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ORIGINAL ARTICLE

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Targeting BCL-2 overexpression in various human malignancies through NF-κB inhibition by the proteasome inhibitor bortezomib

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Abstract *Background*: BCL-2 overexpression occurs in many cancer types and is associated with chemoresistance and radioresistance. The mechanisms responsible for its aberrant expression are thought to be transcriptionally mediated but remain unclear. We examined the cell type-specific mechanism of BCL-2 gene transcription in various solid organ malignancies. *Methods*: Regulation of BCL-2 gene transcription was examined in seven different human cancer cell lines including two pancreatic (MIA-PaCa-2, PANC-1), two prostate (LNCaP, PC-3), two lung (Calu-1, A549) and one breast (MCF-7) cancer cell line. Cells were treated with inhibitors of phosphatidylinositol-3 kinase (PI3K), MEK/ERK, and p38MAPK. The effect of mutation of a NF-κB site in the BCL-2 promoter was determined, as was the effect of inhibition of NF-κB function using a 26S proteasome inhibitor (bortezomib) on both BCL-2 transcription and induction of apoptosis. *Results*: BCL-2 expression varied both between and within tumor types; four of seven cell lines demonstrated high BCL-2 levels (MIA-PaCa-2, PC-3, Calu-1 and MCF-7). No signaling pathway was uniformly responsible for overexpression of BCL-2; however, mutation of the NF-κB site decreased BCL-2 promoter activity in all cell lines. Inhibition of NF-κB activity decreased BCL-2 protein levels independently of the signaling pathway involved in transcriptional activation of the BCL-2 gene. *Conclusions*: Diverse signaling pathways variably regulate BCL-2 gene expression in a cell type-specific fashion. Therapy to decrease BCL-2 levels in various human cancers would be more broadly applicable if targeted to transcriptional activation rather than signal transduction cascades. Finally, the apoptotic efficacy of proteasome inhibition with bortezomib paralleled the ability to inhibit NF-κB activity and decrease BCL-2 levels.

Keywords BCL-2 · PI-3 Kinase · MEK/ERK · p38MAPK · NF-κB

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Introduction

BCL-2 was originally discovered as a result of the t(14;18) chromosomal translocation in B-cell lymphoma [1]. In these cells, the BCL-2 gene is translocated and transcriptionally activated by fusion to the IgG regulatory region, leading to elevated protein levels. BCL-2 is overexpressed in 50–70% of solid organ malignancies including colorectal, prostate, pancreas, breast, and lung [2–6], though the mechanism does not appear to be due to a translocation event; rather, the overexpression may be due to activation of the transcriptional factors that regulate BCL-2 transcription. Aberrant activation of signal transduction pathways with subsequent constitutive activation of transcription factors is common in cancer, though whether there is a unifying mechanism for BCL-2 overexpression among various cancer types is unclear.

Overexpression of BCL-2 enhances tumorigenic and metastatic capability and also decreases the efficacy of ionizing radiation and chemotherapies [7]. Experimental models in which BCL-2 protein levels are reduced or its function inhibited have been shown to restore sensitivity to the cytotoxic effects of chemotherapy and radiotherapy [8, 9]. One of the most commonly used experimental methods to specifically decrease BCL-2 levels has been through antisense techniques. This approach of targeting BCL-2 has led to the development of G3139, a BCL-2 antisense oligonucleotide (Genasense, Genta, Berkeley Heights, N.J.) for use in humans. Several phase I trials have shown its safety and tolerability, and have also demonstrated decreased BCL-2 protein levels in tumor biopsies or peripheral leukocytes obtained after

administration of G3139 [10–15]. Although clinical and radiological responses have been observed in these trials, information on the efficacy of this novel targeted therapy is awaiting conclusion of ongoing phase II/III clinical trials.

The molecular targeting of specific signaling events in cancer is an emerging field that has already demonstrated significant promise. The specific signaling cascade(s) and transcription factor(s) responsible for BCL-2 overexpression in cancer remain largely unknown. Furthermore, it remains unclear if a common mechanism is responsible for the BCL-2 overexpression observed in the majority of cancers. Of the more ubiquitous transcription factors, NF-κB has been demonstrated to be involved in regulation of BCL-2 gene transcription [16–19]; furthermore, NF-κB is frequently constitutively activated in various types of cancer [20–22]. Various signaling pathways have also been coupled to activation of BCL-2 gene transcription in various tissues, including PI3K [23, 24], MEK/ERK [25, 26], and p38MAPK [27].

