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Raf-1/bcl-2 Phosphorylation: A Step from Microtubule Damage to Cell Death

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ABSTRACT

Recent studies have shown that paclitaxel leads to activation of Raf-1 kinase and have suggested that this activation is essential for bcl-2 phosphorylation and apoptosis. In the present study, we demonstrate that, in addition to paclitaxel, other agents that interact with tubulin and microtubules also induce Raf-1/bcl-2 phosphorylation, whereas DNA-damaging drugs, antimetabolites, and alkylating agents do not. Activation of Raf-1 kinase by paclitaxel is linked to tubulin polymerization; the effect is blunted in paclitaxel-resistant cells, the tubulin of which does not polymerize following the addition of paclitaxel. In contrast, vincristine and vinblastine, drugs to which the paclitaxel-resistant cells retain sensitivity were able to bring about Raf-1 phosphorylation. The requirement for disruption of microtubules in this signaling cascade was strengthened further using paclitaxel analogues by demonstrating a correlation between tubulin polymerization, Raf-1/bcl-2 phosphorylation, and cytotoxicity. Inhibition of RNA or protein synthesis prevents Raf-1 activation and bcl-2 phosphorylation, suggesting that an intermediate protein(s) acts upstream of Raf-1 in this microtubule damage-activating pathway. A model is proposed that envisions a pathway of Raf-1 activation and bcl-2 phosphorylation following disruption of microtubular architecture, serving a role similar to p53 induction following DNA damage.

INTRODUCTION

Activation of Raf-1 begins with the recruitment of Raf-1 by Ras on the plasma membrane and provides a mechanism for signal transduction by receptor-coupled tyrosine kinases, PKC,[2] and a variety of other protein kinases leading to cell proliferation or differentiation (for review, see Refs. 1–3). Raf-1 can also interact with bcl-2 protein, thus performing a different function at a distinct cellular location (4). Recent studies have shown that treatment of human carcinomas with PTX can activate Raf-1 (5). This activation does not depend on PMA-regulated PKC, leads to bcl-2 phosphorylation, and results in cell death (6).

PTX binds to and stabilizes microtubules, preventing depolymerization. This results in G_2-M arrest, microtubular bundling, and cell death (7–12). In contrast, the *Vinca* alkaloids bind monomeric tubulin, preventing microtubule assembly and blocking progression through the cell cycle. This results in cell death, which has been shown to depend on new protein synthesis (13, 14).

Since its original isolation, bcl-2 expression has been documented in a variety of normal and transformed cell types (15, 16). In some models, bcl-2 can promote cell survival by inhibiting the process of programmed cell death or apoptosis (17, 18). Phosphorylation of bcl-2 was shown originally in leukemic cells; treatment with phosphatase inhibitors resulted in cell death, suggesting that phosphorylation of bcl-2 inhibits its function (19). These observations were extended to prostate cancer cells and led to speculation that phosphorylation could result in the acquisition of apoptotic functions (20).

Recent studies have suggested that phosphorylation of bcl-2 following PTX treatment depends on Raf-1 activation (6). Raf-1 activation could be a consequence of tubulin binding or could be mediated through interaction of PTX with other targets. To address this question, we examined PTX-resistant cells the tubulin of which is unresponsive to PTX, to determine whether phosphorylation of Raf-1 or bcl-2 could occur in the absence of tubulin polymerization. We also compared PTX analogues with different potencies for their ability to induce Raf-1/bcl-2 phosphorylation in MCF-7 cells. We conclude that Raf-1 activation is dependent on the interaction of PTX with the microtubular network. Similar observations with other microtubule-active agents suggests that Raf-1/bcl-2 phosphorylation occurs in response to microtubule damage. Activation of this pathway mediates the cytotoxicity of microtubule-active drugs.

MATERIALS AND METHODS

Materials. PTX, vincristine, PMA, daunomycin, Adriamycin, wortmannin, and chelerythrine, were obtained from Sigma Chemical Co. and dissolved in DMSO as stock solutions. Actinomycin D, mitomycin C, vinblastine, melphalan, cycloheximide, and methotrexate were also Sigma Chemical Co. products and were prepared as stock solutions in water or 0.1% bicarbonate (methotrexate). Cisplatin was a Bristol-Myers product and was dissolved in water. Jasplakinolide, podophyllotoxin, and maytansine were obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute and were dissolved in DMSO. Sodium orthovanadate was an Aldrich product and was dissolved in water. Epothilones A and B were provided by E. Hamel (Laboratory of Molecular Pharmacology, National Cancer Institute) and were dissolved in DMSO. PTX analogues were prepared from PTX by the methods described previously (21). Murine monoclonal anti-bcl-2 antibody (DAKO) anti-p53 (PAb 1801, Oncogene Science), anti-α-tubulin (Sigma Chemical Co.) and rabbit polyclonal anti-Raf-1 antibody (Santa Cruz) were used for immunoblotting. Enhanced chemiluminescence immunoblotting detection reagents were obtained from Amersham Corp.

