

# Bcl2 Is the Guardian of Microtubule Integrity<sup>1</sup>

Subrata Haldar,<sup>2</sup> Aruna Basu, and Carlo M. Croce

Kimmel Cancer Center, Jefferson Medical College, Philadelphia, Pennsylvania 19107

## Abstract

We have investigated the ability of several drugs commonly used in the treatment of human cancer to induce bcl2 phosphorylation and cell death in human cell lines derived from acute leukemia, lymphoma, breast cancer, and prostate cancer. The results of this analysis indicate that drugs affecting the integrity of microtubules induce bcl2 phosphorylation, whereas anticancer drugs damaging DNA do not. Comparison of the effects of taxol and its analogue, taxotere, indicates that taxotere is capable of inducing bcl2 phosphorylation and apoptotic cell death at 100-fold lower concentrations than taxol. Induction of cancer cell death through phosphorylation of bcl2 thus provides an opportunity not only for more refined targeting of therapeutic drugs but for understanding of an important pathway leading to apoptosis.

Phosphorylation of bcl2 in drug-treated cancer cells occurs in G<sub>2</sub>-M, the phase of the cell cycle in which this class of drugs is active. No induction of bcl2 phosphorylation occurs in chronic lymphocytic leukemia cells that overexpress bcl2 but are blocked at G<sub>0</sub>-G<sub>1</sub>. Thus, prevention of polymerization or depolymerization of cellular microtubules by this class of cancer therapeutic drugs causes phosphorylation of bcl2, abrogating the normal antiapoptotic function of bcl2 and initiating the apoptotic program in the cycling cancer cells; these results are consistent with a normal physiological role of bcl2 as "guardian of microtubule integrity."

## Introduction

A variety of anticancer drugs kill cancer cells through induction of apoptosis (reviewed in Ref. 1). During the past few years, it has been possible to identify genes that are involved in the control of apoptosis in various organisms from nematodes to humans (2, 3). Studies of *Caenorhabditis elegans* have provided important insights into the pathways and genes involved in programmed cell death (4). The nematode *ced3* gene product, required along with *ced4* to complete the nematode death pathways, is homologous to mammalian cysteine proteases (ICE<sup>3</sup>; Refs. 5 and 6). Interestingly, the *BCL2* gene, isolated more than 10 years ago because of its juxtaposition to the immunoglobulin heavy chain locus in follicular lymphoma (7-9), is capable of prolonging cell survival (10) and is homologous to and complements the antiapoptotic *ced9* gene of *C. elegans* (4). Several *BCL2* mammalian homologues have been isolated that have either antiapoptotic (11) or proapoptotic functions (12, 13). Interestingly, bcl2 and related antiapoptotic proteins seem to dimerize with a proapoptotic molecule, bax, inhibiting its function (14).

Overexpression of *BCL2* has been observed, not only in follicular lymphoma where the *BCL2* gene is deregulated by chromosomal translocation (7, 8), but also in a variety of very common human

malignancies, including breast cancer, lung cancer, and hormone therapy-insensitive prostate cancer (15-18). These malignancies are responsible for most cancer deaths in the United States and Western Europe.

We have shown that phosphorylation of bcl2 at serine residues leads to loss of bcl2 antiapoptotic function (19) and that the anticancer drug taxol (1-10 μM) induces bcl2 phosphorylation, followed by apoptosis, in acute leukemia and prostate cancer cell lines (19, 20). Recent reports have confirmed these studies (21). Interestingly, phosphorylation of bcl2 results in decreased bcl2 binding to the proapoptotic bax protein (20). We have, therefore, speculated that stabilization of microtubules in cancer cells by taxol induces bcl2 phosphorylation, followed by an increase in free bax and apoptosis (20).

Because taxol inhibits microtubule depolymerization (22) in the G<sub>2</sub>-M phase of the cell cycle, our finding that it induces bcl2 phosphorylation, which is accompanied by loss of function (19, 20), suggests that microtubule damage may lead to cancer cell apoptosis through a mechanism involving bcl2 phosphorylation and that this pathway may be important in normal physiological elimination of cells with damaged mitotic apparatus.