Therefore, we sought to determine whether there are common mechanisms of BCL-2 gene transcription in various cancers, specifically focusing on the PI3K, MEK/ERK and p38MAPK signaling pathways and the NF-κB transcription factor. We furthermore sought to determine whether inhibition of NF-κB using the 26S proteasome inhibitor bortezomib alters BCL-2 transcription and whether this effect correlates with induction of apoptosis. These data would be helpful to determine whether an upstream signaling target (e.g. a signaling kinase or transcription factor) may be a broadly applicable target to decrease BCL-2 protein levels in various cancers and restore apoptotic sensitivity.

Materials and methods

Materials

All chemical reagents were purchased from Sigma Chemical Company (St. Louis, Mo.) unless otherwise specified. Signal transduction inhibitors used in these experiments were: PI3K inhibitor LY294002 (New England BioLabs, Beverly, Mass.), the MEK/ERK inhibitor PD98059 (CalBiochem, San Diego, Calif.), or the p38MAPK inhibitor SB203580 (CalBiochem). The 26S proteasome inhibitor bortezomib has been shown to inhibit NF-κB function [28, 29] and was kindly supplied by Dr. Julian Adams (Millennium Pharmaceuticals, Cambridge, Mass.). Cell culture supplies and media were purchased from Becton Dickinson (San Diego, Calif.) and Gibco/BRL Life Technologies (Gaithersburg, Md.), respectively. Plasmids for transfection experiments were purified using Qiagen's maxi kit (Valencia, Calif.). Antibodies included a monoclonal antibody to BCL-2, (BD Pharmingen, San Diego, Calif.), a polyclonal antibody to phospho-AKT (serine-473) (Biosource International, Camarillo, Calif.), a monoclonal antibody to phospho-ERK (Cell Signaling Technology, Beverly, Mass.), a monoclonal antibody to phospho-p38 (Cell Signaling Technology), and a polyclonal antibody to actin (Santa Cruz Biotechnology, Santa Cruz, Calif.). The luciferase assay kit was purchased from Promega Corporation (Madison, Wis.).

Cell culture

All human cancer cell lines were obtained from the American Type Culture Collection (Rockville, Md.) and included the pancreatic cancer lines MIA-PaCa-2 and PANC-1, the prostate cancer lines LNCaP and PC-3, the non-small-cell lung cancer lines A549 and Calu-1, and the breast cancer line MCF-7. All cell lines were cultured in appropriate medium supplemented with 10% fetal calf serum, sodium pyruvate, nonessential amino acids, L-glutamine, and penicillin/streptomycin antibiotics. Cells were maintained in a humidified incubator under an atmosphere containing 10% CO_2 at 37°C.

Tetrazolium dye methylthiotetrazole (MTT) cytotoxicity assay

For cytotoxicity assays, cells were seeded onto 96-well microculture plates at 1×10^4 cells/well and allowed to adhere for 24 h. The medium was removed and replaced with fresh medium with or without increasing concentrations of cytotoxic agents. The cells were then incubated for an additional 48 h. The medium was again removed and replaced with fresh medium without additional cytotoxic agents and the cells incubated for 24 h. Cell survival was then quantified using the tetrazolium dye methylthiotetrazole (MTT) as described previously [30]. Each experimental data point represents the average value obtained from six replicates, and each experiment was repeated at least three times.

Western blotting

Levels of BCL-2, AKT, phospho-AKT, ERK, phospho-ERK, p38MAPK and phospho-p38MAPK were determined by the Western blot technique. In brief, cells in logarithmic growth were harvested by trypsinization (trypsin 0.25% w/v, 1 m*M* ethylenediaminetetraacetic acid) and lysed in a buffer containing 150 m*M* NaCl, 1% Triton X-100 and 25 m*M* Tris (pH 7.5). Debris was sedimented by centrifugation for 5 min at 12,000 *g*, and the supernatants were solubilized for 5 min at 100°C in Laemmli's sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 100 m*M* dithiothreitol. Protein concentrations of the lysates were determined with a protein quantitation kit (Bio-Rad Laboratories, Hercules, Calif.), and 50 µg of each sample was separated on a 10% SDS-PAGE gel. Separated polypeptides were then electrophoretically

transferred to 0.2-mm nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.). Membranes were blocked for 1 h in Tris-buffered saline-Tween (TBS-T; 25 m*M* Tris, pH 8.0, 150 m*M* NaCl, and 0.05% Tween-20) containing 5% (w/v) nonfat dried milk. Blots were then probed overnight with primary antibodies and developed using species-specific secondary and tertiary antisera. Immunoreactive material was detected by the enhanced chemiluminescence technique (Amersham).

