0026-895X/01/6002-290–301$3.00
MOLECULAR PHARMACOLOGY
Vol. 60, No. 2
908/918654
Mol Pharmacol 60:290–301, 2001
Printed in U.S.A.

Selective Potentiation of Paclitaxel (Taxol)-Induced Cell Death by Mitogen-Activated Protein Kinase Kinase Inhibition in Human Cancer Cell Lines

HAYLEY M. MCDAID and SUSAN BAND HORWITZ

Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York

Received February 23, 2001; accepted April 25, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Activation of the mitogen-activated protein kinase (MAPK) pathway in HeLa and Chinese hamster ovary cells after treatment with paclitaxel (Taxol) and other microtubule interacting agents has been investigated. Using a *trans*-reporting system, the phosphorylation of the nuclear transcription factors Elk-1 and c-jun was measured. Concentration- and time-dependent activation of the Elk-1 pathway, mediated primarily by the extracellular signal-regulated kinase (ERK) component of the MAPK family, was observed. Inactive drug analogs and other cytotoxic compounds that do not target microtubules failed to induce similar levels of activation, thereby indicating that an interaction between these drugs and the microtubule is essential for the activation of MAPKs. Evaluation of the endogenous levels of MAPK expression revealed cell-dependent expression of the ERK, c-jun N-terminal kinase, and p38 pathways. In the case of HeLa cells, time-dependent activation of ERK coincided with increased poly(ADP-ribose) polymerase (PARP) cleavage, phosphatidylserine externalization, and increased accumulation of cells in G_2M. In both cell lines, inhibition of ERK activity potentiated paclitaxel-induced PARP cleavage and phosphatidylserine externalization, suggesting that ERK activity coincided with, but did not mediate, the cytotoxic effects of paclitaxel. We evaluated the nature of the interaction between paclitaxel and the MAPK kinase inhibitor U0126 in three cell lines, on the basis of a potential chemotherapeutic advantage of paclitaxel plus ERK inhibition. Our data confirmed additivity in those cells lines that undergo paclitaxel-induced ERK activation, and antagonism in cells with low ERK activity, suggesting that in tumors with high ERK activity, there may be an application for this strategy in therapy.

Paclitaxel (Taxol) is a widely used cancer chemotherapeutic drug that exhibits clinical activity in a range of human malignancies (Arbuck et al., 1993) and is being extensively evaluated in ongoing trials as a single agent (Malingre et al., 2000) and in combination therapy (Piccart et al., 2000). The binding site for paclitaxel on β-tubulin has been assigned from photoaffinity labeling experiments (Rao et al., 1994, 1995, 1999) and from the electron crystallography model in which the α- and β-tubulin dimer is fitted to a 3.7-Å density map (Nogales et al., 1998). In addition to its ability to bind to microtubules, paclitaxel also has been shown to interact with bcl-2 (Rodi et al., 1999) and to phosphorylate both bcl-2 and Raf-1 (Blagosklonny et al., 1997; Torres and Horwitz, 1998), events that have been related to the accumulation of cells in G_2M. One major problem with paclitaxel chemotherapy is the acquisition of clinical resistance, which causes chemotherapeutic failure leading to progressive disease. Potential mechanisms of taxane resistance include the overexpression of P-glycoprotein and/or mutations in tubulin, both of which may impair the ability of the drug to bind efficiently to its target. These factors have motivated a search for other natural products that target the microtubule and have superior or equivalent activity compared with paclitaxel but without the associated problems. Recently, three additional compounds that fulfill these criteria have been isolated from diverse natural sources. These include the epothilones (Su et al., 1997), which are currently under clinical evaluation, eleutherobin (Chen et al., 1998), and discodermolide (Smith et al., 2000).

In addition to the well-documented effects on microtubules, a number of reports have described activation of components of the mitogen-activated protein kinase (MAPK) pathway in response to paclitaxel treatment. MAPKs are serine-threonine protein kinases that are activated in response to a

This work was supported in part by United States Public Health Service Grants CA39821 and CA77263 (to S.B.H.) and Cancer Core Support Grant CA13330. H.M.D. was supported by postdoctoral fellowship 99–3054 from the Susan B. Komen foundation.

ABBREVIATIONS: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-jun NH_2-terminal kinase; MAPKK, mitogen-activated protein kinase kinase; MAPKKK, mitogen-activated protein kinase kinase kinase CHO, Chinese hamster ovary cells; PBS, phosphate-buffered saline; GFP, green fluorescent protein; mAb, monoclonal antibody; pAb, polyclonal antibody; CI, combination index; PARP, poly(ADP-ribose) polymerase.

diverse range of stimuli, including growth factors, hormones, neurotransmitters, and cellular stress. So far, five subfamilies have been identified; however, the three major MAPK families are the extracellular signal-regulated kinases (ERKs), the c-Jun NH_2-terminal kinases (JNKs), and the p38 kinases (Widmann et al., 1999). MAPK kinase cascades are characterized by a sequential signaling cascade in which MAPK (MAPKKKs) kinase kinases phosphorylate and activate downstream MAPK kinases (MAPKKs) that ultimately activate MAPKs. Through these specific intracellular signaling cascades, a variety of extracellular stimuli are transduced through cells in a tightly regulated manner. In general, the ERKs are activated after growth factor and differentiation stimuli (Widmann et al., 1999) and are the best characterized pathway in mammalian cells. Several microtubule-associated proteins are substrates for ERK1 and 2, including microtubule-associated proteins 1, 2, and 4 and Tau (Seger and Krebs, 1995), and activated ERKs have been localized to kinetochores of mitotic cells (Zecevic et al., 1998). JNK and p38 are more commonly activated in response to stress and cellular damage (Mendelson et al., 1996; Niisato et al., 1999). Cellular redox state, tyrosine kinases, and phosphatases are thought to be involved in the activation of stress responses; however, the mechanisms for regulation remain unknown. In contrast, the response of the JNK pathway to extracellular ligands is well characterized. The latter are activated by the TNF receptor family, cytokine receptors, and tyrosine kinase receptors (Minden and Karin, 1997; Chainy et al., 2000). Hence, the different kinase members achieve specificity by signaling to unique and common downstream targets.

Several studies have described the activation of various components of the MAPK family in different cell models after treatment with paclitaxel and other microtubule interacting agents (Lieu et al., 1998; Shtil et al., 1999; Wang et al., 1999; Yujiri et al., 1999; Stone and Chambers, 2000). These studies suggest that the activation of various family members is variable and depends on the cell model. Hence, the relevance of MAPK activation to paclitaxel-induced death has not been fully resolved, and the functional relationship between paclitaxel-induced death and other signal transduction events, such as bcl-2 and Raf-1 phosphorylation, remains unclear. The aims of this study were to evaluate the effects of paclitaxel and other microtubule interacting agents on the activation of the MAPK family, initially in HeLa and Chinese hamster ovary (CHO) cells, and to relate these findings to paclitaxel-induced cytotoxicity. A pharmacological evaluation of the combination of MAPKK inhibition and paclitaxel treatment in three cell lines showed additivity in cells exhibiting paclitaxel-induced ERK activation and antagonism in cells with low endogenous ERK activity that did not display paclitaxel-induced ERK activation. These data suggest that the inhibition of ERK signaling coupled with paclitaxel therapy may be a useful clinical combination that requires additional validation.

Materials and Methods

Cell Culture and Reagents. GRC+ LR-73 Chinese hamster ovary (CHO) cells and HeLa cells were cultured in minimum essential medium, supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). CHO cells (Pollard and Stanners, 1979) were the kind gift of Dr. J.W. Pollard (Albert Einstein College of Medicine, Bronx, NY). Exponentially growing cells were used in all experiments. Paclitaxel and baccatin III were obtained from the Drug Development Branch of the National Cancer Institute (Bethesda, MD) and docetaxel (Taxotere) was a gift from Aventis (Strasbourg, France). The epothilones (Meng et al., 1997), eleutherobin (Chen et al., 1998), their structural analogs, and discodermolide (Smith et al., 2000) have been described previously. The Pathdetect in vivo *trans* reporting system was purchased from Stratagene (Austin, TX). The inhibitors PD98059 and SB203580 and the caspase inhibitor Z-VAD-FMK, were purchased from Calbiochem Inc. (La Jolla, CA) and U0126 was purchased from Promega (Madison, WI). All drugs and inhibitors were dissolved in 100% dimethyl sulfoxide, the final concentration of which was kept below 0.1% in all experiments. The pcDNA3-FLAG-JNK1-APF plasmid contains a catalytically inactive dominant-negative mutant of JNK1 with an N-terminal FLAG tag (Gupta et al., 1995) and was kindly provided by Dr. R. J. Davis (Howard Hughes Medical Institute Research Laboratories, University of Massachusetts Medical Center, Worcester, MA). All other reagents were from Sigma Chemicals (St. Louis, MO).

