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13. ABSTRACT (Maximum 200 Words) Previously we showed that MAP4, MRP and stathmin are regulated by the tumor suppressor protein, p53. These proteins play roles in microtubule dynamics as well as transport of chemotherapeutic drugs. In the past year we focused on stathmin's role in cell cycle control and drug sensitivity. Stathmin destabilizes microtubule polymers through interactions with tubulin heterodimers. Since several chemotherapeutic agents used in the treatment of breast cancer target microtubule components, stathmin may play a role in determining sensitivity to these drugs. This is particularly important since stathmin is often upregulated in breast cancer. Our current studies show that overexpression of stathmin leads to decreased microtubule polymerization and decreased sensitivity to both vinblastine and paclitaxel. This was intriguing given the concurrent increased cellular association of vinblastine. Cell lines overexpressing stathmin were more likely to enter G2 but less likely to enter mitosis as measured by mitotic figure counts. These data suggest that stathmin-mediated arrest may block chemotherapeutic drugs that are active in mitotic cells. These studies also suggest that mutant p53 breast cancer exhibiting high levels of stathmin may not be fully responsive to antimicrotubule agents in the face of an intact G2/M check point.				
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INTRODUCTION

Taxanes and *vinca* alkaloids are antimicrotubule drugs that are among the most commonly used chemotherapeutic drugs (1). Taxanes, such as paclitaxel, belong to the class of antimicrotubule drugs that stabilizes microtubules, whereas *vinca* alkaloids, such as vinblastine, belong to the class that destabilizes them (2, 3). These drugs promote cell death by affecting microtubule dynamics in a way that interferes with the function of the mitotic spindle, which appears to be crucial for cell survival (1, 4).

Antimicrotubule drugs are commonly used to treat breast cancer, but are still less than 50% successful even in previously untreated patients (5). Therefore, many patients receive these cytotoxic drugs without receiving the anticancer benefits. The choice of most chemotherapeutic regimens is based on tumor origin alone without considering genetic differences amongst tumors. Therefore, by considering distinct tumor characteristics it may be possible to more accurately predict the most effective drug for an individual patient.

We and others demonstrated that the functional status of p53 can regulate the sensitivity to chemotherapeutic drugs (6, 7). p53 alters the sensitivity to antimicrotubule drugs by controlling proteins that affect the dynamic equilibrium of microtubule assembly. For example, Murphy et al., found that p53 transcriptionally repressed expression of MAP4 (6) and stathmin (8, 9), two genes that can affect the polymerization state of microtubules (10, 11, 12, 13, 14). We recently found that the regulation of MAP4 by p53 markedly affected the sensitivity to taxanes and *vinca* alkaloids (15, 16).

Stathmin is an 18 kDa cytosolic phosphoprotein known to function in microtubule dynamics by stabilizing tubulin heterodimers and promoting microtubule depolymerization (13, 14). The activity of stathmin is regulated during the cell cycle through changes in stathmin protein content and phosphorylation. During interphase, decreased stathmin levels reduce microtubule depolymerization (17) whereas overexpression of stathmin reduces microtubule polymer mass (18, 19). Prior to metaphase, phosphorylation of stathmin inactivates the protein (18, 19, 20, 21, 22). In fact, stathmin is implicated in regulating the assembly of the mitotic spindle (20, 21). Finally, it has recently been shown that stathmin is often upregulated in breast cancer (23). Therefore, we evaluated the effect of stathmin expression on the sensitivity of breast cancer cell lines to antimicrotubule drugs as a potential determinant of chemotherapeutic effectiveness.

BODY:

Task 1. To determine whether loss of p53 function leads to increased expression of proteins that regulate microtubule dynamics in human breast cancer.

Task 2. To determine whether overexpression of proteins that regulate microtubule dynamics is predictive of response to tubulin targeting drugs.

In previous reports we demonstrated that breast cancer cell lines with mutant p53 more reliably demonstrated increased expression of stathmin in contrast to MAP4 (see Figure 2, 2000 report). This suggests that the control of MAP4 may involve other molecules besides p53. However, we have found that DNA-damage induced p53 was capable of leading to repression of MAP4 (see Figure 2, 1999 report and (24) provided in appendix), decreased microtubule polymerization and increased sensitivity to *vinca* alkaloids in a breast cancer cell line. These studies led to a phase I protocol in breast cancer patients treated with sequential doxorubicin/vinorelbine with p53 and MAP4 levels examined in blood and tumor samples (submitted paper is appended). In the past year we have also extended our studies on the microtubule dynamics and drug sensitivity of cells with endogenous mutant p53 and high levels of stathmin. Additionally, to more directly gain an understanding of the role that stathmin plays in drug sensitivity, we studied the effect of overexpression of stathmin in breast cell lines. The details of these experiments are described below.

A panel of mutant p53- containing human breast cancer cell lines (BT20, BT474, BT549, MDA MB231, MDA MB468, and T47D) was examined by Western blot for stathmin content. The non-transformed breast cell line, MCF10a, harboring wild-type p53 was used as a control. All mutant p53 cell lines overexpressed stathmin when compared to MCF10a cells (Fig.1). The increase in stathmin expression ranged from ~10- fold in BT20 cells to ~50- fold in BT549 cells. To investigate the functional significance of stathmin expression in these breast cancer cell lines, we studied the effect of stathmin on microtubule depolymerization. BT20 and BT549 were compared by double immunofluorescence for stathmin content and microtubule polymerization. BT20 cells exhibited relatively low amounts of staining for stathmin and a higher degree of microtubule polymerization (Fig 2A, top panel). In contrast, BT549 cells exhibited relatively more staining for stathmin and a lesser degree of microtubule polymerization (Fig. 2A, bottom panel). We next produced transient stathmin transfectants to analyze the acute effects of forced stathmin expression on the same parameters. Figure 2b demonstrates that BT20 transfectants had greater stathmin and less microtubule polymerization as compared to the vector transfected control.

To evaluate the effect of stathmin expression on the interaction between paclitaxel and vinblastine with microtubules, we used fluorescein- conjugated drugs to visualize the drug-target interactions in isogenic cell lines overexpressing stathmin. BT20 cells were transfected with a stathmin expression vector; two clones transfected with empty vector (BT20V1 and BT20V3) and two clones transfected with vector containing stathmin (BT20ST1 and BT20ST3) were isolated. Western blot analysis demonstrated that the stathmin transfectants showed a five to 10- fold increase in expression of stathmin as compared to the parental line and empty vector- transfected controls (Fig 3). Live BT20V1, BT20V3, BT20ST1, and BT20ST3 cells were stained with fluorescein- labeled paclitaxel or vinblastine for one hour and assayed for fluorescent intensity. Figure 4 demonstrates that overexpression of stathmin had marked effects on paclitaxel binding. BT20V1 and BT20V3 cells exhibited binding of fluorescent paclitaxel to the microtubule network (Fig. 4A,B), whereas paclitaxel binding to microtubules was markedly decreased in cells overexpressing stathmin (Fig. 4C,D). BT20ST1 and BT20ST3 cells were

examined under visible light to confirm the presence of intact cells (data not shown) in the same image fields as those represented in Figure 4. Figure 5 demonstrates the effects of stathmin expression on the binding of vinblastine. Both vector controls and stathmin overexpressing cell lines exhibited binding of fluorescent vinblastine to tubulin. However, BT20V1 and BT20V3 cells showed less binding of vinblastine (Fig. 5A and B) compared to that of BT20ST1 and BT20ST3 cells (Fig. 5C and D).

We next determined the effect of stathmin expression on drug sensitivity. BT20 and BT549 cells were treated with increasing concentrations of paclitaxel and vinblastine. Cell viability was measured by an MTT assay. BT549 cells having higher levels of stathmin with respect to BT20 cells were less sensitive to both paclitaxel and vinblastine. BT549 cells ($IC_{50} = \sim 2nM$) were 1000- fold less sensitive to paclitaxel than BT20 cells ($IC_{50} = \sim 2pM$) (Fig. 6A). BT549 cells ($IC_{50} = \sim 10pM$) were 5- fold less sensitive to vinblastine than BT20 cells ($IC_{50} = \sim 2pM$) (Fig. 6B).

We next determined the effect of stathmin expression on drug sensitivity using the isogenic cell lines BT20V1, BT20V3, BT20ST1, and BT20ST3. BT20 transfectants overexpressing stathmin were less sensitive to both classes of antimicrotubule drugs. BT20ST1 and BT20ST3 were significantly less sensitive to paclitaxel at concentrations ranging from 0.01 to 1nM (Fig. 7A) when compared to BT20V1 and BT20V3 cells, and significantly less sensitive to vinblastine from 0.0001 to 10nM (Fig. 7B).

Next we examined whether or not the changes in drug sensitivity as a consequence of stathmin overexpression was specific for antimicrotubule drugs. We treated BT20V1, BT20V3, BT20ST1, and BT20ST3 cells with increasing concentrations of doxorubicin or camptothecin and determined cell viability by an MTT assay. Figure 8 demonstrates that stathmin expression had no significant effect on the sensitivity to non-antimicrotubule drugs; these data are summarized in Table 1.

Stathmin affects the dynamic equilibrium of microtubule polymerization and its degree of expression could have a profound effect on the cell cycle. Therefore, BT20V1, BT20V3, BT20ST1, and BT20ST3 were analyzed by flow cytometry for cell cycle distribution (Table 2). BT20ST1 ($9.0\% \pm 0.7$) and BT20ST3 ($9.2\% \pm 0.3$) had significantly more cells in G2/M ($p = 0.003$ and $p = 0.00002$, respectively) than BT20V1 ($6.5\% \pm 0.2$) and BT20V3 ($5.7\% \pm 0.8$) cells). To determine the progression of cells through G2 into mitosis, we analyzed the mitotic index in these cell lines. Figure 9 demonstrates that BT20V1 and BT20V3 had 2 to 3- fold more mitotic cells (13% and 12%, respectively) than BT20ST1 and BT20ST3 (5% and 6%, respectively).

KEY RESEARCH ACCOMPLISHMENTS:

- Demonstrated that stathmin levels in either mutant p53 containing breast cell lines or in transient transfected cell lines affects levels of microtubule polymerization.
- Stable overexpression of stathmin led to increased binding of vinblastine and decreased binding of paclitaxel with a decreased sensitivity to both drugs measured by an MTT assay.

- Different stathmin levels do not affect the sensitivity to non-antimicrotubule drugs.
- Although cells overexpressing stathmin show larger numbers of cells in G2, the cells appear to be blocked from entering M phase where antimicrotubule drugs act.

REPORTABLE OUTCOMES:

Manuscripts

Zhang CC, Yang YM, White E, Murphy M, Levine A, Hait WN: The role of MAP4 expression in the sensitivity to paclitaxel and resistance to *vinca* alkaloids in p53 mutant cells. *Oncogene* 16: 1617-1624. 1998.

Zhang CC, Yang JM, Bash-Babula J, White E, Murphy M, Levine A and Hait WN: DNA damage increases sensitivity to taxanes through p53-dependent repression of microtubule-associated protein-4. *Cancer Res* 59: 3663-3670. 1999.

Bash-Babula J, Toppmeyer D, Labassi M, Reidy J, Orlick M, Senzon R, Alli E, Kearney T, Hait WN: Induction of p53 and repression of microtubule-associated protein 4 by DNA damage in breast cancer patients: a pilot clinical trial of sequential doxorubicin and vinorelbine. Submitted.

Alli E, Yang JM, Bash-Babula J, Hait WN: Effect of stathmin, a p53-regulated gene-product, on the sensitivity to antimicrotubule chemotherapeutic drugs in human breast cancer. Submitted.

Abstracts

Zhang C, Bash JE, Hait WN: DNA damaging agents increase wild type p53, suppress microtubule associated protein 4 (MAP4), sensitize cells to *vinca* alkaloids and render cells resistant to taxanes. *Proc Amer Assoc Cancer Res* 40: 633, 1999.

Bash-Babula JE, Alli EL, Toppmeyer DL, Hait WN: Effect of Doxorubicin treatment on p53 and microtubule-associated protein 4 (MAP4) expression in patients with breast cancer. *Proc Amer Assoc Cancer Res* 41, 2124, 2000.

Alli E, Bash-Babula J, Hait WN: Examination of p53-repressed genes in human breast cell lines. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, 62, 2000.

Bash-Babula JE, Alli E, Hait WN, Toppmeyer D: A phase I/II clinical trial of doxorubicin and vinorelbine: effects on p53 and microtubule-associated protein 4 (MAP4) expression in patients with advanced breast cancer. *Proc Amer Assoc Cancer Res* 644, 2001.

Alli E, Bash-Babula J, Yang JM, Ghanim A, Hait WN: Potential role for stathmin in modulation of chemosensitivity. Proc Amer Assoc Cancer Res 1948, 2001.

Degrees obtained from this work:

- Elizabeth Alli has been supported by this grant and will receive an M.S. degree from this work.

Development of cell lines:

- Created two stathmin transfectants in BT20 cells (BT20ST1 and BT20ST3).

CONCLUSIONS:

In conclusion, we found that overexpression of stathmin decreases the sensitivity of breast cancer cells to antimicrotubule drugs. This effect was seen in both mutant p53 human breast cancer cell lines containing different levels of endogenous stathmin and in isogenic cell lines transfected with stathmin. This was anticipated for taxanes based on the alteration of tubulin dynamics and the markedly decreased binding of paclitaxel, but was completely unexpected for vinblastine since stathmin overexpression increased the binding of this *vinca* alkaloid (fig 5). To understand the mechanism by which cells could remain resistant to *vinca* alkaloids despite an increased drug-target interaction, we focused on the effects of stathmin expression on the distribution of cells throughout the cell cycle and found that stathmin increased the number of cells in G2/M (Table 2). However, enumeration of cells in mitosis by direct counting of mitotic figures revealed a decrease in the number of cells in mitosis in stathmin transfectants. Therefore, it appears that despite the increased interaction of vinblastine with microtubules in stathmin overexpressing breast cancer cells, an intact G2/M check point can block the cytotoxic effects by preventing cells from progressing from G2 into mitosis. Stathmin presumably prevents entry into M phase by inhibiting the polymerization and formation of the mitotic spindle resulting in decreased sensitivity to antimicrotubule agents. In summary, we identified stathmin-mediated G2/M arrest and its probable involvement in altering chemosensitivity to antimicrotubule agents. Given that stathmin is overexpressed in mutant p53-containing tumors, this data suggests that stathmin may be an important determinant of response.

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	<u>BT20V1</u>	<u>BT20V3</u>	<u>BT20ST1</u>	<u>BT20ST3</u>	<u>p-value</u>
DOX	180 nM	280 nM	270 nM	325 nM	0.358
CPT	7 nM	9 nM	10 nM	4 nM	0.805

Table 1. IC₅₀ values for non-antimicrotubule drugs. IC₅₀ values of BT20 transfectants were determined from Fig. 8. p-value is determined by comparing BT20V1 and BT20V3 values with BT20ST1 and BT20ST3 values using t-test statistical analysis. DOX, doxorubicin; CPT, camptothecin.

Cell Line	Cells in G2/M	p-value [†]
BT20V1	6.47% ± 0.23	n/a
BT20V3	5.70% ± 0.77	n/a
BT20ST1	9.03% ± 0.65	0.003**
BT20ST3	9.21% ± 0.27	0.00002**

Table 2. Quantitation of cells in G2/M determined by flow cytometry. BT20V1, BT20V3, BT20ST1, and BT20ST3 cells were analyzed for G2/M arrest by dual parameter flow cytometry as described in Materials and Methods. Vector control values were combined for comparison with each individual over-expressing clone to determine p-value. The table is representative of three separate experiments performed in triplicate. †p-value was determined using t-test statistics. **p<0.01; highly significant.