*BCL2* codes for a protein of 239 amino acids with a hydrophobic carboxyl terminus (9). Recently, X-ray diffraction and nuclear magnetic resonance studies have indicated that the structure of the family of bcl2 proteins consists of two central, primarily hydrophobic α-helices, surrounded by amphipathic helices, and that a 60-residue loop connects the two helices (23). This loop is flexible, corresponds to a region of low sequence homology and variable size among bcl2 family members, and is not essential for antiapoptotic activity (23). Deletion of the bcl2 loop region, which includes several potential serine phosphorylation sites, has been reported to increase the antiapoptotic activity of bcl2 (24).

## Materials and Methods

**Cells and Culture Conditions.** Human lymphoid cells 697 [pre-B cells having a t(1;19) translocation] and RS11846 [pre-B cells having t(14;18) and a t(8;14) translocation] were maintained in RPMI 1640 with 10% FBS and 50 μg/ml gentamicin. Breast cancer cells MCF-7 were grown in Iscove's modified Dulbecco's media supplemented with 10% FBS (Hyclone) and 25 μg/ml gentamicin. The human hormone-independent prostate cancer cells DU.145 or PC-3 were cultured in MEM supplemented with 10% FBS and gentamicin. In the case of hormone-dependent prostate cancer cells LNCaP, RPMI 1640 was used instead of MEM. Leukemic lymphocytes from three patients with chronic lymphocytic leukemia (25) were also treated for expression of bcl2 and bcl2 phosphorylation following treatment of taxol or taxotere.

**Reagents.** Monoclonal bcl2 antibody was purchased from Genosys (Woodland, TX). PARP antibody was a kind gift of Professor Nathan Berger (Case Western Reserve University). Enhanced chemiluminescence Western blot detection reagents were obtained from Amersham Corp. (Arlington Heights, IL). Antineoplastic drugs (taxotere, vincristine, cisplatin, doxorubicin, methotrexate, 5'-fluorouracil, and etoposide) were kindly provided by Dr. Robert L. Comis (Kimmel Cancer Center, Thomas Jefferson University). Taxol was purchased from Sigma Chemical Co. Vinblastine was purchased from CBI Technology (Cambridge, MA). All reagents used were of ultrapure grade.

**Treatment of Cells with Antineoplastic Drugs.** Cells were seeded on tissue culture dishes and were treated with specified concentrations of taxotere

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<sup>2</sup> To whom requests for reprints should be addressed, at Kimmel Cancer Center, Bluemle Life Sciences Building, Rm. 1032, 233 South 10th Street, Philadelphia, PA 19107-5799.

<sup>3</sup> The abbreviations used are: ICE, interleukin-1 converting enzyme; bcl2, B-cell leukemia/lymphoma-2; FBS, fetal bovine serum; PARP, poly(ADP-ribose) polymerase; CLL, chronic lymphocytic leukemia.

