

Differential Effect of Anti-apoptotic Genes Bcl-xL and c-FLIP on Sensitivity of MCF-7 Breast Cancer Cells to Paclitaxel and Docetaxel

ZHUO WANG¹, ROBERT GOULET III¹, KATIE J. STANTON¹,
MIRAL SADARIA¹ and HARIKRISHNA NAKSHATRI^{1,2,3,4}

¹Department of Surgery, ²Department of Biochemistry and Molecular Biology and
³Walther Oncology Center, Indiana University School of Medicine, Indianapolis, Indiana 46202;
⁴Walther Cancer Institute, Indianapolis, Indiana 46208, U.S.A.

Abstract. *Background:* Intrinsic or acquired resistance to chemotherapy is a major clinical problem leading to the fatality of patients with advanced and metastatic breast cancer. The overexpression of anti-apoptotic genes is believed to play a role in the resistance to chemotherapy. In the present study, we tested the sensitivity of MCF-7 breast cancer cells overexpressing anti-apoptotic genes TRAF-1, c-FLIP, Bcl-xL, cIAP-2 or Mn-SOD to paclitaxel and docetaxel. *Materials and Methods:* MTT and trypan blue dye exclusion assays were performed to examine the sensitivity of different cell lines to docetaxel and paclitaxel. Cell cycle analysis and carboxyfluorescein FLICA assay were employed to determine whether defects in the cell cycle arrest or apoptotic pathway are responsible for the resistance of cells overexpressing Bcl-xL or c-FLIP. Caspase 8 and 9 activities were measured in cells overexpressing Bcl-xL or c-FLIP exposed to docetaxel and paclitaxel using fluorescent substrate cleavage assay. *Results:* MCF-7 cells overexpressing Bcl-xL but not TRAF-1, cIAP-2 or Mn-SOD were less sensitive to both paclitaxel and docetaxel compared to vector-transfected control cells. Resistance of Bcl-xL-overexpressing cells to taxanes correlated with the

failure to activate caspase 9. 2-Methoxyantimycin A3, a chemical inhibitor of Bcl-xL, sensitized Bcl-xL-overexpressing cells to paclitaxel and docetaxel, which suggests the drugs that inhibit Bcl-xL activity can be used as sensitizers to taxanes. MCF-7 cells overexpressing c-FLIP were less sensitive to paclitaxel but not to docetaxel. Paclitaxel failed to induce caspase 8 in c-FLIP-overexpressing cells. *Conclusion:* Because c-FLIP inhibits the extrinsic pathway of cell death whereas Bcl-xL inhibits the intrinsic pathway of cell death, these results suggest that overexpression of anti-apoptotic genes that inhibit either the extrinsic or intrinsic cell death pathways can reduce sensitivity of cancer cells to paclitaxel, whereas anti-apoptotic genes that inhibit only the intrinsic pathway reduce sensitivity to docetaxel.

Adjuvant chemotherapy is the most crucial treatment in reducing mortality in patients with advanced and metastatic breast cancer (1, 2). Taxanes, docetaxel (taxotere) and paclitaxel (taxol), are the common chemotherapeutic agents used in breast cancer (2-4). Docetaxel and paclitaxel display few overlapping as well as distinct properties. Both are microtubule-stabilizing agents but induce a different set of genes. They exert cytotoxicity by inducing mitotic arrest followed by the activation of the mitochondrial (intrinsic) and/or death receptor-dependent (extrinsic) pathways of cell death (5-7).

Intrinsic or acquired resistance to chemotherapy is a major clinical problem. Several factors including overexpression of multidrug resistance and anti-apoptotic genes are believed to play a role in resistance to chemotherapy. The expression of a number of anti-apoptotic genes including cIAP-1, cIAP-2, TRAF-1, TRAF-2, Bcl-2, Bfl-1/A1, Bcl-xL, c-FLIP and manganese superoxide dismutase (Mn-SOD) is regulated by the transcription factor NF- κ B (8-11). NF- κ B is an extracellular signal activated transcription factor, which usually resides in the cytoplasm of resting cells due its

Abbreviations: NF- κ B, nuclear factor kappaB; IAP, inhibitor of apoptosis; TRAF, tumor necrosis factor receptor associated factor; Mn-SOD, manganese superoxide dismutase; TNF, tumor necrosis factor; MTT, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; c-FLIP, Fas Ligand Interacting Protein.

Correspondence to: Harikrishna Nakshatri, R4-202 Indiana Cancer Research Institute, 1044 West Walnut Street, Indianapolis, IN 46202, U.S.A. Tel: 317 278 2238, Fax: 317 274 0396, e-mail: hnakshat@iupui.edu

Key Words: Breast cancer, NF- κ B, Bcl-xL, c-FLIP, paclitaxel, docetaxel.

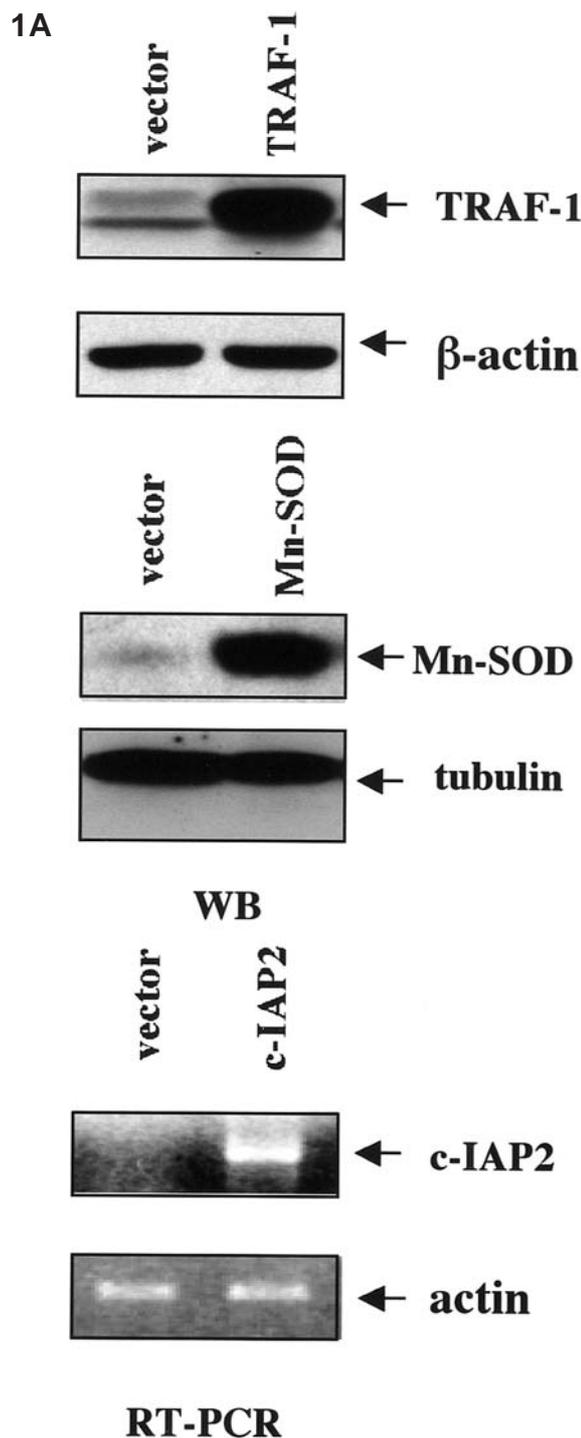
association with the inhibitor of kappaB (I κ B) proteins (8, 11). Upon exposure of cells to cytokines and growth factors such as interleukin 1 (IL-1), tumor necrosis factor (TNF) or epidermal growth factor, a series of signaling events trigger phosphorylation and degradation of I κ Bs. NF- κ B, liberated from I κ B, translocates to the nucleus, binds specific response elements in the promoter region of target genes and regulates gene expression.

Constitutive activation of NF- κ B is observed in a number of cancers including breast cancer (10, 12). The constitutively active NF- κ B in these cancers may contribute to chemotherapeutic resistance by up-regulating the expression of anti-apoptotic genes (9). We and others have shown that inhibition of NF- κ B through either overexpression of I κ Bs or prior exposure to chemical NF- κ B inhibitors leads to increased sensitivity of cancer cells to chemotherapeutic drugs such as paclitaxel, docetaxel and CPT-11 (13-15).