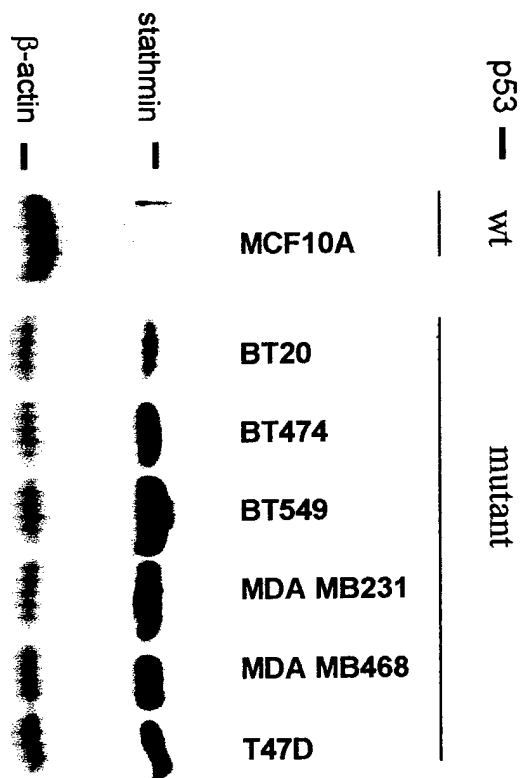


Fig. 1. Stathmin levels in human breast cancer cell lines. Whole cell extracts of mutant p53-containing cell lines were compared to that of wild-type p53-containing MCF10a cells by western blot analysis. 75µg of protein was loaded to determine endogenous stathmin levels. β-actin is used as a loading control.

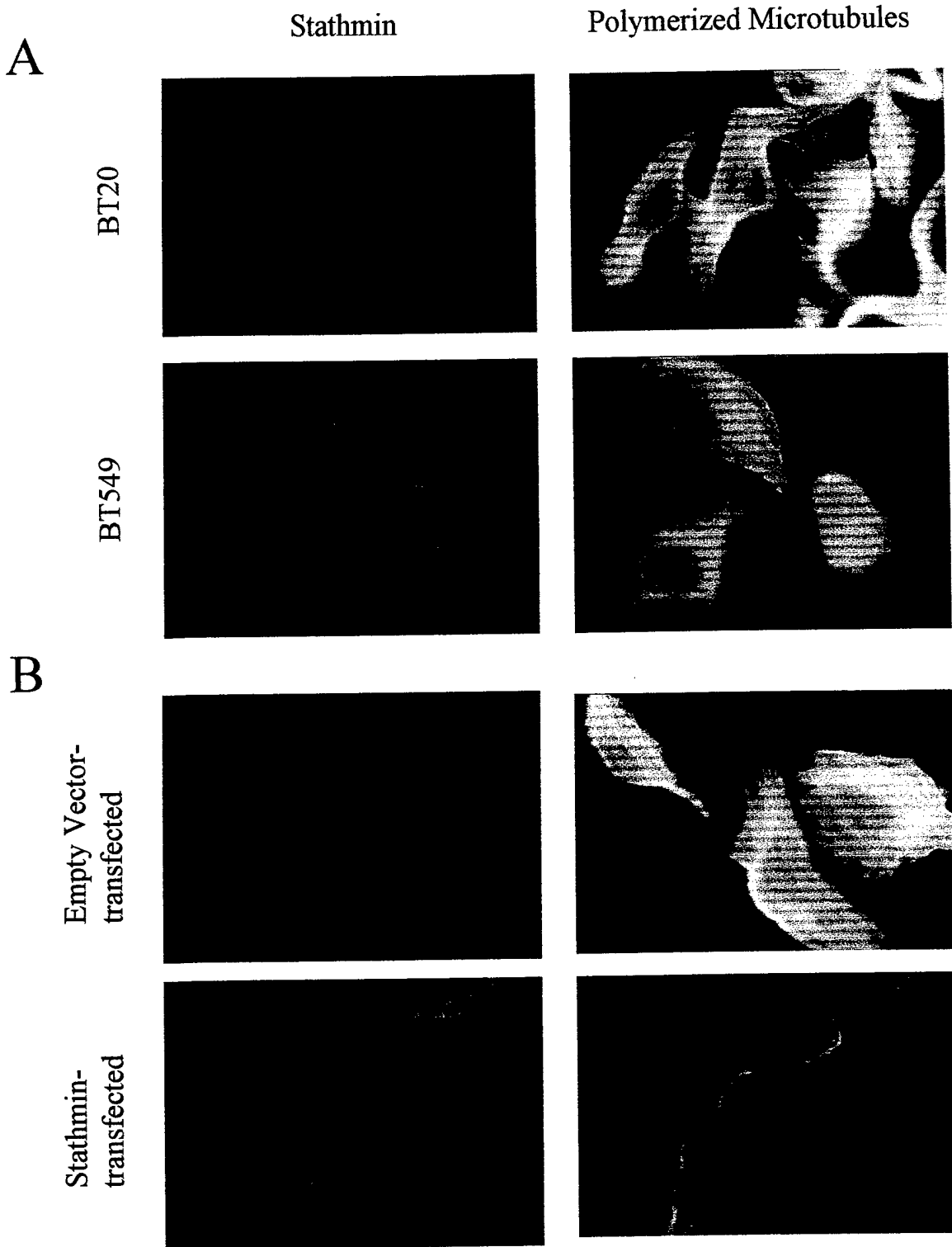


Fig. 2. Stathmin expression correlates with microtubule depolymerization. BT20 (A, top panel), BT549 (A, bottom panel), empty vector-transfected BT20 (B, top panel) and stathmin-transfected BT20 (B, bottom panel) cells were permeabilized and fixed with methanol, double stained for stathmin and α -tubulin using CY3- and FITC- conjugated secondary antibodies respectively, and visualized with a fluorescent microscope under 100x oil immersion objective. The images are representative of two separate experiments.

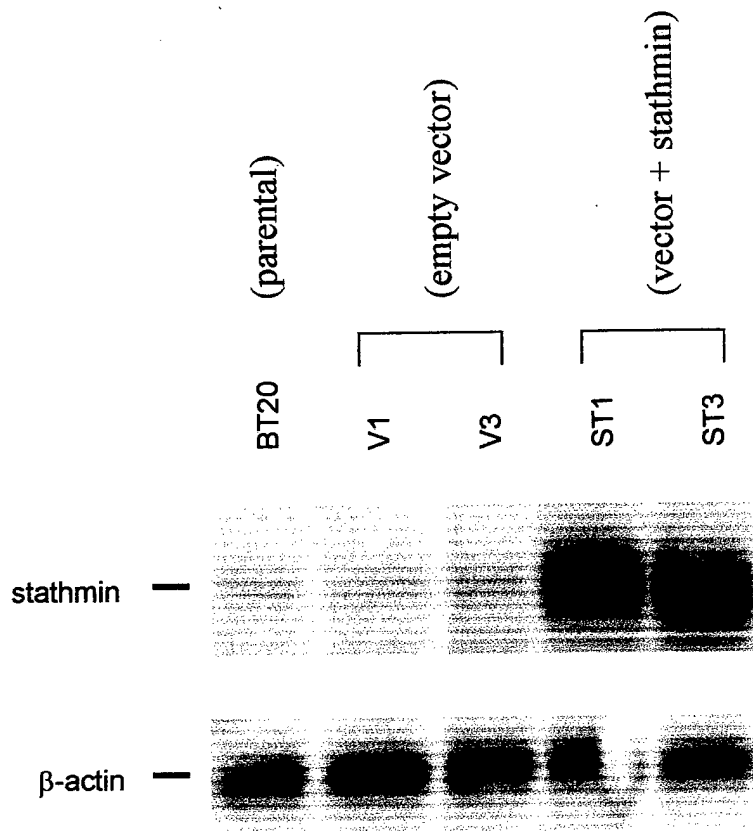


Fig. 3. Stathmin expression in BT20 stable transfectants. Western blot of stathmin expression in BT20 parental cells, two clones transfected with vector alone (BT20V1 and BT20V3) and two clones transfected with vector containing stathmin (BT20ST1 and BT20ST3). 50 μ g of protein was loaded. β -actin is used as a loading control. The figure is representative of three separate experiments.

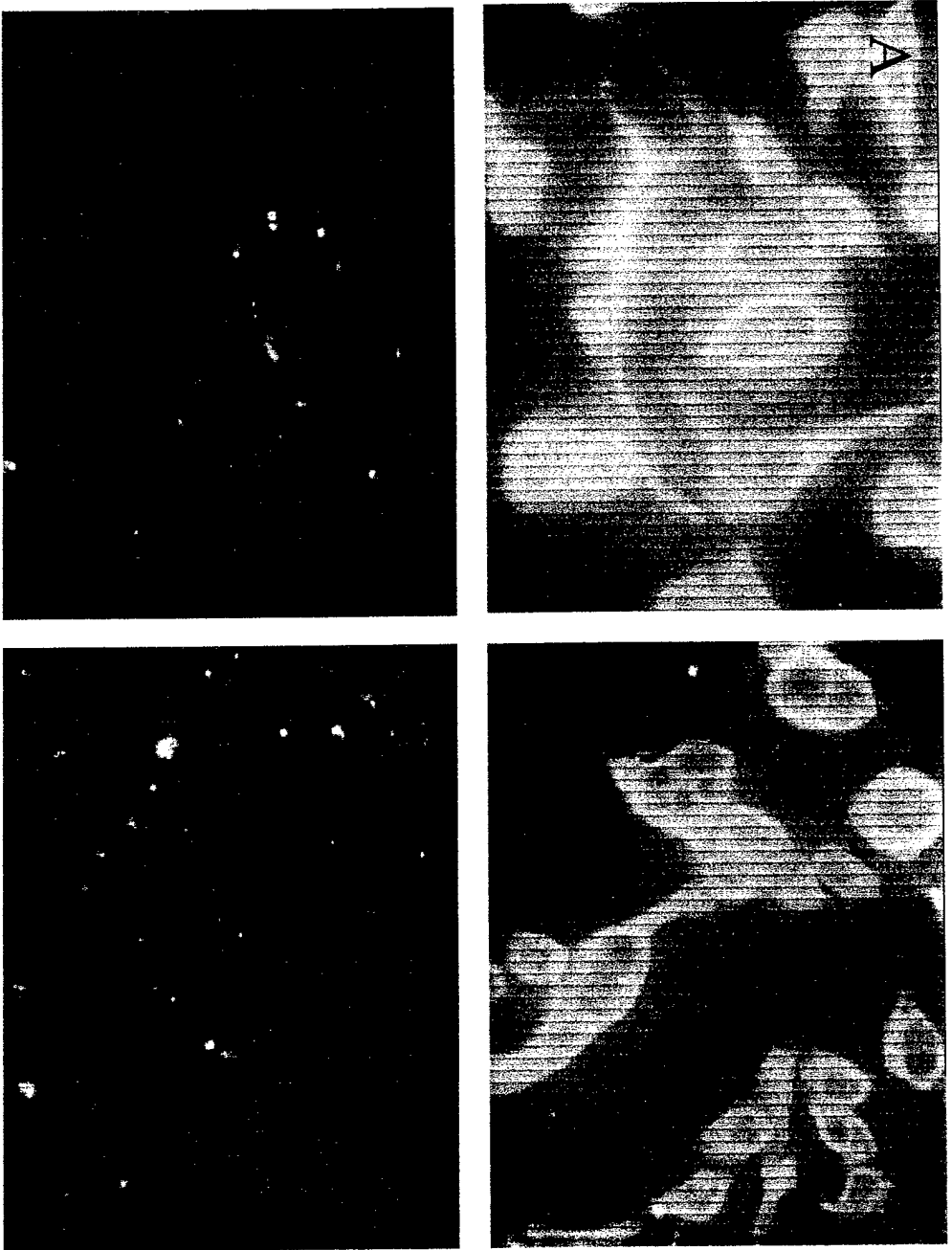


Fig. 4. Decreased binding of paclitaxel in cells overexpressing stathmin. BT20V1 (A), BT20V3 (B), BT20ST11 (C), and BT20ST3 (D) cells were treated with fluorescein-conjugated paclitaxel as described in Materials and Methods. Live cells were viewed with a fluorescent microscope under 100x oil immersion objective. The images are representative of three separate experiments.

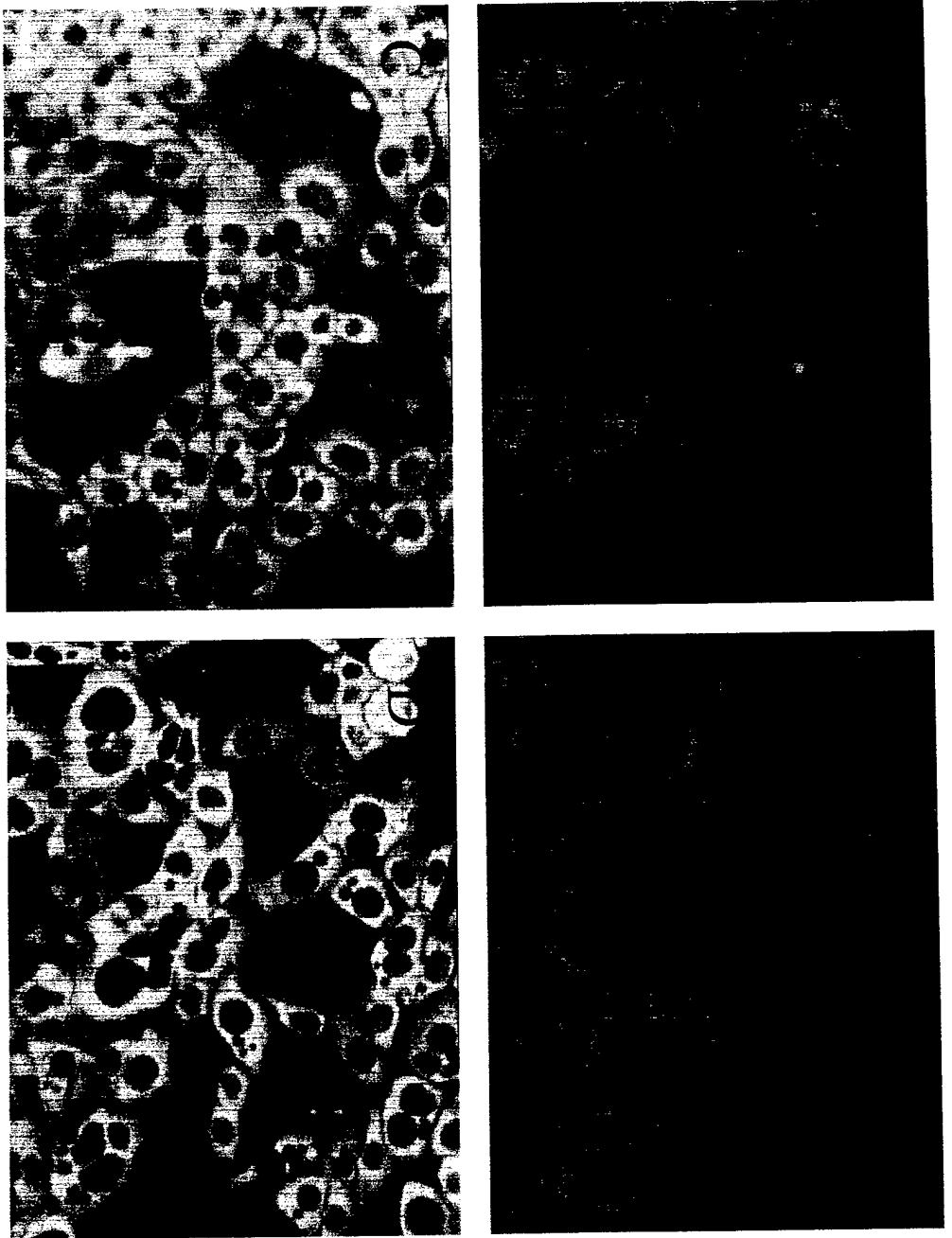


Fig. 5. Increased binding of vinblastine in cells overexpressing stathmin. BT20V1 (A), BT20V3 (B), BT20ST1 (C), and BT20ST3 (D) cells were treated with fluorescein-conjugated vinblastine as described in Materials and Methods. Live cells were viewed with a fluorescent microscope under 40x magnification. The images are representative of two separate experiments.