Measurement of In Vivo Signal Transduction. The Pathdetect system was used to measure the signal transduction events after exposure to microtubule interacting agents. This system uses vectors that express chimeric *trans*-activator proteins containing the DNA binding domain of GAL4 and the *trans*-activation domain of either Elk-1 or c-jun. These domains become phosphorylated if an upstream signal transduction pathway is activated and subsequently activates the transcription of a luciferase gene from a reporter plasmid that is driven by a promoter containing five GAL4 binding sites. Negative control experiments were performed using a plasmid encoding only the GAL4 DNA binding domains, which did not result in luciferase transcription, thereby demonstrating that luciferase expression is dependent on the in vivo phosphorylation of Elk-1 or c-jun (data not shown). All experiments were optimized initially in a Chinese hamster ovary-derived cell line, GRC^+ LR-73, which undergoes growth arrest in low concentrations of serum without impaired cellular viability (Pollard and Stanners, 1979). CHO and HeLa cells were transiently transfected using calcium phosphate precipitation and lipofectin (Invitrogen), respectively. Approximately 3×10^5 cells were seeded into six-well culture plates 24 h before transfection. Each well was transfected with 1 μg of pFR-Luc, 50 ng of pFA-Elk or pFA-cjun, and 0.2 μg of CMV-β-galactosidase. For positive control experiments, cells were also cotransfected with pFC-MEK1 or pFC-MEKK1, upstream activators of the ERK and JNK pathways, respectively. Because there is no commercially available inhibitor of the JNK pathway, a catalytically inactive dominant negative mutant of JNK1 (pcDNA3-FLAG-JNK1-APF) was used to inhibit c-jun *trans*-activation. Where required, an equivalent quantity of empty vector (pcDNA3) was cotransfected to ensure a constant quantity of DNA. Cells were transfected for 8 to 12 h, rinsed with PBS and incubated with drug diluted in media containing 0.3% serum for the times indicated. Because activation of Elk-1 is serum dependent, all experiments using the reporter system were carried out with culture media supplemented with 0.3% serum. Cells were lysed in 500 μl of extraction buffer (40 mM tricine, pH 7.8, 50 mM NaCl, 2 mM EDTA, pH 7.8, 1 mM $MgSO_4$, 5 mM dithiothreitol, and 1% Triton X-100) and 50 μl was used to measure luciferase and β-galactosidase activities. All luciferase values were normalized to β-galactoside and expressed relative to basal control levels, which were assigned a value of 1.

For the experiments described in Fig. 4F, 1×10^5 HeLa cells were transfected for 6 h with 6 μg of either pcDNA3 or pcDNA3-FLAG-JNK1-APF, and 6 μg of GFP. After the transfection period, cells were washed and incubated in standard media containing 10% heat-inactivated fetal bovine serum for 2 h before treatment with paclitaxel. The percentage of GFP-positive cells was scored after transfection, but before paclitaxel treatment, by counting the number of cells exhibiting green fluorescence per 200 cells.

Flow Cytometry. Flow cytometry was performed on a FACScan (BD Biosciences, San Jose, CA) using CellQuest software. For cell

cycle analysis, 1×10^6 cells (including nonadherent cells) were fixed in 70% ethanol for at least 20 min. The cell pellets were washed in cold PBS and incubated for 30 min in PBS containing 10 μg/ml propidium iodide and 1 μg/ml RNase A (Boehringer Mannheim, Indianapolis, IN) at 37°C. Propidium iodide fluorescence was quantified using the FL2 detector and cellular aggregates gated from all samples. For the detection of phosphatidylserine externalization, adherent and nonadherent cells were harvested by trypsinization and 1×10^6 cells/ml were stained with 5 μL of annexin-V Fluor-488 labeled antibody (Molecular Probes Inc., Eugene, OR) in annexin binding buffer (10 nM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4) according to the manufacturer's instructions and analyzed by flow cytometry on the FL1 detector.

Immunoblotting. Cell extracts were prepared from adherent and nonadherent cells after drug treatment by lysis in a buffer composed of 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and 0.1% Nonidet P-40 containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 2 μg/ml aprotinin, and 2 μg/ml leupeptin) and phosphatase inhibitors (50 mM NaF and 1 mM sodium orthovanadate). Cellular debris was removed by centrifugation and the protein was quantified using the Bio-Rad method (Bio-Rad, Hercules, CA). Samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Ponceau S staining before immunoblotting confirmed equal loading of samples. Membranes were blocked for 1 h in 3% bovine serum albumin and incubated in primary and secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. All antibodies were used at a 1:1000 dilution in 1× Tris-buffered saline containing 0.1% Tween-20 and 1 to 2% bovine serum albumin and were as follows: Bcl-2 mAb (DAKO, Glostrum, Denmark), phospho-ERK mAb, phospho-JNK mAb, JNK pAb (New England Biolabs, Beverly, MA), ERK2 pAb, MEKK1 pAb C-22, (Santa Cruz Biotechnology Inc., Santa Cruz, CA), and PARP mAb (P76420), c-Raf-1 mAB (BD Pharmingen, San Diego, CA). An additional PARP antibody, Anti-PARP p85 fragment pAB (Promega, G7341), specific for only the 85-kDa cleavage product, was used at a dilution of 1:750. Membranes were developed using enhanced chemiluminescence reagent (Amersham).

Multiple Drug Effect Analysis. Cells were seeded in triplicate into 24-well plates and, after adherence, serial dilutions of paclitaxel, U0126, or both were added for 72 h. The drug concentrations evaluated were based on the IC_{50} value for each individual drug so that combinations of paclitaxel and U0126 were assessed at their equipotent ratio (i.e., at the ratio of each respective IC_{50} value). Drug combinations were evaluated concurrently or sequentially, wherein paclitaxel or U0126 was given for 24 h before the other drug. The effect of each drug treatment was determined by counting the number of attached viable cells after the 72-h incubation period and expressing this number as a ratio of the number of cells treated with dimethyl sulfoxide alone. This ratio was applied to the combination index (CI) method of Chou and Talalay (1984), using the software Calcusyn (Biosoft, Cambridge, UK) to analyze the nature of the interaction between paclitaxel and U0126. This software applies a mathematical model that computes a CI ratio for various levels of cytotoxicity (fractional inhibition), such that a CI value of 1 indicates additivity, $CI < 1$ indicates synergism, and $CI > 1$ indicates antagonism. For this analysis, the more conservative assumption of mutual nonexclusion (dissimilar mechamisms of action for paclitaxel and U0126) was applied to the derivation of CI ratios.

Results

Activation of the Nuclear Transcription Factors Elk-1 and c-jun by Microtubule-Interacting Agents. A MAPK reporter system was used to determine the ability of various microtubule-interacting drugs to phosphorylate the nuclear transcription factor Elk-1 via activation of the ERK, JNK, and p38 pathways (Yang et al., 1998). The system employs a fusion construct that contains a GAL4 DNA binding domain and the *trans*-activation domain of Elk-1 to induce expression of a luciferase reporter that has five GAL4 binding domains. After MAPK activation, the *trans*-activation domain of Elk-1 becomes phosphorylated and subsequently binds to and induces transcriptional activation of the luciferase reporter.

trans-Activation of Elk-1 results after phosphorylation of the ERK, JNK, and p38 MAP kinases. To delineate which pathway(s) were responsible for Elk-1 activation, PD98059 and SB203580, specific inhibitors of the ERK and p38 pathways, respectively, were used. Because there is no commercially available specific inhibitor of the JNK family, cells were transfected with a plasmid construct containing the *trans*-activation domain of c-jun, so that activity of the JNK pathway could be monitored.

Figure 1A illustrates that paclitaxel and epothilone B (Table 1), two drugs that stabilize microtubules, induced *trans*-activation of Elk-1, which was mediated by the ERK pathway, because treatment of cells with 50 μM PD98059 completely inhibited the effect. Treatment with the p38 inhibitor SB203580 had little effect on Elk-1 activity, suggesting that this pathway was not responsible for the observed *trans*-activation in CHO cells. Exposure to PD98059 and SB203580 alone had no effect of Elk-1 *trans*-activation (data not shown). To ensure the functionality of the Elk-1 transcriptional assay, cells were routinely cotransfected with plasmids containing *trans*-activation domains of MEK1 and MEKK1, upstream components of the ERK and JNK kinase pathways, respectively. Cotransfection of MEKK1 resulted in higher transcriptional activation of Elk-1 compared with cotransfection with MEK1 (25- – 30-fold activation for MEKK1, compared with 10- – 20-fold for MEK1). This finding is consistent with MEKK1 being an activator of both the ERK and JNK pathways (Lange-Carter, 1993) and probably reflects dual activation of Elk-1 via both pathways, compared with transcriptional activation by only the ERK pathway in those cells cotransfected with MEK1.

To evaluate the effect of treatment with paclitaxel on the activation of the JNK pathway, cells were transfected with a c-jun fusion plasmid and the lysates assayed for luciferase activity after drug treatment (Fig. 1B). Cotransfection of cells with MEKK1 was used as a positive control. Paclitaxel induced a time-dependent transcriptional activation of c-jun in CHO cells (data not shown), but only at concentrations of paclitaxel ≥500 nM. This activation was also observed with the other microtubule-stabilizing agents. The mean activation levels for 500 nM concentrations of each drug were as follows: 5-fold for epothilone A, 8-fold for epothilone B, 1.5-fold for eleutherobin, and 2-fold for discodermolide; hence, the degree of activation was correlated with the cytotoxic potency of each drug. Cotransfection of cells with the plasmid pcDNA3-FLAG-JNK1-APF, containing a catalytically inactive dominant negative mutant of JNK1, completely inhibited paclitaxel-induced c-jun *trans*-activation, indicating that this *trans*-activation of c-jun is mediated via JNK1 activation.

Activation of Elk-1 Requires an Interaction with Microtubules. Fig. 2A depicts the concentration-dependent activation of Elk-1 by both microtubule stabilizing and depolymerizing agents in CHO cells after 48 h of drug treatment. The degree of activation for each drug was related to its

cytotoxic potency. Treatment with high concentrations of epothilone B (500 nM) resulted in decreased activation of Elk-1 (compared with the effect at 50 nM) and is probably related to cell death because of a lethal concentration of drug, given that epothilone B is the most active of the drugs. Transcriptional activation of Elk-1 was observed for both microtubule stabilizing and depolymerizing agents; however, other cytotoxic agents, such as cisplatin, 5-FU, and etoposide, resulted in insignificant levels of activation. The effect of inactive analogs of microtubule-stabilizing drugs (Table 1) in both CHO and HeLa cells were evaluated; compounds that were deemed inactive by microtubule polymerization assays (Su et al., 1997; McDaid et al., 1999) also failed to activate Elk-1 (Fig. 2B). This strongly suggests a relationship between the perturbation of microtubule dynamics and the nuclear *trans*-activation of Elk-1. The data shown are for CHO cells. Similar results were also obtained for HeLa cells.