Effect of signal transduction inhibition on BCL-2 promoter function and NF-κB activity

Cells were treated with various doses of a PI3K inhibitor (LY294002), a MEK/ERK inhibitor (PD98059), or a p38MAPK inhibitor (SB203580) for 6 h and then transfected with a BCL-2 promoter/luciferase plasmid. Transcriptional regulation of the BCL-2 gene was determined using a BCL-2 promoter/luciferase construct described previously [30]. In addition, a NF-κB site at −736 nt has been previously characterized and a site-directed mutant has been developed that abolishes NF-κB binding [31]. The NF-κB luciferase plasmid contains four tandem copies of the NF-κB consensus sequence fused to a TATA-like promoter (Clontech, Palo Alto, Calif.). Following a 6-h pretreatment with the various inhibitors, the BCL-2 promoter/luciferase reporter plasmid (2.5 μg) and the control Renilla promoter/reporter, pRL-TK (0.25 μg), were transiently transfected in cells using lipofectin reagent (Life Technologies). Fresh medium was applied after the cells had been in the transfection reagent for 8–12 h. Cells were harvested 24–48 h after transfection by gentle scraping and resuspended in luciferase lysis buffer. Dual luciferase assays were performed according to the manufacturer's protocol (Promega). A 20-μl aliquot of sample supernatant was mixed with 100 μl luciferase assay reagent II followed by a 10-s reading of firefly luciferase activity using an analytical luminescence laboratory luminometer (Monolight 2010) followed by addition of 100 μl of Stop and Glo reagent (Promega). Dual luciferase assays were expressed as the ratio of firefly/Renilla RLUs and then normalized to untreated samples. All experiments were performed in triplicate and repeated on three separate occasions.

Determination of apoptotic cells by FACS analysis

Cell cycle analysis and quantification of apoptosis were carried out as described previously using propidium iodide (PI) staining and fluorescence-activated cell sorting (FACS) [32]. In brief, following treatment cells were collected by gentle trypsinization, washed in phosphate-buffered saline (PBS), and pelleted by centrifugation. Cells were fixed in 70% ethanol, washed twice in PBS and resuspended in PBS containing RNAse A (20 μg/ml). Cells were stained with propidium iodide (final concentration 10 μg/ml) for 10 min at room temperature. Samples were analyzed by FACS (FL-3 channel) using a Beckman Coulter Counter Epics XL flow cytometer (Beckman Coulter, Miami, Fl.). For each sample, 10,000 events were collected and stored for subsequent analysis using EXPO software (version 2.0; Applied Cytometry Systems, Sheffield, UK). Data were elaborated using the Autofit feature of the Multicycle for Windows software (version 3.0, University of Washington, Wash.) and expressed as fraction of cells in the different cycle phases. The percentage of cells in the sub-G_0 phase was quantitated as an estimate of cells undergoing apoptosis.

Results

Activation of signaling cascades and BCL-2 levels in various human cancer cell lines

Elevated levels of BCL-2 protein were observed in four of the seven cell lines examined (Fig. 1a). Basal levels of BCL-2 protein varied both between tumor types and

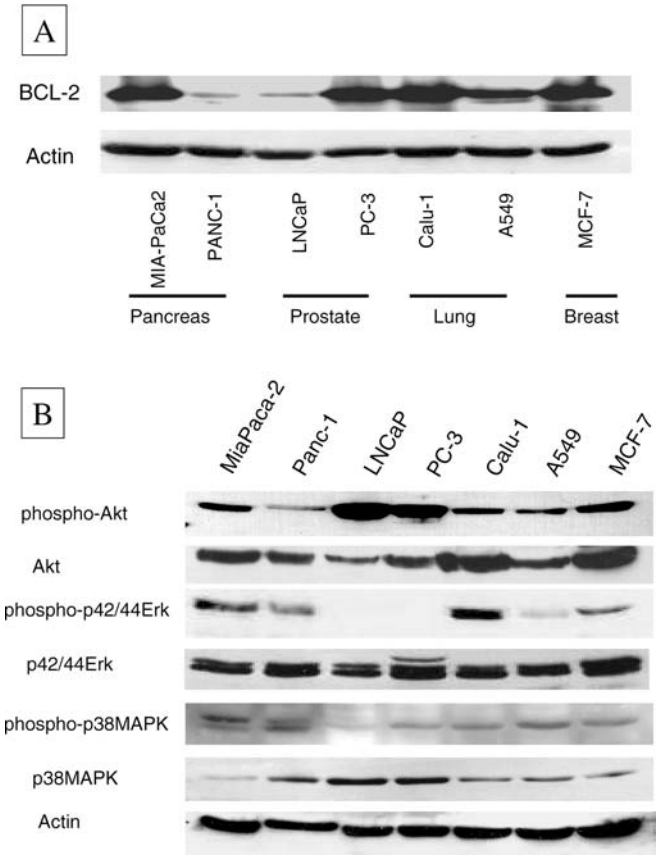

Fig. 1 **a** Levels of the BCL-2 protein as determined by Western blotting in seven human cancer cells lines representing four different types (pancreas, prostate, lung and breast cancer). **b** Degree of activation of AKT, p42/44 ERK and p38MAPK as determined by Western blotting for the phosphorylated (activated) form and total protein level in the seven human cancer cell lines. Immunoblotting for β-actin is shown to demonstrate loading equivalency