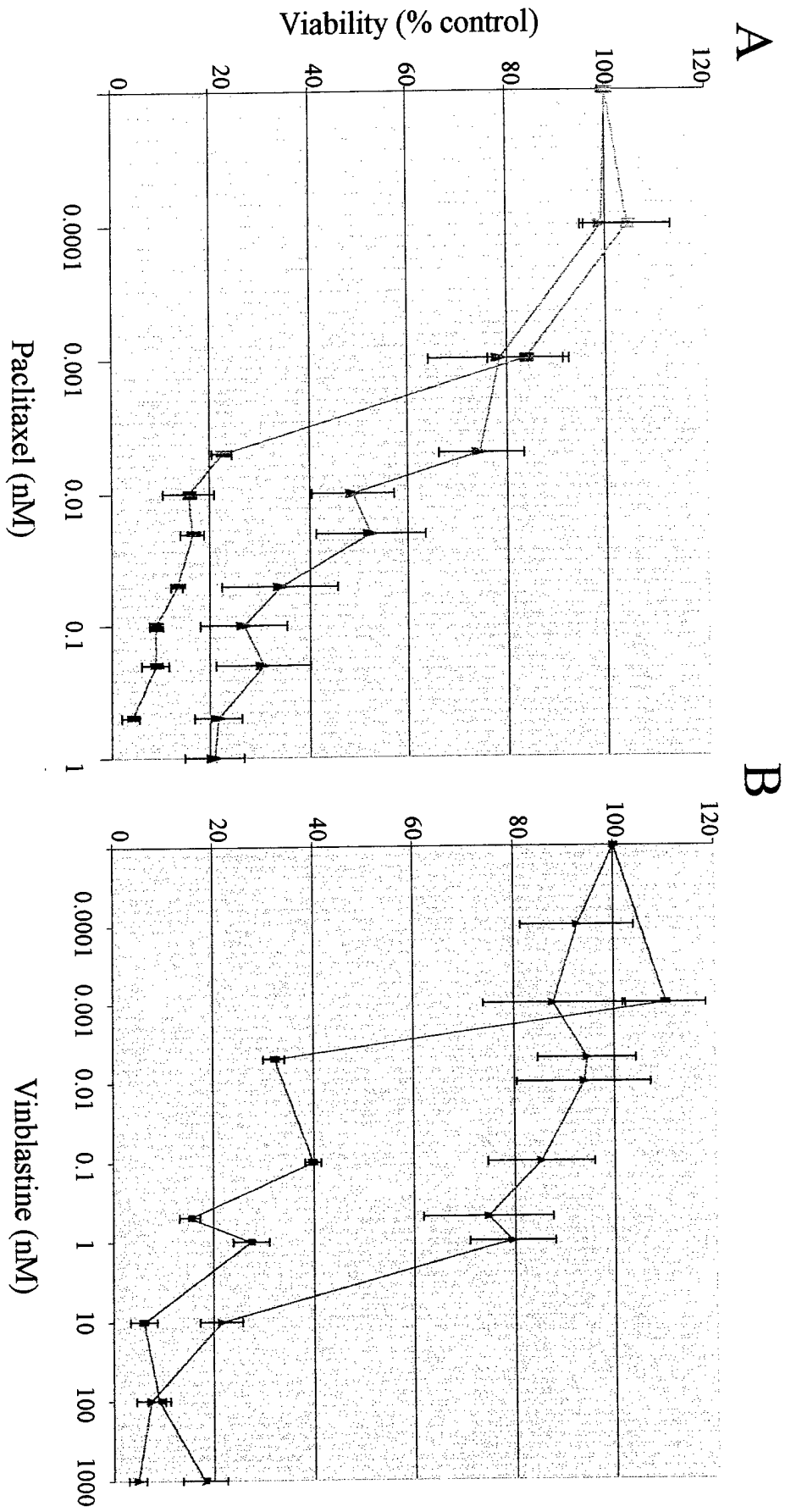
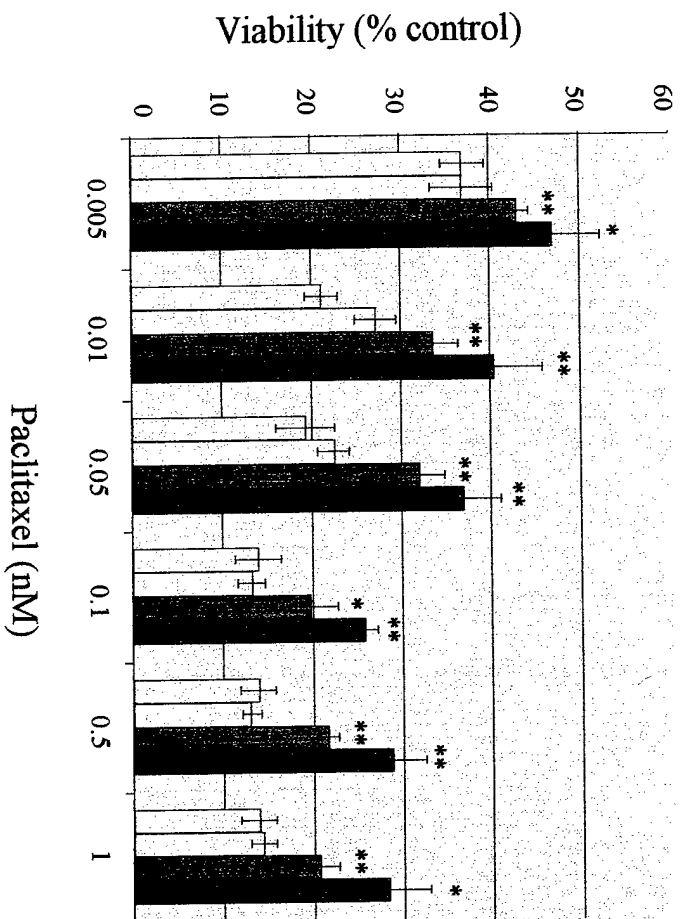


Fig. 6. Effect of stathmin expression on the sensitivity to antimicrotubule drugs. BT20 (□) and BT549 (△) cells were treated with paclitaxel (A) and vinblastine (B) as described in Materials and Methods. Drug sensitivity was determined by MTT assay.

A



B

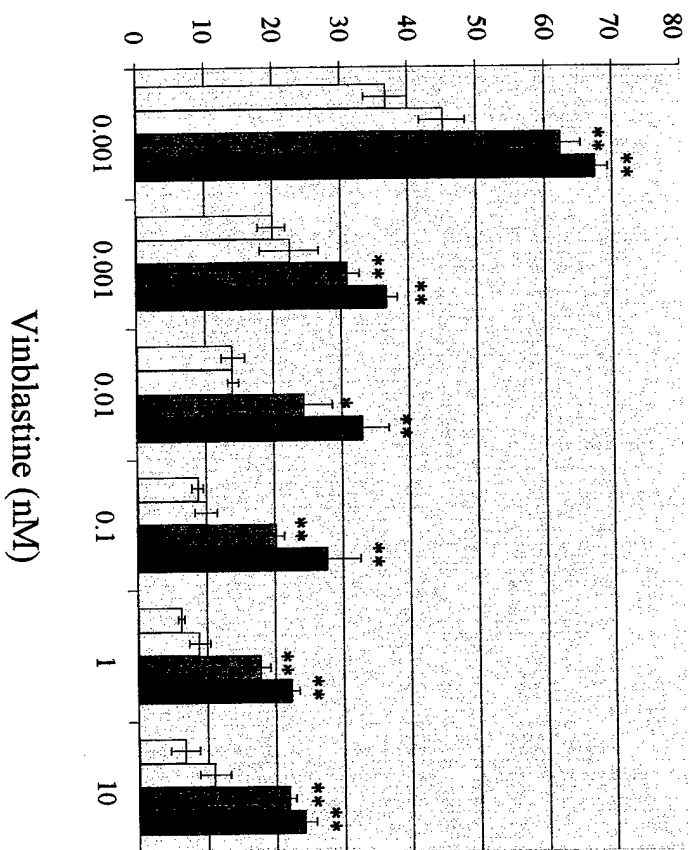
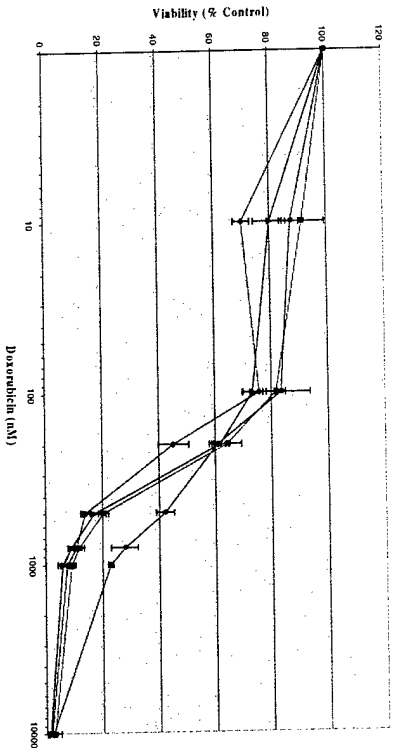


Fig. 7. Decreased sensitivity of statmin overexpressing cells to antimicrotubule agents. BT20V1 (○), BT20V3 (□), BT20ST1 (■), and BT20ST3 (■) cells were treated with paclitaxel (A) and vinblastine (B) as described in Materials and Methods. Drug sensitivity was determined by MTT assay. Values shown are means \pm SD from quadruplicate readings. The figure is representative of three separate experiments. t-test was used to determine statistical significance. * $p < 0.05$; ** $p < 0.01$.

A



B

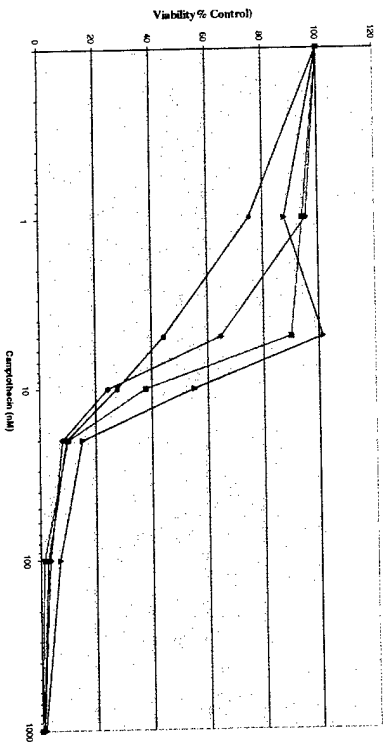


Fig. 8. Sensitivity of stathmin overexpressing cells to non-antimicrotubule drugs. BT20V1 (—◆—), BT20V3 (—■—), BT20ST1 (—▲—), and BT20ST3 (—●—) cells were treated with doxorubicin (A), or camptothecin (B). Drug sensitivity was determined by MTT assay. Values shown are from quadruplicate readings. The figure is representative of two separate experiments.

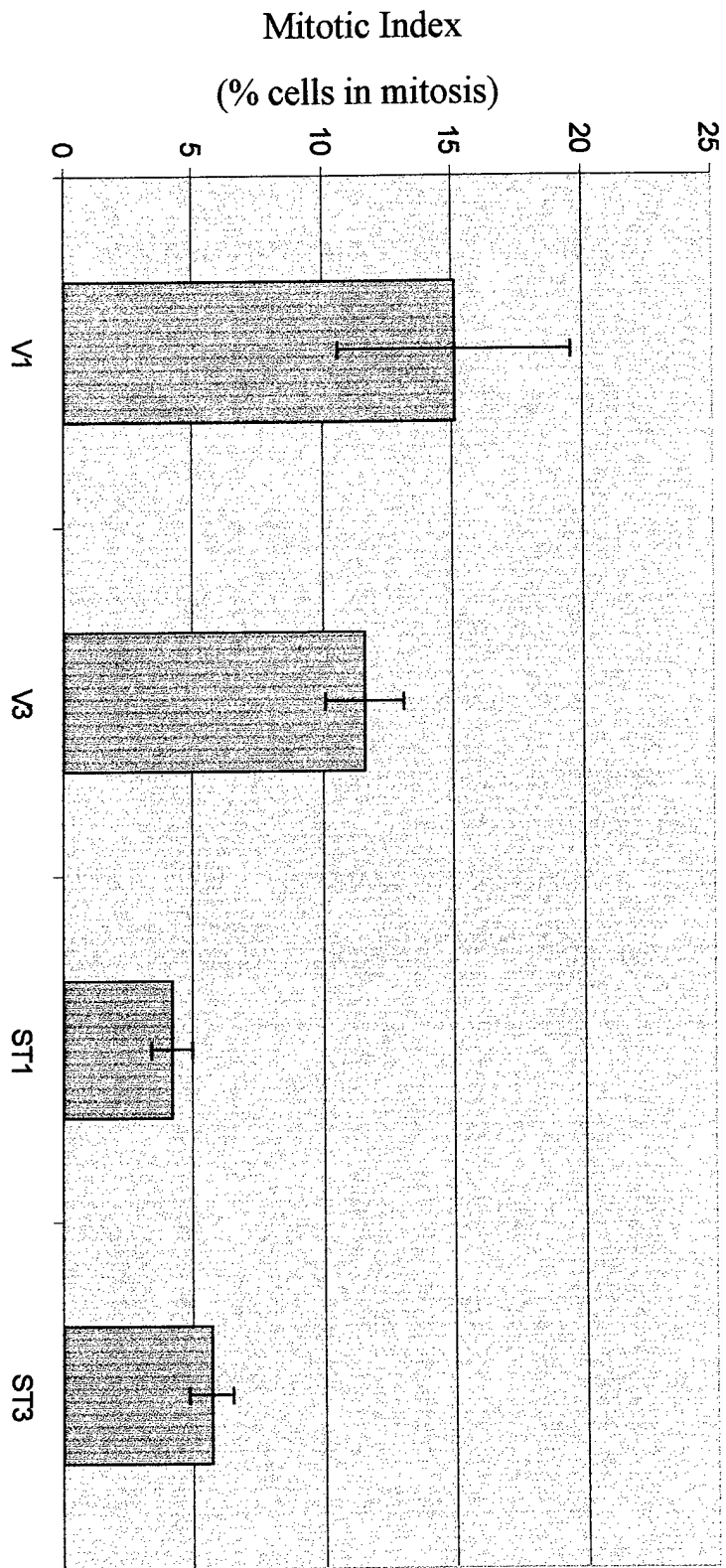


Fig. 9. Quantitation of cells in M phase determined by mitotic index. BT20 statmin transfectants were scored for mitotic cells after DAPI staining as described in materials and methods. Percentages are expressed as averages of four individual experiments \pm SE.