Cell Culture. MCF-7 is a human breast cancer cell line. A2780(1A9) is a single-cell clone of the human ovarian carcinoma cell line A2780. 1A9(PTX22) is a PTX-resistant subline derived from A2780(1A9) cells. The PTX22 subline was isolated initially as a single clone in a single-step selection by exposing A2780(1A9) cells to 5 ng/ml PTX and 5 μg/ml verapamil. Verapamil, an antagonist of the Pgp drug efflux pump, was included in the selection to preclude the isolation of a subline overexpressing Pgp. The resistant subline does not express Pgp, and its tubulin does not polymerize when exposed to PTX *in vivo* or *in vitro* (22). The cells are cultured in media containing 15 ng/ml PTX and 5 μg/ml verapamil. The drug-resistant phenotype and lack of tubulin polymerization have been shown to be stable over 2 years in the absence of drug. For the experiments described herein, drugs were removed 2 weeks prior to an experiment. The DU145 prostate cancer cell line was obtained from American Type Culture Collection (Rockville, MD). Cells were cultivated in either DMEM (MCF-7) or RPMI 1640 [A2780(1A9), 1A9(PTX22), and DU145] media supplemented with 10% fetal bovine serum.

Immunoblotting. Cells in log phase were washed in PBS and lysed in TNESV buffer (50 mM Tris 7.5, 2 mM EDTA, 100 mM NaCl, 1 mM vanadate, and 1% NP40) as described previously (6). Protein concentrations were determined using the bicinchoninic acid reagent (Pierce Chemical Co.). Equal amounts of protein were separated on 7.5% (Raf-1 and p53) or 12.5% (bcl-2) polyacrylamide gels, transferred, and probed with antibodies (5).

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[2] The abbreviations used are: PKC, protein kinase C; PTX, paclitaxel; PMA, phorbol 12-myristate 13-acetate; Pgp, P-glycoprotein; DAPI, 4,6 diamino-2-phenylindole; IL, interleukin.

Cytotoxicity Assay. Cytotoxicity assays were performed in 96-well microtiter plates by seeding 500 cells per well as described previously (23). Untreated control wells were assigned a value of 100%. IC_{50} values are the concentration at which cell growth was inhibited by 50%, and LC_{50} values represent the concentration of drug lethal to 50% of the cells seeded originally.

Tubulin Polymerization Assay. Tubulin polymerization was quantitated using a simple assay modified from Minotti *et al.* (24). Cells grown to confluency in 24-well plates were treated, washed twice with PBS, lysed for 5 min with 100 μl of hypotonic buffer and transferred to Eppendorf tubes (hypotonic buffer: 1 mM $MgCl_2$, 2 mM EGTA, 0.5% NP40, 2 mM phenylmethylsulfonyl fluoride, 200 units/ml aprotinin, 100 μg/ml soybean trypsin inhibitor, 5 mM ε-amino-caproic acid, 0.01 nM benzamidine and 20 mM Tris-HCl, pH 6.8). The wells were rinsed with an additional 100 μl, and this was pooled with the lysates. The samples were centrifuged at 14,000 rpm for 10 min at room temperature, and the 200 μl of supernatants containing soluble (cytosolic) tubulin were separated from the pellets containing polymerized (cytoskeletal) tubulin. The pellets were resuspended in 200 μl of hypotonic buffer. Both fractions were mixed with 70 μl of 4× SDS-PAGE sample buffer (4× sample buffer: 45% glycerol, 20% β-mercaptoethanol, 9.2% SDS, 0.04% bromphenol blue, and 0.3 M Tris-HCl, pH 6.8), heated for 5 min at 95°C, and analyzed by SDS-PAGE on a 10% resolving gel with a 3% stacking gel. Immunoblotting was performed using a primary antitubulin antibody and enhanced chemiluminescence (Amersham Corp.).

Apoptotic Assay. Nuclear fragmentation was visualized using DAPI staining by UV microscopy as described (25). With this protocol, significant staining of DNA with DAPI is obtained only in dead cells.

RESULTS

Drugs Targeting Microtubules Phosphorylate Raf-1 and bcl-2. Previous studies showed that treatment of MCF-7 cells with PTX but not mitomycin C resulted in Raf-1/bcl-2 phosphorylation (6). To extend these observations, we examined additional DNA-damaging drugs, alkylating agents, antimetabolites, an actin-binding drug, and compounds that interact with tubulin or microtubules. Phosphorylation of Raf-1 kinase or bcl-2 delays mobility and can be detected after electrophoresis (19). Fig. 1 shows the results with cellular extracts from MCF-7 cells harvested after a 16-h drug exposure. Drug concentrations were comparably cytotoxic. Fig. 1*A* shows that, as with mitomycin C, phosphorylation of Raf-1 or bcl-2 was not observed with other DNA-damaging agents, including daunomycin, actinomycin D, and Adriamycin under conditions that result in the accumulation of p53. Phosphorylation of Raf-1 or bcl-2 was also not observed with melphalan, cisplatin, 5-fluorouracil, and methotrexate. Nor was it observed after treatment with jasplakinolide, which induces actin polymerization, or orthovanadate, which causes G_2-M arrest as shown in Fig. 1*B* (26, 27). At the concentrations used, the latter increased the fraction in G_2-M from about 14 to 39% (data not shown). In contrast, like PTX treatment, exposure to a broad concentration range of either vincristine or vinblastine led to phosphorylation of Raf-1 and bcl-2, as shown in Fig. 1*C*. In the experiment shown, Raf-1/bcl-2 phosphorylation occurred at concentrations as low as 0.01 μM PTX, vincristine, or vinblastine, and the results were not enhanced by higher concentrations. This correlates with the cytotoxic activity of these drugs, which demonstrate near-maximal toxicity at concentrations of 0.01 μM or less.