Table 1 Doses of either gemcitabine or paclitaxel that inhibited growth of the five indicated cell lines by 50% (IC_{50}) with notation of cellular BCL-2 level determined by immunoblotting

	MIA-PaCa-2	PANC-1	Calu-1	A549	MCF-7
BCL-2 level	High	Low	High	Intermediate	High
Gemcitabine	100 n*M*	500 n*M*	20 n*M*	10 n*M*	
Paclitaxel	80 n*M*	60 n*M*		3 n*M*	200 n*M*

within tumor types; MIA-PaCa-2, PC-3, and Calu-1 showed the highest basal level while PANC-1 and LNCaP showed the lowest level. The chemosensitivity of five of the cell lines was examined following exposure to gemcitabine or paclitaxel to determine whether BCL-2 content alone was sufficient to predict response to chemotherapy. There was no correlation between BCL-2 level and cytotoxicity following treatment with either gemcitabine or paclitaxel (Table 1). For example, PANC-1 has low levels of BCL-2, but was more resistant to the cytotoxic effects of gemcitabine than other cell lines with higher BCL-2 levels. Similarly, A549 has intermediate levels of BCL-2 but was the most sensitive cell line tested for response to paclitaxel. Therefore, BCL-2 levels alone are insufficient to predict cytotoxic response among cell lines, though lowering or raising BCL-2 levels within a cell line is sufficient to predict response [9, 13].

We also determined the relative status of the PI3K/AKT, MERK/ERK and p38MAPK signaling pathways by determining the presence of the phosphorylated form of the kinase under basal growth conditions (Fig. 1b). Six of the seven cell lines demonstrated significant levels of phospho-AKT, the downstream signaling partner of PI3K. Two of the seven cell lines did not have any detectable phospho-ERK, while the other five had varying levels of the activated form. Finally, all seven cell lines had very low, nearly undetectable levels of phospho-p38MAPK. Therefore, activation of the ERK signaling pathway was most variable, as nearly all cell lines demonstrated activation of PI3K, while all cell lines had very low levels of activated p38MAPK. Combining the data of BCL-2 levels and status of signaling cascade, there does not appear to be a predictable correlation of an activated signaling pathway with overexpression of BCL-2. While MIA-PaCA-2, PC-3 and MCF-7 all have high levels of BCL-2 and activated AKT, LNCaP also has activated AKT but with very low levels of BCL-2. Conversely, PC-3 and Calu-1 have similar levels of BCL-2, though dramatically different levels of activated ERK.

Effect of PI3K, MEK/ERK or p38MAPK inhibitors on BCL-2 promoter activity

As we wished to identify a common mechanism involved in BCL-2 overexpression, we focused our further investigation on the four cells lines with the highest level of BCL-2 expression (MIA-PaCa-2, PC-3, Calu-1 and MCF-7). We then examined the effect of inhibition of these three major signaling pathways on the function of the BCL-2 promoter using a promoter/luciferase reporter construct. Inhibition of PI3K with LY294002 decreased BCL-2 promoter activity in MIA-PaCa-2 and PC-3 while it increased BCL-2 promoter activity in MCF-7 and Calu-1 (Fig. 2a, Table 2). Inhibition of the MEK/ERK pathway with PD98059 increased BCL-2 promoter activity in MCF-7 cells, had no effect on MIA-PaCa-2 and PC-3, and inhibited BCL-2 promoter activity in Calu-1 (Fig. 2b, Table 1). Inhibition of p38MAPK with SB203580 increased BCL-2 promoter activity in Calu-1 without a significant effect on the three other cell lines. These data would suggest that there is not a dominant signaling pathway that is responsible for BCL-2 overexpression. Furthermore, a single signaling