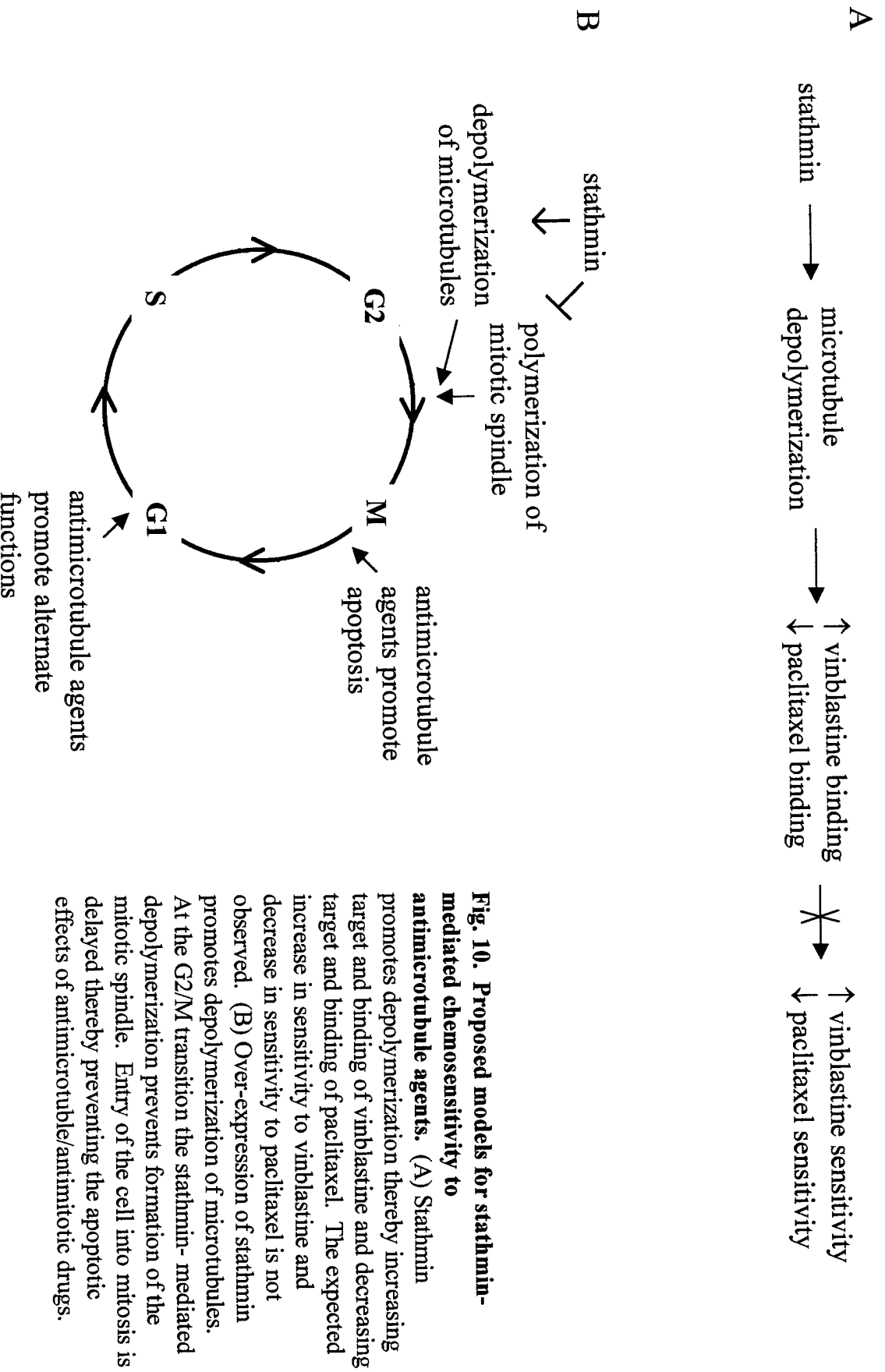


Fig. 10. Proposed models for stathmin-mediated chemosensitivity to antimicrotubule agents. (A) Stathmin promotes depolymerization thereby increasing target and binding of vinblastine and decreasing target and binding of paclitaxel. The expected increase in sensitivity to vinblastine and decrease in sensitivity to paclitaxel is not observed. (B) Over-expression of stathmin promotes depolymerization of microtubules. At the G2/M transition the stathmin-mediated depolymerization prevents formation of the mitotic spindle. Entry of the cell into mitosis is delayed thereby preventing the apoptotic effects of antimicrotubule/antimitotic drugs.

DNA Damage Increases Sensitivity to *Vinca* Alkaloids and Decreases Sensitivity to Taxanes through p53-dependent Repression of Microtubule-associated Protein 4¹

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ABSTRACT

Taxanes and *Vinca* alkaloids are among the most active classes of drugs in the treatment of cancer. Yet, fewer than 50% of previously untreated patients respond, and clinicians have few ways of predicting who will benefit from treatment and who will not. Mutations in p53 occur in more than half of human malignancies and may alter the sensitivity to a variety of anticancer therapies. We have shown that the transcriptional status of p53 determines the sensitivity to antimicrotubule drugs and that this is mediated through the regulation of microtubule-associated protein 4 (MAP4). Expression of MAP4 is transcriptionally repressed by wild-type p53. Increased expression of MAP4, which occurs when p53 is transcriptionally inactive, increases microtubule polymerization, paclitaxel binding, and sensitivity to paclitaxel, a drug that stabilizes polymerized microtubules. In contrast, overexpression of MAP4 decreases microtubule binding and sensitivity to *Vinca* alkaloids, which promotes microtubule depolymerization. To determine whether induction of endogenous wild-type p53 by DNA-damaging agents alters the expression of MAP4 and changes the sensitivity to antimicrotubule drugs, we assayed cell lines with wild-type or mutant p53 for the expression of MAP4 and drug sensitivity before and after DNA damage. UV irradiation, bleomycin, and doxorubicin increased wild-type p53 expression and decreased MAP4 expression. These changes were associated with decreased sensitivity to paclitaxel and increased sensitivity to vinblastine. These changes in drug sensitivity were no longer observed when p53 and MAP4 returned to baseline levels. Changes in drug sensitivity following DNA-damaging agents were associated with decreased binding of paclitaxel and increased binding of *Vinca* alkaloids. In contrast, DNA damage did not alter the sensitivity to non-microtubule-active drugs, such as 1- β -D-arabinofuranosylecytosine and doxorubicin. Changes in drug sensitivity following DNA-damaging drugs were not observed in cells with mutant p53. These studies demonstrate that induction of wild-type p53 by DNA-damaging agents can affect the sensitivity to antimicrotubule drugs through the regulation of MAP4 expression and may have implications for the design of clinical anticancer therapies.

INTRODUCTION

Treatment of cancer is often empirical, based more on clinical findings and histological appearance of the tumor than on an understanding of the molecular mechanism(s) of drug sensitivity. For example, taxanes, such as paclitaxel and docetaxel, and *Vinca* alkaloids, such as vinblastine and vincristine, are among the most active drugs in the treatment of breast, lung, and other cancers. Yet only 30–50% of previously untreated patients respond to these individual agents (1).

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Despite this, clinicians have few ways of predicting who will respond and who will not. As a result, many patients are exposed to highly toxic drugs and suffer the side effects without reaping the benefits.

Recently, important gene products have been discovered that affect the action of cancer chemotherapeutic agents (2). For example, p53 may determine the way cells respond to genomic and nongenomic cellular injury, including radiation and chemotherapy (3). Because p53 is frequently mutated in human malignancies, we focused our attention on how this protein affects drug sensitivity.

A variety of mechanisms might explain the altered drug sensitivity of cells with mutant p53. Because p53 is a transcription factor, the expression or loss of expression of p53-dependent gene products was of considerable interest. Murphy *et al.* (4) demonstrated that MAP4³ is transcriptionally repressed by wild-type p53. We have previously shown that the transcriptional status of p53 determines the sensitivity to antimicrotubule drugs and that these changes in sensitivity are mediated through the transcriptional regulation of MAP4 (5). MAP4 is the major microtubule-associated protein in nonneuronal tissues. Increased expression of MAP4, which occurs when p53 is transcriptionally inactive, increases microtubule polymerization and paclitaxel binding. These changes produce increased sensitivity to paclitaxel and decreased sensitivity to *Vinca* alkaloids (5). Furthermore, cell lines that overexpress MAP4 following transfection show the same drug sensitivity profile as do those with mutant p53 (5). Thus, p53-regulated changes in the expression of MAP4 can alter microtubule dynamic stability and influence the sensitivity to drugs whose mechanism of action is mediated through the polymerization or depolymerization of microtubules.

The expression of wild-type p53 protein can be increased by DNA damage (6). Murphy *et al.* (4) demonstrated that wild-type p53 can repress the expression of MAP4 at the level of transcription. Because changes in the expression of MAP4 alter sensitivity to microtubule-active drugs, repression of MAP4 through induction of wild-type p53 would provide a plausible means to manipulate sensitivity to taxanes and *Vinca* alkaloids, depending upon the mutant or wild-type state of p53 in cancer cells. Therefore, in these studies, we sought to determine whether or not induction of endogenous wild-type p53 by DNA-damaging agents altered the expression of MAP4 and changed the sensitivity to antimicrotubule drugs.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. C127 cells are mouse ductal epithelial carcinoma cells that are transformed by BPV-4 (7); products of BPV-1 gene expression do not bind or promote the degradation of p53. C127 cells were maintained at 37°C in monolayer in DMEM supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μ g/ml streptomycin.

The BRK p53-An1 cell line has been described previously (8). Briefly,

³The abbreviations used are: MAP4, microtubule-associated protein 4; BPV-1, bovine papilloma virus type 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

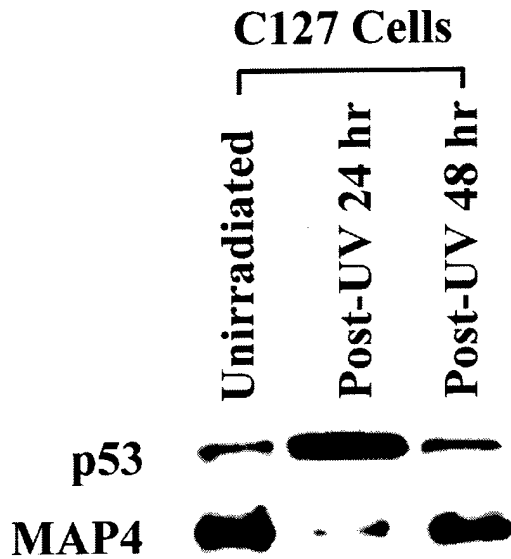


Fig. 1. Effect of UV irradiation on induction of wild-type p53 and MAP4 protein expression. Exponentially growing C127 cells were treated with 10 J/m^2 of UV irradiation. Total protein was collected and analyzed 24 and 48 h following irradiation. Loading was normalized to protein concentration, and equal transfer was measured by Coomassie Blue staining of the transferred gels. p53 and MAP4 expression was assayed by Western analysis as described in "Materials and Methods" using monoclonal antibodies Pab240 and IF5 MAP4, respectively. The Western blot shown is a representative of three separate experiments.

plasmid pmsvegval135 was introduced into primary BRK epithelial cells with the adenovirus *E1A* gene to generate p53-An1 (8). These cells were transfected with the murine *tsp53*(Val135), a temperature-sensitive p53 protein that assumes the mutant conformation at the restrictive temperature (38.5°C) but is predominantly in the wild-type conformation at the permissive temperature (32°C ; Ref. 8). BRK cells were maintained at 38.5°C and grown in monolayer in DMEM supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and $50 \mu\text{g/ml}$ streptomycin.

LnCAP cells and PA-1 cells were obtained from American Type Culture Collection (Manassas, VA). LnCAP is a human prostate cancer cell line that has been described previously (9, 10), and it carries wild-type alleles of *p53* (11). LnCAP cells were maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) with 2 mM L-glutamine adjusted to contain 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. PA-1 is a human ovarian carcinoma cell line (12, 13) with wild-type p53 (14, 15). PA-1 cells were maintained in monolayer in DMEM supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and $50 \mu\text{g/ml}$ streptomycin.

All cell lines were grown in an atmosphere of 95% air-5% CO_2 , routinely checked, and found to be free of contamination by *Mycoplasma* and fungi.

UV Irradiation. UV irradiation was administered as described previously (4) using a dose of 10 J/m^2 at 254 nm with a germicidal lamp (Fisher Scientific, Springfield, NJ). This dose had minimal effect upon the growth and distribution of these cells.

Drugs. Bleomycin and doxorubicin were obtained from Bristol-Myers Squibb (Princeton, NJ) and Sigma Chemical Co. (St. Louis, MO), respectively, and stock solutions were made freshly for each experiment in sterile dH_2O . For drug treatment with bleomycin or doxorubicin, cells were exposed for 24 h to a concentration of each drug that produced <15% cell killing over a 72-h time period. Paclitaxel (Taxol) was prepared as a 1 mM stock in absolute ethanol.

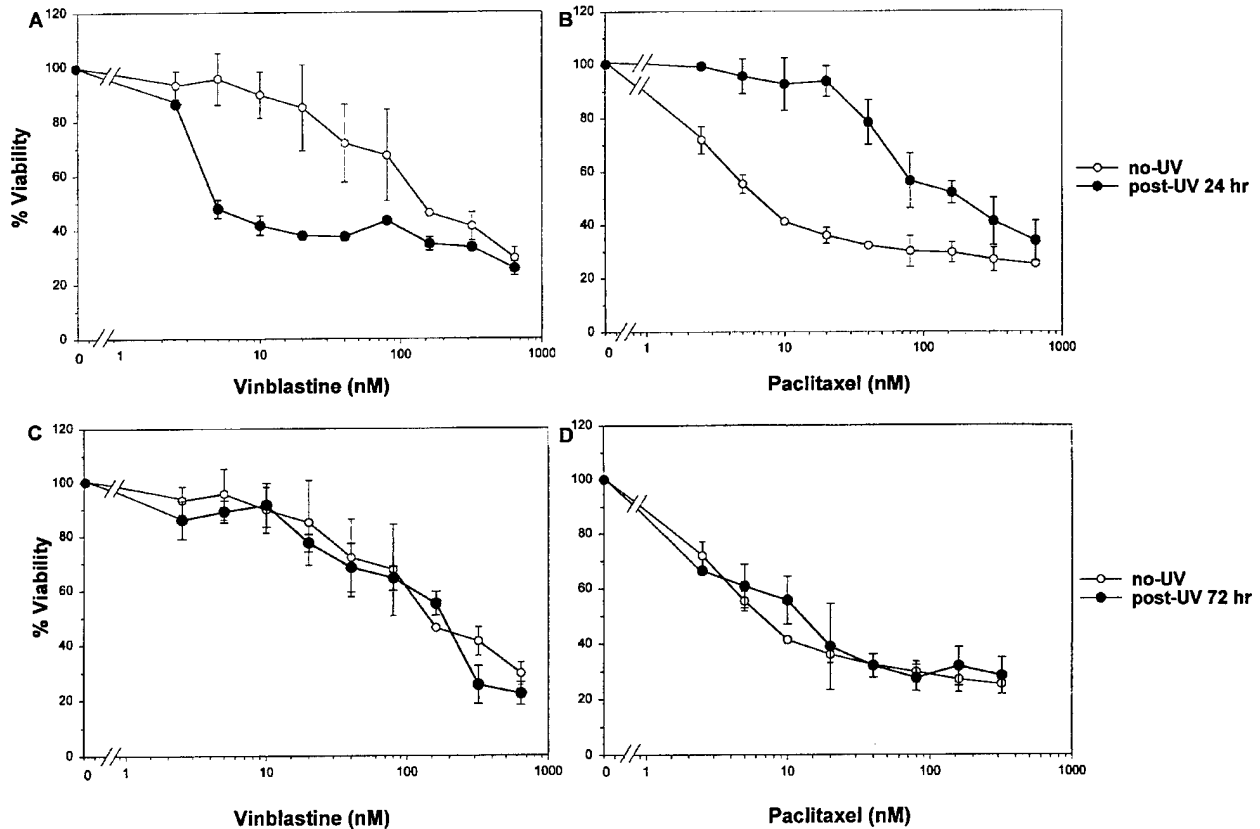


Fig. 2. Effect of UV irradiation on the sensitivity to microtubule-active drugs in C127 breast carcinoma cells. Exponentially growing C127 cells were treated with 10 J/m^2 of UV irradiation. Twenty-four (A and B) and 72 (C and D) h following irradiation, cells were exposed to various concentrations of paclitaxel and vinblastine for an additional 72 h. Cell viability was measured with the MTT assay, as described in "Materials and Methods." Data points, means from three separate experiments; bars, SD.