Interaction with Microtubules Is Required for the Phosphorylation of Raf-1 and Bcl-2. Since the data established a relationship between the *trans*-activation of Elk-1 and perturbations of microtubule dynamics, we evaluated the effects of three microtubule-stabilizing agents and their inactive analogs on cell-cycle kinetics and the phosphor-

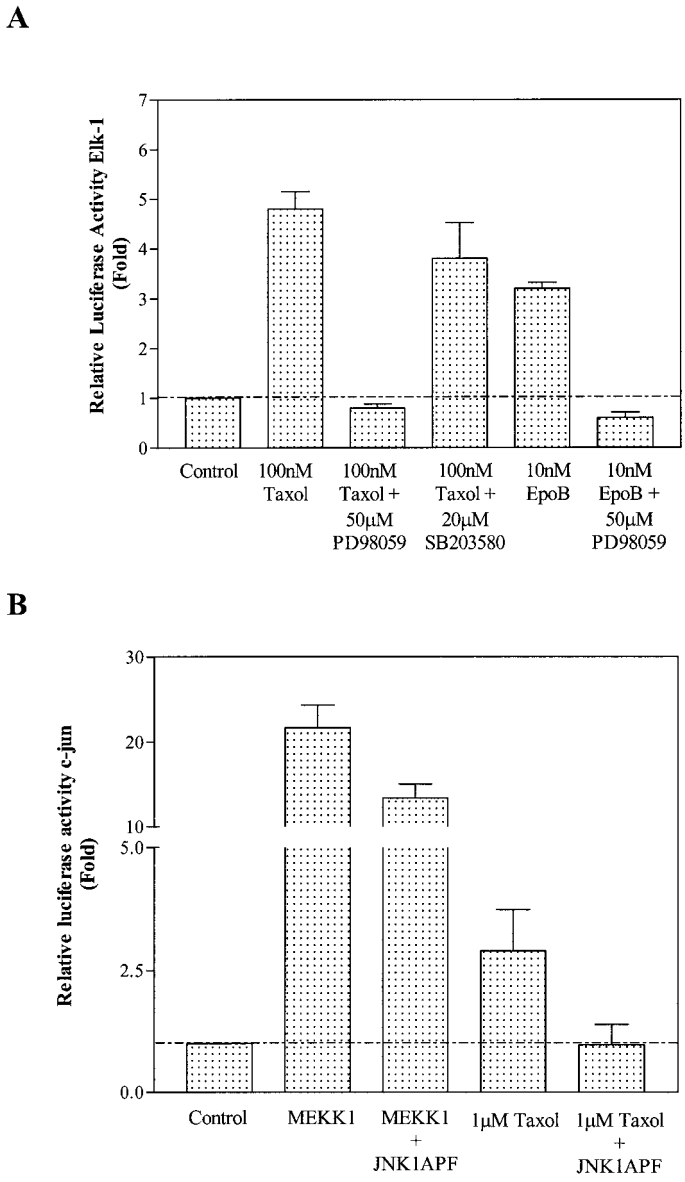


Fig. 1. A, Elk-1 activation by paclitaxel (Taxol) and epothilone B in CHO cells. Cells were transiently cotransfected with the *trans*-activation domain of Elk-1, a luciferase reporter, and β-galactosidase. After transfection, cells were exposed to paclitaxel or epothilone B in the presence or absence of the MEK1/2 inhibitor PD98059 for 48 h. Fold luciferase activation levels were normalized to β-galactosidase expression and the data presented summarize the mean (± S.E.) of three independent experiments. B, c-jun activation by paclitaxel in CHO cells. Cells were transiently cotransfected with the *trans*-activation domain of c-jun, a luciferase reporter, and β-galactosidase. A dominant negative mutant of JNK1 (pcDNA3-FLAG-JNK1-APF) was used to inhibit c-jun activation. After transfection, cells were exposed to 1 μM paclitaxel for 48 h. Positive control cells were also cotransfected with the *trans*-activation domain of MEKK1, an upstream activator of JNK. Fold luciferase activation levels were normalized to β-galactosidase expression and the data presented summarize the mean (±S.E.) of three independent experiments and suggest that the JNK pathway is activated in response to paclitaxel treatment in CHO cells.

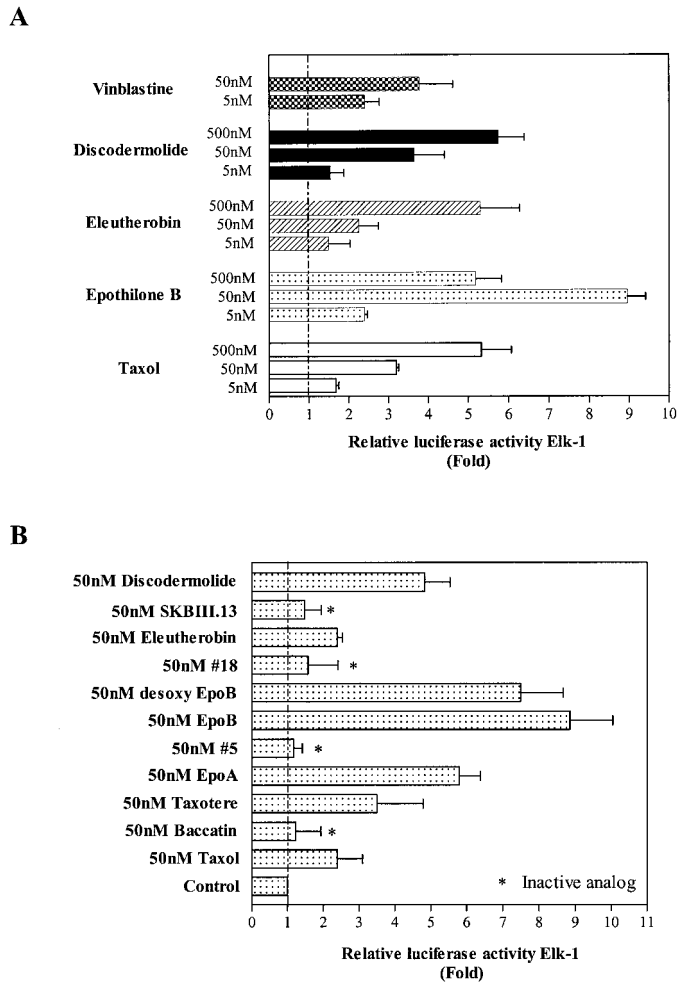
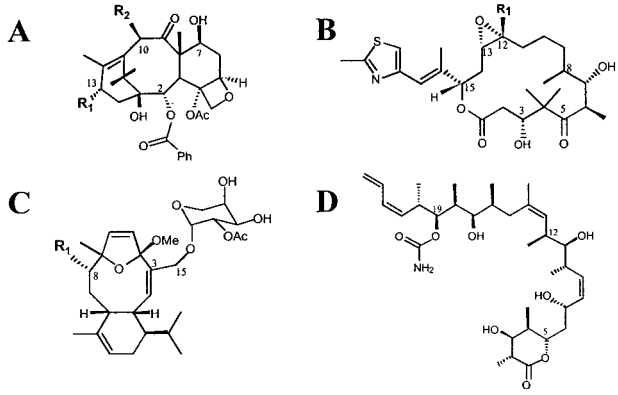
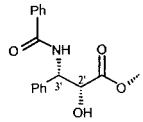
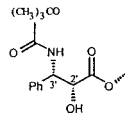
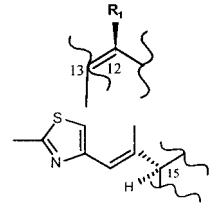
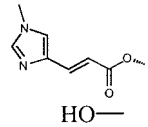


Fig. 2. A, dose-dependent activation of Elk-1 by microtubule stabilizing and depolymerizing agents in CHO cells. Cells were transiently transfected with the *trans*-activation domain of Elk-1, the luciferase reporter, and β-galactosidase and, after transfection, was exposed to various concentrations of the microtubule-interacting compounds paclitaxel (Taxol), epothilone B, eleutherobin, discodermolide, and vinblastine for 48 h. Fold luciferase activity was determined after normalization to β-galactosidase. B, drugs that interact directly with microtubules mediate Elk-1 *trans*-activation in CHO cells. Cells were transiently transfected and exposed to 50 nM concentrations of various microtubule-interacting agents or their inactive analogs for 48 h. *trans*-Activation of Elk-1 was observed only in those compounds that had previously been determined to be active with the use of in vitro microtubule polymerization assays.

TABLE 1
Chemical structures of microtubule polymerizing agents and related compounds



	Name	R_1	R_2	Other	Activity
A	Paclitaxel				+
	Docetaxel				+
	Baccatin III				–
B	Epothilone A	H—			+
	Analog 5	H—			–
	Epothilone B	Me—			+
	Desoxyepothilone B	Me—			+
	Analog 18	Propyl			–
C	Eleutherobin				+
	SKBIII.13				–
D	Discodermolide				+

ylation status of Raf-1 and Bcl-2 in HeLa cells. We also evaluated cleavage of the nuclear repair enzyme PARP as a marker of cell death (Nosseri et al., 1994), using a monoclonal antibody derived from the N terminus of PARP; hence, this antibody recognizes the intact protein and the 24-kDa cleavage product derived from the N-terminal. The phosphorylation of Raf-1 and Bcl-2 in response to treatment with microtubule-interacting drugs is well documented (Blagosklonny et al., 1997) and these events are known to be associated with the accumulation of cells in mitosis (Scatena et al., 1998; Laird et al., 1999).