Because PTX and the *Vinca* alkaloids have disparate effects on the microtubular network, the observations were extended further by examining additional drugs known to target microtubules: (*a*) epothilones A and B, which promote tubulin polymerization (28); and (*b*) colchicine, podophyllotoxin, and maytansine, which depolymerize

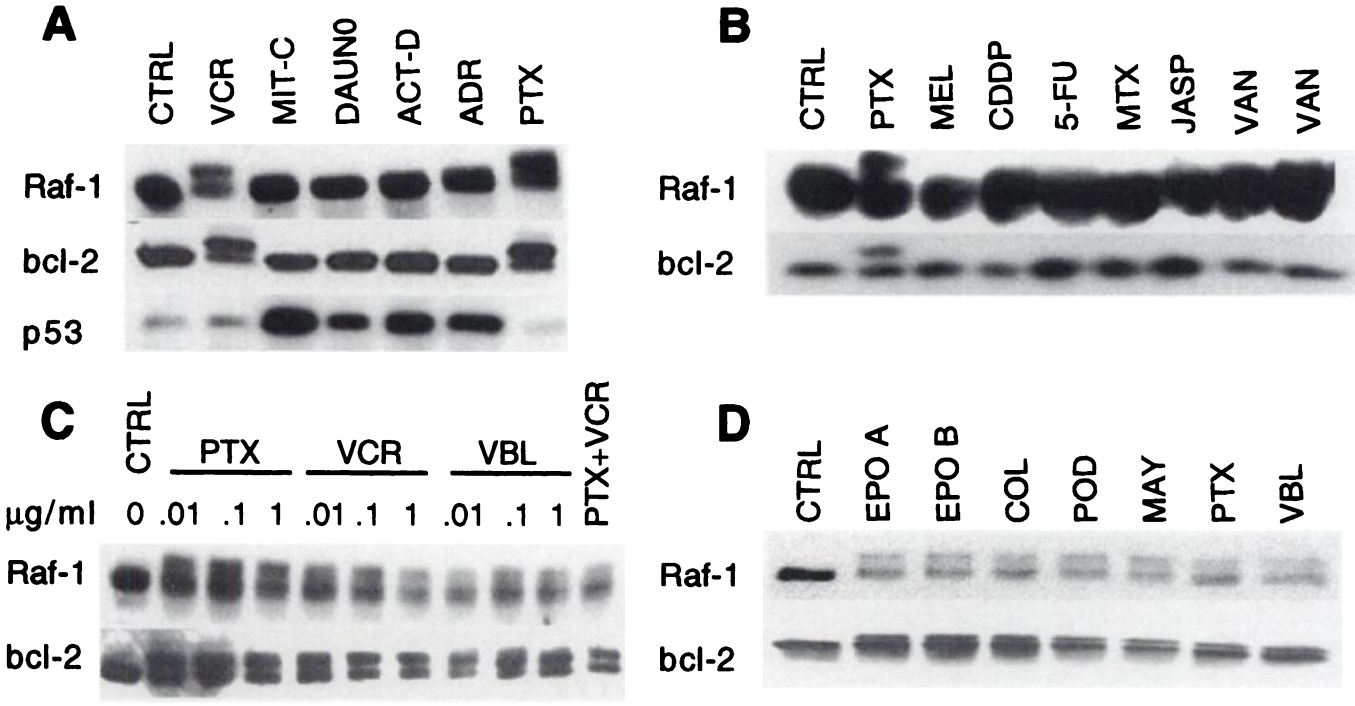

Fig. 1. Microtubule-active drugs induced Raf-1/bcl-2 phosphorylation. *A*, phosphorylation of Raf-1/bcl-2 and induction of p53: DNA-damaging agents. Immunoblot of untreated MCF-7 cells (*CTRL*) or MCF-7 cells treated with DNA-damaging agents or microtubule-active drugs for 16 h. Drug concentrations: 1 μg/ml vincristine (*VCR*); 10 μg/ml mitomycin C (*MIT-C*); 2 μg/ml daunomycin (*DAUNO*); 2 μg/ml actinomycin D (*ACT-D*); 5 μg/ml Adriamycin (*ADR*); or 1 μg/ml PTX (*PTX*). *B*, phosphorylation of Raf-1/bcl-2: agents with diverse mechanisms of cytotoxicity. Untreated (*CTRL*) MCF-7 cells or MCF-7 cells treated with drugs for 16 h. Drug concentrations: 0.25 μM PTX (*PTX*); 50 μM cisplatin (*CDDP*); 25 μM melphalan (*MEL*); 250 μM 5-fluorouracil (*5-FU*); 250 μM methotrexate (*MTX*); 2.5 μM jasplakinolide (*JASP*); 30 or 50 μM orthovanadate (*VAN*). *C*, phosphorylation of Raf-1/bcl-2: dose response. MCF-7 cells treated with PTX (*PTX*), vincristine (*VCR*), or vinblastine (*VBL*) for 16 h. Drug doses: 0.01 μg/ml; 0.1 μg/ml, and 1 μg/ml. Untreated cells (*CTRL*) and cells treated with a combination of PTX (0.1 μg/ml) and vincristine (0.1 μg/ml; *PTX + VCR*) are also included. D, phosphorylation of Raf-1/bcl-2: microtubule-active agents. Untreated MCF-7 cells (*CTRL*) and cells treated with seven microtubule-active drugs for 16 h. Drug concentrations: 0.1 μM epothilones A (*EPO A*) and B (*EPO B*); 0.25 μM colchicine (*COL*), podophyllotoxin (*POD*), maytansine (*MAY*), PTX (*PTX*), and vinblastine (*VBL*).

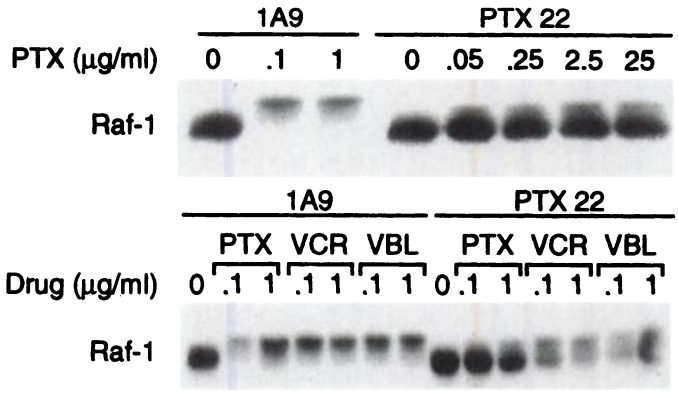

Fig. 2. PTX does not induce Raf-1 phosphorylation in PTX-resistant cells. *Top*, dose-dependent Raf-1 phosphorylation. Immunoblot of protein from parental A2780(1A9) cells and the PTX-resistant clone, 1A9(PTX22), treated with a range of PTX concentrations. *Bottom*, effect of the microtubule-active agents, vincristine (*VCR*) and vinblastine (*VBL*), on Raf-1 phosphorylation in parental and PTX-resistant cells: comparison with PTX (*PTX*). Immunoblot of protein from untreated cells (*0*) and cells treated with 0.1 μM and 1 μM drug for 16 h.