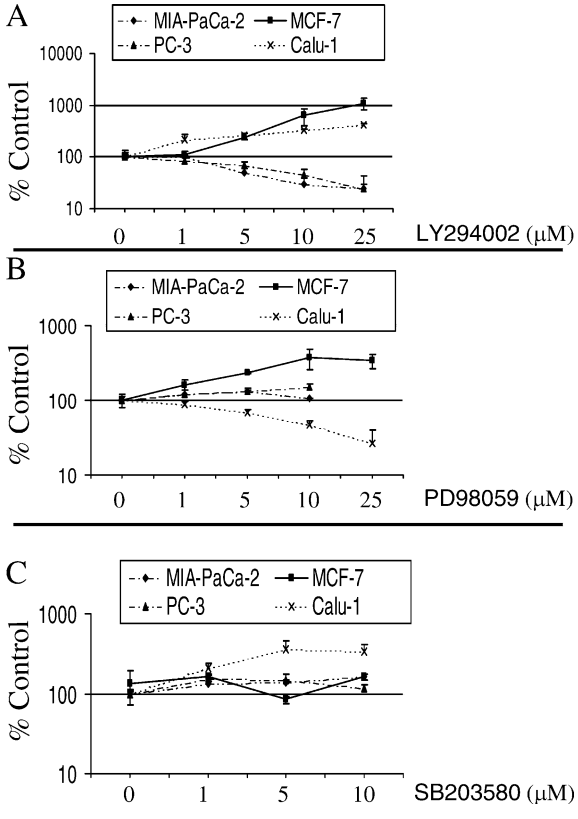

Fig. 2 Effect of inhibition of (**a**) the PI3K/AKT pathway with LY294002, (**b**) the MEK/ERK pathway with PD98059 or (**c**) the p38MAPK/MAPK pathway with SB203580 on the function of the BCL-2 promoter in representative cell lines as denoted. The results shown are the BCL-2 promoter luciferase activity normalized to the activity of the cotransfected Renilla luciferase at the indicated doses of the kinase inhibitors compared to control

Table 2 Summary of basal protein levels of BCL-2 and changes in BCL-2 promoter activity following inhibition of three signal transduction pathways (*NC* no change)

Cell line	Basal BCL-2 level	Signal transduction pathway		
		PI3K	MEK/ ERK	P38 MAPK
MIA-PaCa-2	High	↓	NC	↑
PC-3	High	↓	↑	NC
Calu-1	High	↑	NC	↓
MCF-7	High	↑	↑	↓

pathway can have opposite effects on BCL-2 promoter activity depending on the specific cell line. Reconciling these data with those of the basal activation of these signaling cascades from Fig. 1b, the basal level of kinase activation does not appear to predict the effect on BCL-2 promoter function. Therefore, there does not appear to be a uniform signaling cascade responsible for BCL-2 overexpression, nor can kinase profiling provide any guidance on the cell-specific mechanism of BCL-2 overexpression.

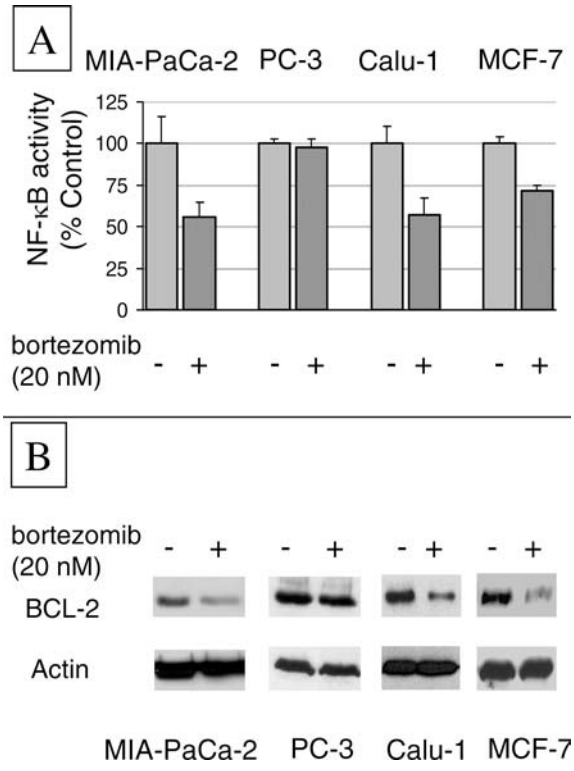

Fig. 4 Effect of treatment with the 26S proteasome inhibitor bortezomib on (**a**) NF-κB transcriptional activity or (**b**) BCL-2 protein levels in the MIA-PaCa-2, PC-3, Calu-1 and MCF-7 cell lines. Immunoblotting for β-actin is shown to demonstrate loading equivalency