Figure 3A illustrates the profiles of HeLa cells exposed to the various drugs for 16 h. Cells were treated with the minimal concentration of drug known to cause mitotic arrest. In the case of the inactive analogs (baccatin III, analogs 5 and 18), cells were treated with micromolar or submicromolar concentrations that have been shown previously to have no cytotoxic effects (Su et al., 1997; Miller et al., 1999). Agents

known to interact with microtubules (paclitaxel and the epothilones) caused mitotic arrest coupled with Raf-1 and Bcl-2 phosphorylation and the release of a 24-kDa cleavage product of PARP (Fig. 3B). In contrast, exposure of cells to the inactive analogs of these drugs showed little, if any, change in their cell cycle distribution and no change in their Raf-1, Bcl-2, or PARP status, confirming that the interaction with microtubules, leading to an accumulation of cells in mitosis, is related to these molecular changes.

Differences in the Expression of Endogenous Levels of MAPK in CHO and HeLa Cells in Response to Paclitaxel Treatment. Because an association between exposure to microtubule-interacting agents and activation of MAPK has been demonstrated, the endogenous levels of MAPK expression were evaluated in control and drug-treated cells. Lysates were subjected to immunoblotting using monoclonal antibodies specific for Thr183/Tyr185 of stress-activated protein kinase/JNK and Thr202/Tyr204 of ERK1/2. Dual phosphorylation of the respective residues in each pathway has been determined to be essential for kinase activation. Figure 4A depicts the expression of phospho-ERK and phospho-JNK in CHO cells after exposure to paclitaxel at both 10 nM and 1 μM concentrations. No detectable activation of p38 was observed in this cell line. Total levels of MAPK proteins were unchanged throughout the drug treatments (data not shown). There was transient activation of phospho-ERK 4 h after drug exposure and dephosphorylation of phospho-ERK after 24 h at 1 μM paclitaxel that coincided with an increase in the proportion of cells in G_2M (Fig. 4B). No significant alterations in the cell cycle profile were observed with 10 nM paclitaxel treatment. CHO cells exhibited some activation of phospho-JNK, occurring predominantly after 1 μM paclitaxel treatment. This data is consistent with the *trans*-activation of c-jun described in Fig. 1B. Transient altered electrophoretic mobility of MEKK1, a 196-kDa kinase that acts upstream of JNK, occurred concomitant with phospho-JNK activation observed with 1 μM paclitaxel exposure. UV-irradiated cells were used as positive control cells and exhibited strong activation of both the ERK and JNK pathways and degradation of MEKK1, as anticipated.

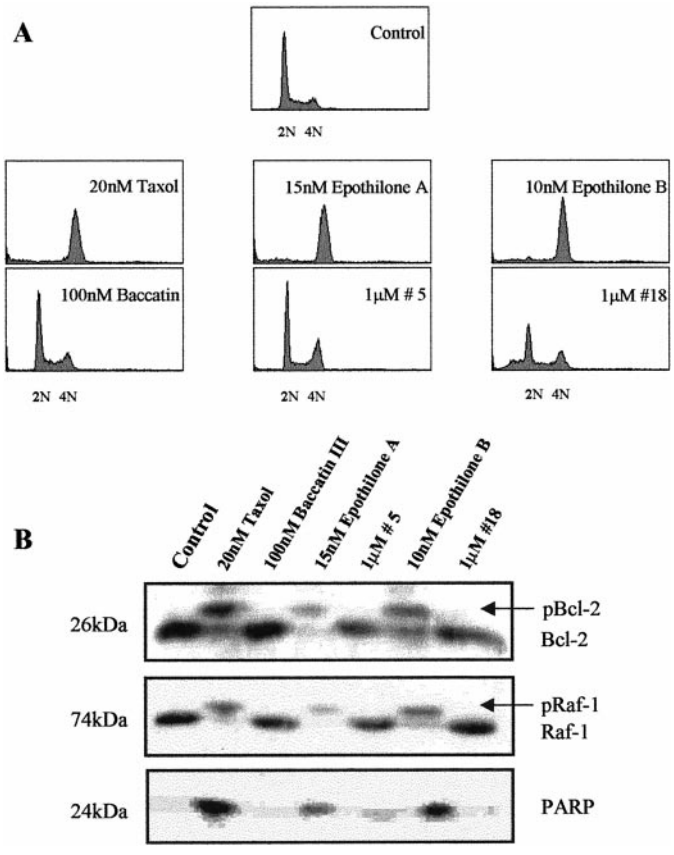


Fig. 3. A, the cell cycle profile of HeLa cells exposed to various concentrations of microtubule active and inactive agents for 16 h. Adherent and nonadherent cells were harvested and DNA content was determined by flow cytometric analysis after fixation and propidium iodide staining, as described under *Materials and Methods*. B, Western blotting for expression of Bcl-2, Raf-1, and the 24-kDa cleavage product of PARP in total HeLa cell lysates exposed to microtubule active and inactive agents for 16 h. Data are representative of two independent experiments, both yielding identical results.

HeLa cells exhibited time-dependent activation of phospho-ERK, phospho-JNK, and p38 at both paclitaxel concentrations evaluated (Fig. 4C). These coincided with increases in the proportion of cells in the G_2M phase of the cell cycle and the appearance of hypodiploid cells from flow cytometric analysis (Fig. 4D). Interestingly, the cell cycle profiles at 48 h after paclitaxel-treatment were essentially identically for 10 nM and 1 μM concentrations of drug in HeLa cells, despite a 2-fold log difference in drug concentration. In addition, we also observed an electrophoretic mobility shift of MEKK1 from the $\approx$196-kDa full-length form to multiple higher molecular mass species that we assume to be phosphorylated (Fig. 4C).

An altered electrophoretic mobility of MEKK1 has been reported previously in response to UV irradiation (Widmann et al., 1998a,b). In addition to hyperphosphorylation, MEKK1 is cleaved by activated caspases into a 91-kDa kinase fragment that becomes an apoptosis inducer. This occurs after exposure to UV irradiation or in response to DNA-damaging agents. This classical cleavage of MEKK1 was not observed after paclitaxel treatment, in CHO and HeLa cells, or in Jurkat, human embryonic kidney 293 (Gibson et al., 1999), T47D breast carcinoma, or mouse ES cells (Yujiri et al., 1999), all of which exhibit MEKK1 cleavage in response to DNA-damaging agents. In paclitaxel-treated HeLa cells, the altered electrophoretic mobility shift of MEKK1 is apparent 8 h after exposure to paclitaxel. This coincides with significant JNK activation and with substantial increases in the proportions of cells in G_2M. At later time points, the expression of MEKK1 diminishes until it is no longer apparent by 48 h after paclitaxel treatment. Furthermore, we also observed a cleavage product of an estimated 170 kDa, the expression of which intensifies concomitantly with the altered electrophoretic mobility shift. These paclitaxel-induced effects are observed in the presence of Z-VAD-FMK, a broad-range irreversible caspase inhibitor (Fig. 4E), suggesting that they are not related to the caspase regulating properties of MEKK1. In addition, the cleavage of PARP to its 24- and 85-kDa fragments by paclitaxel is reduced only slightly in the presence of the inhibitor, suggesting that paclitaxel-induced cell death is predominantly independent of caspase processing.

Because there have been several suggestions that the JNK pathway may regulate the apoptotic response (Wang et al., 1998, 1999), HeLa cells were transiently transfected with the pcDNA3-FLAG-JNK1-APF plasmid that contains a catalytically inactive dominant-negative mutant of JNK1. This ap-

proach was used in the absence of a commercially available JNK inhibitior. Cells were cotransfected with GFP and the percentage of GFP-positive cells were counted by immunofluorescence. Transfection efficiencies of 40 to 45% were achieved. Subsequent treatment of the transfected cells with 10 nM and 1 μM paclitaxel for 16 h followed by Western blot analysis demonstrated little if any change in the degree of PARP cleavage to the 85 kDa form (Fig. 4F), thereby suggesting that in HeLa cells, paclitaxel-induced cell death is predominantly independent of JNK activation. Transfected cells exhibited PARP cleavage in untreated control samples, presumably because of the toxic effects of transfection.

Inhibition of the ERK Pathway Enhances Paclitaxel-Induced Apoptosis. Because the time-dependent activation of phospho-ERK in HeLa cells coincides with the accumulation of cells in the G_2M phase of the cell cycle and with increases in the proportion of hypodiploid cells (Fig. 4D), we questioned whether the ERK pathway is mediating paclitaxel-induced cell death. Figure 5A illustrates the effect of the MEK inhibitors PD98059 and U0126 on phospho-ERK expression and the cleavage of PARP in the presence and absence of paclitaxel in CHO cells. Phospho-ERK activity was partially down-regulated by PD98059 and U0126 in both the presence and the absence of paclitaxel; however, both inhibitors augmented paclitaxel-induced PARP cleavage. Identical observations were noted in HeLa cells (Fig. 5B). Note that the expression of the 24-kDa cleavage fragment of PARP was comparable for both cell lines, but in Fig. 5A, the Western blot is overexposed.

The effects of these inhibitors on the externalization of phosphatidylserine, an early marker of apoptosis, were also investigated in HeLa cells using an annexin-V antibody (Fig. 5C). The number of cells positive for annexin-V cells increased in a concentration-dependent manner after exposure to increasing concentrations of both paclitaxel or the MEK inhibitior U0126, indicating increasing numbers of cells undergoing apoptosis (similar results were also obtained with PD98059). The cytotoxic effects of U0126 were apparent at concentrations of 5 μM and greater in HeLa cells. The combination of U0126 and paclitaxel significantly increased the proportion of annexin-V positive cells, compared with treatment with either drug alone. This combination also caused an increase in the proportion of hypodiploid cells, without other significant alterations to the cell cycle distribution (data not shown). These data suggest that the ERK pathway does not mediate paclitaxel-induced cytotoxicity but may actually protect cells from death, because inactivation of this pathway by U0126 enhances the cytotoxic effects of paclitaxel.