In the present study, we sought to identify NF- κ B-regulated anti-apoptotic genes involved in resistance to taxanes. MCF-7 breast cancer cells are ideal for this study because NF- κ B is not constitutively active in these cells and inducible NF- κ B is less active due to interaction with estrogen receptor (10, 16). Thus, it is possible to generate derivatives of this cell line overexpressing individual NF- κ B-regulated anti-apoptotic genes and evaluate the contribution of individual genes in resistance to specific chemotherapeutic drugs. The sensitivities of MCF-7 cells with vector alone or overexpressing cIAP-2, TRAF-1, Mn-SOD, Bcl-xL or c-FLIP to docetaxel and paclitaxel were compared. We observed reduced sensitivity of cells overexpressing Bcl-xL to both docetaxel and paclitaxel. Cells overexpressing c-FLIP showed partial resistance to paclitaxel but not to docetaxel. The cells overexpressing cIAP-2, TRAF-1 and Mn-SOD were as sensitive as control cells to both drugs. 2-Methoxyantimycin A3, a chemical inhibitor of Bcl-xL (17), increased the sensitivity of Bcl-xL-overexpressing cells to docetaxel and paclitaxel. Based on a recent report showing an important role for Bcl-xL in survival and growth of breast cancer cells at sites of metastasis (18), and our results highlighting the importance of this gene in reducing the sensitivity of cancer cells to commonly used chemotherapeutic drugs, we propose that inhibitors of Bcl-xL function are necessary to reduce metastasis as well as to increase the sensitivity of metastatic cancer cells to chemotherapy.

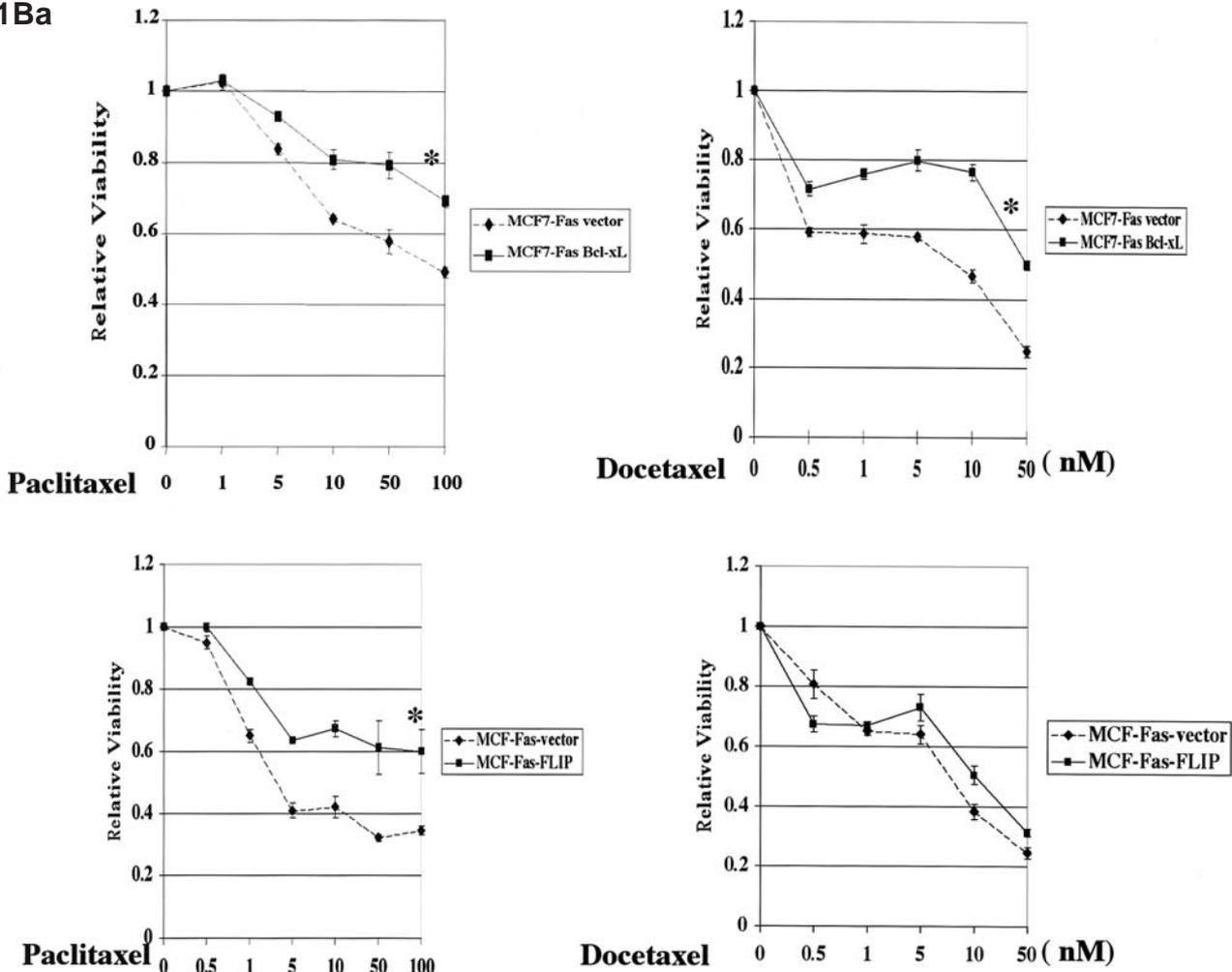
Materials and Methods

Cell culture. MCF-7 breast cancer cells stably overexpressing cIAP-2 and TRAF-1 were generated by transfecting pTRE2hyg cIAP-2 and TRAF-1 cDNA constructs into MCF-7 cells, followed by selection with 150 μ g/ml hygromycin B. MCF-7 stably overexpressing Mn-SOD cells were generated by transfecting pcDNA3-Mn-SOD cDNA construct into MCF-7 cells, followed by selection with 600 μ g/ml G418. Empty vector pTRE-2hyg



(Clontech, Palo Alto, CA, USA) or pcDNA3 (Invitrogen, Carlsbad, CA, USA) was transfected into MCF-7 cells to generate control cells. MCF-7 cells overexpressing Bcl-xL and c-FLIP, along with corresponding control cells of similar passage number, were a generous gift of Dr. Marcus E. Peter (University of Chicago, Chicago, IL, USA) (19, 20) and were originally

1Ba



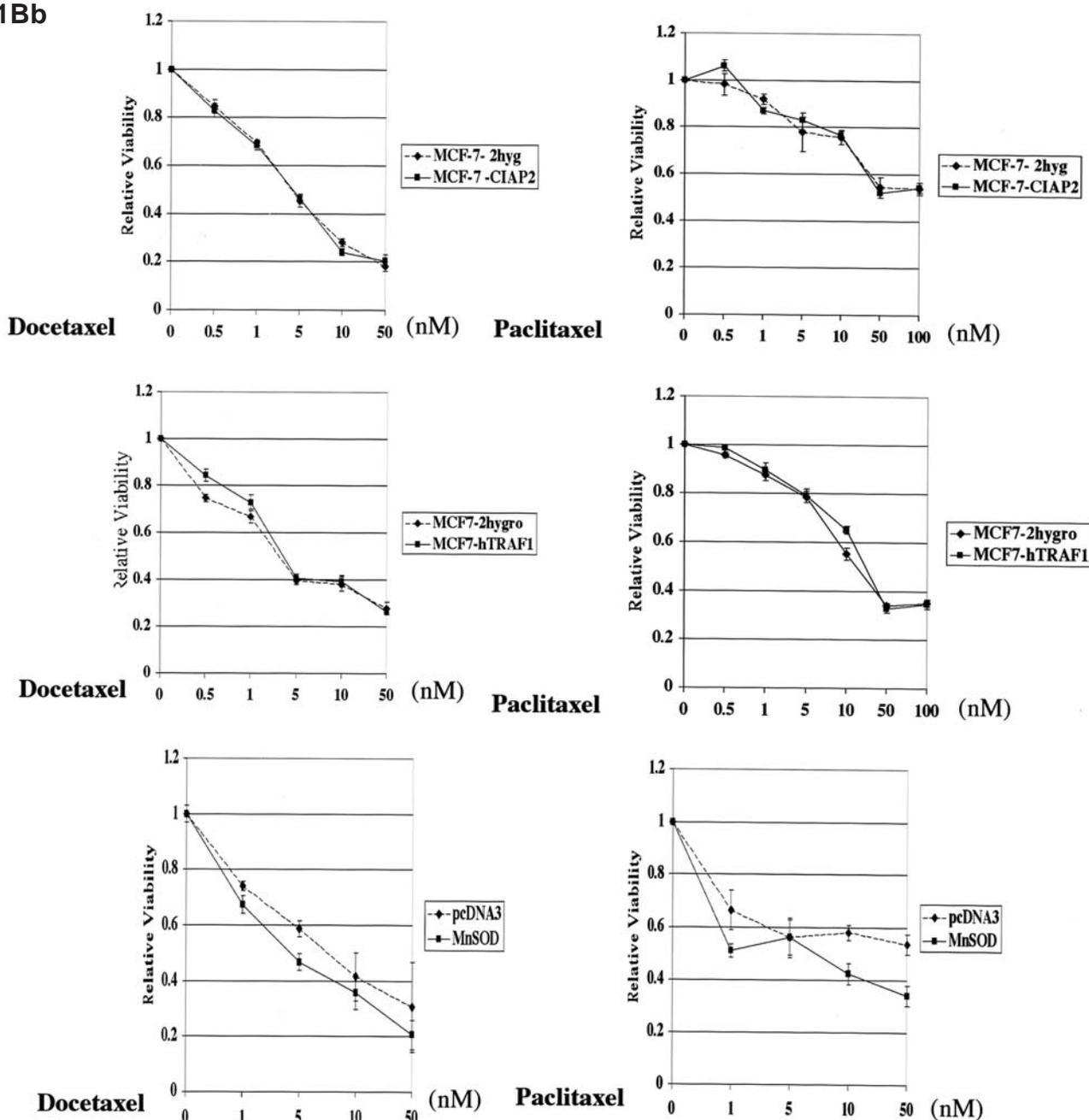
generated in Drs. Vishva Dixit's (Genentech, San Francisco, CA, USA) and Xiaolu Yang's (University of Pennsylvania) laboratories. Note that these cells originated from different laboratories and there are some differences in the growth media and consequently behavior of control cells. Cells overexpressing cIAP-2, TRAF-1 and Mn-SOD were maintained in Eagle Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS). Bcl-xL cells were maintained in RPMI 1640 media supplemented with 10% FBS. c-FLIP cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS.