Function and significance of NF-κB on the transcriptional activation of BCL-2

We have previously demonstrated a functional NF-κB site in the BCL-2 promoter at −736 nt; site-directed mutagenesis abolishes NF-κB binding and eliminates NF-κB-mediated transcriptional activation [31]. Mutation of this NF-κB site significantly decreased BCL-2 promoter activity in all cell lines (Fig. 3a). These results indicate that diverse signaling pathways converge to NF-κB to specifically activate BCL-2 expression in the cell lines assayed. To support these observations of the importance of NF-κB in transcriptional activation of BCL-2 independent of specific upstream signaling pathway, we used a pharmacological NF-κB inhibitor to block activation of NF-κB [33, 34]. Interestingly, inhibition of NF-κB transcriptional activity with bortezomib decreased BCL-2 promoter function in three of the cell lines (MIA-PaCa-2, MCF-7, Calu-1), but had no effect on BCL-2 promoter activity in PC-3, despite the observation that mutation of the NF-κB site reduced promoter activity by nearly 80% (Fig. 3). Although the inhibitory effect of mutation of the NF-κB site was about the same in all cell lines, bortezomib treatment was not as effective,

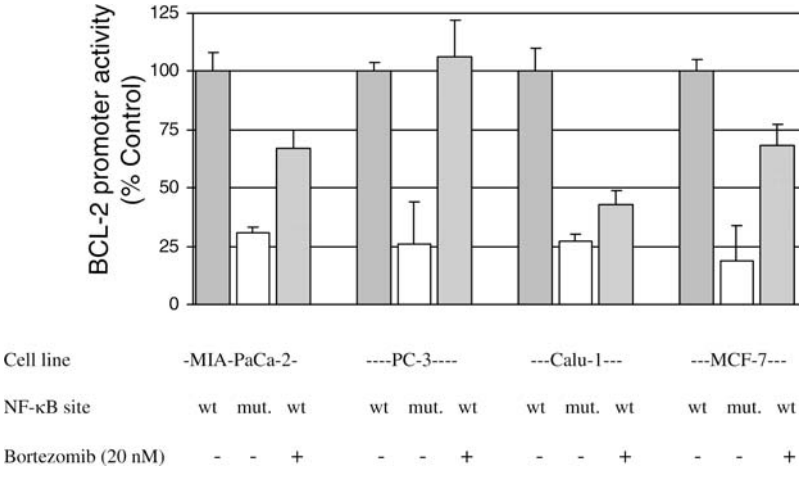

Fig. 3 Effect of mutation of the NF-κB site on BCL-2 promoter activity examined using a full-length BCL-2 promoter/ luciferase reporter containing a site-specific mutation in the consensus NF-κB site at −736 nt (*mut.*) compared to the wild-type BCL-2 promoter (*wt*) in the four human cancer cell lines. Also shown is the effect of treatment with bortezomib (20 n*M*) on the wild-type BCL-2 promoter activity

nor was the effect to the same degree in all cell lines, possibly reflecting pharmacological differences.

Effect of bortezomib treatment on transcriptional activation of BCL-2 and subsequent cellular consequences

To further explore these data, we examined the effect of bortezomib treatment on NF-κB function in these four cell lines. Similar to the effect observed on the BCL-2 promoter, bortezomib treatment reduced NF-κB transcriptional activity in three of the cell lines (MIA-PaCa-2, MCF-7, Calu-1), but without effect in PC-3 (Fig. 4a). These results would suggest that the transcriptional regulation of BCL-2 in PC-3 is dependent on NF-κB, but bortezomib treatment does not inhibit the specific NF-κB isoform or mechanism responsible for transcriptional regulation of the BCL-2 in PC-3 cells. We further examined BCL-2 protein levels following bortezomib treatment in these cell lines and noted similar results. Bortezomib treatment decreased BCL-2 protein levels in the same three cell lines (MIA-PaCa-2, MCF-7, Calu-1), but had no effect in PC-3 (Fig. 4b). These results would suggest that the regulation of BCL-2 in PC-3 does not follow the usual paradigm of NF-κB function, though there clearly are various isoforms of NF-κB as well as mechanisms of transcriptional activation independent of I-κB phosphorylation.

Finally, we examined the cellular effect of bortezomib treatment on these four cell lines that have high levels of BCL-2. We noted a G_2/M cell cycle arrest in all cell lines,