The Combination of Paclitaxel and U0126 Is Additive. To extend our observations that the combination of paclitaxel with U0126 (and PD98059) potentiated paclitaxel-induced PARP cleavage and annexin-V externalization, we evaluated the nature of this interaction using the multiple drug-effect analysis of Chou and Talalay (1984), which computes the level of additivity, synergism, or antagonism at discrete levels of cytotoxicity. The combination indices (CI values) were derived using the mutually nonexclusive assumption of interaction between both drugs. Table 2 summarizes the findings from three cell lines in which the concurrent combination of paclitaxel and U0126 was evaluated. Additivity was confirmed in HeLa and A549 cells, both of which express phospho-ERK, which is induced in a time- and concentration-dependent manner after paclitaxel treatment

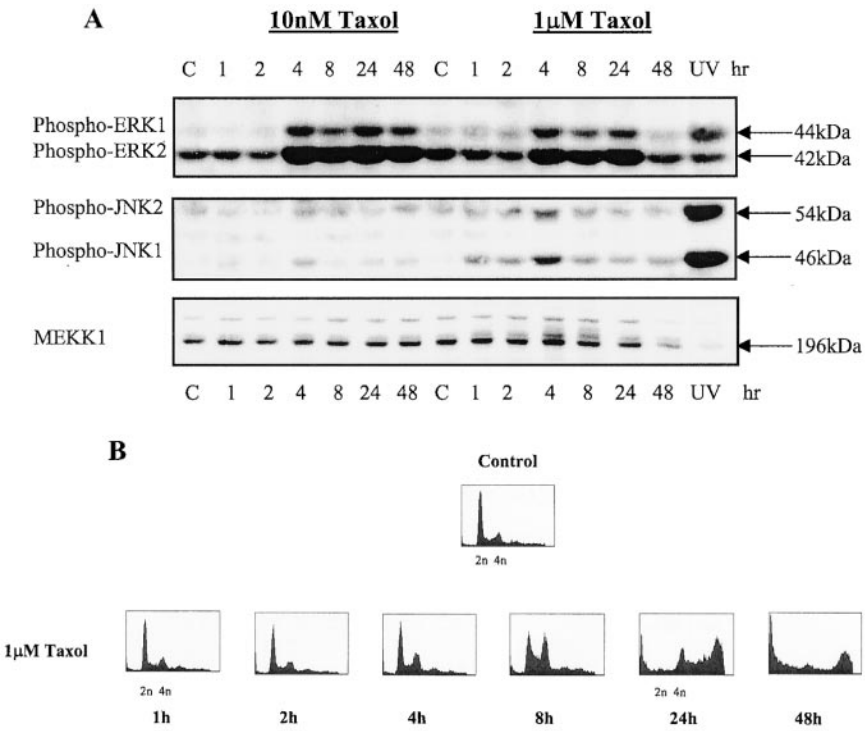


Fig. 4. A, expression of phospho-ERK1/2, phospho-JNK1/2 and MEKK1 proteins in CHO cell lysates after exposure to 10 nM and 1 μM paclitaxel (Taxol). Cells exposed to UV irradiation for 30 min were used as positive control cells. Data is representative of three independent experiments, each yielding identical results. B, Time course of CHO cell cycle distribution after exposure to 1 μM paclitaxel. Adherent and nonadherent cells were harvested, fixed, and stained with propidium iodide as described under *Materials and Methods*.

(Yang and Horwitz, 2000). MCF-7 cells were used as a control because they express low levels of phospho-ERK (Fig. 6) and do not exhibit paclitaxel-induced ERK activation (Shtil et al., 1999). The combination of paclitaxel with U0126 was antagonistic in MCF-7 cells. Hence, these results prove additivity between concurrent paclitaxel and MEK inhibition in cell lines exhibiting ERK activation in response to paclitaxel and provide an explanation for the potentiation of paclitaxel-induced cell death observed in the presence of U0126. Overall, the data supports a survival function for the ERK/MEK signaling pathway.

In addition, the effect of sequencing the drugs was evaluated in HeLa cells in which treatment with either paclitaxel or U0126 for 24 h before administration of the other drug resulted in ablation of the additivity observed with concurrent treatment, although the effect was more pronounced when U0126 treatment preceded paclitaxel. These results imply that sustained inactivation of ERK is necessary for the enhancement of paclitaxel cytotoxicity by U0126.

Discussion

Microtubules form a scaffolding and transport network in eukaryotic cells and their unique polymerization/depolymerization dynamics are critical for multiple cellular functions. Perturbation of normal microtubule dynamics by microtubule-interacting agents can be regarded as an intracellular stress that leads to cell death. A correlation between the potency of microtubule inhibitors and the induction of mitotic arrest is well documented; however, the signal transduction events that mediate the cytotoxic effects of these drugs are poorly defined. The data presented here indicate that interactions between such drugs and the microtubule are essential for MAPK activation; however, there is specific involvement of defined MAPK subfamilies in different cell lines. For example, activation of ERK in response to microtubule binding agents has been reported in A549 (human lung carcinoma) (Yang and Horwitz, 2000), HL-60 (human myeloid leukemic; Blagosklonny et al., 1999), U937 (human T-cell leukemic; Lieu et al., 1998), HeLa, and CHO cells. Conversely, JNK activation in response to paclitaxel and other microtubule inhibitors has been observed, in Chinese hamster ovary and HeLa in this report, in MCF-7 (human breast cancer; Shtil et al., 1999), KB-3 (human epidermal; Stone and Chambers, 2000), BR (Wang et al., 1999), 67R (both human ovarian), and human fibroblast and SAOS2 (human osteosarcoma) cells (Wang et al., 1998). P38 activation in response to

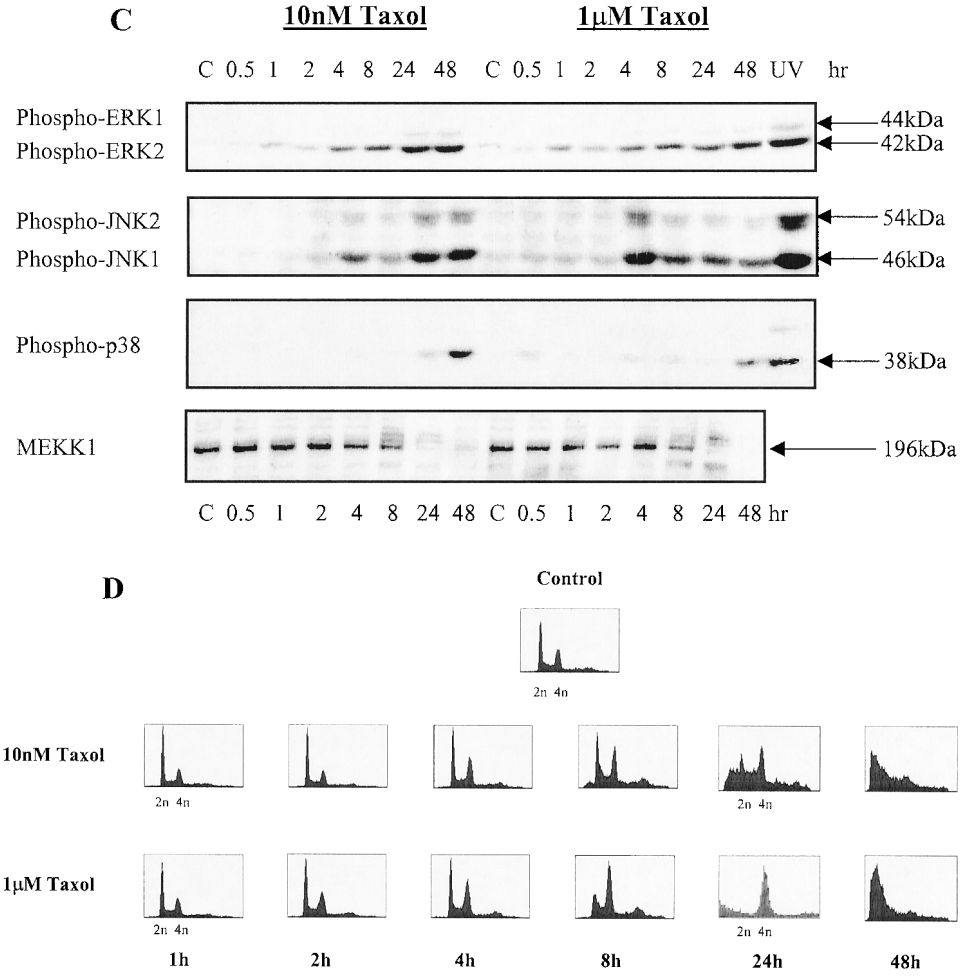


Fig. 4 *(continued).* C, expression of endogenous MAPK levels in HeLa cells after paclitaxel treatment. Time course of phospho-ERK1/2, phospho-JNK1/2, p38, and MEKK1 expression in HeLa cells after exposure to 10 nM and 1 μM paclitaxel. D, time course of HeLa cell cycle distribution after exposure to 10 nM and 1 μM paclitaxel.

paclitaxel has been observed in MCF-7 cells (Shtil et al., 1999) and in HeLa cells (this report). One must assume that there is a high level of redundancy and compensation occurring between these signaling pathways in different cell lines.

Despite the complexity of these observations, one query that requires clarification is how disruption of microtubules transduces the activation of these signaling cascades. It is estimated that 40% of MAPKs are associated with microtubules (Reszka et al., 1995), presumably making the kinases more accessible to substrates and activators. Furthermore, ERKs have been localized to the kinetochores in mitotic HeLa cells (Shapiro et al., 1998), are involved in the regulation of the microtubule organizing center during metaphase, and function in spindle assembly in *Xenopus laevis* egg extracts (Verlhac et al., 1993). MAPKs also regulate cyclin D1 promoter activity and protein expression (Lavoie et al., 1996), and phosphorylate cyclin B, resulting in cyclin B/cdc2 translocation to the nucleus, where this complex is dephosphorylated and activated by cdc25c. Overall, these various activities make MAPK a likely regulator of mitotic cell progression. Indeed the activation of ERK that we observe in HeLa cells coincides with increasing proportions of cells in the G_2M phase of the cell cycle after paclitaxel treatment.