MTT assay. Cells were plated at 2000 cells per well in 96-well tissue culture plates. Different concentrations of docetaxel or paclitaxel were added one day after culturing and replaced after three days. After six days, the number of viable cells was determined using the celltiter non-radioactive cell proliferation kit (Promega, Madison, WI, USA), according to the instruction of the manufacturer. In each experiment, cells in eight wells were treated with the same drug. Each data point is the average of results obtained from three independent experiments.

Cell cycle analysis. Cells were grown in culture for 48 h before treatment with vehicle or different drugs. The cells were trypsinized and collected in media, and washed twice with PBS and resuspended in 125 μ l of 2 μ g/ml RNase and 300 μ l of propidium iodide containing buffer (50 μ g/ml propidium iodide, 1 mg/ml sodium citrate, 0.03% NP40). The cells were analyzed on a FACScan flow cytometer (Becton Dickon, Franklin Lakes, NJ, USA). The cell cycle distribution was determined using the ModFit computer software. All cell cycle assays were performed three times and representative data is presented in the text.

Cell death assay. The carboxyfluorescein FLICA apoptosis detection kit (Immunohistochemistry Technologies, LLC, Bloomington, MN, USA) was used to measure typical apoptosis, atypical apoptosis and necrosis (21, 22). Briefly, 2×10^5 cells grown overnight on a 60-mm plate were exposed with the indicated drugs for 3 days. Both floating and attached cells were collected by trypsinization and incubated with carboxyfluorescein-labelled pan-caspase inhibitor FAM-VAD-FMK for 2 h at 37°C. The labelled cells were rinsed twice in washing buffer and resuspended in 400 μ l of the washing

1Bb



buffer containing 0.4 µg of propidium iodide. Apoptotic cells were identified by FACSscan analysis as described previously (22). All apoptosis assays were performed three times and representative data is presented in the text.

Western blot analysis. Cell lysates prepared in radioimmunoassay buffer were subjected to Western blot, as described previously (13).

Caspase 8 and 9 activities assay. Caspase 8 or 9 activities were measured using fluorogenic caspase 8 or 9 substrate, respectively

(Ac-IEPD-AMC or Ac-LEHD-AMC, Alexis, San Diego, CA, USA). Briefly, 1x10⁶ cells, grown overnight in 100-mm dishes, were treated with the indicated drugs for 48 h. Both floating and attached cells were collected by trypsinization and lysed in 150 µl of lysis buffer (50 mM PIPES, pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂ and 1 mM DTT). After incubation on ice for 30 minutes, the lysate was collected by centrifugation. Fifty µl of cell lysate was incubated with 50 µl of 2 x reaction buffer (20 mM HEPES, pH7.5, 20% sucrose, 0.2% CHAPS, 0.2 mg/ml BSA and 20 mM DTT) and 5 µl of 1 mM caspase 8 or 9 substrate for 1 h at

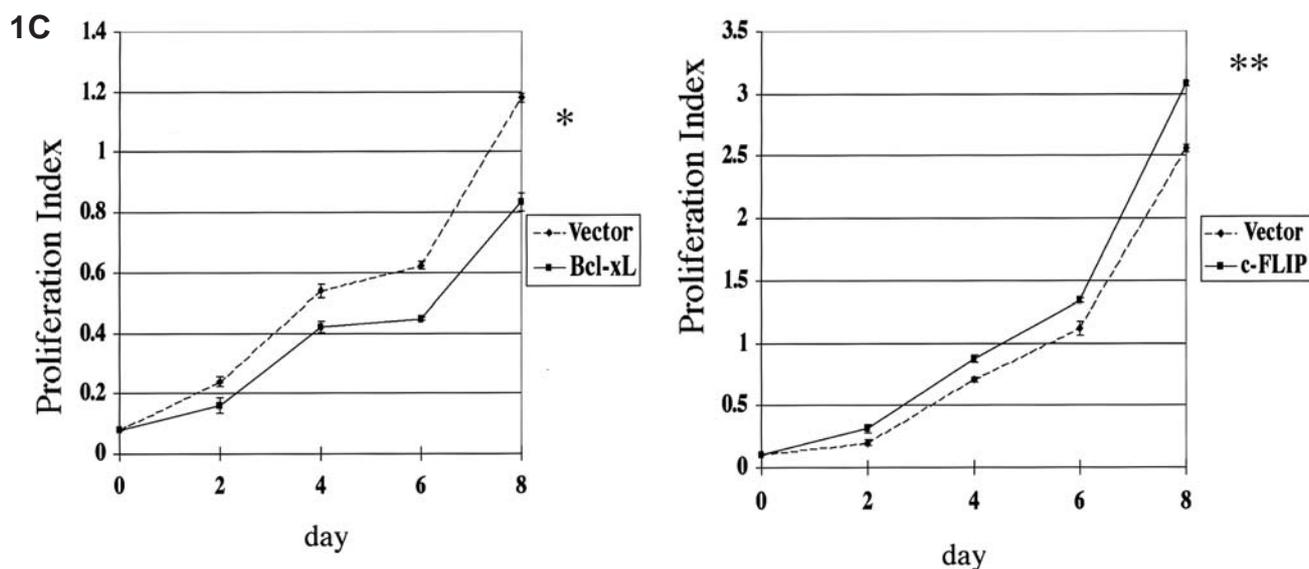


Figure 1. Sensitivity of MCF-7 cell derivatives to docetaxel and paclitaxel. A) Expression levels of Mn-SOD, TRAF-1 and cIAP-2 in overexpressing cells. Mn-SOD and TRAF-1 expression was determined by Western blotting, whereas cIAP-2 expression was measured by RT-PCR. B) Sensitivity of cells to various concentrations of docetaxel and paclitaxel was measured by MTT assay as described in Materials and Methods. * $p < 0.001$ C) Proliferation rate of control, Bcl-xL- and c-FLIP-overexpressing cells. * $p < 0.001$ ** $p = 0.08$

37°C. Caspase 8 or 9 activities were measured at an excitation wavelength of 380 nm and emission wavelength of 460 nm. Caspase 8 or 9 activities were normalized to protein concentration and basal activity in untreated cells was set as one unit. All assays were performed three times.

RNAse protection assay. RNAse protection assay with human APO2 probe (BD Biosciences) was performed as described previously (13). RNA from untreated MCF-7 and MDA-MB-231 cells for 4 h was used in the RNAse protection assay.

Statistical analysis. Data were analyzed using GraphPad software (Graphpad.com). Analysis of variance was employed to determine p values between mean measurements. A p value < 0.05 was deemed significant. Error bars on all histograms in this text represent the standard deviation between measurements from duplicate experiments.