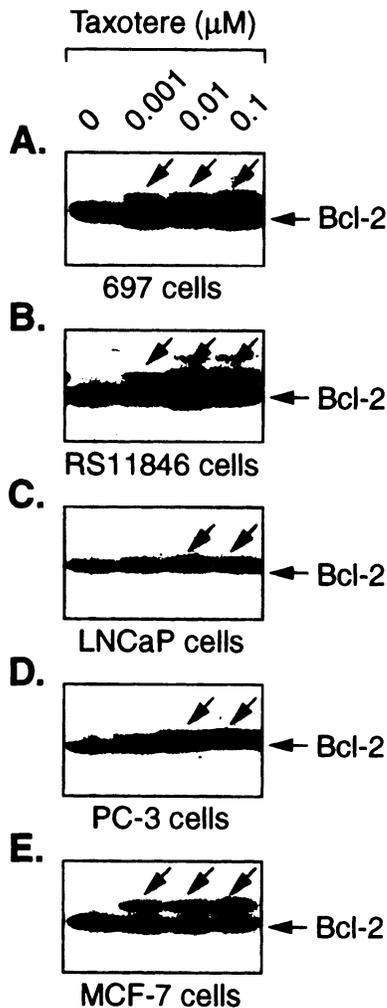


Fig. 1. Taxotere induces phosphorylation of bcl2 protein. A, 697 cells derived from a human pre-B cell leukemia with a t(1;19) chromosomal translocation; B, RS11846 cells derived from a human B-cell lymphoma carrying a t(14;18) and a t(8;22) chromosomal translocation; C, LNCaP cells derived from a human metastatic prostate adenocarcinoma; D, PC-3 cells, derived from a human grade IV prostate adenocarcinoma; E, MCF-7 cells derived from an estrogen receptor-positive human breast adenocarcinoma. The cell lines were treated with 1–100 nM taxotere (docetaxel) for 24 h in their respective culture media. Following treatment, total cellular proteins were isolated as described previously (19, 20). Equal amounts of protein were fractionated by SDS-PAGE followed by immunoblotting using a monoclonal antibody against bcl2. Arrows, modified (phosphorylated) forms of bcl2 protein. Note that even 1 nM taxotere can trigger a significant degree of bcl2 phosphorylation in MCF-7 breast cancer cells, and more than 75% of the bcl2 protein is in its phosphorylated form in PC-3 or LNCaP cells at concentrations of 100 nM taxotere. Control experiments were performed by treating the same cancer cell lines with 1–10  $\mu$ M taxol. The control results were identical to those reported previously (Ref. 20; data not shown).

(1–100 nM), taxol (1–5  $\mu$ M), vincristine (0.1  $\mu$ M), vinblastine (0.1  $\mu$ M), 5'-fluorouracil (250  $\mu$ M), methotrexate (250  $\mu$ M), cisplatin (50  $\mu$ M), doxorubicin (0.34  $\mu$ M), and the etoposide VP16 (1–5  $\mu$ M) for 24 h in a 5% CO<sub>2</sub> incubator with humidified conditions at 37°C. Following treatment, total proteins were extracted as described previously (19, 20).

**Assessment of bcl2 Phosphorylation.** Equal amount of proteins extracted from control or drug-treated cells were subjected to 12% SDS-PAGE followed by transfer on nitrocellulose sheets. Transferred proteins were further subjected to immunoblotting using anti-bcl2 monoclonal antibody. The phosphorylated form of bcl2 protein was detected as a slower mobility form as described previously (19–21).

**Assessment of Apoptosis.** The assessment of apoptosis was carried out by DNA fragmentation analysis and by determining PARP degradation. For PARP degradation, the cells were harvested following the scheduled exposure to the designated concentration of the drugs. The protein content by the total cellular extract was determined by bicinchonic acid protein assay reagent

(Pierce). Equivalent amounts of protein from each sample were electrophoresed on 5–15% gradient SDS-PAGE, followed by immunoblotting with PARP monoclonal antibody. DNA fragmentation analyses were carried out by extracting total DNA from  $\sim 10^8$  cells. Following treatment with drugs,  $10^8$  cells were suspended in 1 ml of digestion buffer containing 100 mM NaCl, 10 mM Tris (pH 8.0), 25 mM EDTA, 0.5% SDS, and 0.2  $\mu$ g/ml proteinase K and incubated with shaking in a 50°C waterbath for 12–18 h. DNA extraction was carried out with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) and precipitated with ammonium acetate and ethanol. Approximately 20  $\mu$ g of DNA were loaded on each lane of a 2% agarose gel containing 1  $\mu$ g/ml ethidium bromide.

**Cell Sorting.** Control or drug-treated human pre-B leukemic cells 697 (unfixed) were stained with Hoechst 33342 (Sigma) at a concentration of 15  $\mu$ g/ml for 1 h at 37°C. 3,3'-Dipentylloxycarbocyanine iodide (Molecular Probes) was added at a concentration of 0.2  $\mu$ g/ml at the same time with Hoechst stain to increase resolution of DNA distribution. Cells were sorted by a fluorescence activated cell sorter (EPIC) at the Institute's Core Facility.

