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Sequence-Dependent Potentiation of Paclitaxel-Mediated Apoptosis in Human Leukemia Cells by Inhibitors of the Mitogen-Activated Protein Kinase Kinase/Mitogen-Activated Protein Kinase Pathway

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ABSTRACT

Effects of inhibitors of the mitogen-activated protein kinase kinase/mitogen-activated protein kinase (MEK/MAPK) cascade have been examined in relation to paclitaxel-induced apoptosis in human monocytic leukemia cells (U937). Cells treated with paclitaxel (250 nm; 6 h) followed by PD98059 (40 μM; 15 h) exhibited a significant increase in mitochondrial dysfunction (e.g., cytochrome *c* release), caspase activation, poly ADP-ribose polymerase cleavage, and apoptosis, whereas pretreatment of cells with PD98059 reduced lethality. Similar results were obtained with other MEK/MAPK inhibitors (e.g., U0126 and PD184352). Subsequent exposure of paclitaxel-treated cells to PD98059 did not enhance dephosphorylation/activation of $p34^{cdc2}$ but diminished expression of the antiapoptotic protein Mcl-1. The caspase inhibitor ZVAD-fmk opposed potentiation of paclitaxel-induced loss of mitochondrial membrane potential ($\Delta\psi_m$) and apoptosis by PD98059, but not cytochrome *c* release. Paclitaxel treatment induced sustained phosphorylation/activation of MAPK, an effect prevented by subsequent, but not prior, exposure to PD98059. Paclitaxel treatment also induced c-Jun N-terminal kinase phosphorylation, but this effect was enhanced only slightly by subsequent PD98059 administration. Although paclitaxel alone failed to induce p38 MAPK activation, subsequent (but not prior) exposure to PD98059 induced a dramatic increase in p38 MAPK phosphorylation. Moreover, coadministration of the p38 MAPK inhibitors SB203580 and SB202190 abrogated the increase in paclitaxel-mediated apoptosis induced by PD98059. Finally, subsequent PD98059 exposure increased, whereas prior exposure decreased inhibition of clonogenicity by paclitaxel. Together, these findings suggest that subsequent exposure of paclitaxel-treated U937 cells to MEK/MAPK inhibitors induces perturbations in signaling pathways, particularly the p42/44 MAPK and p38 MAPK cascades, that lower the threshold for mitochondrial injury and induction of cell death.

Paclitaxel (Taxol) is a member of the taxane class of antineoplastic agents and exhibits activity against both solid tumors as well as hematologic malignancies (Rowinsky et al., 1992). In contrast to microtubule disaggregating agents, paclitaxel induces microtubular stabilization, leading to arrest of cells in G_2M and ultimately, apoptosis (Bhalla et al., 1993). The mechanism(s) by which paclitaxel and related agents trigger the cell death process is not known with certainty, but has been variously attributed to dysregulation of signal transduction pathways (Wang et al., 1999a), induction of cell cycle perturbations (Shen et al., 1998), or phosphorylation/inactivation of the antiapoptotic protein Bcl-2 (Yamamoto et al., 1999). Paclitaxel can also mimic the actions of lipopolysaccharides in stimulating TNF release (Ding et al., 1990).

The mitogen-activated protein kinase (MAPK) pathways provide a mechanism by which signals are transduced from the cell surface to the nucleus, leading to activation of genes involved in cell cycle progression, survival, differentiation, proliferation, and cell death. Three major subfamilies have been identified: c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPKs), extracellular signal-related kinases (p42/44 MAPK; ERK1/2), and p38 MAPK. Of these, JNKs and p38 MAPK are activated by a variety of noxious stimuli, including inflammatory cytokines, endotoxins, heat shock, alterations in osmolarity, and genotoxic stresses (Minden and Karin, 1997). In contrast, the ERK1/2 module is

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ABBREVIATIONS: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; PKC, protein kinase C; MEK, mitogen-activated protein kinase kinase; FCS, fetal calf serum; DMSO, dimethyl sulfoxide; TNF, tumor necrosis factor; PBS, phosphate-buffered saline; 7-AAD, 7-amino actinomycin D; PBS-T, phosphate-buffered saline-Tween 20; NP-40, Nonidet P-40.

highly responsive to mitogens and growth factors and has been implicated in cell proliferation, differentiation, and cell cycle progression (Cobb, 1999). Although exceptions exist, the bulk of evidence suggests that activation of the SAPK and p38 MAPK cascades promote apoptosis (Cross et al., 2000), whereas activation of ERK1/2 exerts a cytoprotective effect (Xia et al., 1995; Kang et al., 2000). Efforts to understand the functional role of the ERK1/2 pathway in various cellular functions have been greatly facilitated by the recent development of several specific pharmacologic inhibitors of the MAPK kinase (MEK1/2), including PD98059 and PD184352 (Alessi et al., 1995; Sebolt-Leopold et al., 1999). The latter compound has attracted considerable interest in view of its capacity to inhibit the ERK1/2 pathway when administered in vivo (Sebolt-Leopold et al., 1999).