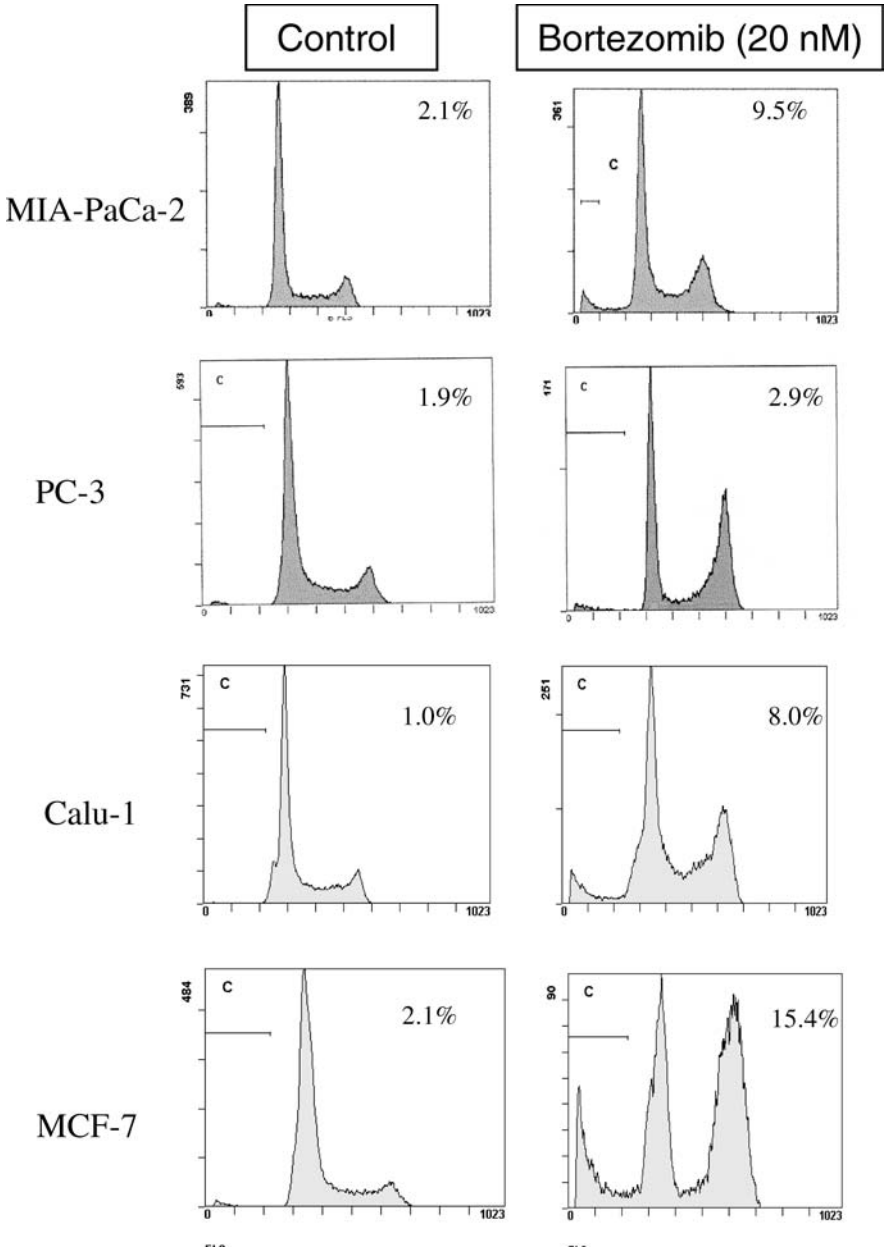

Fig. 5 FACS analysis following 24 h of treatment with the 26S proteasome inhibitor bortezomib (20 n*M*) in the MIA-PaCa-2, PC-3, Calu-1 and MCF-7 cell lines (*right column*) compared to no treatment (*left column*). Also shown are the relative percentages of cells in the sub-G_0 phase of cell distribution, which corresponds to the fraction undergoing apoptosis

as well as induction of apoptosis in three of the cell lines (MIA-PaCa-2, MCF-7, Calu-1), but no effect in PC-3 (Fig. 5). These findings are very fascinating, as a cellular effect is observed in all cells in terms of cell cycle arrest, but the induction of apoptosis occurred in only those cells in which NF-κB activity was reduced and BCL-2 levels decreased by bortezomib. Therefore, the absence of an effect of bortezomib on either NF-κB function of BCL-2 promoter activity is not due to inability of bortezomib to enter the cell or alter proteasome function, as a cellular consequence (i.e. G_2/M arrest) was observed in PC-3 cells consistent with that reported for other cells [31–33]. These results suggest that bortezomib inhibits NF-κB activity resulting in reduction of BCL-2 levels and induction of apoptosis in most, but not all, cell lines that overexpress BCL-2. In addition, assaying the NF-κB response or BCL-2 levels following treatment with bortezomib may be appropriate surrogate markers for the induction of apoptosis within the tumor. Whether these events are mechanistically involved awaits further investigation.