JNK is perceived as a mediator of stress signaling, and models have been proposed in which microtubule disruption induces JNK via upstream signaling components involving ASK1 and MKK7. The ASK1/JNK pathway is normally activated in the G_2M phase of the cell cycle in Jurkat cells and is

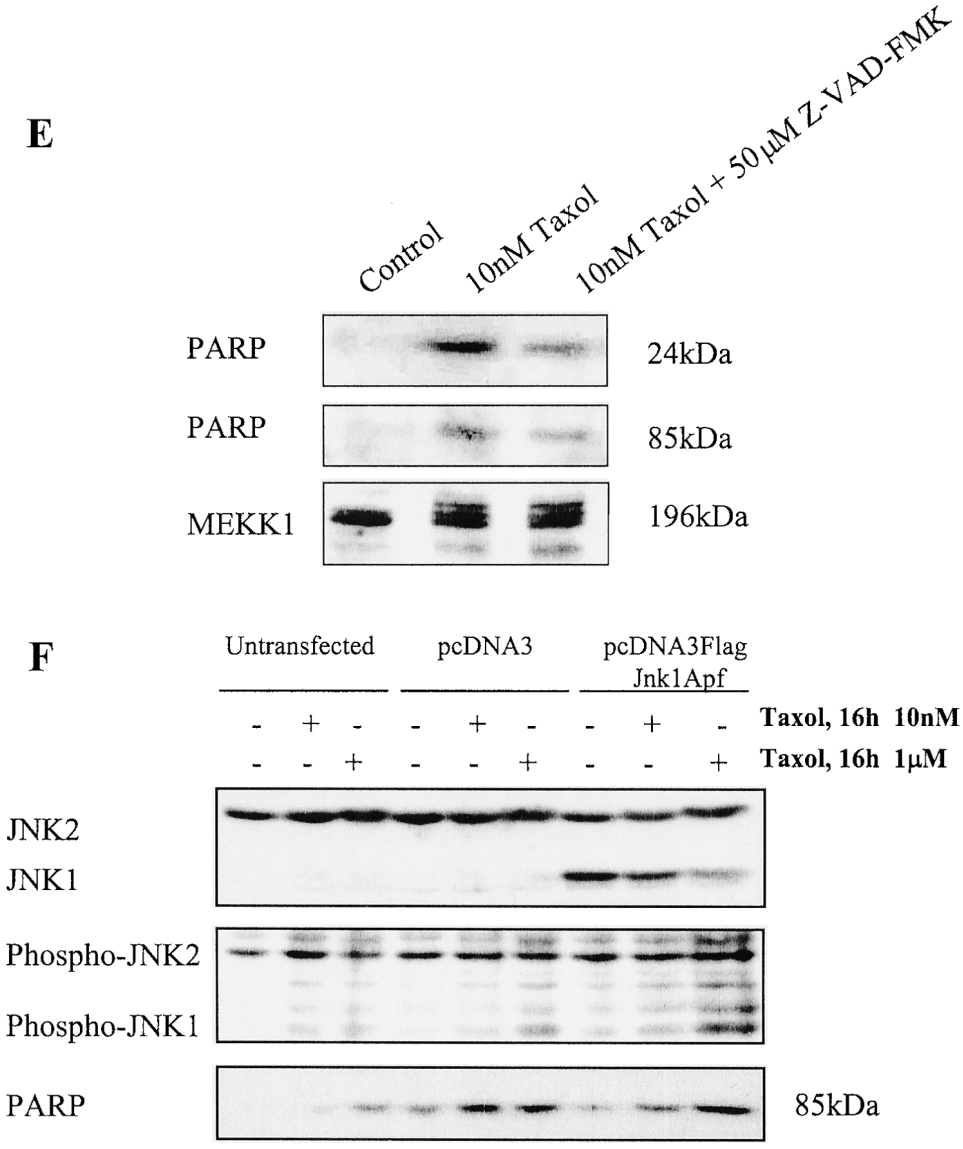


Fig. 4 *(continued).* E, caspase inhibition has a moderate effect on paclitaxel-induced PARP cleavage and no effect on the altered electrophoretic mobility of MEKK1 induced by paclitaxel. Cells were treated with either 10 nM paclitaxel for 16 h or 50 μM Z-VAD-FMK and 10 nM paclitaxel for 16 h. Adherent and nonadherent cells were harvested, cell lysates were prepared, and immunoblot analysis was performed on the indicated proteins. Two antibodies for PARP expression were evaluated, as indicated, in an N-terminal derived antibody that recognizes the 24-kDa cleavage fragment and one that recognizes only the cleaved 85-kDa fragment. The data shown are representative of three independent experiments. F, suppression of JNK by transient transfection of a dominant negative mutant of JNK1 (pcDNA3-FLAG-JNK1-APF) does not modulate the degree of paclitaxel-induced PARP cleavage in HeLa cells. Cells were transiently transfected as described under *Materials and Methods* and exposed to paclitaxel for 16 h. Adherent and nonadherent cells were harvested, lysates were prepared, and immunoblot analysis was performed on the proteins indicated. The results are representative of three independent experiments yielding an average transfection efficiency of 40 to 45%.

thought to mediate paclitaxel-induced bcl-2 phosphorylation (Yamamoto et al., 1999). In ovarian carcinoma cells, a biphasic activation of JNK in response to paclitaxel has been observed; however, neither phase of JNK activity mediates paclitaxel-induced bcl-2 phosphorylation (Wang et al., 1999). The same study concludes that the majority of paclitaxel-induced cell death is independent of JNK activity. Indeed, inhibition of JNK signaling in HeLa cells (this report) using a catalytically inactive dominant-negative mutant of JNK1 does not modulate the degree of paclitaxel-induced cell death.

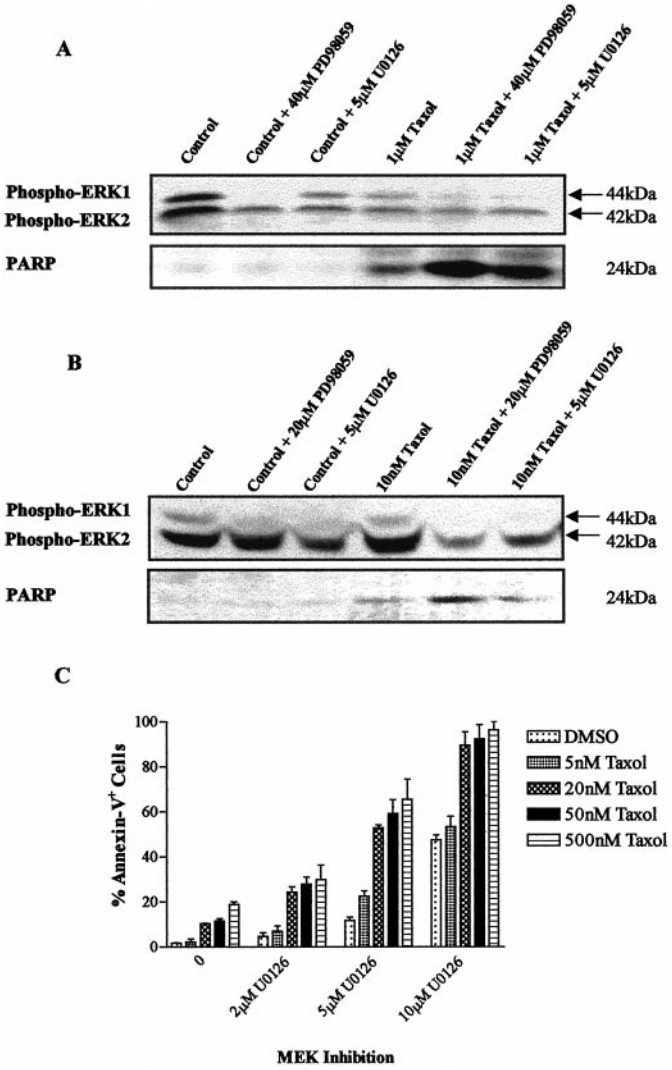


Fig. 5. A, inhibition of ERK potentiates paclitaxel (Taxol)-induced PARP cleavage in CHO cells. Western blot analysis of phospho-ERK and the 24-kDa cleavage product of PARP after 16 h paclitaxel treatment with the MEK1 inhibitors, PD98059 and U0126. B, inhibition of ERK potentiates paclitaxel-induced PARP cleavage in HeLa cells. C, inhibition of ERK potentiates paclitaxel-induced phosphatidylserine externalization in HeLa cells. Cells were exposed to paclitaxel and U0126 at the various concentrations indicated for 24 h. Cells were harvested and incubated with annexin-V-488 fluorochrome as described under *Materials and Methods*. The percentage of annexin-V–positive cells was determined from the flow cytometric evaluation of stained cells.