tubulin (29). As shown in Fig. 1*D*, treatment of MCF-7 cells with these drugs (16 h, 0.25 μM) led to phosphorylation of Raf-1 and bcl-2. Thus, in contrast to DNA-damaging agents, drugs that target tubulin induce phosphorylation of Raf-1/bcl-2.

PTX Treatment Does Not Result in Raf-1 Phosphorylation in PTX-resistant Cells. To clarify the role of microtubules in this pathway, parental A2780(1A9) ovarian carcinoma cells and three PTX-resistant sublines were examined. PTX resistance in these cells is not associated with reduced PTX accumulation, but it is associated with a failure of polymerization following PTX treatment of either cells, crude extracts, or purified tubulin (22). The resistant sublines are 20 to 30-fold less sensitive to PTX, but they retain sensitivity to the *Vinca* alkaloids (cross-resistance: 0.25–0.5, indicating the PTX-resistant cells are 2–4 fold more sensitive). PTX treatment resulted in Raf-1 phosphorylation in parental A2780(1A9) cells as shown in the upper panel of Fig. 2. In this cell line, complete phosphorylation of Raf-1 occurs following PTX, with the disappearance of the faster migrating band. In contrast, significant Raf-1 phosphorylation was not observed in the resistant cells (results with 1A9(PTX22) clone shown, similar results observed with two others); at higher doses of PTX, minimal retardation was observed, and this did not increase at higher concentrations. Under these conditions, tubulin polymerization does not occur in the resistant cells (not shown).

The lack of phosphorylation was observed only with PTX, as shown in Fig. 2 (*bottom*), which demonstrates the changes when parental cells and the 1A9(PTX22) clone were treated with either PTX, vincristine, or vinblastine (similar results were observed with two other clones). In parental cells, Raf-1 phosphorylation can be seen after exposure to each drug, whereas in the resistant subline, an effect is seen clearly only with vincristine and vinblastine. These results indicate that the pathway leading to Raf-1 activation is intact in the resistant cells and suggest that phosphorylation of Raf-1 requires drug interaction with microtubules. Low bcl-2 levels precluded accurate quantitation of bcl-2 phosphorylation in these cells.