Results

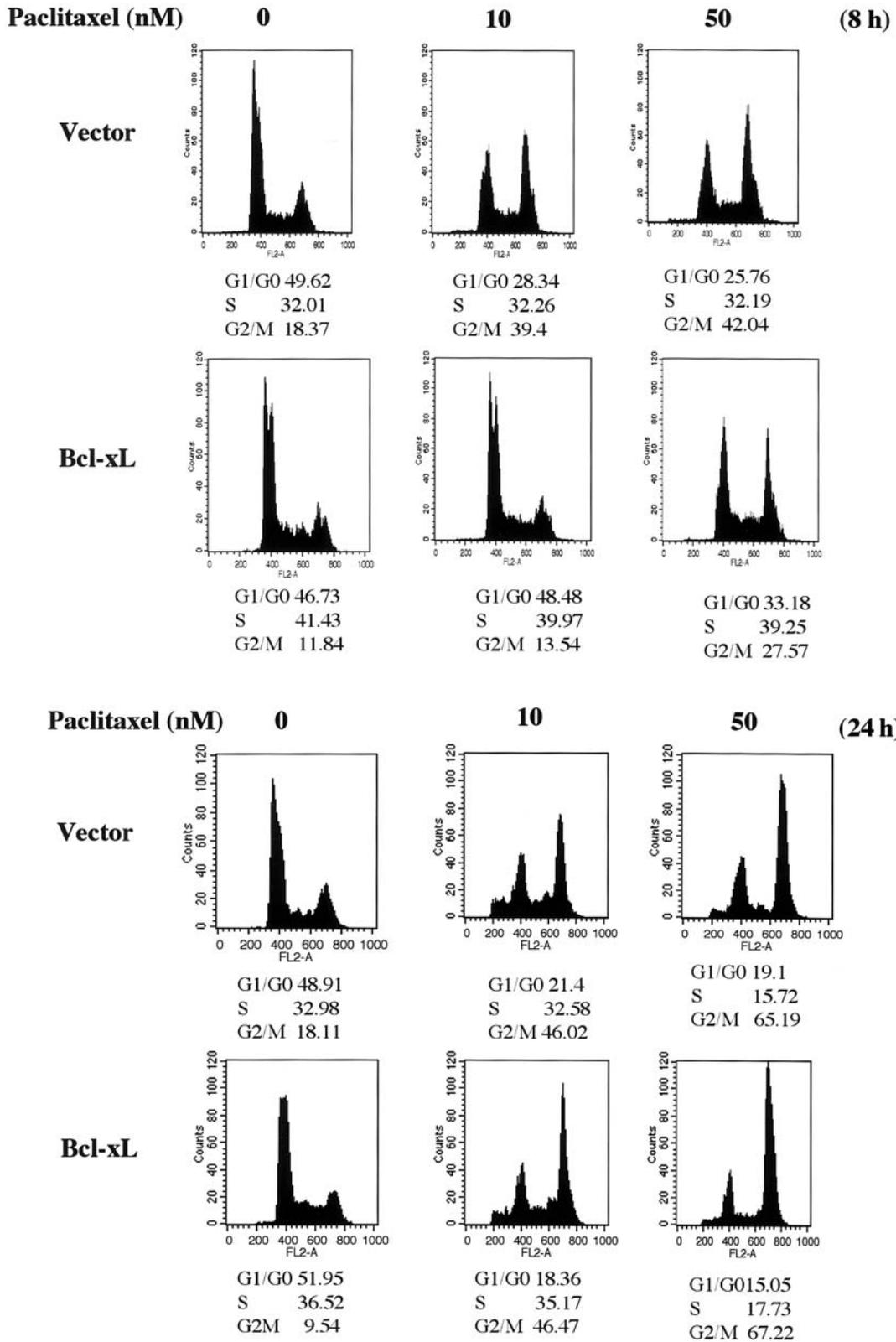
Overexpression of Bcl-xL leads to reduced sensitivity of MCF-7 breast cancer cells to docetaxel or paclitaxel. We sought to identify NF- κ B regulated anti-apoptotic genes involved in resistance to taxanes using MCF-7 breast cancer cells overexpressing TRAF-1, c-FLIP, c-IAP2, Mn-SOD or Bcl-xL (8, 9). The expression of TRAF-1, Mn-SOD or cIAP-2 was confirmed by Western blotting or reverse transcriptase-polymerase chain reaction (RT-PCR) (Figure 1A). Overexpression of these genes had no effect on basal NF- κ B DNA binding activity (data not shown). MTT assay was performed to examine the sensitivity of different cell lines to

docetaxel and paclitaxel. As shown in Figure 1B, only cells overexpressing Bcl-xL showed reduced sensitivity to docetaxel and paclitaxel compared to vector control cells. The cells overexpressing c-FLIP were modestly resistant to paclitaxel but not to docetaxel. Results from the trypan blue dye exclusion assay were consistent with those from MTT assay (data not shown). In addition, docetaxel was effective at a lower concentration than paclitaxel, which is consistent with previous studies (23, 24).

To determine whether the differential sensitivity of Bcl-xL- and c-FLIP-overexpressing cells to taxanes compared to vector control cells is due to changes in proliferation rate, the rate of cell proliferation was measured by MTT assay (Figure 1C). The cells overexpressing Bcl-xL grew slower than control cells, while the cells overexpressing c-FLIP showed slightly faster growth than control cells, suggesting that there is no correlation between the cell proliferation rate and drug sensitivity.

Delayed G2/M arrest in cells overexpressing Bcl-xL but not c-FLIP upon taxane treatment. Taxanes bind and stabilize microtubules leading to mitotic arrest followed by apoptosis (6). To assess whether reduced sensitivity of Bcl-xL cells to taxanes is due to defects in cell cycle arrest, we performed cell cycle analysis of untreated and drug-treated cells. As summarized in Figure 2, G2/M arrest was delayed in Bcl-xL-overexpressing cells compared to control cells. For example, after 8-h drug treatment, only control cells were arrested at G2/M. However, at 24 h post-treatment, both control and Bcl-xL-overexpressing