The data described here, and from other studies, report activation of components of MAPK families that coincides with both mitotic arrest and an increased proportion of cells undergoing cell death. Hence, the interpretation of data regarding the effect of microtubule inhibitors on MAPK is confounded by the dual effect of these drugs (that is, by the induction of both mitotic arrest and cell death, both of which are probably regulated by MAPK activity). The ERK, the JNK, and the p38 kinases have been implicated in the regulation of apoptosis and of proliferation and differentiation, depending on the cell type and stimulus. In some cell systems, there is good correlation between ERK activation and the proliferation of cells, as is observed with epidermal or platelet-derived growth factors (Seger and Krebs, 1995). Inhibition of ERKs by antisense oligonucleotides or dominant-negative Raf-1 kinase inhibits cellular proliferation, whereas activation of ERKs may provide protection against apoptosis in other cell types (Widmann et al., 1999). Conversely, in some cell systems, apoptosis is associated with ERK activation; for example, in Jurkat cells, ERKs are transiently activated after Fas stimulation (Widmann et al., 1998b). A significant observation made in HeLa cells in response to paclitaxel are that alterations in MAPK activity relate to the increased accumulation of cells in mitosis, in contrast to the rapid responses observed in growth factor mediated signaling. Others and we have documented the phosphorylation of Raf-1 in response to mitotic arrest, an observation that is distinct from the Raf-1 response to growth factors. Furthermore, the inhibition of ERK in HeLa cells does not prevent paclitaxel-induced Raf-1 phosphorylation (data not shown); hence, Raf-1 phosphorylation during exposure to microtubule inhibitors is not transduced through the classic ERK/MEK pathway.

Despite the positive correlation between phospho-ERK activity and increased proportions of hypodiploid and annexin-V–positive cells, and PARP cleavage, the inhibition of the ERK pathway by specific inhibitors of MEK did not prevent paclitaxel-induced cell death but in fact potentiated cell death. This observation led us to analyze the nature of the interaction between paclitaxel and U0126 using the combination index method of Chou and Talalay (1984) in three cell lines. In cell lines that exhibited paclitaxel-induced ERK activation (A549 and HeLa), this drug combination was additive. Conversely, in MCF-7 cells that have low activation levels of the ERK/MEK pathway and do not exhibit activation in response to paclitaxel treatment, the nature of the interaction between paclitaxel and U0126 is antagonistic, presumably because of low substrate concentrations for

TABLE 2
Mean CI at various levels of fractional inhibition (FA)

Cell Line	Taxol Dose Evaluated	CI (± SE) at FA				
		0.10	0.25	0.50	0.75	0.95
	nM					
A549	0.01–40	3.185 (0.707)	2.162 (0.325)	1.604 (0.099)	1.312 (0.064)	1.169 (0.286)
HeLa	0.01–40	1.424 (0.632)	0.934 (0.087)	0.826 (0.113)	0.865 (0.176)	1.087 (0.218)
MCF-7	0.01–40	9.803 (5.767)	5.922 (2.147)	4.618 (0.762)	4.576 (0.210)	6.472 (0.525)

U0126. A recent report has described findings that suggest that this drug combination is greater than additive (MacKeigan et al., 2000) and the data described in our study suggest additivity between paclitaxel and MEK inhibition, although this interaction is limited to those cells lines expressing high levels of activated ERK.

This study indirectly supports a role for ERK in survival signaling that has been previously documented (Widmann et al., 1999). The mechanism by which ERK inhibition potentiates paclitaxel-induced apoptosis remains to be delineated, although we speculate that inhibiting the proliferative signaling function of ERK renders cells more susceptible to the toxic effects of paclitaxel. In addition, the inhibition of MEK1/2 by U0126 may regulate the activity of the JNK pathway via MEKK1, which is an activator of both the ERK and JNK pathways (Lange-Carter, 1993). We have demonstrated altered MEKK1 expression in both CHO and HeLa cells after paclitaxel treatment, although modification of JNK activity by a dominant negative mutant has no effect on paclitaxel-induced cell death. It should also be considered that MEKK1 activity might regulate the activity of other proteins that affect cell death, such as nuclear factor-κB. Indeed, it has been shown that paclitaxel significantly down-regulates I-κB, thereby increasing the nuclear translocation of nuclear factor-κB (Huang et al., 2000). There also exists the possibility that ERKs directly modulate the expression of Bcl-2 family members via phosphorylation of Bad (Scheid et al., 1999) or down-regulation of Bcl-2, Bcl-X_L, and Mcl-1 (Boucher et al., 2000). It remains to be determined whether these are plausible mechanisms for the potentiation of paclitaxel-induced cell death by U0126.

In conclusion, we speculate that this drug combination would be attractive only as a potential chemotherapeutic combination in tumors that have confirmed high levels of ERK. Various tumor types have been reported to have high levels of ERK/MEK activity (Loda et al., 1996; Schmidt et al., 1997), although there is also a high degree of heterogeneity within a tumor. Hence, it must be considered that for some patients, this drug combination will not enhance tumor cell death compared with paclitaxel alone, with a detrimental effect of increased toxicity from two drugs in combination. This study, however, does endorse the importance of targeting signal transduction pathways aberrantly activated in human cancer. This strategy can be used to modulate the efficacy of cytotoxic drugs used currently in chemotherapy by the addition of cytostatic agents, as in the case of herceptin (Slamon et al., 2001) and epidermal growth factor receptor (Sirotnak et al., 2000), both of which are being evaluated clinically with paclitaxel. Unfortunately, both commercially available MEK inhibitors, PD98059 and U0126, are cytotoxic at high concentrations; consequently, any potential clinical applications for this drug combination will require the development of effective MEK/ERK inhibitors with low toxicity for use in clinical medicine and will need extensive in vivo testing.

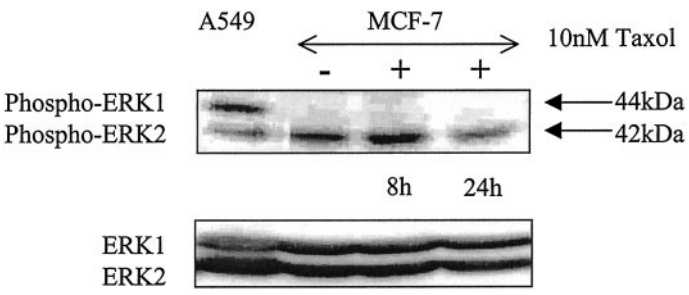


Fig. 6. Expression of ERK and phospho-ERK in paclitaxel (Taxol)-treated MCF-7 cells. Cells were treated with 10 nM paclitaxel for the indicated times and 20 μg of total cell lysate was used for immunoblot analysis, together with 20 μg of total cell lysate from A549 cells. Compared with A549 cells, MCF-7 cells expressed low levels of phospho-ERK and did not exhibit paclitaxel-induced ERK activation.

Acknowledgments

The authors thank Professors S. J. Danishefsky and A. B. Smith III and Dr. C.-P. H. Yang for helpful discussions. Dr. R. J. Davis provided the pcDNA3-FLAG-JNK1-APF plasmid.

References

Arbuck SG, Christian MC, Fisherman JS, Cazenave LA, Sarosy G, Suffness M, Adams J, Canetta R, Cole KE and Friedman MA (1993) Clinical development of Taxol. *Natl Cancer Inst Monogr* **15:**11–24.

Blagosklonny MV, Chuman Y, Bergan RC and Fojo T (1999) Mitogen-activated protein kinase pathway is dispensable for microtubule-active drug-induced Raf-1/Bcl-2 phosphorylation and apoptosis in leukemia cells. *Leukemia* **13:**1028–1036.

Blagosklonny MV, Giannakakou P, el-Deiry WS, Kingston DG, Higgs PI, Neckers L and Fojo T (1997) Raf-1/bcl-2 phosphorylation: a step from microtubule damage to cell death. *Cancer Res.* **57:**130–135.

Boucher MJ, Morisset J, Vachon PH, Reed JC, Laine J and Rivard N (2000) MEK/ERK signaling pathway regulates the expression of Bcl-2, Bcl-X(L) and Mcl-1 and promotes survival of human pancreatic cancer cells. *J Cell Biochem* **79:**355–369.

Chainy GB, Manna SK, Chaturvedi MM and Aggarwal BB (2000) Anethole blocks both early and late cellular responses transduced by tumor necrosis factor: effect on NF-kappaB, AP-1, JNK, MAPKK and apoptosis. *Oncogene* **19:**2943–2950.

Chen X-T, Zhou B, Bhattacharya SK, Gutteridge CE, Pettus TRR and Danishefsky SJ (1998) The total synthesis of eleutherobin: a surprise ending. *Agnew Chem Int Ed Engl* **37:**789–792.

Chou T and Talalay P (1984) Quantitative analysis of dose-effect relationships: the combined effect of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* **22:**27–55.

Gibson S, Widmann C and Johnson GL (1999) Differential involvement of MEK kinase 1 (MEKK1) in the induction of apoptosis in response to microtubule-targeted drugs versus DNA damaging agents. *J Biol Chem* **274:**10916–10922.

Gupta S, Campbell D, Derijard B and Davis RJ (1995) Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science (Wash DC)* **267:**389–393.

Huang Y, Johnson KR, Norris JS and Fan W (2000) Nuclear factor-kappaB/IkappaB signaling pathway may contribute to the mediation of paclitaxel-induced apoptosis in solid tumor cells. *Cancer Res* **60:**4426–4432.

Laird AD, Morrison DK and Shalloway D (1999) Characterization of Raf-1 activation in mitosis. *J Biol Chem* **274:**4430–4439.

Lange-Carter CA, Pleiman CM, Gardner AM, Blumer KJ and Johnson GL (1993) A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. *Science (Wash DC)* **260:**315–319.

Lavoie JN, L'Allemain G, Brunet A, Muller R and Pouyssegur J (1996) Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. *J Biol Chem* **271:**20608–20616.

Lieu CH, Liu CC, Yu TH, Chen KD, Chang YN and Lai YK (1998) Role of mitogen-activated protein kinase in Taxol-induced apoptosis in human leukemic U937 cells. *Cell Growth Differ* **9:**767–776.

Loda M, Capodieci P, Mishra R, Yao H, Corless C, Grigioni W, Wang Y, Magi-Galluzzi C and Stork PJ (1996) Expression of mitogen-activated protein kinase phosphatase-1 in the early phases of human epithelial carcinogenesis. *Am J Pathol* **149:**1553–1564.