Active PTX Analogues Phosphorylate Raf-1 and bcl-2. To further probe the relationship between PTX-induced tubulin polymerization and Raf-1/bcl-2 phosphorylation, we compared 14 PTX analogues. Previous studies have shown that these analogues vary in cytotoxicity and in potency as measured by *in vitro* polymerization assays using purified brain tubulin (21).[3] In MCF-7 cells, a broad range of toxicity and induced polymerization was observed (Fig. 3). The ability to induce Raf-1/bcl-2 phosphorylation (0.1 μm, 16 h) correlated with both drug potency, as measured by cytotoxicity, and tubulin polymerization. The analogues that induced Raf-1/bcl-2 phosphorylation were the more toxic compounds that resulted in nearly total tubulin polymerization (90–99%). Those analogues that induced Raf-1/bcl-2 phosphorylation increased the percentage of polymerized tubulin by 66.6 ± 2.5% compared to the inactive analogues that only increased polymerization by 18.8 ± 6.5% ($P \leq 0.000003$, two-tailed, non-paired Student *t* test).

Raf-1 Activation Depends on New Protein Synthesis. Although the results indicate that PTX-induced tubulin polymerization is necessary for Raf-1 activation, previous studies demonstrated that this interaction did not result in immediate Raf-1 activation (6). In MCF-7 cells, phosphorylation of Raf-1/bcl-2 was not observed until 4 h of PTX treatment; a maximal effect was observed after 12–16 h. Such a delay may reflect the time elapsed before a critical level of microtubule disruption occurs, or it may indicate that an unidentified step(s) occurs proximal to Raf-1 phosphorylation. A possible role for PKC in Raf-1 phosphorylation appears unlikely. Previous experiments demonstrated that chronic PMA exposure with down-regulation of PKC did not prevent PTX-induced Raf-1/bcl-2 phosphorylation (15). This was confirmed in the present study, as shown in Fig. 4*A*. Furthermore, adding the specific PKC inhibitor, chelerythrine (1 μM) with PTX did not inhibit Raf-1/bcl-2 phosphorylation. Likewise, involvement of PI-3 kinase was excluded by the lack of effect of 10 μM wortmannin. In contrast, it appears that Raf-1 activation by PTX requires both new RNA and new protein synthesis, because the addition of either actinomycin D or cycloheximide simultaneously with PTX inhibited Raf-1/bcl-2 phosphorylation. A time course demonstrated that treatment with cycloheximide for 4 h abrogated the PTX-induced activation, as shown in Fig. 4*B*.

Raf-1 Activation by PTX Occurs Independent of bcl-2 Levels. Previous experiments and those described herein demonstrate that bcl-2 phosphorylation by PTX requires activation of Raf-1 (6). We sought to determine whether bcl-2 was required for Raf-1 activation. As shown in Fig. 2, Raf-1 activation can occur in cells expressing low or undetectable levels of bcl-2 [A2780(1A9) and its PTX-resistant sublines]. Additionally, similar results were observed in DU145 prostate carcinoma cells in which bcl-2 is undetectable (Fig. 4). PTX treatment induced phosphorylation of Raf-1 in DU145 as shown in Fig. 4*C*. Moreover, as in MCF-7 cells, this effect was abrogated by the simultaneous addition of cycloheximide or actinomycin D, and unaffected by pretreatment with 1 μM PMA for 20 h, or cotreatment with 10 μM wortmannin or 1 μM chelerythrine. In addition, we have observed similar Raf-1 phosphorylation following PTX in MDA-MB231 parental cells and in a bcl-2-transfected clone with very high levels of bcl-2 expression (data not shown).

Cycloheximide or Actinomycin D Inhibit PTX Cytotoxicity. Finally, both cycloheximide and actinomycin D, the agents that abrogated Raf-1 activation and bcl-2 phosphorylation, also blocked cytotoxicity induced by PTX. As shown in Fig. 5, cotreatment with either agent protects against PTX-induced apoptosis.

DISCUSSION

Studies with PTX reporting an increasing number of cellular effects suggest that this drug has complex interactions beyond stabilization of microtubules (14, 30–32). Although the toxicity of PTX correlates with its effect on microtubules (9–11), other targets or secondary pathways may lead to cell death. Recent studies have demonstrated that PTX treatment activates Raf-1 kinase, leading to bcl-2 phosphorylation and apoptosis (6, 20). The present investigations demonstrate

[3] E. Hamel, personal communication.