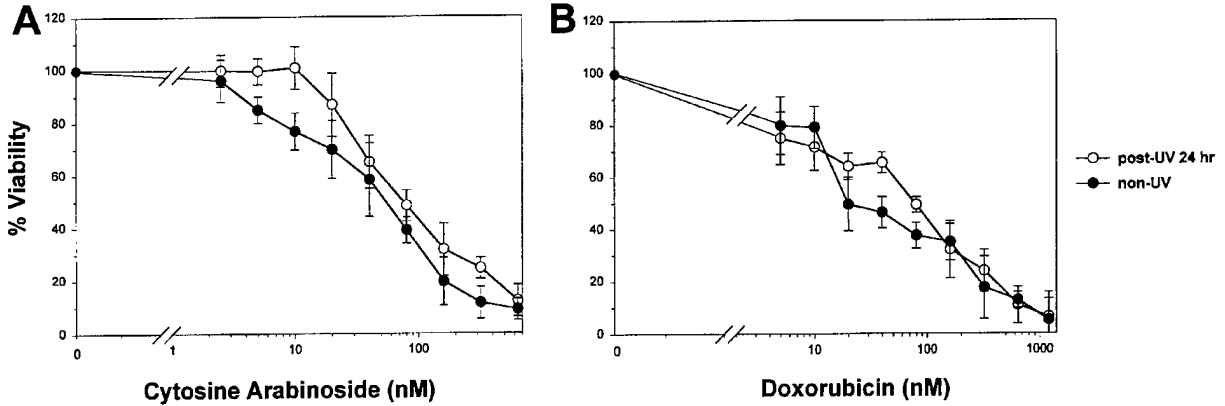


Fig. 3. Effect of UV irradiation on the sensitivity to non-microtubule-active drugs in C127 breast carcinoma cells. Exponentially growing C127 cells were treated with 10 J/m² of UV irradiation. Twenty-four h following irradiation, cells were exposed to various concentrations of 1- β -D-arabinofuranosylcytosine (A) or doxorubicin (B) for 72 h. Cell viability was measured with the MTT assay as described in "Materials and Methods." Data points, means from three separate experiments; bars, SD.

Vinblastine was dissolved in sterile distilled water. Stock solutions of all drugs were freshly prepared for each experiment and diluted to appropriate concentrations in vehicle immediately before adding to the cells.

All chemicals were purchased from Sigma unless otherwise indicated.

Cell Viability. Cell viability was assessed by the MTT assay (16, 17). MTT (Sigma) was freshly prepared as a 5 mg/ml stock in PBS. The MTT-formazan product was dissolved in 100% DMSO after a 4-h incubation, as described previously (16, 17). Absorbance was measured at 550 nm using a Dynatech

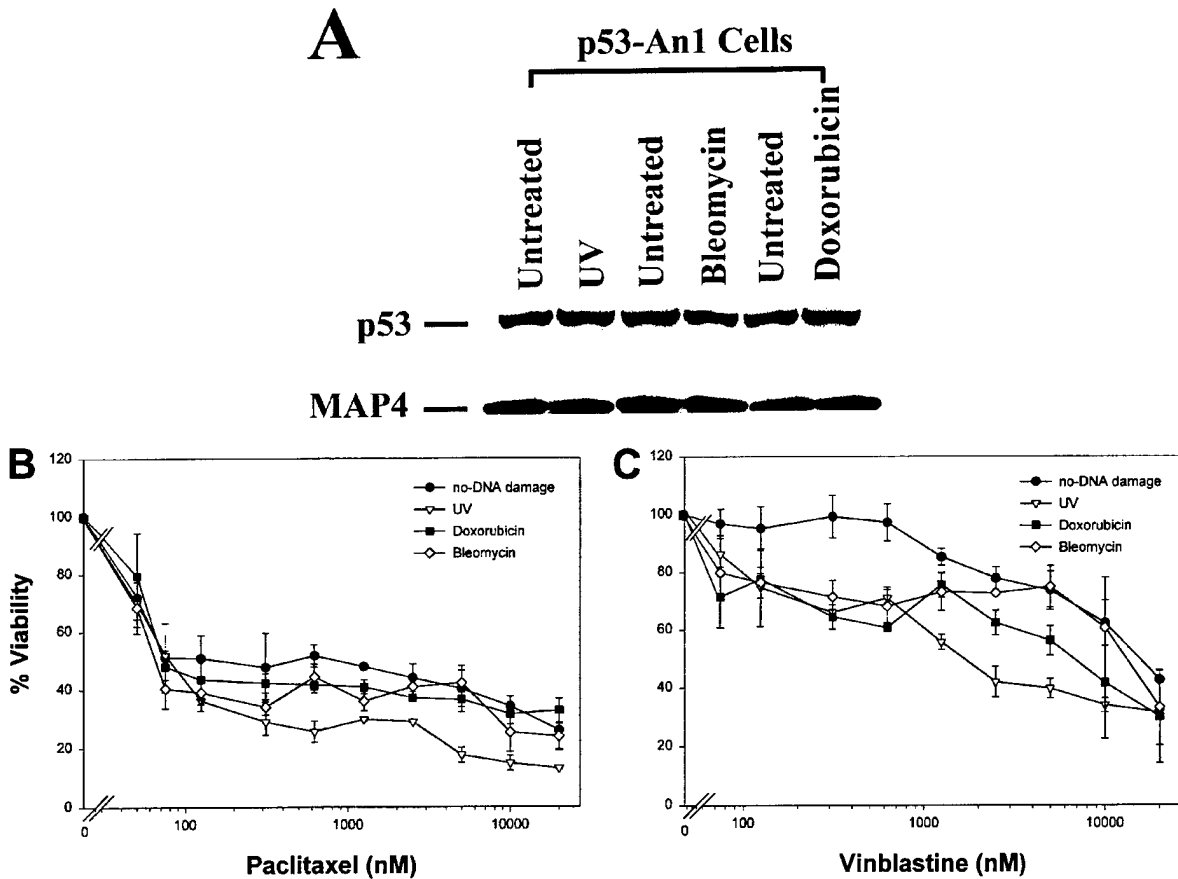


Fig. 4. Effect of DNA-damaging agents on p53 and MAP4 expression and on the sensitivity to microtubule active drugs in cells with mutant p53. p53 An-1 cells were grown at 38.5°C and exposed to 10 J/m² of UV irradiation, doxorubicin, or bleomycin, as described in "Materials and Methods." A, total protein was collected and analyzed 24 h following drug exposure. p53 and MAP4 expression were assayed by Western analysis as described in Fig. 1. The Western blot shown is a representative of three separate experiments. B and C, 24 h following DNA damage, cells were exposed to various concentrations of paclitaxel or vinblastine for an additional 72 h. Cell viability was measured with the MTT assay as described in "Materials and Methods." Data points, means from three separate experiments; bars, SD.

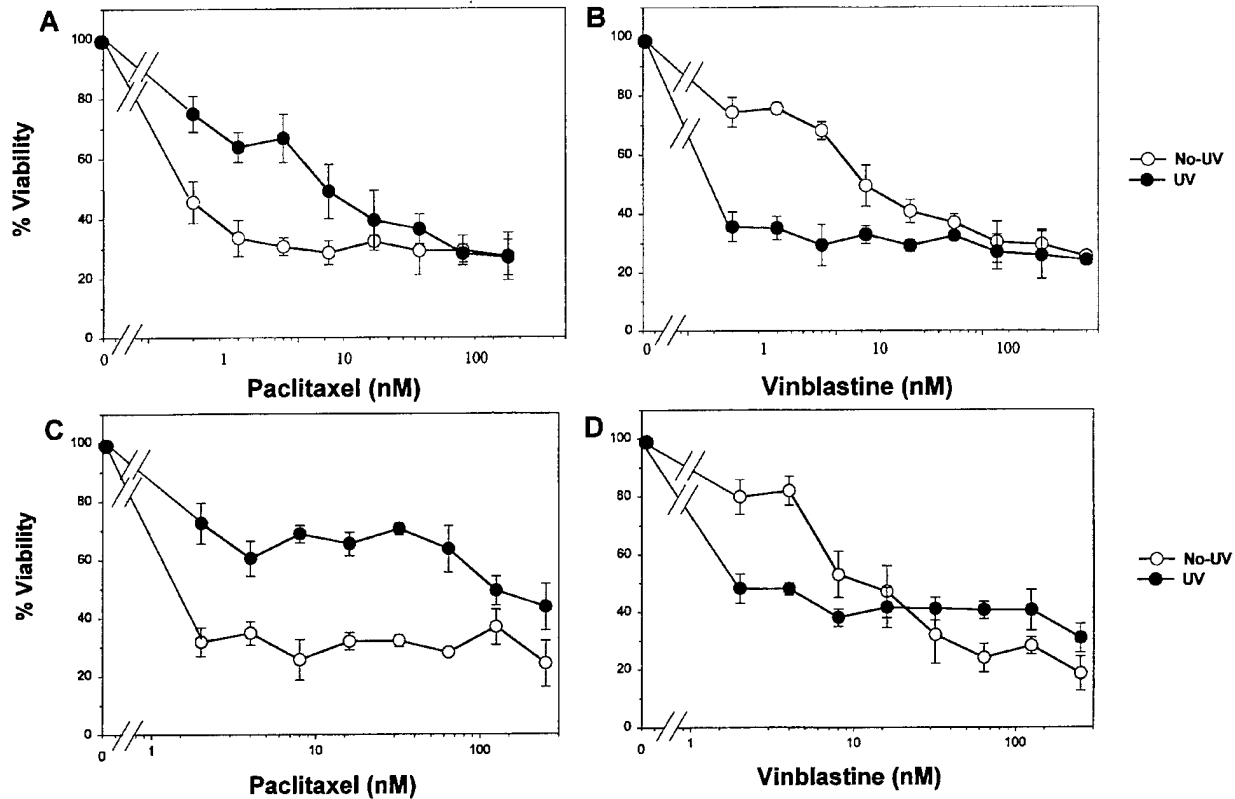


Fig. 5. Effect of UV irradiation on the sensitivity to microtubule-active drugs in human cancer cells with endogenous wild-type p53. PA-1 human ovarian cancer cells (A and B) and LnCAP human prostate cancer cells (C and D) were UV-irradiated at 10 J/m^2 . Twenty-four h following irradiation, cells were exposed to various concentrations of paclitaxel or vinblastine and assayed for cell viability by the MTT assay as described in "Materials and Methods." Data points, means from three separate experiments; bars, SD.

microplate reader MR5000 (Dynatech Laboratory Inc., Chantilly, VA). Cell viability was expressed as the percentage of the absorbance of drug-treated cells, relative to that of the vehicle-treated controls. IC_{50} was defined as the concentration of drug that produced a 50% decrease in cell viability relative to the vehicle-treated controls.

Exponentially growing cells were plated into 96-well tissue culture plates. Various concentrations of diluted drugs were added to quadruplicate wells (final vehicle concentration, $\leq 1\%$). For experiments carried out after DNA damage, cells in logarithmic phase of growth were either UV-irradiated at 10 J/m^2 or treated with bleomycin or doxorubicin for 24 h to allow the induction of wild-type p53 before plating into 96-well tissue culture plates (18, 19). Cells were then exposed to various concentrations of paclitaxel or vinblastine for 72 h.

Western Blotting. Measurement of p53 by immunoblotting was performed as described previously, using mouse monoclonal antibody PAb240 (Santa Cruz Biotechnology, Santa Cruz, CA), which is equally reactive with denatured wild-type and mutant p53 proteins of murine and human origin (20). PAb240 was diluted in Blotto (5% milk-2% BSA, PBS, and 0.05% Tween 20) to a final concentration of $5 \mu\text{g/ml}$. A 1:10,000 dilution in PBSBT (PBS, 1% BSA, and 0.05% Tween 20) of goat-antimouse IgG (Sigma) was used as the secondary antibody in a direct chemiluminescence system (Amersham, Arlington Heights, IL).

Measurement of MAP4 by immunoblotting was performed as described previously (4), using the IF5 rat-antimouse MAP4 monoclonal antibody kindly supplied by Dr. Joanna Olmsted (University of Rochester). Rabbit-antirat IgG (Sigma) was diluted 1:10,000 dilution in PBSBT and used as the secondary antibody, which was visualized by direct chemiluminescence (Amersham).

Loading of lanes was normalized to protein concentration and complete transfer was validated by staining of the posttransfer gels with Coomassie Blue. Bands were quantitated by phosphorimaging using a Bio-Rad Molecular Imager System (5).

Binding of Fluoresceinated Drugs. Cells were grown either on glass coverslips or culture dishes before and after DNA damage followed by incubation with either 200 nM fluorescein-conjugated paclitaxel (Bodipy-FL Taxol®; Molecular Probes Inc., Eugene, OR) or fluorescein-conjugated vinblastine (Bodipy-FL Vinblastine; Molecular Probes). After 60 min of incubation, cells were washed free of unbound drug and visualized under a fluorescent microscope. Fluorescence intensity of bound drug was quantitated by flow cytometry and profiles were generated on a Becton Dickinson FACSscan analyzer with laser excitation wavelength at 488 nm (5).

Statistical Analysis. Statistical analysis of each dose-response curve was performed by the method of Finney (21). Accordingly, mean IC_{50} values \pm SDs for the inhibition of proliferation by drugs under different experimental conditions were determined by linear regression analysis of the logit-transformed data. The differences between IC_{50} s were determined using the Student's *t* test (two-tailed).

RESULTS

Induction of Wild-Type p53 by UV Irradiation Leads to Decreased MAP4 Expression and Altered Sensitivity to Microtubule-active Drugs.

To determine the effect of DNA damage on p53 and MAP4 expression, we exposed C127 cells to 10 J/m^2 of UV irradiation. As shown in Fig. 1, UV irradiation increased the expression of wild-type p53 by 5-fold and decreased the expression of MAP4 by 5–7-fold. C127 cells were 32-fold more sensitive to vinblastine and 20-fold less sensitive to paclitaxel 24 h after UV irradiation (Fig. 2, A and B). Forty-eight h postirradiation, MAP4 and p53 protein expression returned to baseline (Fig. 1). After that period of time, the differences in drug sensitivity were no longer seen (Fig. 2,

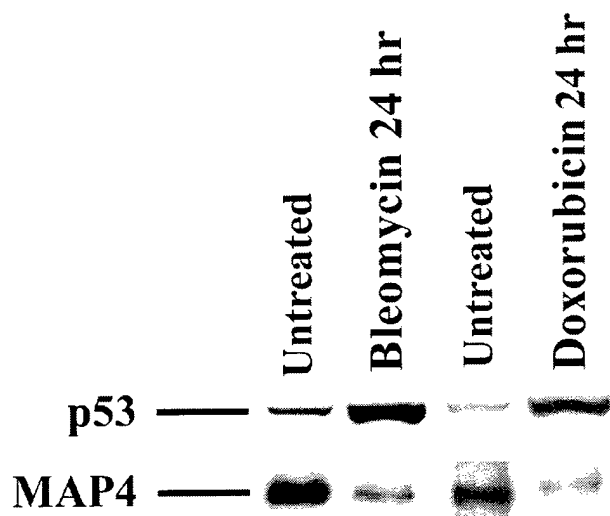


Fig. 6. Effect of induction of wild-type p53 by bleomycin and doxorubicin on the expression of MAP4 protein. Exponentially growing C127 cells were treated with bleomycin (200 nM) or doxorubicin (20 nM) for 24 h. Total protein was collected and analyzed at different time points following treatment. p53 and MAP4 expression were assayed by Western analysis as described in Fig. 1 using the PAb240 p53 monoclonal antibody and the IF5 MAP4 monoclonal antibody. The Western blot shown is a representative of three separate experiments.

C and D). UV irradiation had no effect on the sensitivity to 1- β -D-arabinofuranosylcytosine and doxorubicin, drugs that do not affect microtubule function (Fig. 3, A and B).

To exclude the effect of UV irradiation alone on drug sensitivity, we performed identical assays using BRK p53-An1 cells, which express a temperature-sensitive p53 mutant protein that is transcriptionally silent at 38.5°C (8). As shown in Fig. 4A, DNA damage by UV irradiation, doxorubicin, or bleomycin did not alter the expression of p53 and MAP4 in cells with mutant p53. DNA-damaging agents, including UV irradiation, doxorubicin, and bleomycin, did not affect the sensitivity to paclitaxel ($P > 0.1$; Fig. 4B). In addition, doxorubicin and bleomycin did not affect the sensitivity of p53 An-1 cells to vinblastine ($P > 0.1$; Fig. 4C). However, p53-An1 cells were more sensitive to vinblastine 24 h after UV irradiation ($P = 0.004$; Fig. 4C).

To determine whether the effect of UV irradiation on sensitivity to antimicrotubule drugs was a more general phenomenon, we studied several human cancer cells containing wild-type p53. Fig. 5 demonstrates that UV irradiation increased the sensitivity to *Vinca* alkaloids and decreased the sensitivity to taxanes in PA-1 human ovarian carcinoma cells (12–15) and LnCAP human prostate carcinoma cells (9–11).