## Results

### Taxotere Is More Active Than Taxol in the Induction of bcl2 Phosphorylation and Apoptosis of Cancer Cells.

We found previously that 1–10  $\mu$ M taxol induces bcl2 phosphorylation and apoptosis of 697 human acute leukemic pre-B cells (19) and prostate carcinoma cells, LNCaP and PC-3, whereas bcl2-negative DU145 prostate carcinoma cells fail to respond to 1–10  $\mu$ M taxol (20). We have now tested the taxol analogue, taxotere, for its ability to induce bcl2 phosphorylation in human tumor cell lines derived from acute leukemia (697), B-cell lymphoma (RS 11846), prostate carcinoma (LNCaP and PC-3), and breast carcinoma (MCF7). As shown in Fig. 1, taxotere induced bcl2 phosphorylation in cancer cells at concentrations of 0.001 and 0.01  $\mu$ M, indicating that it is much more active than taxol in inducing bcl2 phosphorylation and cell death. Previous experiments with taxol indicated that only 30–50% of bcl2 molecules were phosphorylated in taxol-treated prostate cancer cells, whereas more than 75% of bcl2 protein is phosphorylated in PC-3 prostate carcinoma cells treated with 0.1  $\mu$ M taxotere. In previous experiments, 10  $\mu$ M taxol induced bcl2 phosphorylation after 4 h of treatment of PC-3 cells with the peak at 18–24 h. As shown in Fig. 2, phosphorylation of bcl2 occurred at 2 h after the addition of taxotere, and at

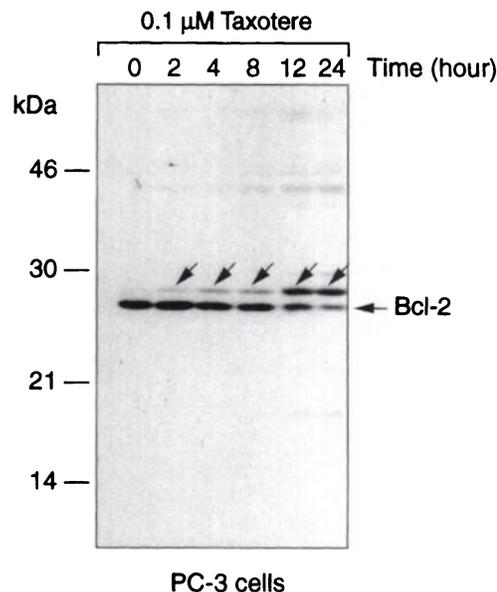


Fig. 2. Kinetics of taxotere induced bcl2 phosphorylation. PC-3 cells were exposed to taxotere for several time periods between 0 and 24 h in a humidified 5% CO<sub>2</sub> incubator as described previously (19–20). Immunoblotting using monoclonal antibody against bcl2 was performed as described in the legend to Fig. 1. Arrows, phosphorylated bcl2 protein.