2A



2B

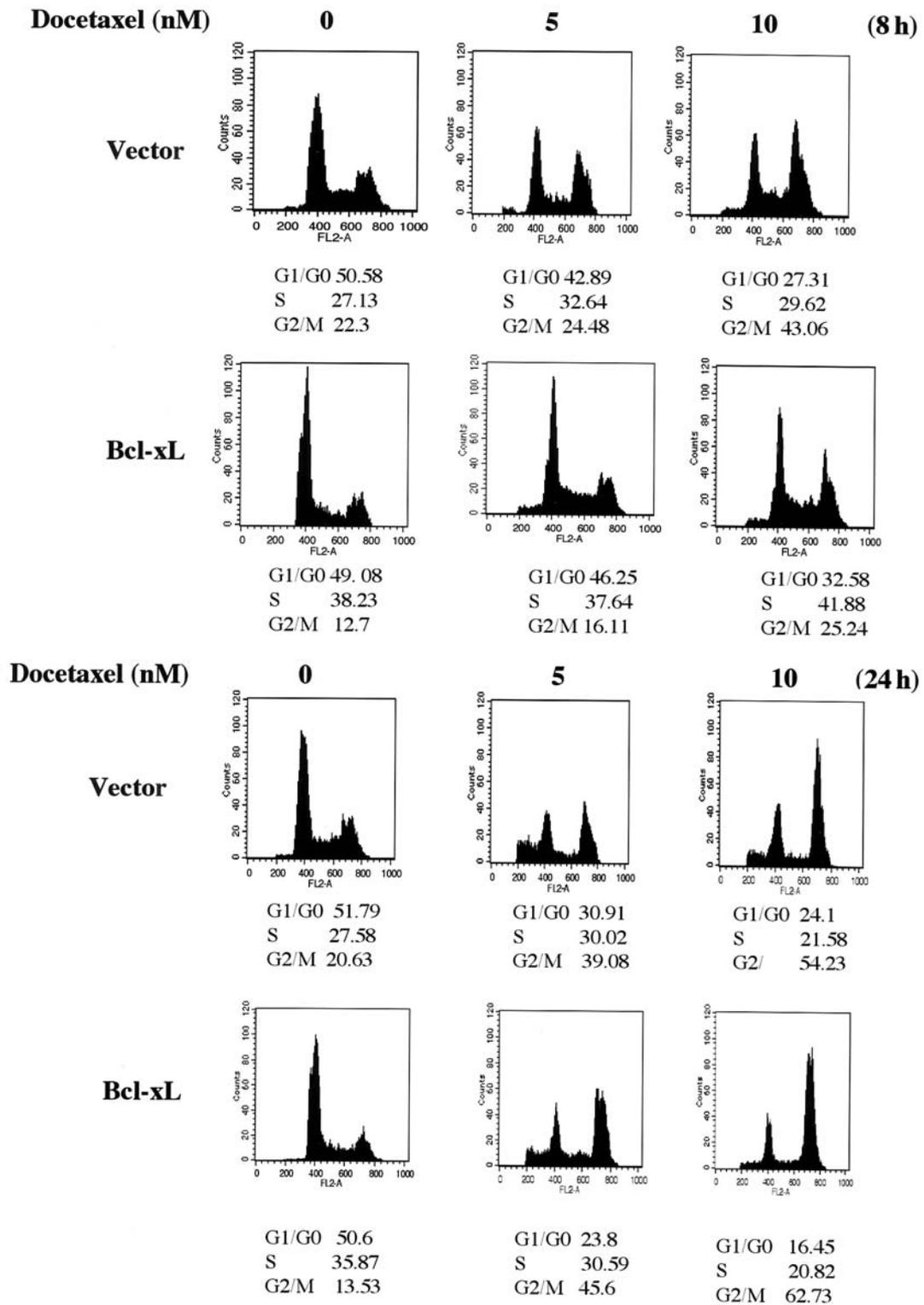
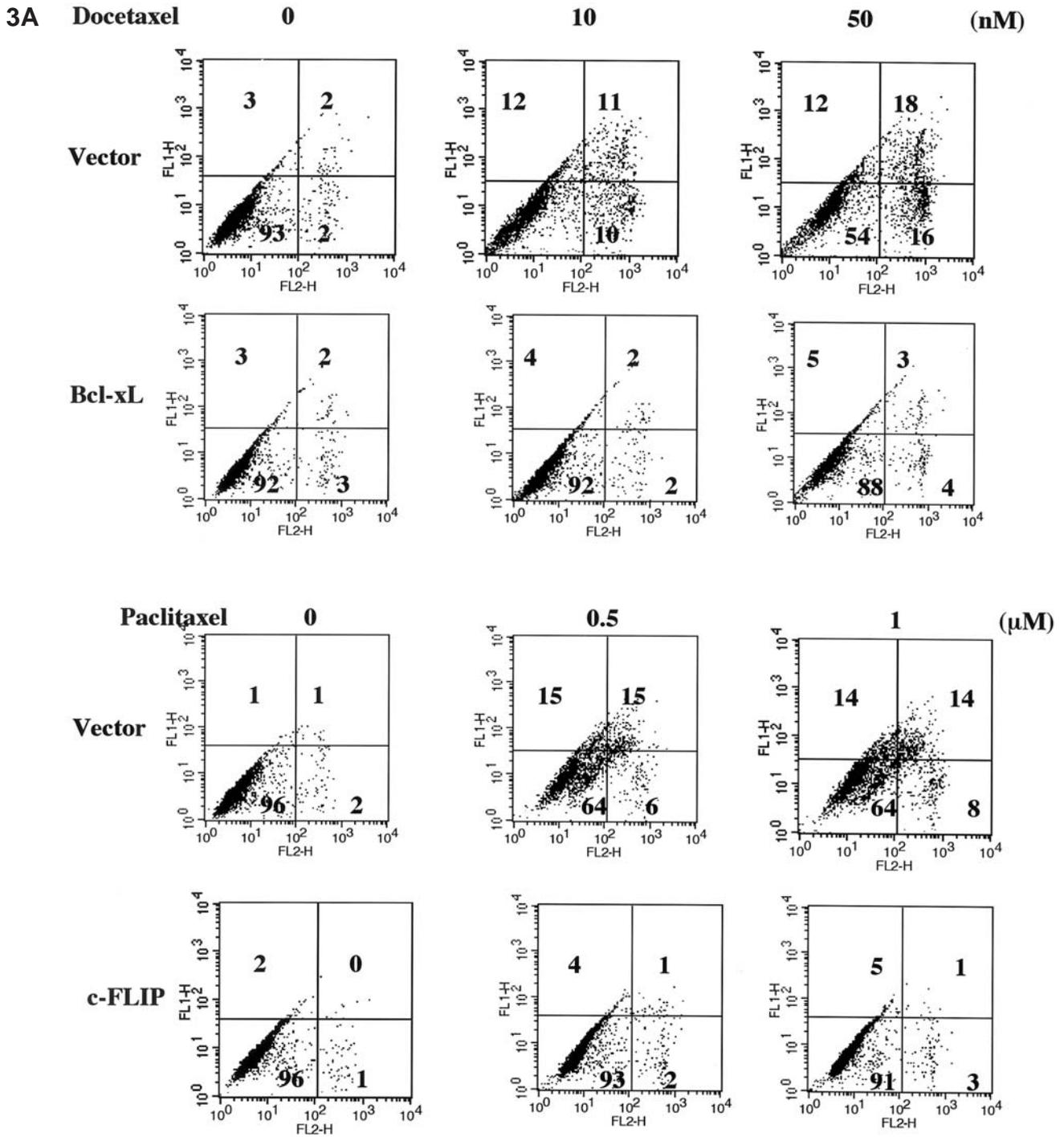


Figure 2. The effect of docetaxel and paclitaxel on cell cycle distribution of control and Bcl-xL-overexpressing cells. Cells were treated with drugs for the indicated times and cell cycle distribution was measured by flow cytometry. Representative data from three experiments is shown.



cells accumulated at G2/M-phase at a similar level. Interestingly, the proportion of untreated Bcl-xL cells in S-phase was always higher compared to untreated control cells, which suggests that Bcl-xL-overexpressing cells take a longer time to complete S-phase or that Bcl-xL delays

the entry of cells from S-phase to G2/M. The delay in taxane-induced G2/M arrest of Bcl-xL-overexpressing cells could be a consequence of prolonged S-phase in these cells. Docetaxel-induced G2/M arrest was not affected in cells overexpressing cIAP-2, TRAF-1, Mn-SOD or c-FLIP (data not shown).

