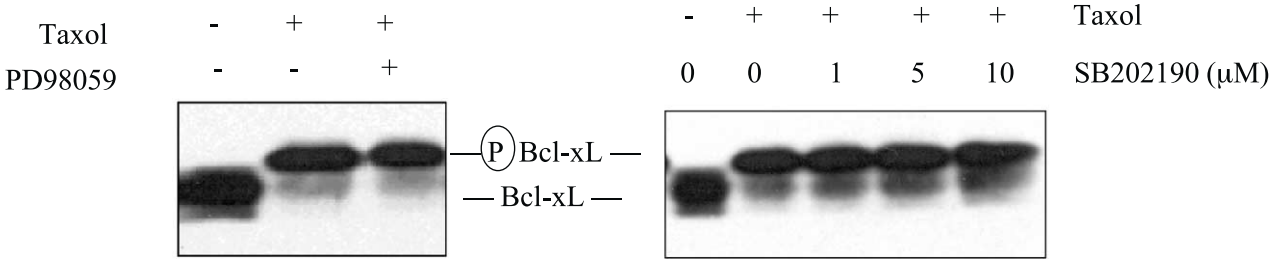
W-34 cells

C.



DU145-2 cells

D.



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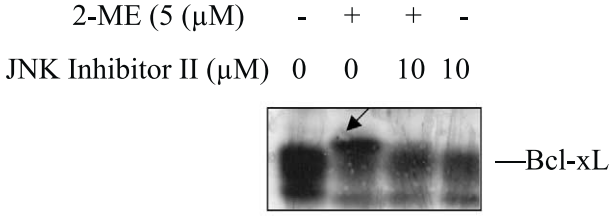

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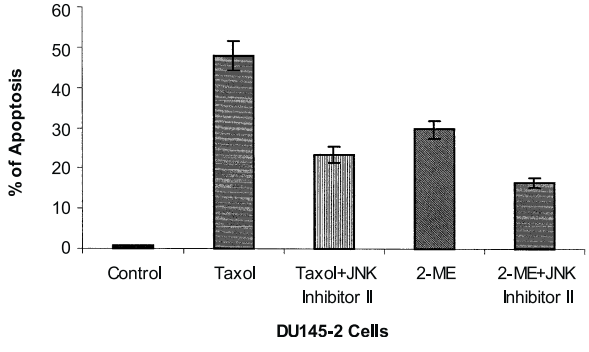

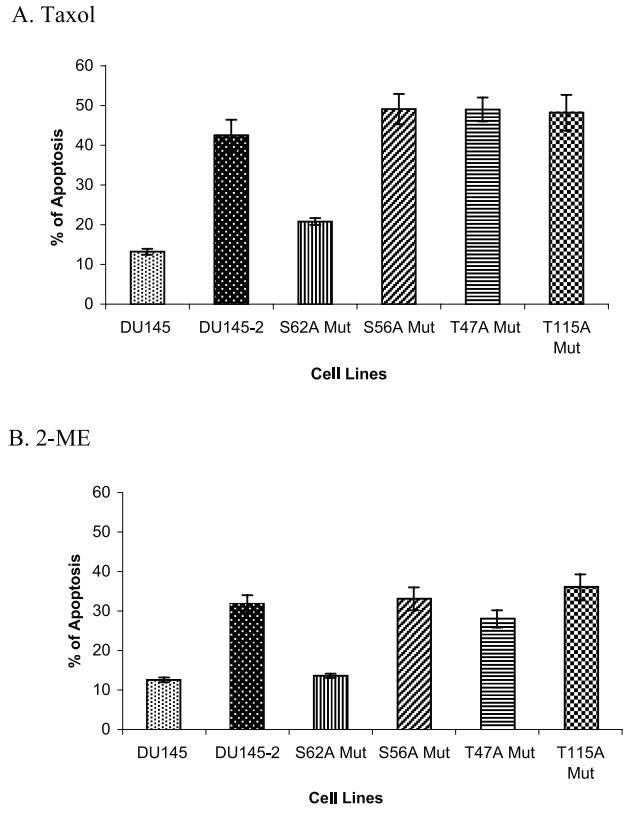

Fig. 4. Ser62Ala mutant blocks taxol- or 2-ME-induced apoptosis. A: 10 nM taxol and B: 5 μM 2-ME treatment for 16 h. Cells were fixed with 4% paraformaldehyde followed by staining with DAPI. Each value represents mean ± S.D. of three independent experiments.

as well as on the concomitant apoptosis establishes the role of SAPK/JNK signaling in Bcl-xL phosphorylation. But our studies do not rule out the possibility of the involvement of other upstream kinase of JNK as reported earlier in the case of p53-mediated Bcl2 phosphorylation and its inactivation [27]. Interestingly, like Bcl-xL, not only Bcl2 but also another anti-apoptotic member of Bcl2 family, Mcl-1, is inactivated by JNK-mediated phosphorylation in response to oxidative stress [21]. It is worth mentioning that parental DU145 and DU145-2 cells do not express other anti-apoptotic members such as Bcl2. Moreover, Bcl-xL is completely phosphorylated by taxol or 2-ME in these cells. The lack of expression of Bcl2 and inactivation of Bcl-xL by phosphorylation can be the key factors for DU145-2 cells to die. If phosphorylated form of Bcl-xL could protect, one would note similar or lesser extent of cell death than parental DU145 cells. But we have rather observed the greater extent of cell death (Fig. 4) in wild-type Bcl-xL overexpressing cells. In contrast, Ser62Ala mutant expressing clone but not other Ser/Thr to Ala mutant clones were relatively resistant to taxol- or 2-ME-induced cell death. Our observation clearly suggests that Bcl-xL phosphorylation in response to taxol or 2-ME permits cells to undergo apoptosis. Conceivably, the greater extent of the presence of Bcl-xL containing Ser62 residue is more responsive to taxol- or 2-ME-induced apoptosis of prostate cancer cells. Our observation is quite concordant with a recent study on Bcl2 phosphorylation with a population of breast tumors. Intriguingly, 83% of breast tumors with high phospho-Bcl2 expression were sensitive to treatment to paclitaxel or docetaxel [43]. In contrary, 57% of tumors with low phospho-Bcl2 expression did not respond to taxane therapy. Together these findings suggest that pS70 Bcl2 or pS62 Bcl-xL might be a predictive factor for prognosis and sensitivity to 2-ME or paclitaxel treatments.

The susceptibility to apoptosis is an essential prerequisite for the successful elimination of cancer cells [44]. Apoptosis is the outcome of the activation of a family of cysteine proteases called caspases [45,46]. Two different major pathways basically activate caspases: (a) one pathway is linked to TNF family of death receptors [47], (b) another is mediated through mitochondria [45,48]. Both taxol and 2-ME elicited apoptosis occurs through mitochondria [13,30]. Apparently, activated JNK also acts on mitochondria and induces apoptosis through the release of cytochrome *c* into cytosol [49]. Thus the members of Bcl2 family located in mitochondria are the potential targets of translocated phospho-JNK. It was reported earlier that activated phospho-JNK can translocate to mitochondria to associate with Bcl-xL in response to phorbol ester [50].

The studies depicted here have potential clinical significance. The anti-apoptotic protein Bcl-xL plays an important role in tumor cell survival. The overexpression of Bcl-xL in chemo-resistant tumors is well known [1]. The potential apoptosis yielding effects of phospho-Bcl-xL by tubulin binding drugs to render its inactivation will be definitely helpful to identify another molecular target.

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