Discussion

We demonstrated cell type-specific differences in BCL-2 gene regulation and protein levels. Inhibition of three signaling pathways (PI3K/AKT, MEK/ERK, p38MAPK) was able to inhibit, stimulate or have no effect on BCL-2 gene activity dependent on the cell line examined. However, mutation of a NF-κB site in the BCL-2 promoter dramatically reduced promoter activity in all cell lines examined. Constitutive activation of NF-κB has been frequently observed in various cancer types, though it is unclear if NF-κB is the dominant transcription factor integrating diverse signals to regulate BCL-2 transcription in these cells. Furthermore, whether the signaling pathways that do alter BCL-2 transcription mediate this effect through NF-κB is also unknown. Various other transcription factors (e.g. CRE, cdx-1, p53) have been shown to regulate BCL-2 transcription in cancer cells and therefore it is unlikely that NF-κB is the sole point of integration of various signaling cascades to regulate BCL-2 levels. We did not demonstrate the coupling of the signaling cascades to NF-κB function and it is possible that these cascades utilize other transcription factors to regulate BCL-2 gene expression. Nevertheless, inhibition of NF-κB activity with the proteasome inhibitor bortezomib decreased NF-κB function, BCL-2 promoter function and BCL-2 protein levels, and induced apoptosis in three of the four cell lines that demonstrated BCL-2 overexpression. Therefore, inhibition of NF-κB function may be a mechanism to reduce BCL-2 levels and induce apoptosis independently of the signaling cascade that mediates BCL-2 overexpression.

These results demonstrate that diverse signaling pathways contribute to the high BCL-2 levels frequently observed in cancer. Reduction in BCL-2 levels is an active area of research as a means to re-establish apoptotic sensitivity in cancer cells [35, 36]. In addition to the antisense techniques, small molecule agents are being developed to inhibit the function of BCL-2 [37]. While various kinases inhibitors are currently in development, if alteration of BCL-2 is to be a primary modality of therapy in human malignancies, it seems unlikely that inhibitors of signal transduction kinases will be uniformly applicable. However, inhibition of NF-κB may be a more reliable mechanism to universally reduce BCL-2 levels. The further investigation of the use of bortezomib to inhibit NF-κB function as an additional approach to target BCL-2 is warranted.

The lack of effect of bortezomib on NF-κB activity, BCL-2 promoter function, BCL-2 protein levels or induction of apoptosis in the PC-3 cells further supports the coupling of NF-κB function with BCL-2 gene transcription and resistance to apoptosis. NF-κB is in fact a family of proteins that function as transcriptional regulators through both homodimerization and heterodimerization [38, 39]. Although phosphorylation of I-κB and subsequent release of NF-κB allowing nuclear translocation and gene transcriptional regulation is the predominant mechanism of NF-κB activation, alternative mechanisms independent of I-κB exist, such as cleavage of p100 allowing dimerization of the p50 subunit [40, 41]. Our previous work, as well as that of others, suggests that the NF-κB isoform responsible for regulation of BCL-2 gene transcription is the p65/p50 heterodimer, which is normally sequestered in the cytoplasm by I-κB in an inactive state [31, 41, 40]. However, as mutation of the NF-κB at −736 nt in the BCL-2 promoter reduced promoter activity in all cell lines, including PC-3, it is likely that various isoforms of NF-κB can bind at this site and regulate BCL-2 gene transcription independently of the mechanism of NF-κB activation. In fact, p100 processing to generate p50 homodimers can transactivate the BCL-2 promoter [19, 42].

We showed that the induction of apoptosis correlates with inhibition of NF-κB function and decreased BCL-2 levels. However, this is unlikely to be the sole mechanism, as 26S proteasome inhibition can alter levels of other proteins involved in the regulation of apoptosis, such as BAX, BAK and BAD [43, 44]. As the majority of these proteins homodimerize and heterodimerize, the effect of bortezomib may not be solely coupled to changes in the protein levels of BCL-2, but more importantly the ratio of antiapoptotic to proapoptotic proteins. The relative ratio of BCL-2 to BAX has been shown to be a better predictor of apoptotic sensitivity to chemotherapy than individual assessment of the protein levels of either BCL-2 or BAX [45].

In conclusion, we showed that the mechanisms responsible for overexpression of BCL-2 in various cancer types is cell specific through diverse signaling pathways that converge to NF-κB. The proteasome inhibitor bortezomib is an effective method to inhibit the NF-κB-mediated overexpression of BCL-2 and

induce apoptosis in most, though not all, cancer cell types. Finally, the effect of bortezomib on both NF-κB and BCL-2 levels is independent of the signaling cascade that mediates the overexpression of BCL-2 and therefore represents a therapy that may be broadly effective at directly targeting BCL-2 in cancer.

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