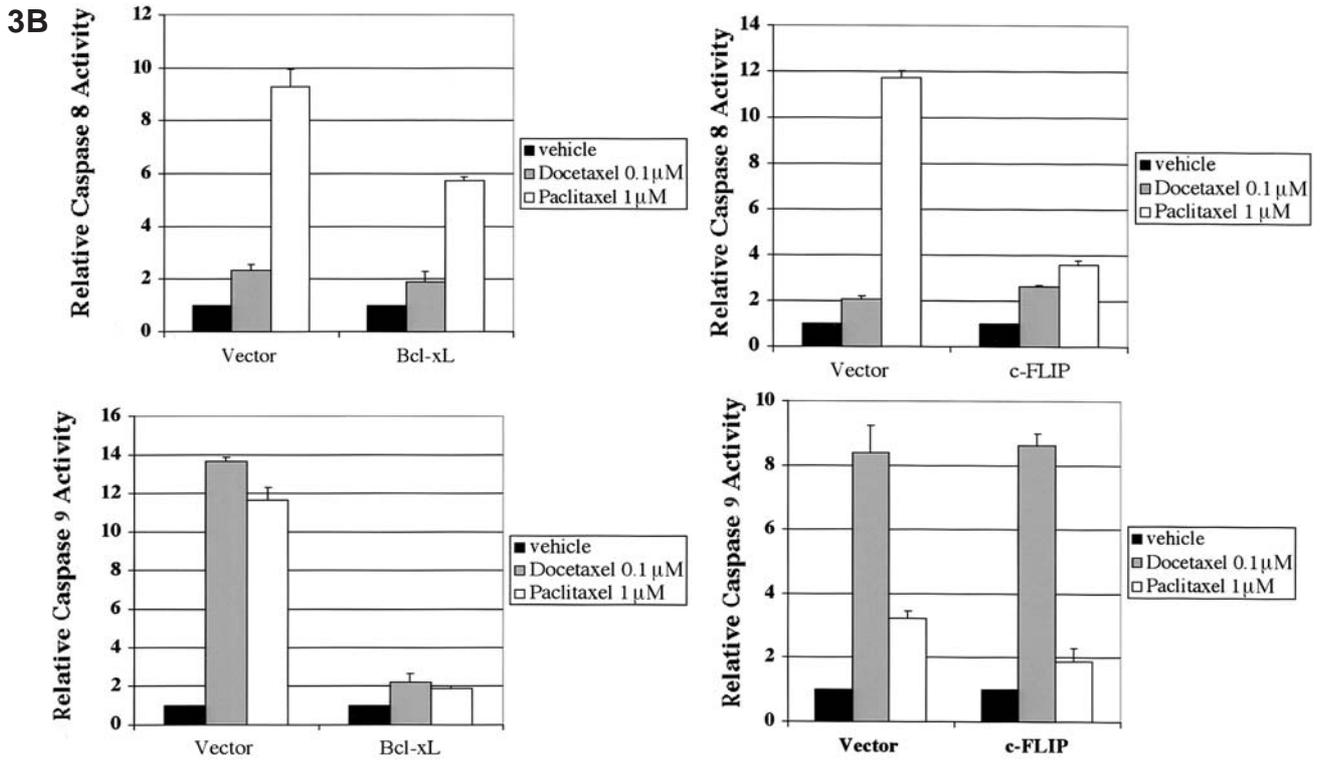
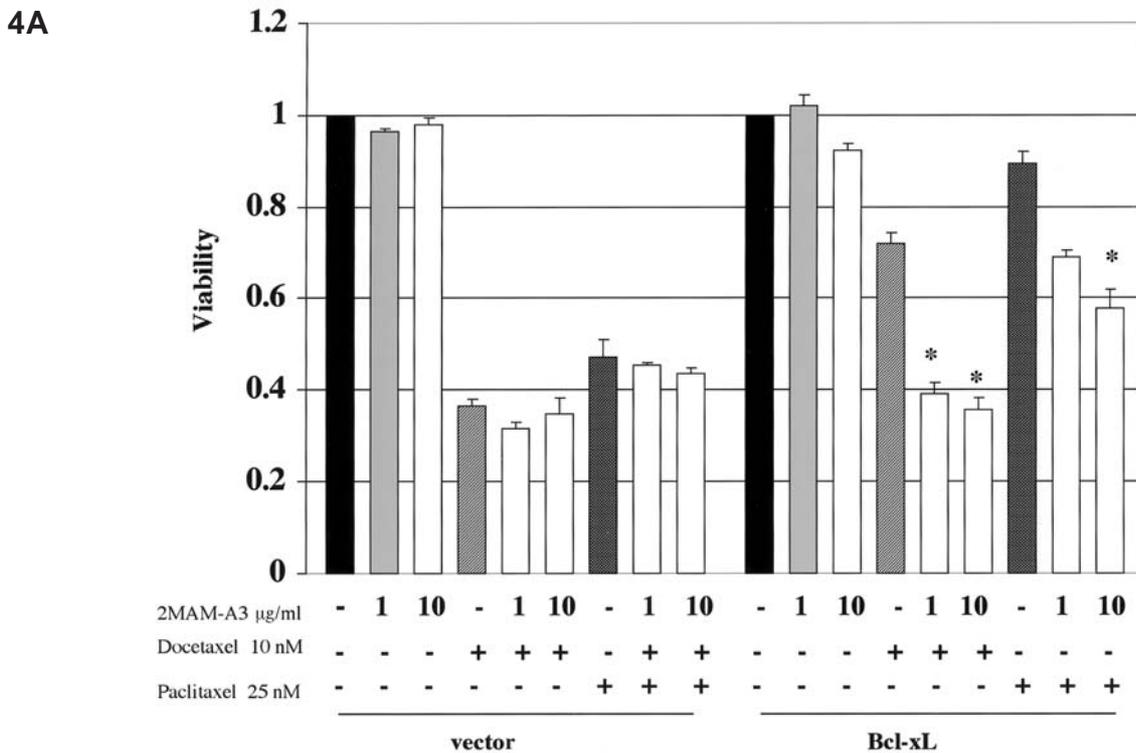
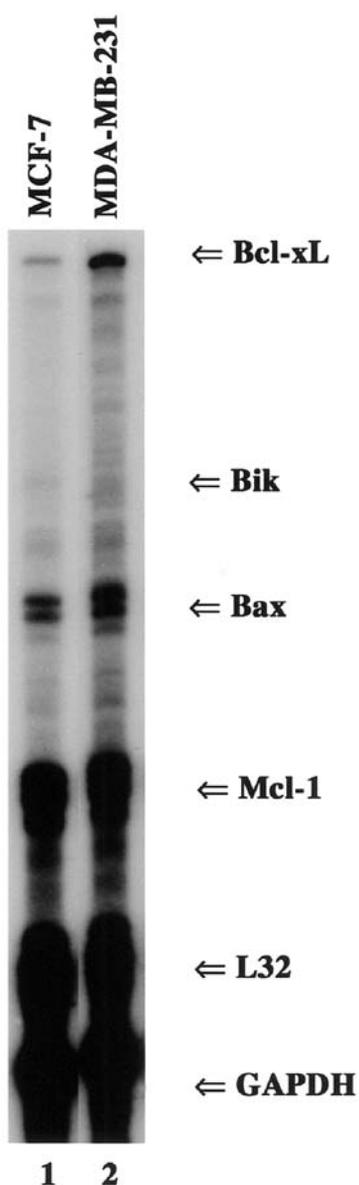


Figure 3. The effect of docetaxel and paclitaxel on apoptosis of control, Bcl-xL- and c-FLIP-overexpressing cells. A) Apoptosis was measured using carboxyfluorescein FLICA. Apoptosis was measured three days after treatment. X-axis, propidium iodide staining; Y-axis, active caspase staining; lower left, live cells; upper left, apoptotic cells, lower right, necrotic cells and upper right, atypical apoptotic cells. Representative data from three experiments is shown. B) Caspase 8 and 9 activities in cells treated with docetaxel or paclitaxel for 48 h. Caspase 8 or 9 activities were measured using the substrate Ac-IEPD-AMC or Ac-LEHD-AMC, respectively, as described in Materials and Methods.



4B



Defects in taxane-induced apoptosis in cells overexpressing Bcl-xL and c-FLIP. To further determine whether defects in the apoptotic pathway are responsible for the resistance of cells overexpressing Bcl-xL and c-FLIP to taxanes, apoptosis of drug-treated cells was measured using the carboxy-fluorescein FLICA assay. This assay simultaneously measures the level of active caspases and the loss of plasma membrane integrity, thus allowing quantitation of live, apoptotic, atypical apoptotic (with loss of plasma membrane integrity) and necrotic cells (21, 22). Since the FLICA assay is less sensitive than the MTT assay employed in Figure 1B, higher concentrations ($\geq 0.5 \mu\text{M}$) of paclitaxel were used to detect significant apoptosis. Cells overexpressing Bcl-xL or

c-FLIP showed significantly decreased typical and atypical apoptosis and increased cell survival when treated with paclitaxel at different concentrations, while cells overexpressing Bcl-xL but not c-FLIP displayed increased cell survival when treated with docetaxel (Figure 3A, data not shown). Thus, the resistance to paclitaxel and/or docetaxel appears to be due to an impaired apoptotic pathway in cells overexpressing c-FLIP or Bcl-xL.

To determine whether taxanes are less efficient in inducing caspase activity in cells overexpressing Bcl-xL or c-FLIP, we measured the activities of caspase 8 and 9, which are major caspases in the extrinsic and/or intrinsic apoptosis pathways using a fluorescent substrate cleavage assay (Figure 3B). We did not examine caspase 3 activity because MCF-7 cells lack the caspase 3 gene (25). Paclitaxel was more efficient in inducing caspase 8, whereas docetaxel was more efficient in inducing caspase 9 in control cells (Figure 3B). Note that, as with cell death assays, a higher concentration of paclitaxel compared to docetaxel was required to observe caspase activation. Despite the lower concentration, docetaxel was still more efficient than paclitaxel in inducing caspase 9. Both paclitaxel and docetaxel failed to induce caspase 9 in Bcl-xL-overexpressing cells. Paclitaxel-induced caspase 8 activity was lower in Bcl-xL-overexpressing cells compared to control cells. While c-FLIP overexpression had no effect on docetaxel-induced caspase 9 activation, c-FLIP reduced paclitaxel-induced caspase 8 and caspase 9 activation. Thus, Bcl-xL and c-FLIP protect cancer cells against taxanes by reducing caspase activation.

2-Methoxyantimycin A3, a chemical inhibitor of Bcl-xL, sensitizes cancer cells with high levels of Bcl-xL to paclitaxel and docetaxel. To confirm that Bcl-xL is responsible for taxane resistance, we treated cells with taxanes along with 2-methoxyantimycin A3, which selectively inhibits Bcl-xL function by binding to its hydrophobic groove (17). As shown in Figure 4 A, 2-methoxyantimycin A3 alone had no effect on both control and Bcl-xL-overexpressing cells. Paclitaxel and docetaxel in combination with 2-methoxyantimycin A3 caused significant cytotoxicity to Bcl-xL-overexpressing cells. Since MCF-7 cells lack caspase 3 expression, we sought to further investigate if 2-methoxyantimycin A3 sensitizes MDA-MB-231 cells, which contains caspase 3 and constitutively active NF- κ B activity, to taxanes. The RNase protection assay showed that MDA-MB-231 cells express a higher Bcl-xL mRNA level compared to MCF-7 cells (Figure 4B). MDA-MB-231 cells showed increased typical and atypical apoptosis when treated with a combination of 2-methoxyantimycin A3 and paclitaxel or docetaxel (Figure 4C). These results suggest that inhibitors of Bcl-xL can be used to increase the sensitivity of cancer cells to taxanes.