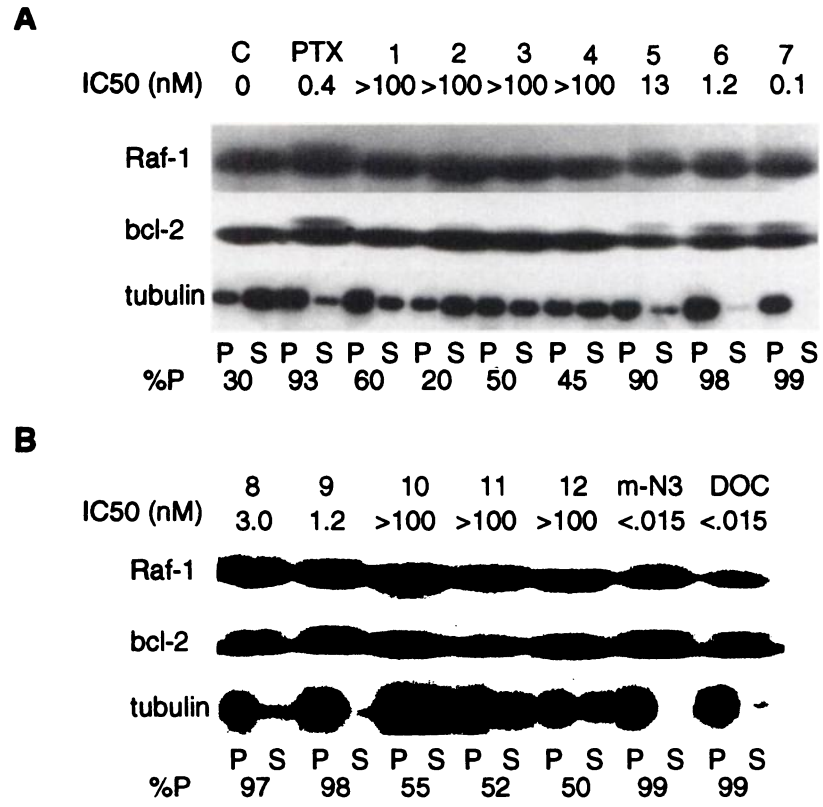

Fig. 3. Effect of PTX analogues on Raf-1/bcl-2 phosphorylation and microtubule polymerization. MCF-7 cells were left untreated (*CTRL*) or were treated with 0.1 μM PTX (*A, PTX*) or a PTX analogue (*B, 1–12, m-N3*) or docetaxel (*DOC*) for 16 h. The PTX analogues were all 2-debenzoyl-2-acyl derivatives of PTX and are identified by the acyl group: *1*, 3,5-dinitrobenzoyl; *2*, 4-nitrobenzoyl; *3*, valeryl; *4*, 2,3-dimethoxybenzoyl; *5*, 3-trifluoromethylbenzoyl; *6*, 3-fluorobenzoyl; *7*, 3,5-difluorobenzoyl; *8*, 2,3-difluorobenzoyl; *9*, 3,5-dichlorobenzoyl; *10*, 3-furoyl; *11*, 2-furoyl; *12*, 3-propoxybenzoyl; *m-N_3*, 3-azidobenzoyl. The number above each lane is the IC_{50} (nM), defined as the concentration that inhibited growth by 50% in a 96-h assay. Phosphorylation of Raf-1 and bcl-2 is shown. Cells in separate wells were processed for quantitation of tubulin polymerization. The soluble (*S*) and polymerized (*P*) fractions were loaded in adjacent wells, and immunoblotting was performed with an anti-α-tubulin antibody.

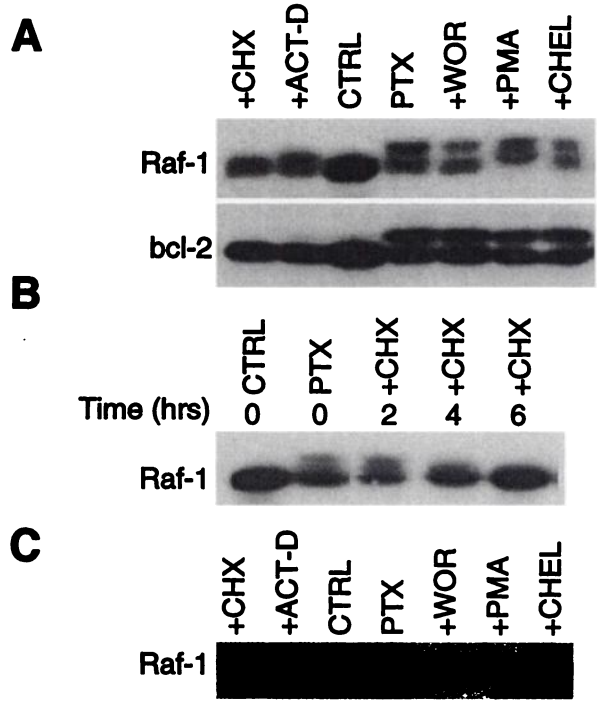

Fig. 4. Effect of inhibitors of RNA synthesis, protein synthesis, and cellular kinases on PTX-induced Raf-1/bcl-2 phosphorylation in MCF-7 cells; comparison with DU145 cells without detectable bcl-2. *A*, Raf-1/bcl-2 phosphorylation: inhibitors of protein synthesis, RNA synthesis, and cellular kinases. Untreated MCF-7 cells (*CTRL*) and MCF-7 cells treated with drug(s) for 12 h. Cells were treated with 0.25 μM PTX alone (*PTX*) or simultaneously with PTX and either 100 μg/ml cycloheximide (*+CHX*), 5 μg/ml actinomycin D (*+ACT-D*), 10 μM wortmannin (*+WOR*), or 1 μM chelerythrine (*+CHEL*). Cells in the *+PMA lane* were pretreated for 20 h with 1 μM PMA before the addition of PTX. *B*, Raf-1 phosphorylation. Time course of cycloheximide effect: MCF-7 cells were treated with 250 nM PTX for 16 h. Cycloheximide (100 μg/ml) was added for the last 2, 4, or 6 h as shown. The results are compared to untreated cells (*CTRL*) and cells treated only with PTX (*PTX*). *C*, Raf-1 activation does not depend on bcl-2; Raf-1 activation in DU145 cells. Cells were treated as in *A*.