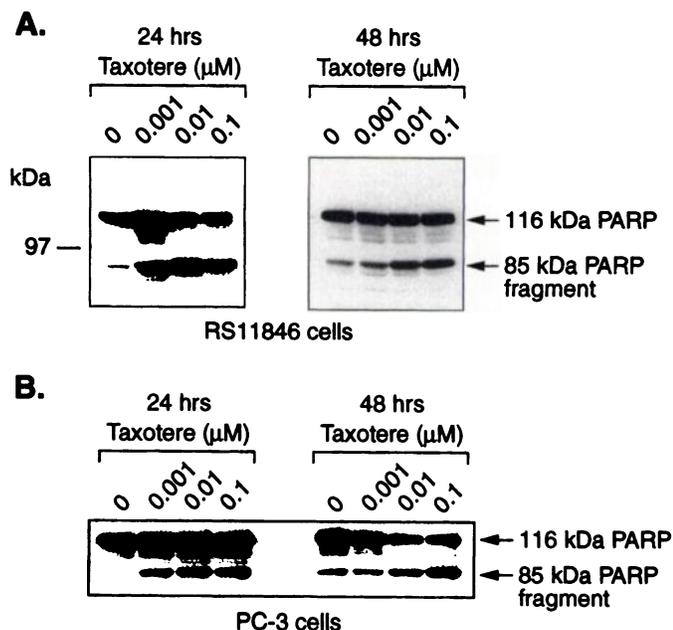


Fig. 3. Taxotere-induced PARP degradation; Western blots of taxotere-treated cell lysates using monoclonal antibody against PARP. RS11846 and PC-3 cells were treated with taxotere for 24–48 h. Note that the intensity of the  $M_r$  85,000 signature PARP degradation fragment increased with the concentration of taxotere. The presence of a low amount of the  $M_r$  85,000 fragment in control cells indicates the occurrence of normal apoptosis in these cells.

24 h, more than 75% of the bcl2 protein was in the phosphorylated form. As shown in Fig. 3, treatment of PC-3 prostate carcinoma cells and RS11846 B-cell lymphoma cells with taxotere resulted in enhancement of cleavage of the  $M_r$  116,000 PARP, the substrate of several ICE-like proteases. The members of the ICE family of proapoptotic proteins could perhaps be activated as a consequence of bcl2 phosphorylation.

At present, taxol is the drug of choice in the treatment of breast cancer (22). As shown in Fig. 1, treatment of MCF-7 breast carcinoma cells results in robust expression of phosphorylated bcl2 at taxotere concentrations as low as 0.001  $\mu\text{M}$  to 0.1  $\mu\text{M}$ . Similar treatment of five additional breast carcinoma cell lines resulted in bcl2 phosphorylation and cell death (data not shown).

**Anticancer Drugs Damaging Microtubules Induce bcl2 Phosphorylation, Whereas Anticancer Drugs Damaging DNA Do Not.** Because taxotere and taxol damage microtubules by inhibiting microtubule depolymerization, it was important to determine if drugs affecting microtubule polymerization also induce bcl2 phosphorylation. As shown in Fig. 4, vincristine and vinblastine, two vinca alkaloids that bind monomeric tubulin, preventing microtubule assembly (22, 26), also induced bcl2 phosphorylation in 697 leukemia cells and PC-3 prostate carcinoma cells, whereas the DNA-damaging agents 5'-fluorouracil, methotrexate, cisplatin, and doxorubicin did not induce bcl2 phosphorylation in the same cell lines. Treatment of pros-

tate cancer cells with inhibitors of topoisomerase II (etoposides) also failed to induce bcl2 phosphorylation (data not shown). We concluded that drugs that affect microtubule integrity by inhibiting polymerization or depolymerization induce loss of bcl2 antiapoptotic function through phosphorylation, whereas anticancer drugs that damage DNA do not.

**Induction of bcl2 Phosphorylation Occurs at  $G_2$ -M.** To determine when in the cell cycle bcl2 phosphorylation occurs, vincristine (0.1  $\mu\text{M}$ )-treated 697 leukemia cells were sorted after 24 h into pools of cells in  $G_0$ -S/ $G_1$ -S or  $G_2$ -M with the aid of a fluorescence-activated cell sorter. The sorted cells were then tested for the presence of phosphorylated bcl2. As shown in Fig. 5, phosphorylated bcl2 was not detected in the vincristine-treated cells at  $G_0$ -S/ $G_1$ -S, whereas abundant phosphorylated bcl2 was detected in the  $G_2$ -M fraction of treated 697 cells. These results indicated that anticancer drugs that cause  $G_2$ -M arrest by affecting microtubule integrity induce bcl2 phosphorylation in the  $G_2$ -M phase of the cell cycle.