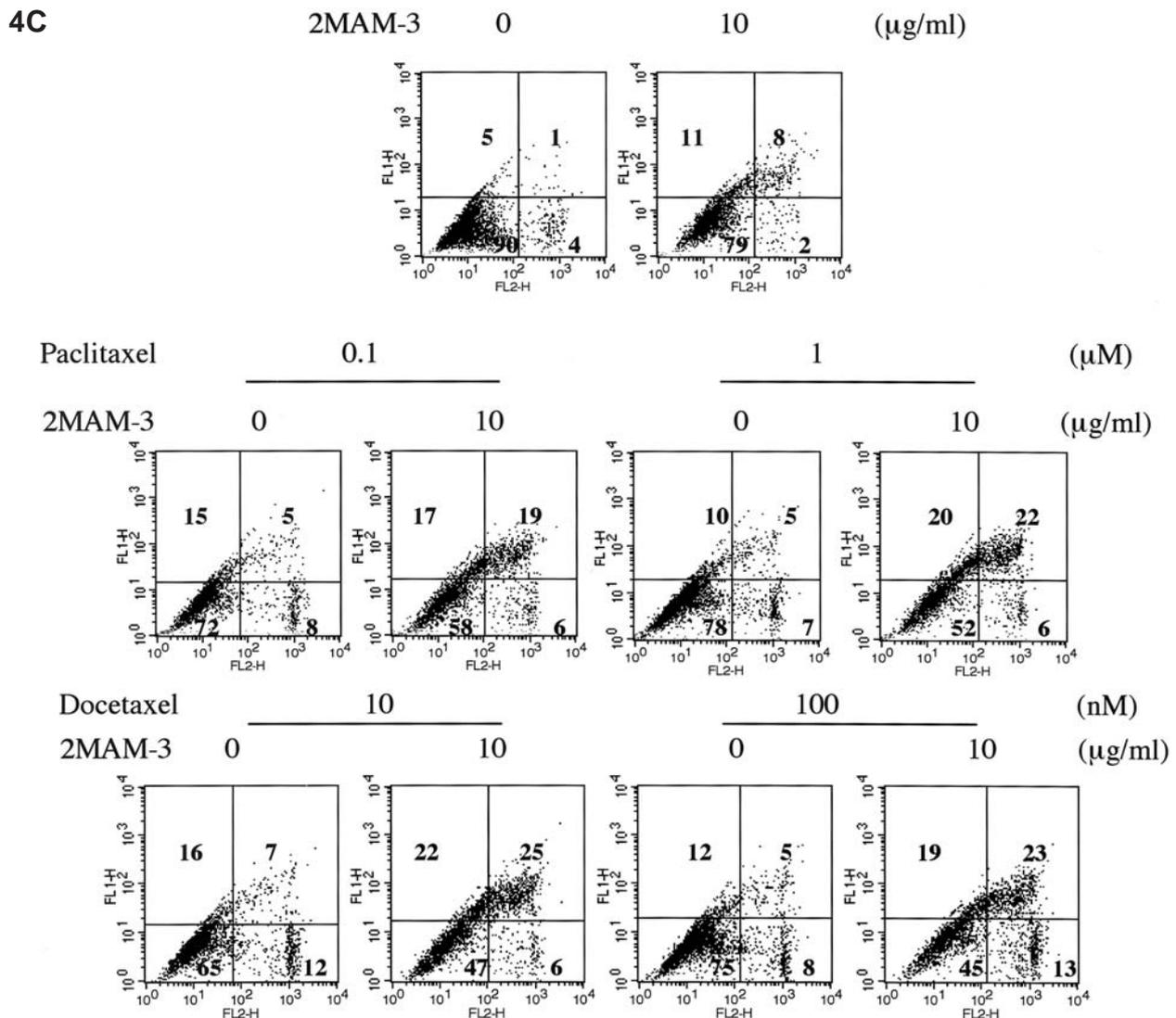


Figure 4. 2-Methoxyantimycin A3, a chemical inhibitor of Bcl-xL, reverses resistance of cancer cells with high levels of Bcl-xL to taxanes. A) MCF-7 cells overexpressing Bcl-xL were treated with indicated drugs for three days and cell death was measured by MTT assay * $p < 0.001$. B) RNA from MCF-7 and MDA-MB-231 cells for 4 h was subjected to RNase protection assay. C) MDA-231 cells were treated with a combination of 10 µg/ml 2-methoxyantimycin A3 and paclitaxel or docetaxel for 48 h and apoptosis was measured as in Figure 3A.

Discussion

Both paclitaxel and docetaxel are considered to be microtubule-targeted tubulin-polymerizing agents (MTPAs) (6). Previous studies suggest that MTPAs induce G2/M arrest and subsequent apoptosis through the mitochondrial pathway (6, 7). Bcl-xL but not c-FLIP caused a delay in docetaxel-induced G2/M arrest. The adaptor protein c-FLIP serves as an anti-apoptotic gene in the death receptor pathway (26). It competes with caspase 8 for binding to FADD and inhibits caspase 8 activation, which is an essential step in initiation of the extrinsic death pathway. Moos and

Fitzpatrick demonstrated that paclitaxel, but not docetaxel stimulates TNF α production (5). Neutralizing antibodies against TNF α reduced paclitaxel- but not docetaxel-induced cell death, suggesting the TNF α -induced extrinsic apoptosis is an integral part of paclitaxel-mediated cell death (5). Consistent with this possibility, we observed elevated caspase 8 activation by paclitaxel compared to docetaxel, which was inhibited by c-FLIP (Figure 3B). Thus, the resistance of c-FLIP-overexpressing cells to paclitaxel is due to inefficient activation of the extrinsic cell death pathway. Activated caspase 8 may be involved in feed-forward activation of the intrinsic cell death pathway in paclitaxel-treated cells,

because paclitaxel failed to induce caspase 9 in both Bcl-xL- and c-FLIP-overexpressing cells (Figure 3B). Because docetaxel-induced cell death is dependent mostly on the intrinsic cell death pathway, c-FLIP-overexpressing cells were still sensitive to this drug. Although TRAF-1 is an inhibitor of the extrinsic pathway of cell death (27), it was unable to confer resistance to paclitaxel. TRAF-1 is mostly involved in signaling by CD40 and its anti-apoptotic function may be signal-dependent (28).

The failure of cIAP-2 and Mn-SOD, both of which antagonize the intrinsic cell death pathway, to protect against taxanes is rather surprising (29, 30). The anti-apoptotic function of cIAP-2 in the intrinsic cell death pathway is downstream of mitochondria (30). It inhibits activation of caspase 9 in the apoptosome and inhibits the activity of processed caspase 3. It is possible that once the mitochondria are damaged, apoptosis is irreversible and the anti-apoptotic proteins functioning downstream of mitochondria are not potent enough to prevent apoptosis. Mn-SOD scavenges reactive oxygen species and, thus, reduces mitochondrial damage (31). Paclitaxel- and possibly docetaxel-induced cell death in our assays less likely involve oxidative stress because previous studies have shown that paclitaxel up to one μM does not induce oxidative stress in MCF-7 cells (32). This may explain the differences in our results with that of Manna *et al.* (33), who showed enhanced survival of MCF-7 cells overexpressing Mn-SOD treated with 25 μM paclitaxel.

Cells selected for paclitaxel resistance *in vitro* have been shown to overexpress Bcl-2 or Bcl-xL (6, 34). Bcl-xL-mediated resistance to taxanes may involve defects in cell cycle arrest, apoptosis or both. We observed a delay in taxane-induced G2/M arrest of Bcl-xL cells compared to control cells (Figure 2). The consequences of this delay on ultimate cell death are not known. It is possible that Bcl-xL-mediated delay in G2/M arrest may have an impact on drug sensitivity *in vivo* depending on the rate of drug clearance. Alternatively, Bcl-xL-mediated delay in G2/M arrest has no consequence on cell death, but it blocks the release of cytochrome c from the mitochondria and subsequent activation of caspases (6). The apoptosis assay clearly indicates reduced caspase activation in Bcl-xL-overexpressing cells compared to control cells (Figure 3).

In addition to its role in drug resistance, Bcl-xL has been identified recently as an enhancer of metastasis, but not primary tumor growth (18). This is consistent with our observation that cells overexpressing Bcl-xL are refractory to treatment with paclitaxel and docetaxel, but display slower growth compared to control vector cells. Overexpression of Bcl-xL correlates with increased nodal involvement and higher tumor grade in human breast cancer patients (34). In metastatic human breast tumor cell lines, overexpression of Bcl-xL can increase cell survival in the

vasculature and enhance the formation of distant metastases (18). Our results suggest that chemotherapeutic drugs may efficiently eliminate tumor cells that express a low level of Bcl-xL at primary tumor sites as well as at sites of metastasis and few tumor cells that overexpress Bcl-xL survive. Regrowth of these Bcl-xL-overexpressing tumor cells leads to recurrence of the tumor at metastatic sites and a poor prognosis. A significant observation of this present study is that inhibition of Bcl-xL expression by a specific inhibitor 2-methoxyantimycin A3 dramatically sensitized the Bcl-xL-overexpressing cells and cells with high levels of Bcl-xL to paclitaxel and docetaxel, implying that a promising strategy of Bcl-xL inhibitor or anti-sense molecule in combination with paclitaxel or docetaxel may benefit patients with chemotherapy-resistant metastatic breast cancers.