Induction of Wild-Type p53 by DNA Damage Other Than UV Irradiation Produces Decreased Expression of MAP4 and Altered Sensitivity to Microtubule-active Drugs. To evaluate other DNA-damaging agents, we studied the effect of bleomycin and doxorubicin on the expression of wild-type p53 and MAP4 proteins. As shown in Fig. 6, treatment of C127 cells with 200 nM bleomycin or 20 nM doxorubicin (concentrations that caused <15% cell kill, data not shown) produced a 2–5-fold increase in the expression of p53 and a 5-fold decrease in MAP4, as measured by phosphorimaging.

C127 cells were then exposed to paclitaxel or vinblastine for 72 h with and without pre-exposure to the DNA-damaging agents. Fig. 7A shows that C127 cells were >20-fold more resistant to paclitaxel ($IC_{50} = 160$ nM) after bleomycin or doxorubicin treatment, compared to cells not previously exposed to the DNA-damaging drugs ($IC_{50} = 7$ nM). In contrast, Fig. 7B demonstrates that C127 cells were 30-fold ($IC_{50} = 5$ nM) and 60-fold ($IC_{50} = 2.5$ nM) more sensitive to

vinblastine after doxorubicin and bleomycin treatment, respectively, compared to cells not previously exposed to the DNA-damaging drugs ($IC_{50} = 160$ nM).

To rule out an effect of bleomycin or doxorubicin on sensitivity to antimicrotubule drugs independent of wild-type p53, we carried out identical assays using BRK p53-An1 cells. Treatment of p53-An1 cells with bleomycin or doxorubicin grown at 38.5°C (mutant p53) had no significant effect on the sensitivity to paclitaxel (Fig. 4B) or vinblastine (Fig. 4C).

Induction of Wild-Type p53 by UV Irradiation Leads to Altered Cellular Binding of Microtubule-active Drugs. To determine whether the increased sensitivity to vinblastine and decreased sensitivity to paclitaxel after DNA damage was associated with changes in cellular binding of drugs, we incubated live C127 cells with fluoresceinated paclitaxel or fluoresceinated vinblastine before and after UV irradiation. Binding was visualized by immunofluorescence microscopy. Twenty-four h after UV irradiation, C127 cells bound more fluoresceinated vinblastine than did untreated cells (Fig. 8, AC and AD). In contrast, binding of fluoresceinated-paclitaxel was decreased after UV irradiation (Fig. 8, AA and AB). No significant changes in drug binding were observed with p53-An1 cells (mutant p53) before or after UV irradiation (Fig. 8, Aa–Ad). Quantitative analysis of

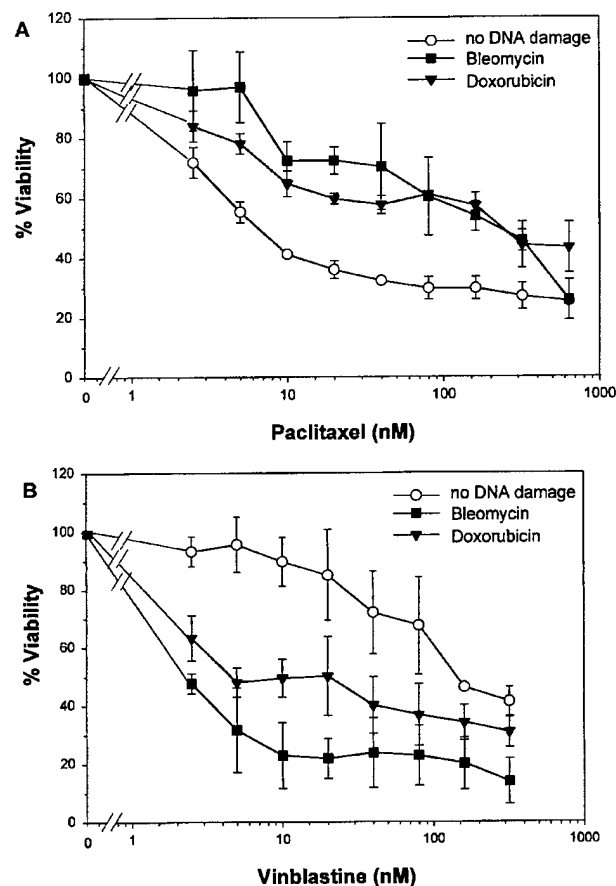


Fig. 7. Effect of treatment with DNA-damaging agents on the sensitivity to microtubule-active drugs. Exponentially growing C127 cells were treated with bleomycin (200 nM) or doxorubicin (20 nM) for 24 h followed by exposure to various concentrations of paclitaxel (A) and vinblastine (B) for 72 h. Cell viability was measured by the MTT assay as described in "Materials and Methods." Data points, means from three separate experiments; bars, SD.

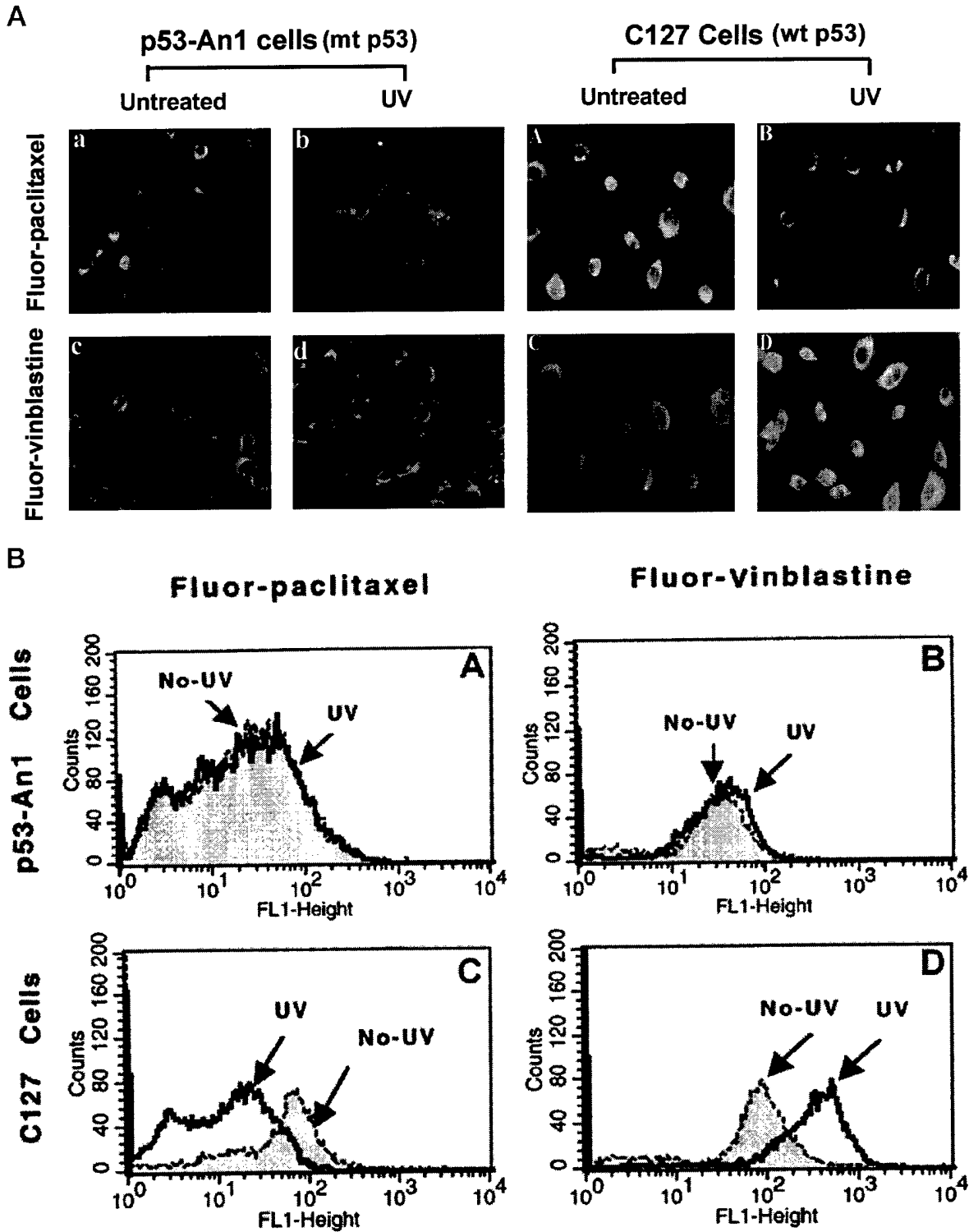


Fig. 8. Effect of UV irradiation on cellular binding of microtubule-active drugs. *A*, live C127 cells (AA-AD) and BRK p53-An1 cells (Aa-Ad) were UV-irradiated at 10 J/m² 24 h before incubation with fluorescein-conjugated paclitaxel (200 nM) or fluorescein-conjugated vinblastine (200 nM) for 60 min. Images were taken from a fluorescent microscope under a 40× oil-immersion objective. *B*, cellular binding of microtubule-active drugs to live p53-An1 cells (*A* and *B*) and C127 cells (*C* and *D*) was quantitated by fluorescent-activated cell sorting as described in "Materials and Methods." The results are representative of two individual experiments.

changes in drug binding was performed by flow cytometry and are shown in Fig. 8B.

DISCUSSION

Sensitivity to microtubule-active drugs is influenced by various factors, including P-glycoprotein-mediated mechanisms of drug efflux (22), altered tubulin structure and function (23–26), and the ability of cells to survive lethal damage due to the expression of antiapoptotic gene products (27). Several laboratories, including our own, found that mutant p53 increased the sensitivity to paclitaxel in a tissue-specific manner (5, 28–31). Our previous studies demonstrated that one of the mechanisms by which mutant p53 increases sensitivity to paclitaxel is through derepression of MAP4 (5). Because MAP4 promotes polymerization of microtubules by binding to the COOH terminus of α - and β -tubulin (32–34), p53-regulated changes in the expression of MAP4 can influence the sensitivity to drugs with mechanisms of action that affect the state of microtubule polymerization. We went on to demonstrate that overexpression of MAP4 in mouse embryo fibroblasts fully mimicked the drug sensitivity profile observed in cells with mutant p53 (5). Accordingly, we hypothesized that repression of MAP4 through induction of wild-type p53 would provide a plausible means to manipulate sensitivity to taxanes and *Vinca* alkaloids in cancers with endogenous wild-type p53.

MAP4 Expression Is Repressed by Induction of Wild-Type p53 through DNA Damage. p53 is known to be induced by numerous DNA-damaging agents, including UV irradiation and cancer chemotherapeutic drugs (18, 19, 35, 36). p53 regulates G₁-S cell cycle arrest and may allow time for DNA repair following genomic damage (36–39). Murphy *et al.* (4) recently reported that MAP4 expression was transcriptionally repressed when wild-type p53 was induced by UV irradiation. This led us to investigate the influence of induction of p53 by DNA-damaging agents on MAP4 expression, microtubule polymerization, and the sensitivity to antimicrotubule drugs.

C127 murine breast carcinoma cells are transformed by BPV-1, which does not produce gene products that bind to or promote the degradation of the p53 protein (7). As shown in Fig. 1, UV irradiation of C127 cells increases expression of wild-type p53 and decreases expression of MAP4. Because UV irradiation produces a myriad of cellular responses, we investigated whether other DNA-damaging agents would produce similar effects. Accordingly, we found that bleomycin and doxorubicin both increase p53 expression and repress the expression of MAP4 (Fig. 6). In contrast, UV irradiation, bleomycin, and doxorubicin do not alter the expression of p53 or MAP4 in cells containing mutant p53 (Fig. 4A).

Repression of MAP4 by p53 Decreases Sensitivity to Paclitaxel and Increases Sensitivity to the *Vinca* Alkaloids. *Vinca* alkaloids have been used to augment the effects of radiation in a variety of human malignancies, including prostate cancer (40, 41). In contrast, the effect of paclitaxel on the sensitivity to radiation is not universal. In certain cell lines, paclitaxel treatment rendered cells more sensitive to radiation (42–45), whereas in other cells, paclitaxel did not have a significant effect (46–48).

Repression of MAP4 by p53 following UV irradiation renders C127 cells 32-fold more sensitive to vinblastine and 20-fold less sensitive to paclitaxel (Fig. 2, A and B). By 48 h postirradiation, MAP4 and p53 protein expression return to baseline (Fig. 1). After that period of time, the differences in drug sensitivity are no longer seen (Fig. 2, C and D). We also found increased sensitivity to vinblastine and decreased sensitivity to paclitaxel after UV irradiation in several human carcinoma cell lines that harbor wild-type p53, including PA-1 ovarian carcinoma cells and LnCAP prostate carcinoma cells (Fig. 5). These results suggest that this mechanism of

altering drug sensitivity may be applicable to several types of human cancers.

The UV-induced decrease in sensitivity to paclitaxel appears to be dependent on a transcriptionally competent p53 because no significant changes in sensitivity were observed in BRK p53-An1 cells before or after UV exposure (Fig. 4B). In contrast, the UV-induced increase in sensitivity to vinblastine in p53-An1 cells suggests that both p53-dependent and -independent mechanisms exist (Fig. 4C).

Doxorubicin and bleomycin have similar effects in C127 cells; they increase the sensitivity to vinblastine and decrease the sensitivity to paclitaxel (Fig. 7). The effects of these drugs are not observed in p53An-1 cells, suggesting dependence on transcriptionally competent p53 (Fig. 4, B and C).

MAP4 Expression May Explain Changes in Sensitivity to Microtubule-active Drugs following DNA Damage. Taxanes bind to and stabilize polymerized microtubules, leading to altered microtubular function. MAP4 promotes polymerization of microtubules by binding to the negatively charged COOH termini of α - and β -tubulin (49). We previously found that overexpression of MAP4 in mouse embryo fibroblasts increased polymerization of microtubules and cellular binding of fluoresceinated paclitaxel (5).

Vinca alkaloids bind to tubulin monomers and inhibit microtubule polymerization (50). Repression of MAP4 by p53 promotes a shift in tubulin dynamics toward depolymerization (5). In parental mouse embryo fibroblasts with lower levels of MAP4, there were more depolymerized paracrystalline tubulin structures after treatment with *Vinca* alkaloids (5). Twenty-four h after UV treatment, when MAP4 expression is suppressed, C127 cells bound more vinblastine (Fig. 8, AC and AD) and less paclitaxel (Fig. 8, AA and AB) than untreated cells. There was no significant change in fluorescence intensity of bound drugs in p53-An1 cells before and after UV irradiation in the presence of transcriptionally inactive p53 (Fig. 8, Aa–Ad).

In summary, our studies demonstrate that induction of wild-type p53 by DNA damage can alter the sensitivity to antimicrotubule drugs and suggest that this is mediated through p53-induced repression of MAP4. These changes in drug sensitivity are consistent with the effects of MAP4 on microtubule polymerization. Because the expression of wild-type p53 and MAP4 can be regulated by treatment with DNA-damaging agents, these studies provide a rationale for the future design of combination anticancer therapies.

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Induction of p53 and Repression of Microtubule-Associated Protein 4 by DNA Damage in Breast Cancer Patients: A Pilot Clinical Trial of Sequential Doxorubicin and Vinorelbine.

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Abbreviations: wtp53, wild-type p53; MAP4, microtubule-associated protein 4; PBMNCs, peripheral blood mononuclear cells.