that Raf-1 activation requires the interaction of PTX with tubulin. Raf-1 activation was diminished markedly in PTX-resistant sublines in which tubulin polymerization does not occur following PTX, although other microtubule-active agents were able to induce Raf-1 phosphorylation. In addition, a correlation was found using PTX analogues between tubulin polymerization, Raf-1 activation, bcl-2 phosphorylation, and cytotoxicity. Furthermore, we show that this pathway of Raf-1 activation is shared by other microtubule-active agents. A model is proposed that envisions a common pathway for microtubule-active agents, which includes Raf-1 activation and bcl-2 phosphorylation leading to cell death. For both microtubule-stabilizing and -destabilizing drugs, this pathway may have a similar role to that of p53 activation following treatment with DNA-damaging drugs.

A drug must interact with tubulin before Raf-1 activation occurs, and previous studies have demonstrated a delay between PTX addition and phosphorylation of Raf-1 (6). Part of this delay may reflect the delay in cytoskeletal disruption; this would be expected if a crucial level of disruption must be reached. In MCF-7 and A2780 cells, time-course experiments demonstrate a delay of 1–2 h in achieving a maximal cytoskeletal effect.[4] However, the data also suggest that additional step(s) exist in this pathway. Raf-1 activation by PTX required both RNA and protein synthesis. Inhibition of transcription or translation prevented Raf-1 phosphorylation. If this were crucial for cell death, one would predict that actinomycin D or cycloheximide would abrogate the toxicity of tubulin-active agents. Indeed, in agreement with previous studies (13, 14) that demonstrated that treatment with cycloheximide or actinomycin D inhibits cytotoxicity, we demonstrated that cycloheximide and actinomycin D block apoptosis induced by microtubule-active drugs (vincristine data not shown). Likewise, depletion of Raf-1 by geldanamycin was shown to inhibit

[4] P. Giannakakou, unpublished observations.