Acknowledgements

We thank M. E. Peter, V. Dixit, X. Yang, L. W. Oberley, D. Donner and K. Orth for various reagents. This work was supported in part by grants from the National Institutes of Health (CA89153) and a grant-in-aid from Aventis Pharmaceuticals to H. N. H. N. is the Marian J. Morrison Investigator in Breast Cancer Research.

References

- 1 Hudis CA: Current status and future directions in breast cancer therapy. *Clin Breast Cancer 4*: S70-75, 2003.
- 2 Garces CA and Cance WG: Neoadjuvant chemotherapy of breast cancer. *Am Surg 70*: 565-569, 2004.
- 3 Nowak A K, Wilcken NR, Stockler MR, Hamilton A and Ghersi D: Systematic review of taxane-containing *versus* non-taxane-containing regimens for adjuvant and neoadjuvant treatment of early breast cancer. *Lancet Oncol 5*: 372-380, 2004.
- 4 Yardley DA: Gemcitabine and docetaxel in metastatic and neoadjuvant treatment of breast cancer. *Semin Oncol 31*:37-44, 2004.
- 5 Moos PJ and Fitzpatrick FA: Taxane-mediated gene induction is independent of microtubule stabilization: induction of transcription regulators and enzymes that modulate inflammation and apoptosis. *Proc Natl Acad Sci USA 95*: 3896-3901, 1998.
- 6 Bhalla KN: Microtubule-targeted anticancer agents and apoptosis. *Oncogene 22*: 9075-9086, 2003.
- 7 Orr GA, Verdier-Pinard P, McDaid H and Horwitz SB: Mechanisms of Taxol resistance related to microtubules. *Oncogene 22*: 7280-7295, 2003.
- 8 Karin M and Lin A: NF-kappaB at the crossroads of life and death. *Nat Immunol 3*:221-227, 2002.
- 9 Baldwin AS: Control of oncogenesis and cancer therapy resistance by the transcription factor NF-kappaB. *J Clin Invest 107*: 241-246, 2001.
- 10 Nakshatri H, Bhat-Nakshatri P, Martin DA, Goulet RJ Jr and Sledge GW Jr: Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth. *Mol Cell Biol 17*: 3629-3639, 1997.
- 11 Ghosh S and Karin M: Missing pieces in the NF-kappaB puzzle. *Cell 109*: S81-96, 2002.

- 12 Karin M, Cao Y, Greten FR and Li ZW: NF-kappaB in cancer: from innocent bystander to major culprit. *Nature Rev Cancer* 2: 301-310, 2002.
- 13 Patel NM, Nozaki S, Shortle NH, Bhat-Nakshatri P, Newton TR, Rice S, Gelfanov V, Boswell SH, Goulet RJ Jr, Sledge GW Jr and Nakshatri H: Paclitaxel sensitivity of breast cancer cells with constitutively active NF-kappaB is enhanced by IkappaBalpha super-repressor and parthenolide. *Oncogene* 19: 4159-4169, 2000.
- 14 Wang CY, Cusack JC Jr, Liu R and Baldwin AS Jr: Control of inducible chemoresistance: enhanced anti-tumor therapy through increased apoptosis by inhibition of NF-kappaB. *Nat Med* 5: 412-417, 1999.
- 15 Zhang H, Morisaki T, Nakahara C, Matsunaga H, Sato N, Nagumo F, Tadano J and Katano M: PSK-mediated NF-kappaB inhibition augments docetaxel-induced apoptosis in human pancreatic cancer cells NOR-P1. *Oncogene* 22: 2088-2096, 2003.
- 16 Bhat-Nakshatri P, Campbell RA, Patel NM, Newton TR, King AJ, Marshall MS, Ali S and Nakshatri H: Tumor necrosis factor and PI3-kinase control oestrogen receptor alpha protein level and its transrepression function. *Br J Cancer* 90: 853-859, 2004.
- 17 Tzung SP, Kim KM, Basanez G, Giedt CD, Simon J, Zimmerberg J, Zhang KY and Hockenbery DM: Antimycin A mimics a cell-death-inducing Bcl-2 homology domain 3. *Nat Cell Biol* 3:183-191, 2001.
- 18 Martin SS, Ridgeway AG, Pinkas J, Lu Y, Reginato MJ, Koh, EY, Michelman M, Daley GO, Brugge JS and Leder PA: Cytoskeleton-based functional genetic screen identifies Bcl-xL as an enhancer of metastasis, but not primary tumor growth. *Oncogene* 23: 4641-4645, 2004.
- 19 Stegh AH, Barnhart BC, Volkland J, Algeciras-Schimmich A, Ke N, Reed JC and Peter ME: Inactivation of caspase-8 on mitochondria of Bcl-xL-expressing MCF7-Fas cells: role for the bifunctional apoptosis regulator protein. *J Biol Chem* 277: 4351-4360, 2002.
- 20 Scaffidi C, Schmitz I, Krammer PH and Peter ME: The role of c-FLIP in modulation of CD95-induced apoptosis. *J Biol Chem* 274: 1541-1548, 1999.
- 21 Pozarowski P, Halicka DH and Darzynkiewicz Z: NF-kappaB inhibitor sesquiterpene parthenolide induces concurrently atypical apoptosis and cell necrosis: difficulties in identification of dead cells in such cultures. *Cytometry* 54A: 118-124, 2003.
- 22 Nakshatri H, Rice SE and Bhat-Nakshatri P: Antitumor agent parthenolide reverses resistance of breast cancer cells to tumor necrosis factor-related apoptosis-inducing ligand through sustained activation of c-Jun N-terminal kinase. *Oncogene* 23: 7330-7344, 2004.
- 23 Grant DS, Williams TL, Zahaczewsky M and Dicker AP: Comparison of antiangiogenic activities using paclitaxel (taxol) and docetaxel (taxotere). *Int J Cancer* 104: 121-129, 2003.
- 24 Lin HL, Liu TY, Chau GY, Lui WY and Chi CW: Comparison of 2-methoxyestradiol-induced, docetaxel-induced, and paclitaxel-induced apoptosis in hepatoma cells and its correlation with reactive oxygen species. *Cancer* 89: 983-994, 2000.
- 25 Janicke RU, Ng P, Sprengart ML and Porter AG: Caspase-3 is required for alpha-fodrin cleavage but dispensable for cleavage of other death substrates in apoptosis. *J Biol Chem* 273: 15540-15545, 1998.
- 26 Debatin KM and Krammer PH: Death receptors in chemotherapy and cancer. *Oncogene* 23: 2950-2966, 2004.
- 27 Ashkenazi A and Dixit VM: Death receptors: signaling and modulation. *Science* 281: 1305-1308, 1998.
- 28 Fotin-Mleczek M, Henkler F, Hausser A, Glauner H, Samel D, Graness A, Scheurich P, Mauri D and Wajant H: Tumor necrosis factor receptor-associated factor (TRAF) 1 regulates CD40-induced TRAF2-mediated NF-kappaB activation. *J Biol Chem* 279: 677-685, 2004.
- 29 Macmillan-Crow LA and Cruthirds DL: Invited review: manganese superoxide dismutase in disease. *Free Radic Res* 34: 325-336, 2001.
- 30 Deveraux QL and Reed JC: IAP family proteins – suppressors of apoptosis. *Genes Dev* 13: 239-252, 1999.
- 31 Li JJ and Oberley LW: Overexpression of manganese-containing superoxide dismutase confers resistance to the cytotoxicity of tumor necrosis factor alpha and/or hyperthermia. *Cancer Res* 57: 1991-1998, 1997.
- 32 Shtil AA, Mandlekar S, Yu R, Walter RJ, Hagen K, Tan TH, Roninson IB and Kong AN: Differential regulation of mitogen-activated protein kinases by microtubule-binding agents in human breast cancer cells. *Oncogene* 18: 377-384, 1999.
- 33 Manna SK, Zhang HJ, Yan T, Oberley LW and Aggarwal BB: Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappaB and activated protein-1. *J Biol Chem* 273: 13245-13254, 1998.
- 34 Olopade OI, Adeyanju MO, Safa AR, Hagos F, Mick R, Thompson C B and Recant WM: Overexpression of BCL-x protein in primary breast cancer is associated with high tumor grade and nodal metastases. *Cancer J Sci Am* 3: 230-237, 1997.

Received March 30, 2005

Accepted April 12, 2005