ABSTRACT Few molecular determinants of sensitivity to cancer chemotherapy exist. In experimental systems, p53 regulates the sensitivity to anti-microtubule drugs through its effect on microtubule-associated protein 4 (MAP4). MAP4 is the major microtubule-associated protein in non-neuronal tissues and promotes microtubule polymerization. We reported that induction of wild-type p53 (wtp53) by the DNA damaging agent doxorubicin in C127 breast cancer cells repressed MAP4, decreased microtubule polymerization and increased *vinca* alkaloid sensitivity. The goal of this pilot clinical trial was to determine if DNA damage by doxorubicin leads to the induction of p53 and repression of MAP4 in patients with breast cancer, thereby increasing sensitivity to the sequential administration of the *vinca* alkaloid, vinorelbine. Peripheral blood mononuclear cells (PBMNCs) and accessible tumor tissue were obtained from sixteen women with locally advanced (stage IIIb) or metastatic (stage IV) breast cancer before doxorubicin treatment and immediately before treatment with vinorelbine 24 or 48 hours later. Following doxorubicin, p53 increased in 12/14 PBMNC and 4/10 tumor samples. MAP4 decreased in 7/12 PBMNC and 3/4 breast cancer specimens in which p53 was induced. Immunohistochemistry confirmed lower MAP4 levels in tumor cells post-doxorubicin treatment. Seven of 16 patients had a partial response to treatment. Sequential administration of doxorubicin and vinorelbine was well tolerated. These data suggest that induction of p53 and repression of MAP4 occurs in normal and malignant tissues in patients treated with a DNA damaging agent, and that an anti-microtubule drug can be safely administered at a time when cells may be more sensitive to treatment.

INTRODUCTION

The choice of cancer chemotherapy is often empirical, based more on the histological appearance of the tumor than on an understanding of the molecular determinants of drug sensitivity. In patients with metastatic breast cancer, the complete response rate is less than 20%, the median duration of response is nine months, and the median survival is 12-24 months. Similarly, the prognosis for inflammatory or locally advanced breast cancer is dismal, with fewer than 50% of diagnosed patients alive at five years (1). Yet, virtually all patients treated with chemotherapy suffer significant side effects. Therefore, we have focused on defining molecular markers of drug sensitivity to help identify those patients who are most likely to respond to therapy and to develop more effective therapeutic strategies.

p53 can regulate the fate of cells injured by radiation and chemotherapy through its effects on cell-cycle kinetics and programmed cell death (2, 3). We found that induction of wild-type p53 (wtp53) following DNA damage increased the sensitivity to *vinca* alkaloids (4). This response was attributed to the ability of p53 to transcriptionally repress microtubule-associated protein 4 (MAP4) (5-7), a protein that regulates the polymerization state of microtubules (8-10). Since p53 is wild-type in approximately 50% of breast cancers (11-13), we sought to determine whether or not patients with breast cancer treated with doxorubicin, a DNA damaging drug, would also demonstrate induction of p53 and repression of MAP4, thereby creating a background of enhanced sensitivity to vinorelbine, a *vinca* alkaloid commonly used in the treatment of this disease

(14-16). We administered doxorubicin to patients with locally advanced or metastatic breast cancer and studied the effects on p53 and MAP4 expression in peripheral blood mononuclear cells (PBMNCs) and in breast cancer biopsies. In addition, we studied whether it would be safe to administer vinorelbine either 24 or 48 hours after doxorubicin treatment. We now report the safety and biological activity of this approach.

METHODS

Eligibility Criteria. Patients with histologically confirmed metastatic (stage IV) or locally advanced breast cancer (stage IIIb) without prior chemotherapy other than in the adjuvant setting were eligible for study. Eligible patients were 18 years of age or older, had an Eastern Cooperative Oncology Group performance status of 0-2, a life expectancy of more than 12 weeks, and provided written informed consent in accordance with institutional review board guidelines. Patients who had previously received doxorubicin were eligible if the total dose had not exceeded 300 mg/m². Prior hormonal therapy and localized radiotherapy for metastatic disease were permitted, but must have been completed three weeks before entry. Measurable disease was defined as follows: (1) any lesion with two measurable perpendicular diameters and (2) computed axial tomography (CT scan) demonstrating defects greater than 1.5 cm in diameter. Stage IIIb disease was assessed by measuring skin changes as well as tumor size. All patients were required to have adequate renal, hepatic, bone marrow, and cardiac function. Pretreatment evaluation performed within two weeks of initiation of therapy included a

complete history and physical examination, complete blood cell count, platelet count, serum chemistries, chest X-ray, electrocardiogram, site-specific imaging, and tumor measurements.

Patients were ineligible if they had a past history of cancer with the exception of non-melanoma skin cancer or treated in situ cervical carcinoma. Other exclusions included evidence of visceral crisis (lymphangitic pulmonary metastases, or liver or marrow replacement sufficient to cause significant dysfunction), or a pre-existing medical condition that would preclude the safe administration of chemotherapeutic drugs.

Treatment Plan. Patients received doxorubicin (40 mg/m^2) on Day 1 and vinorelbine (20 mg/m^2) on Day 2 and Day 9 (odd number enrollees) or on Day 3 and Day 10 (even number enrollees). If only one of the first six patients experienced a dose-limiting toxicity, the dose was increased to the previously established phase II doses of 50 mg/m^2 doxorubicin and 25 mg/m^2 vinorelbine. The second cycle began on Day 22. In patients with stage IIIb breast cancer, doxorubicin and vinorelbine were administered to maximal response defined as stability of disease for two cycles following an initial response. Patients with stage IV disease received doxorubicin and vinorelbine until progression. A multi-gated acquisition scan to assess left ventricular cardiac ejection fraction was obtained before treatment and after a total dose of 400 mg/m^2 of doxorubicin. PBMNCs and biopsies of accessible tumor tissue were obtained pre-treatment and 24 or 48 hr after doxorubicin, i.e., immediately before receiving vinorelbine.

Response and Toxicity Assessment. All treated patients with measurable disease were assessed for response. Tumor measurements were done every cycle by physical examination and every other cycle by tumor imaging. Responses were scored according to World Health Organization criterion. Physical examination and serum chemistries were repeated before each cycle and a complete blood cell count was obtained weekly. Toxicity was assessed using the National Cancer Institute Common Toxicity Criteria. Dose-limiting toxicity was defined as either: 1) < 500 neutrophils/ μl or $< 25,000$ platelets/ μl for > 5 days or febrile neutropenia, or 2) irreversible grade 2 or any grade 3-5 non-hematologic toxicity.

Evaluation of p53 and MAP4. PBMNCs were isolated from eight ml of heparinized blood by Ficoll Hypaque gradient centrifugation using VACUTAINER[®] CPT[™] tubes (Becton Dickinson, Franklin Lakes, NJ). Cells were washed with phosphate buffered saline by centrifugation at $700 \times g$ and lysed in RIPA buffer (10 mM sodium phosphate, pH 7.2, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1% aprotinin, 1 mM sodium orthovanadate, 1mM PMSF, 50 $\mu\text{g}/\text{ml}$ leupeptin). Protein was measured by the method of Bradford (17) (BIO-RAD, Hercules, CA). Proteins (30 μg) were resolved by 7.5% and 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The expression of MAP4, p53, and p21/WAF1 was assayed by Western blotting using the following monoclonal antibodies: MAP4, clone 18 (Transduction Labs, Lexington, KY); p53, clone DO-7 (DAKO Corporation, Carpinteria, CA) and; p21/WAF1, clone EA10 (Calbiochem, San Diego, CA). β -actin was used to

control for protein loading and transfer using clone AC-15 (Sigma, St. Louis, MO). A 1:1000 dilution of horseradish peroxidase-linked mouse IgGs (DAKO Corporation, Carpinteria, CA) in PBST (phosphate-buffered saline, 0.05% Tween-20) plus 1% nonfat milk was used as the secondary antibody in an enhanced chemiluminescence system (Amersham, Piscataway, NJ). Laboratory personnel were blinded to the results of treatment.

Core needle biopsies were used to obtain tumor tissue using a 14 gauge spring-loaded biopsy gun (Bard Inc., Covington, GA) after the area was cleaned with povidone-iodine and anesthetized with 1% lidocaine. Three to four cores were harvested at each time point to account for tumor heterogeneity. Skin lesions were biopsied using a 4 mm punch. One portion of each biopsy was embedded in paraffin; a second portion of the biopsy was immediately frozen in liquid nitrogen for Western blots. This portion was homogenized on ice in 20 mM HEPES, pH 7.4, 100 mM NaCl, 20 mM sodium pyrophosphate, 2 mM EDTA, 0.1 mM PMSF, 10 µg/ml leupeptin, 2 µg/ml aprotinin and 0.1 mM sodium orthovanadate with a Dounce homogenizer. Protein was quantified by the Bradford assay, subjected to 7.5% and 15% SDS-PAGE gels, and transferred to nitrocellulose for Western blot analysis of MAP4, p53, p21/WAF1 and β-actin as described above.

Immunohistochemistry. The monoclonal p53 antibody DO-7 (Dako Corporation, Carpinteria, CA) was used for immunohistochemistry as previously reported by our laboratory (18). Staining for MAP4 was performed using the anti-MAP4 antibody clone

18 (Transduction Labs, Lexington, KY). The anti-p53 antibody was diluted 1:10 in 1% bovine serum albumin/phosphate-buffered saline and anti-MAP4 antibody clone 18 was diluted 1:2000 in 1% bovine serum albumin/phosphate-buffered saline. Staining with mouse ascites fluid was used as a negative control. Staining with hematoxylin and eosin was performed as described previously (18).

RESULTS

We carried out a pilot clinical study to determine whether or not treatment with intravenous doxorubicin, a DNA damaging drug, increased p53 in patients with breast cancer as described previously in pre-clinical models (4), and whether this would lead to repression of MAP4. Patients received doxorubicin on Day 1 and vinorelbine either 24 or 48 hrs later.

Patient Characteristics. The characteristics of the 16 enrolled patients are listed in Table 1. Six of 16 patients had received adjuvant chemotherapy and two had received hormonal therapy for metastatic disease. There was a nearly equal number of patients with locally advanced (stage IIIb) and metastatic (stage IV) disease. All patients had an Eastern Cooperative Oncology Group performance status of 0-1. Four of 16 patients had visceral involvement as the dominant site of disease. A total of 82 cycles of treatment were delivered with a range of 2-10 per patient. Doses of doxorubicin and vinorelbine were escalated in this pilot study to the previously defined phase II doses (15) after the

first six patients were enrolled, as only one patient developed a dose-limiting toxicity (febrile neutropenia).

Induction of p53 and repression of MAP4. To determine whether or not treatment with intravenous doxorubicin increased p53 in patients, tumor and PBMNCs were assayed before and 24 or 48 hours after receiving doxorubicin. As shown in Figures 1 and 2, we found an induction of p53 in the PBMNCs of 12 of 14 patients and in four of 10 breast cancer biopsies following doxorubicin treatment.

We next asked whether or not induction of p53 following DNA damage with doxorubicin led to the repression of MAP4. As shown in Figure 1, we observed repression of MAP4 in PBMNCs from seven of 12 patients in which p53 was induced. Additionally, we found repression of MAP4 in three of the four breast biopsies in which p53 was induced (Figure 2). The majority of patients showing both increased p53 and repressed MAP4 also had increased expression of p21/WAF1, a transactivation target for wtp53 (5/7 PBMNC samples and 3/3 tumor specimens) (19). In two patients who provided blood and tumor samples at both 24 and 48 hours (patients 2 and 3), we found maximal induction of p53 at 24 hours and maximal repression of MAP4 at 48 hours (Figures 1 and 2). However, not all patients exhibited the anticipated changes in protein expression following treatment. For example, patient 5 demonstrated no change in p53 in tumor while patient 15 showed decreased p53 expression in PBMNCs and MAP4 repression. In addition, increased MAP4 was observed in four PBMNCs and one tumor biopsy following doxorubicin treatment.

Immunohistochemistry. To confirm that changes seen on Western blots represented changes in cancer cells rather than contaminating stroma, we analyzed p53 and MAP4 expression by immunohistochemistry. Figure 3 demonstrates increased expression of p53 at 24 hours and repression of MAP4 expression at 48 hours following doxorubicin treatment in the tumor cells of a representative patient (#3). Hematoxylin and eosin staining revealed the presence of infiltrating lobular carcinoma (panels A,B and E,F). In agreement with the short half-life of p53 of approximately 30 minutes in undamaged cells (20), staining for p53 was weak in untreated cancer cells (panel C) whereas MAP4 stained strongly positive in the cytoplasm of untreated cancer cells (panel G). Following doxorubicin treatment, staining for p53 increased in the nucleus (panel D) while cytoplasmic MAP4 decreased in tumor cells (panel H). Control staining with mouse ascites fluid was negative (data not shown).

Toxicities. The incidence of side effects from the 82 cycles of sequential doxorubicin and vinorelbine are listed in Table 2. There were no significant differences in toxicities between the 24 and 48 hours sequence, so these data were pooled. Neutropenia was the most common grade 3 and grade 4 toxicity followed by febrile neutropenia as the most common grade 3 toxicity. Two patients without previous anthracycline exposure developed congestive heart failure at cumulative doxorubicin doses of 350 mg/m² and 400 mg/m². One of these patients entered the study with a baseline ejection fraction of 50%. Four patients experienced grade 3-4 fatigue; one of these patients required a one-week delay in treatment. Three patients developed a thromboembolic event. One patient

was treated for an asymptomatic pulmonary embolism diagnosed on a restaging CT scan. Two additional patients developed a deep venous thrombosis with one complicated further by a pulmonary embolism. Twenty-six episodes of mild (grade 1-2) nausea and vomiting were observed. Only two episodes of grade 3 nausea and vomiting were reported. One patient developed grade 2 mucositis that prevented the administration of vinorelbine on Day 9 in two of four cycles. There was no significant neuropathy.

Responses. Seven of 16 patients had a partial response to treatment (Table 3). There were no complete responses; two patients progressed while on treatment. The response to doxorubicin followed by vinorelbine as a function of MAP4 expression is shown in Table 3. Four of eight patients with decreased MAP4 after doxorubicin responded to treatment while three had disease stabilization, and only one progressed.

DISCUSSION

The treatment of breast cancer improved when molecular markers were identified to predict response. For example, the ability to measure estrogen and progesterone receptors identified a patient population more likely to respond to hormonal therapies such as tamoxifen (21). Similarly, the measurement of HER-2/*neu* overexpression identified a subset of patients more likely to respond to trastuzumab (Herceptin[®]) (22). Yet, we have found few markers of sensitivity to most chemotherapeutic agents. In this pilot study, we examined the role of p53 and MAP4 as molecular determinants of chemosensitivity to drugs commonly used in the treatment of breast cancer.

p53 is wild-type in approximately 50% of human breast cancers (11-13). In response to DNA damage, wtp53 protein is stabilized and transcriptionally activates a series of genes having p53-responsive elements in their promoter regions (23-25). p53 can also repress transcription through an indirect mechanism requiring the cooperation between mSin3a, p53, and histone deacetylase 1 (HDAC1) (7). Several p53 repressible genes have been identified including the anti-apoptotic protein bcl-2, which may account for the propensity of cells with wtp53 to undergo apoptosis in response to DNA damage (3, 26). Murphy and colleagues demonstrated that MAP4 is repressed by wtp53 (5, 7). MAP4 is the major microtubule-associated protein in non-neuronal tissues and promotes polymerization of microtubules through binding to the carboxyl termini of α - and β -tubulin (27). We postulated and demonstrated that repression of MAP4 by wtp53 would increase the sensitivity to the *vinca* alkaloids by favoring the binding of these drugs to the greater mass of depolymerized tubulin heterodimers. In these experiments, cells in which DNA was damaged with irradiation, bleomycin, or doxorubicin increased wtp53, decreased MAP4, decreased tubulin polymerization, and increased binding of and sensitivity to *vinca* alkaloids (4).

These results raised important opportunities for the choice and sequence of chemotherapeutic drugs. However, the ability to regulate genes repressed by p53 in patients had not previously been demonstrated. Therefore, we have begun to translate our laboratory observations into the more complex clinical setting by administering the DNA damaging agent, doxorubicin, followed by the *vinca* alkaloid, vinorelbine.