Human chronic leukemia cells overexpress bcl2, show increased survival, and are blocked at  $G_0$ - $G_1$  (25). Treatments of three human chronic lymphocytic leukemias with either taxol or taxotere did not result in phosphorylation of bcl2 (Fig. 6). These results are consistent with the findings that phosphorylation of bcl2 in response to taxol or taxotere occurs at  $G_2$ -M and cannot be introduced in nondividing cells.

## Discussion

Studies using inhibitors of phosphatases, such as okadaic acid, have shown that bcl2 can be phosphorylated at serine residues and that bcl2 phosphorylation is associated with loss of function (19). We have also shown that taxol, a drug that affects microtubule depolymerization, induces bcl2 phosphorylation in leukemia and prostate carcinoma cell lines (19, 20).

Because taxol is the drug of choice in the treatment of some of the most common human malignancies, such as breast cancer, and very little is known of the mechanisms of action of taxol, we have further investigated the effect of taxol and its analogue, taxotere, on bcl2 in a variety of types of human cancer-derived cell lines. It is clear that taxotere is a more active inducer of bcl2 phosphorylation and apoptotic death in all cell lines tested. Whereas taxol induces bcl2 phosphorylation and cell death at concentrations of 1–10  $\mu\text{M}$ , taxotere induces bcl2 phosphorylation and cell death at concentrations of 1–100 nM.

These findings are important for cancer treatment because they suggest that novel drugs could be tested for their ability to induce bcl2 phosphorylation. Thus, bcl2 could be a very useful target for drug development.

We have also used other anticancer drugs that damage either microtubules or DNA to determine whether bcl2 phosphorylation is a general phenomenon following microtubule damage. The results indicate that not only taxol and taxotere, but also vinblastine and vincristine, drugs that affect microtubule polymerization, induce bcl2

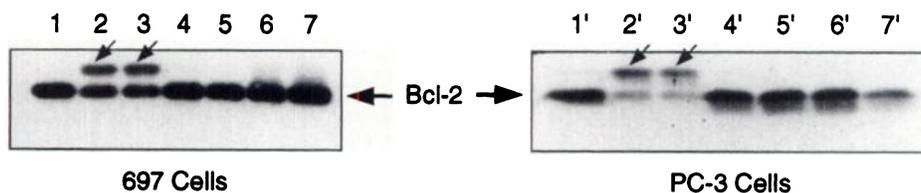


Fig. 4. Comparison of microtubule and DNA-damaging anticancer drugs. The drugs vincristine and vinblastine, which affect microtubule polymerization, induce bcl2 phosphorylation. Lanes 1 and 1', control (no treatment); Lanes 2 and 2', vincristine (0.1  $\mu\text{M}$ ); Lanes 3 and 3', vinblastine (0.1  $\mu\text{M}$ ); Lanes 4 and 4', 5'-fluorouracil (250  $\mu\text{M}$ ); Lanes 5 and 5', methotrexate (250  $\mu\text{M}$ ); Lanes 6 and 6', cisplatin (50  $\mu\text{M}$ ); Lanes 7 and 7', doxorubicin (0.34  $\mu\text{M}$ ). The cells were treated with the respective drugs for 24 h. Arrows, modified (phosphorylated) form of bcl2 protein.