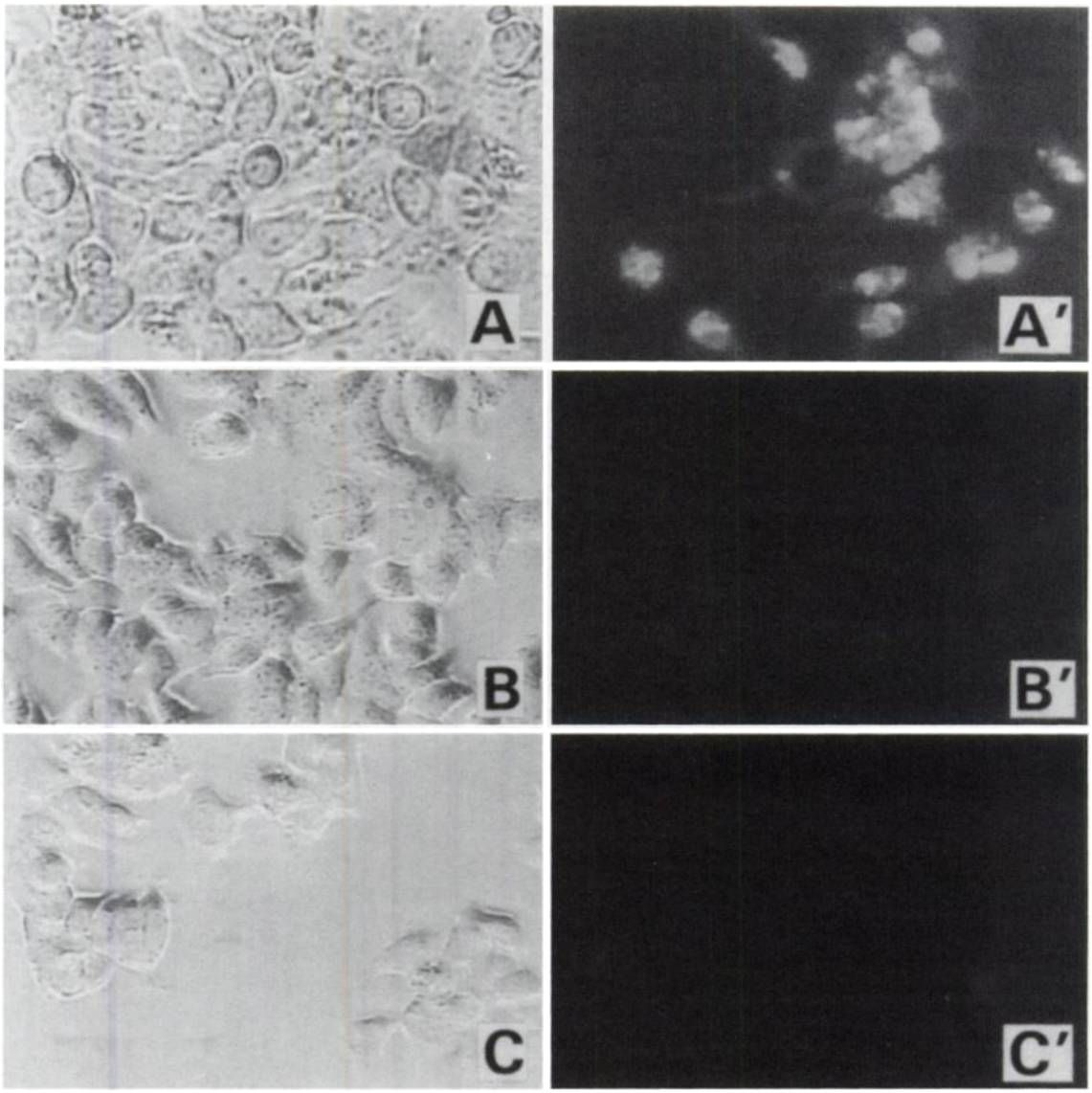

Fig. 5. Prevention of Raf-1/bcl-2 phosphorylation blocks apoptosis induced by PTX. MCF-7 cells were treated with 0.25 μM PTX for 30 h alone (*A* and *A'*) or simultaneously with 50 μg/ml cycloheximide (*B* and *B'*) or 1 μg/ml actinomycin D (*C* and *C'*). Light microscopy (*A–C*); UV microscopy for DAPI staining (*A'–C'*) demonstrates apoptotic cells.

PTX-induced apoptosis (6). Together, these results support a crucial role for Raf-1 in toxicity after microtubule damage.

The mechanism whereby bcl-2 phosphorylation leads to cell death must remain speculative. Previous studies have shown that this phosphorylation disrupts the association of bcl-2 with bax, an effect that could lead to bax-induced apoptosis (20). Studies demonstrating bcl-2 protein localization on chromosomes in mitotic nuclei suggest a possible role for bcl-2 in protection from apoptosis during mitosis (33–35). If bcl-2 phosphorylation results in a loss of function (19), then higher bcl-2 levels may not provide additional protection against apoptosis. Once activated, the pathway leading to phosphorylation of bcl-2 could lead to complete inactivation, irrespective of bcl-2 level. The results with the PTX analogues indicate that some degree of microtubular disruption is tolerated, because the less toxic analogues were able to induce small but measurable changes in tubulin polymerization. However, those analogues that effected microtubular collapse led to Raf-1/bcl-2 phosphorylation. That this effect is not the result of G_2-M arrest is supported by the observations with orthovanadate, which causes G_2-M arrest, but did not induce Raf-1/bcl-2 phosphorylation (26). Furthermore, activation of Raf-1 was not observed with jasplakinolide, which induces actin polymerization, suggesting that induction of Raf-1/bcl-2 phosphorylation occurs as a result of the interaction of a drug with tubulin, not the cytoskeleton (27). It must be stressed that in the present study, phosphorylation of bcl-2 was observed following microtubule disruption. Phosphorylation of bcl-2 on serine residues has been demonstrated previously, and of the 17 serine residues present in bcl-2, several are potential sites of phosphorylation by different kinases (19, 36). Thus, it is possible that the effect of phosphorylation on bcl-2 function may depend on the site of phosphorylation. For example, IL-3 and bryostatin have been shown to result in bcl-2 phosphorylation in an IL-3-dependent cell line. But, in contrast to phosphorylation induced by microtubule-active drugs in the present study, IL-3-induced phosphorylation was associated with cell survival, occurred through PKC, and did not change bcl-2 mobility (36).

p53 has been implicated in checkpoint control leading to G_1 block after transient mitotic arrest (12, 37, 38). Recent studies have demonstrated induction of p53 and $p21^{WAF1/CIP1}$ following PTX treatment (5, 38, 39). In contrast to p53 accumulation induced by DNA-damaging drugs, which is Raf-1 independent, accumulation of p53 following PTX is Raf-1 dependent (5, 40). However, the levels of p53 and p21 induced by PTX are significantly lower than those induced by

DNA-damaging drugs. And, in contrast to Raf-1 activation, p53 induction by PTX depends on the cell line and cell density, (seen in MCF-7 and A549 cells plated at low densities (Ref. 5; data not shown). These observations suggest that cell death induced by PTX is likely to be p53 independent. It has been shown that arrest in prophase by PTX induces the onset of a p53-independent apoptotic pathway, whereas G_1 block is p53 dependent (12).

Considering these observations and the data in the present study, we propose that drug-induced disruption of microtubules initiates a pathway that leads to activation of Raf-1, phosphorylation of bcl-2, and cell death. Similar to p53 induction in response to DNA damage caused by a variety of insults, the Raf-1/bcl-2 pathway can be activated by a variety of drugs that disrupt microtubules. Given the crucial role of microtubules in chromosomal segregation, this pathway provides a means to eliminate cells with aberrant chromosome segregation equivalent to severe DNA damage. Future studies should help to further clarify the biological significance of this pathway.

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