Doxorubicin produces single and double strand DNA breaks through poisoning of topoisomerase II (28). Although the mechanism by which DNA damage leads to increased p53 protein stability is yet to be fully elucidated, it is known that phosphorylation of p53 by several kinases (e.g., ATM, DNA-dependent protein kinase) diminishes the interaction of p53 with MDM2, a protein that promotes p53 degradation (24, 25, 29, 30). We examined whether treatment of patients with doxorubicin increased p53 protein in both normal and tumor tissue. Figures 1 and 2 demonstrate that patients receiving standard doses of doxorubicin increase p53 in both PBMNCs (12/14) and in breast cancer biopsies (4/10). These percentages are consistent with the expectation that PBMNCs contain wtp53 and show increased p53 protein stability in the majority of samples in response to DNA damage by doxorubicin and that only 50% of breast cancers retain wtp53 (11-13); this is in general agreement with our finding that only 40% of the tumor samples demonstrated increased p53 protein stability following treatment with doxorubicin.

The second goal of this study was to determine whether or not doses of doxorubicin that produced increased p53 could also result in repression of MAP4. Figures 1 and 2 demonstrate that the induction of p53 led to the repression of MAP4 in most PBMNCs (7/12) and breast cancer biopsies (3/4). Immunohistochemistry verified that the repression of MAP4 following treatment is found in tumor cells rather than surrounding stroma (Figure 3). Consistent with these observations, the majority of patients showing both increased p53 and repressed MAP4 also have increased expression of p21 (5/7

PBMNC samples and 3/3 tumor specimens). Although the presence of tumor with wtp53 was not a requisite for this pilot study, the increased p21 protein expression in tumor tissue following doxorubicin treatment most likely reflects a wtp53 genotype as doxorubicin treatment fails to induce p21 in the presence of mutant p53 in cells and breast tumors (31, 32).

Not all patients exhibited the anticipated changes in response to doxorubicin. For example, three patients with induced p53 and p21 in PBMNC samples following doxorubicin had increased rather than decreased expression of MAP4 (patients 4, 13, and 14). Furthermore, MAP4 repression was observed in three tumor samples where we failed to detect induction of p53 (patients 5, 9, and 16). There are several possible explanations for these results. First, the failure to detect increased p53 in certain specimens may be due to the timing of sampling, i.e., the transient induction of p53 at an earlier time-point may have been missed (29, 30). It is also possible that other p53 family members compensate for loss of p53 function in these cases. For example, p73 is infrequently mutated in breast cancer and can be induced by DNA damage (33-35). Second, the response of p53 to DNA damage may differ in normal cells from that of cancer cells. To date, the relationship between p53 and MAP4 has been predominantly studied in cycling immortal or transformed tumor cells (4-6), whereas PBMNCs are terminally differentiated, quiescent cells. In this regard, Siu et al., reported that quiescent Swiss 3T3 cells had lower levels of p53 and p21 in response to doxorubicin and had longer survival than proliferating cells (36). Third, the failure to detect the induction of p53 or repression of MAP4 may also be due to the drug concentration and extent of DNA

damage at the time of sampling. However, we found that treatment of PBMNCs in vitro with concentrations of doxorubicin equal to that achieved in patients (37) was able to induce p53, p21 and repress MAP4 in the majority but not all cases (Bash-Babula and Hait, unpublished). Repression of genes such as MAP4 is dependent on both the amount of p53 induced and the duration of time that p53 remains elevated in the target tissue (38). Differences in proteins that degrade p53 such as mdm2 could also account for inter-patient variations. Finally, repression of MAP4 by p53 can be blocked by cellular proteins that can be differentially expressed such as bcl-2 and WT-1 (5, 39, 40). Therefore, in future studies it will be critical to gain a more robust picture of the genomic and proteomic profile of the individual tumor under treatment before one might predict with accuracy the effects of p53 induction on gene expression.

A third goal of this trial was to determine the optimal sequencing of the two chemotherapeutic drugs. We chose 24 and 48 hours post-doxorubicin for the injection of vinorelbine based on pre-clinical models demonstrating maximal repression of MAP4 during this time period (4). In two patients who provided blood and tumor samples at both 24 and 48 hours (patients 2 and 3, respectively) the induction of p53 peaked at 24 hours and the repression of MAP4 was most pronounced at 48 hrs (Figures 1 and 2). This result is consistent with the 14 hour half-life of MAP4 (M. Murphy, personal communication), and the negative feedback on p53 by MDM2 (29, 30).

The induction of p53 and repression of MAP4 in normal tissues could potentially increase the toxicity of *vinca* alkaloids, thereby limiting the therapeutic index of this

combination. Anticipating increased toxicity with sequential versus combined treatment, the first six patients were treated with 40 mg/m² and 20 mg/m² of doxorubicin and vinorelbine treatment, respectively; these were escalated to the phase II established maximum tolerated dose of the doxorubicin/vinorelbine combination of 50 mg/m² and 25 mg/m² after only one of the first six patients experienced significant toxicity. The toxicities seen with the sequential use of doxorubicin and vinorelbine do not appear to be in excess of those seen when doxorubicin and vinorelbine are given together (14-16). As was observed in previously reported phase II studies, febrile neutropenia and neutropenia were the most common grade 3 and grade 4 toxicities, respectively (14-16). The incidence of cardiac dysfunction observed in this study (12.5%) was higher than anticipated. Anthracycline-induced cardiotoxicity is dose dependent and cumulative (41) and is unusual at a cumulative dose of less than 450 mg/m², in the absence of other risk factors (42). The two patients who developed congestive heart failure were at cumulative doses of doxorubicin at 350 mg/m² and 400 mg/m²; both had a history of hypertension and were older than 65 years of age. One of the two patients entered the study with a baseline ejection fraction of 50% and her ejection fraction obtained at 300 mg/m² was unchanged from baseline. The occurrence of thromboembolic events in three patients is consistent with the 2%-18% incidence in patients with advanced stage cancers treated with chemotherapy (43).

The responses to sequential doxorubicin and vinorelbine in this trial (44%) were in the range for those previously reported for this combination. This was observed despite the lower dose of doxorubicin and vinorelbine used to treat the first six patients in

comparison to the established phase II doses (14-16). Six of ten patients responded to treatment when the established phase II doses were reached.

In summary, this is the first trial designed to identify enhanced drug sensitivity through the induction of p53 and alteration of a downstream, p53-regulated, gene product. These data demonstrate that the molecular changes seen in tissue culture can be seen in patients treated with doxorubicin, albeit with significant variability. This study demonstrated the relative safety and feasibility of sequential doxorubicin/vinorelbine and provided important information for a phase II trial exclusively in patients with wtp53 breast cancer.

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TABLE 1. Patient Characteristics

Characteristics	# of	Percentage
Age:		
< 50	5	31%
> 50	11	69%
Median: 61		
Range: 32-86		
ECOG Performance Status:		
0	12	75%
1	4	25%
Ethnic Background/Race:		
Caucasian	10	63%
Hispanic	1	6%
African-American	3	19%
Asian	2	12%
Stage:		
IIIb	9	56%
IV	7	44%
Dominant Site of Disease:		
Visceral involvement	4	25%
Bone	3	19%
Soft tissue	9	56%
Estrogen Receptor Status:		
Positive	8	47%
Negative	8	53%
Progesterone Receptor Status:		
Positive	4	25%
Negative	12	75%
Previous Treatment:		
Surgery	9	56%
Adjuvant Chemotherapy	3	19%
Adjuvant Tamoxifen	2	13%
Adjuvant Chemotherapy/Tamoxifen	3	19%
Radiation	4	25%
None	7	44%

TABLE 2. Overall Incidence of Toxicities

TOXICITY GRADE				
Toxicity (grade)	1	2	3	4
Anemia	1	6	3	
Neutropenia		12	9	10
Febrile Neutropenia			5	
Fever (in the absence of neutropenia)/infection	8	2	1	
Fatigue (lethargy, malaise, asthenia)	8	5	2	2
Anorexia/Poor Appetite	5			
Constipation	4	1	1	
Diarrhea	5	3		
Mucositis (mouthsores-stomatitis/pharyngitis)	1	2		
Heartburn (dyspepsia)	4	3		
Nausea	10	6	1	
Vomiting	4	6	1	
Increase in SGOT (AST)	1	3		
Increase in SGPT (ALT)	3	2		
Neurology Dizziness/lightheadedness		1	1	
Mood alteration - anxiety/depression	1	3		1
Neuropathy - sensory	2			
Arthralgia (joint pain)/Myalgia (muscle pain)	5	3	1	
Headache	4			
Dyspnea (Shortness of breath)	1	4	2	
Deep venous thrombosis			2	
Pulmonary embolism				2
Cardiac Left Ventricular Function			2	

TABLE 3. Response Data as a Function of MAP4

Expression in Tumor Biopsies

Patient	MAP4 expression	Response
1	N/A*	Progression
2	N/A	Stable
3	Decrease	Partial
4	N/A	Stable
5	Decrease	Stable
6	Decrease	Stable
7	Increase	Stable
8	No Change	Partial
9	Decrease	Partial
10	Decrease	Partial
11	N/A	Partial
12	N/A	Partial
13	Decrease	Progression
14	Decrease	Stable
15	N/A	Stable
16	Decrease	Partial

* Tumor tissue was not available from these patients.

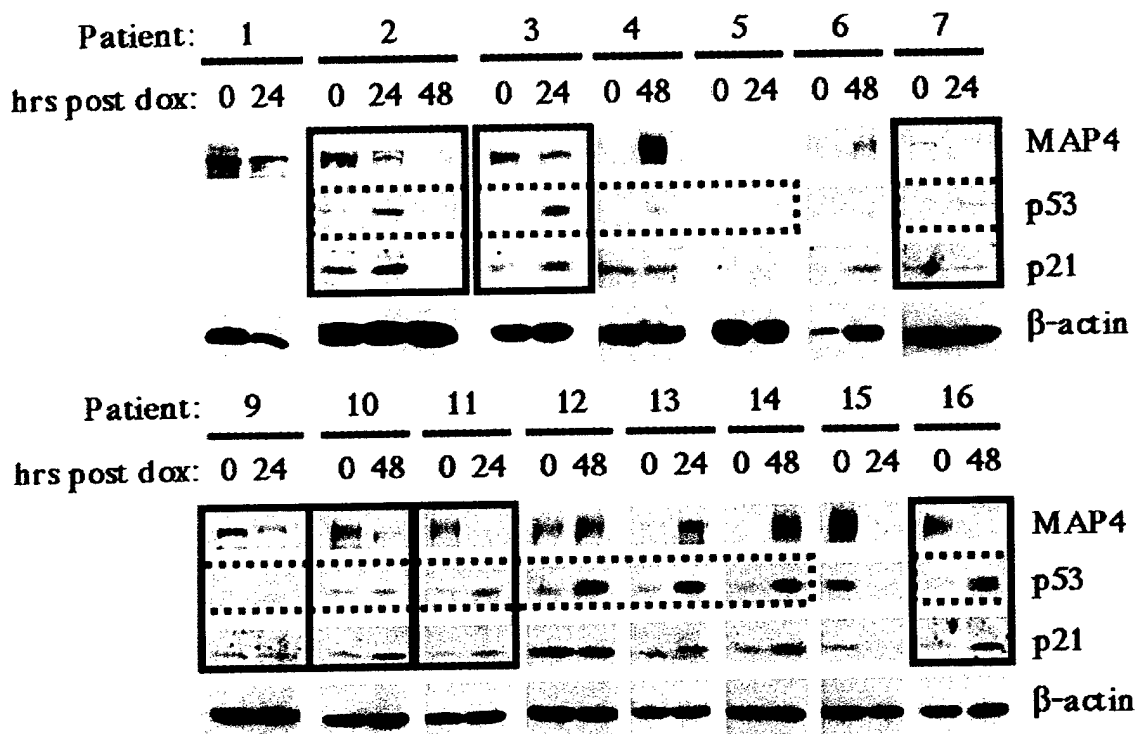


Figure 1. Western blot of MAP4, p53 and p21 expression in PBMNCs before and after doxorubicin. Equal amounts of protein (30 μ g) were loaded onto 7.5% and 15% SDS-PAGE gels and proteins detected by enhanced chemiluminescence as described in methods. Dashed-line boxes highlight patients with increased p53 post-doxorubicin. Black boxes denote patients with both increased p53 and decreased MAP4 post-doxorubicin. Blood was not available from patient 8 and insufficient material precluded analysis of p53 and p21 in patient 1. The blots are representative of two experiments.

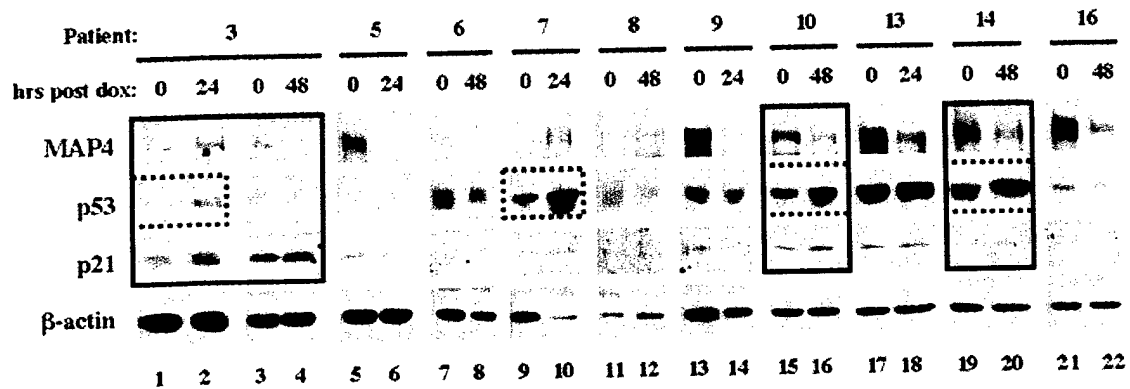


Figure 2. Western blot of MAP4, p53 and p21 expression in tumor samples before and after doxorubicin. Equal amounts of protein (30 μ g) were loaded onto 7.5% and 15% SDS-PAGE gels and proteins detected by enhanced chemiluminescence as described in methods. Dashed-line boxes highlight patients with increased p53 post-doxorubicin. Black boxes denote patients with both increased p53 and decreased MAP4 post-doxorubicin. The blots are representative of two experiments.

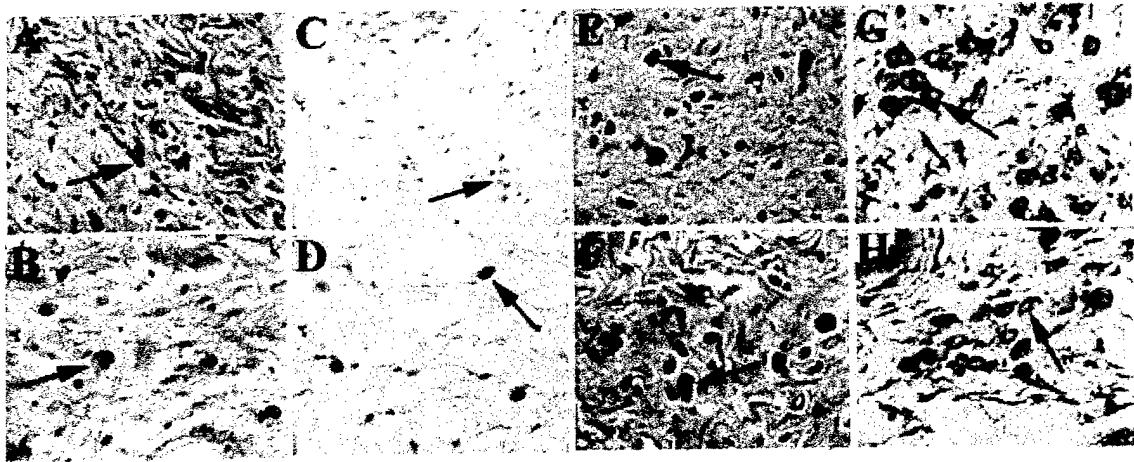


Figure 3. Immunohistochemical staining of p53 and MAP4 in breast tumor tissue. Patient 3 tumor samples were either untreated (A,C) and 24 hours post-doxorubicin treatment (B,D) or untreated (E,G) and 48 hours post-doxorubicin treatment (F,H). Tissues were stained with hematoxylin and eosin (panels A,B,E,F) or immunostained with p53 (panels C,D) and MAP4 antibodies (panels G,H) as described in methods. Tissue was counterstained with toluidine blue and photographed at a magnification of 60X. Arrows denote tumor cells.