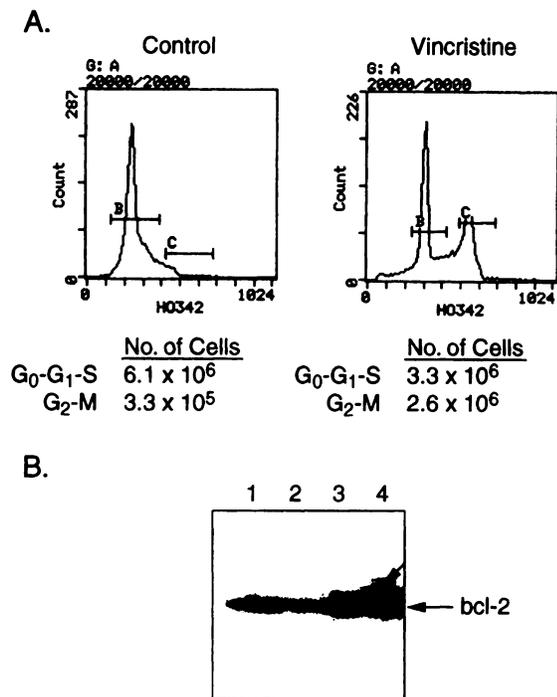


Fig. 5.  $G_2$ -M arrest and bcl2 phosphorylation occur simultaneously in 697 pre-B leukemia cells following vincristine treatment. A, separation of 697 pre-B cells by cell sorting. Fraction B (cells in  $G_0$ -S/ $G_1$ -S) and fraction C ( $G_2$ -M) were separated and analyzed for bcl2 phosphorylation; B: Lane 1, control cells at  $G_0$ -S/ $G_1$ -S stage; Lane 2, control cells at  $G_2$ -M stage; Lane 3, cells at  $G_0$ -S/ $G_1$ -S stage following 0.1  $\mu$ M vincristine treatment; Lane 4, cells at  $G_2$ -M stage after 0.1  $\mu$ M vincristine treatment. Arrow, modified (phosphorylated) form of bcl2 protein.

phosphorylation and cell death. On the contrary, drugs that damage DNA do not induce bcl2 phosphorylation but induce apoptosis through a different, p53-dependent mechanism (21). These results suggest that bcl2, through its phosphorylation, is the guardian of microtubule integrity. Because microtubules play an important role in chromosome segregation, alterations in microtubules could cause genomic instability. Thus, cells with altered microtubules should be eliminated through the induction of bcl2 phosphorylation, loss of bcl2 function, and apoptotic death. Recently, it has been shown that cells overexpressing the apoptosis-inhibitory protein Bcl-x<sub>l</sub> have an increased rate of spontaneous tetraploidization, suggesting that apoptosis may be important in eliminating cells failing to complete mitosis (27).

Taxol, taxotere, and other agents affecting microtubule integrity induce phosphorylation of bcl2 at  $G_2$ -M. Thus, these drugs are unable to kill resting tumor cells, even if they express high levels of bcl2. For example, we did not observe phosphorylation of bcl2 in taxol- or

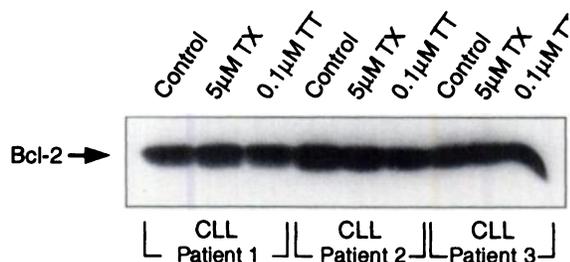


Fig. 6. Taxol or taxotere cannot induce bcl2 phosphorylation in nondividing lymphocytes isolated from CLL patients. Lymphocytes were isolated from three individual CLL patients by the method described earlier (25). Isolated lymphocytes were heated with 0.1  $\mu$ M taxotere (TT) or 5  $\mu$ M taxol (TX) for a period of 24 h. Western blot of total extracted proteins was carried out as described previously (19, 20).

taxotere-treated CLL cells that are blocked in  $G_0$ - $G_1$  (data not shown). A corollary of these findings is that taxol and taxotere are effective in killing bcl2-expressing tumors with high mitotic index. It should be possible, however, to develop methods to induce bcl2 phosphorylation at other stages of the cell cycle. Induction of bcl2 phosphorylation in resting cancer cells could lead to significant progress in cancer treatment.

In summary, our results describe a novel role for bcl2, that of guardian of microtubule integrity. Through its phosphorylation and functional inactivation, bcl2 responds to damage in microtubules by abrogation of its normal antiapoptotic function, leading to induction of apoptotic death of cells exhibiting such alterations.

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