MacKeigan JP, Collins TS and Ting JP (2000) MEK inhibition enhances paclitaxel-induced tumor apoptosis. *J Biol Chem* **275:**38953–38956.

Malingre MM, Terwogt JM, Beijnen JH, Rosing H, Koopman FJ, van Tellingen O, Duchin K, Huinink WW, Swart M, Lieverst J, et al. (2000) Phase I and pharmacokinetic study of oral paclitaxel. *J Clin Oncol* **18:**2468–2475.

McDaid HM, Bhattacharya SK, Chen X-T, He L, Shen HJ, Gutteridge CE, Horwitz SB and Danishefsky SJ (1999) Structure-activity profiles of eleutherobin analogs and their cross-resistance in Taxol-resistant cell lines. *Cancer Chemother Pharmacol* **44:**131–137.

Mendelson KG, Contois LR, Tevosian SG, Davis RJ and Paulson KE (1996) Independent regulation of JNK/p38 mitogen-activated protein kinases by metabolic oxidative stress in the liver. *Proc Natl Acad Sci U S A* **93:**12908–12913.

Meng D, Kamenecka T, Bertinato P, Balog A, Su D-S, Kamencka T, Sorensen E and Danishefsky SJ (1997) The total syntheses of epothilones A and B. *J Am Chem Soc* **119:**10073–10092.

Miller, MC 3rd, Johnson KR, Willingham MC and Fan W (1999) Apoptotic cell death induced by baccatin III, a precursor of paclitaxel, may occur without G_2/M arrest. *Cancer Chemother Pharmacol* **44:**444–452.

Minden A and Karin M (1997) Regulation and function of the JNK subgroup of MAP kinases. *Biochim Biophys Acta* **1333:**F85–F104.

Niisato N, Post M, Van Driessche W and Marunaka Y (1999) Cell swelling activates

stress-activated protein kinases, p38 MAP kinase and JNK, in renal epithelial A6 cells. *Biochem Biophys Res Commun* **266:**547–550.
Nogales E, Wolf SG and Downing KH (1998) Structure of the alpha beta tubulin dimer by electron crystallography. *Nature (Lond)* **391:**199–203.
Nosseri C, Coppola S and Ghibelli L (1994) Possible involvement of poly(ADP-ribosyl) polymerase in triggering stress-induced apoptosis. *Exp Cell Res* **212:**367–373.
Piccart MJ, Bertelsen K, James K, Cassidy J, Mangioni C, Simonsen E, Stuart G, Kaye S, Vergote I, Blom R, et al. (2000) Randomized intergroup trial of cisplatin-paclitaxel versus cisplatin-cyclophosphamide in women with advanced epithelial ovarian cancer: three-year results. *J Natl Cancer Inst* **92:**699–708.
Pollard JW and Stanners CP (1979) Characterization of cell lines showing growth control isolated from both the wild type and a leucyl-tRNA synthetase mutant of Chinese hamster ovary cells. *J Cell Physiol* **98:**571–585.
Rao S, He L, Chakravarty S, Ojima I, Orr GA and Horwitz SB (1999) Characterization of the Taxol binding site on the microtubule. Identification of Arg(282) in beta-tubulin as the site of photoincorporation of a 7-benzophenone analogue of Taxol. *J Biol Chem* **274:**37990–37994.
Rao S, Krauss NE, Heerding JM, Swindell CS, Ringel I, Orr GA and Horwitz SB (1994) 3′-(*p*-azidobenzamido)taxol photolabels the N-terminal 31 amino acids of beta-tubulin. *J Biol Chem* **269:**3132–3134.
Rao S, Orr GA, Chaudhary AG, Kingston DG and Horwitz SB (1995) Characterization of the Taxol binding site on the microtubule. 2-(m-Azidobenzoyl)taxol photolabels a peptide (amino acids 217–231) of beta-tubulin. *J Biol Chem* **270:**20235–20238.
Reszka AA, Seger R, Diltz CD, Krebs EG and Fischer EH (1995) Association of mitogen-activated protein kinase with the microtubule cytoskeleton. *Proc Natl Acad Sci USA* **92:**8881–8885.
Rodi DJ, Janes RW, Sanganee HJ, Holton RA, Wallace BA and Makowski L (1999) Screening of a library of phage-displayed peptides identifies human bcl-2 as a taxol-binding protein. *J Mol Biol* **285:**197–203.
Scatena CD, Stewart ZA, Mays D, Tang LJ, Keefer CJ, Leach SD and Pietenpol JA (1998) Mitotic phosphorylation of Bcl-2 during normal cell cycle progression and Taxol-induced growth arrest. *J Biol Chem* **273:**30777–30784.
Scheid MP, Schubert KM and Duronio V (1999) Regulation of bad phosphorylation and association with Bcl-x(L) by the MAPK/Erk kinase. *J Biol Chem* **274:**31108–31113.
Schmidt CM, McKillop IH, Cahill PA and Sitzmann JV (1997) Increased MAPK expression and activity in primary human hepatocellular carcinoma. *Biochem Biophys Res Commun* **236:**54–58.
Seger R and Krebs EG (1995) The MAPK signaling cascade. *FASEB J* **9:**726–735.
Shapiro PS, Vaisberg E, Hunt AJ, Tolwinski NS, Whalen AM, McIntosh JR and Ahn NG (1998) Activation of the MKK/ERK pathway during somatic cell mitosis: direct interactions of active ERK with kinetochores and regulation of the mitotic 3F3/2 phosphoantigen. *J Cell Biol* **142:**1533–1545.
Shtil AA, Mandlekar S, Yu R, Walter RJ, Hagen K, Tan TH, Roninson IB and Kong AN (1999) Differential regulation of mitogen-activated protein kinases by microtubule-binding agents in human breast cancer cells. *Oncogene* **18:**377–384.
Sirotnak FM, Zakowski MF, Miller VA, Scher HI and Kris MG (2000) Efficacy of cytotoxic agents against human tumor xenografts is markedly enhanced by coadministration of ZD1839 (Iressa), an inhibitor of EGFR tyrosine kinase. *Clin Cancer Res* **12:**4885–4892.
Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, et al. (2001) Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* **344:**783–792.
Smith IA, Kaufman MD, Beachamp TJ, LaMarche MJ and Arimoto H (2000) Gram-scale synthesis of (+)-discodermolide. *Org Lett* **2:**1983–1986.
Stone AA and Chambers TC (2000) Microtubule inhibitors elicit differential effects on MAP kinase (JNK, ERK and p38) signaling pathways in human KB-3 carcinoma cells. *Exp Cell Res* **254:**110–119.
Su D-S, Balog A, Meng D, Bertinato P, Danishefsky SJ, Zheng Y-H, Chou T-C, He L and Horwitz SB (1997) Structure-activity relationships of the epothilones and the first in vivo comparison with paclitaxel. *Agnew Chem Int Ed Engl* **36:**2093–2096.
Torres K and Horwitz SB (1998) Mechanisms of Taxol-induced cell death are concentration dependent. *Cancer Res* **58:**3620–3626.
Verlhac MH, de Pennart H, Maro B, Cobb MH and Clarke HJ (1993) MAP kinase becomes stably activated at metaphase and is associated with microtubule-organizing centers during meiotic maturation of mouse oocytes. *Dev Biol* **158:**330–340.
Wang TH, Popp DM, Wang HS, Saitoh M, Mural JG, Henley DC, Ichijo H and Wimalasena J (1999) Microtubule dysfunction induced by paclitaxel initiates apoptosis through both c-Jun N-terminal kinase (JNK)-dependent and -independent pathways in ovarian cancer cells. *J Biol Chem* **274:**8208–8216.
Wang TH, Wang HS, Ichijo H, Giannakakou P, Foster JS, Fojo T and Wimalasena J (1998) Microtubule-interfering agents activate c-Jun N-terminal kinase/stress-activated protein kinase through both Ras and apoptosis signal-regulating kinase pathways. *J Biol Chem* **273:**4928–4936.
Widmann C, Gerwins P, Johnson NL, Jarpe MB and Johnson GL (1998a) MEK kinase 1, a substrate for DEVD-directed caspases, is involved in genotoxin-induced apoptosis. *Mol Cell Biol* **18:**2416–2429.
Widmann C, Gibson S, Jarpe MB, and Johnson GL (1999) Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol Rev* **79:**143–180.
Widmann C, Gibson S and Johnson GL (1998b) Caspase-dependent cleavage of signaling proteins during apoptosis. A turn-off mechanism for anti-apoptotic signals. *J Biol Chem* **273:**7141–7147.
Yamamoto K, Ichijo H and Korsmeyer SJ (1999) BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G_2/M. *Mol Cell Biol* **19:**8469–8478.
Yang CP and Horwitz SB (2000) Taxol mediates serine phosphorylation of the 66-kDa Shc isoform. *Cancer Res* **60:**5171–5178.
Yang SH, Whitmarsh AJ, Davis RJ and Sharrocks AD (1998) Differential targeting of MAP kinases to the ETS-domain transcription factor Elk-1. *EMBO J* **17:**1740–1749.
Yujiri T, Fanger GR, Garrington TP, Schlesinger TK, Gibson S and Johnson GL (1999) MEK kinase 1 (MEKK1) transduces c-Jun NH2-terminal kinase activation in response to changes in the microtubule cytoskeleton. *J Biol Chem* **274:**12605–12610.
Zecevic M, Catling AD, Eblen ST, Renzi L, Hittle JC, Yen TJ, Gorbsky GJ and Weber MJ (1998) Active MAP kinase in mitosis: localization at kinetochores and association with the motor protein CENP-E. *J Cell Biol* **142:**1547–1558.

Address correspondence to: Dr. Susan B. Horwitz, Department of Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. E-mail: shorwitz@aecom.yu.edu