The relationship between paclitaxel-mediated lethality and the ERK1/2 pathway is unclear. Previous studies have shown that activation of ERK1/2 is required for entry into and progression through G_2M as well as for the proper functioning of the mitotic spindle apparatus (Wang et al., 1997). Thus, it seems plausible that perturbations in the ERK1/2 cascade might influence the response of cell agents, such as paclitaxel, that trigger G_2M arrest. However, attempts to define the relationship between the p42/44 MAPK cascade and paclitaxel-related lethality are complicated by conflicting reports that paclitaxel exposure can induce either an increase (Huang et al., 1999), a decrease (Stone and Chambers, 2000), or no change (Shtil et al., 1999) in ERK1/2 activity, depending on the cell type. Lieu et al. (1998) reported that administration of the specific MEK/MAPK inhibitor PD98059 before and concurrently with paclitaxel reduced lethality in the human myelomonocytic leukemia cell line U937. Such a finding is somewhat difficult to reconcile with observations from our laboratory that the macrocyclic lactone bryostatin 1, which on chronic administration down-regulates protein kinase C (PKC) as well as its downstream target, MEK/MAPK (Jarvis et al., 1998), enhances paclitaxel lethality toward U937 cells in a sequence-dependent manner (Wang et al., 1998). Moreover, the actions of bryostatin were mimicked, at least to an extent, by PD98059. To gain insights into mechanisms responsible for these phenomena, we have examined the schedule-dependent effects of pharmacologic MEK/MAPK inhibitors on the response of leukemic cells to paclitaxel, as well as perturbations in signaling events accompanying these interactions. Our results indicate that subsequent but not prior exposure of paclitaxel-treated leukemic cells to various MEK/MAPK inhibitors leads to potentiation of mitochondrial damage, caspase activation, and apoptosis. Furthermore, such effects are associated with multiple perturbations in signaling and apoptotic regulatory pathways, including inhibition of p42/44 MAPK activation, down-regulation of Mcl-1, and, unexpectedly, activation of the p38 MAPK cascade.

Materials and Methods

Cells. The human monocytic leukemic cell line U937, isolated from the peripheral blood of a patient with diffuse histiocytic lymphoma, was maintained as described previously (Wang et al., 1998). Cells were cultured in RPMI 1640 medium supplemented with sodium pyruvate, minimum essential medium essential vitamins, L-glutamate, penicillin and streptomycin, and 10% heat-inactivated fetal calf serum (FCS; Hyclone, Logan, UT). They were maintained in a 37°C, 5% CO_2, fully humidified incubator, passed twice weekly, and prepared for experimental procedures when in log phase growth (cell density $\leq 4 \times 10^5$ cells/ml). U937 TAM67 cells, which stably express a c-Jun transactivation binding domain-deficient mutant protein, were maintained, along with empty-vector control cells, under selection pressure in medium containing 400 μg/ml G418, as described previously (Grant et al., 1996).

Reagents. Paclitaxel and cycloheximide were purchased from Sigma (St Louis, MO), stored in light-protected containers at −20°C, and dissolved in sterile dimethyl sulfoxide (DMSO; Sigma) before use. PD98059, U0126, SB203580 and 202190, and TNFα were purchased from Calbiochem (La Jolla, CA), and formulated in DMSO as above. Stock solutions were then diluted in RPMI medium to achieve the desired final concentration. In all cases, final concentrations of DMSO were less than 0.1% and did not modify responses of cells to paclitaxel. PD184352 was kindly provided by Dr. Judy Sebolt-Leopold (Parke-Davis Pharmaceuticals, Ann Arbor, MI), and was formulated as above. IETD-fmk and ZVAD-fmk were purchased from Enzyme Products, Ltd., (Livermore, CA) and formulated in sterile water before use.

Experimental Format. As described previously (Wang et al., 1998) experimental design was employed. Logarithmically growing cells were treated with the designated concentration of paclitaxel (generally 250 nM) for 6 h, washed free of drug, and resuspended in medium containing various concentrations of MEK inhibitors for 15 h. Alternatively, cells were first exposed to MEK inhibitors for 15 h, washed, and treated with paclitaxel for 6 h, after which they were washed again, resuspended in drug-free medium, and apoptosis monitored over the ensuing 15 h. Treatment with paclitaxel alone induced apoptosis in 27.8 ± 2.8% of cells when given by this schedule, whereas treatment with MEK inhibitors alone according to this schedule had negligible effects on cell viability or apoptosis. This experimental format allowed comparisons to be made between the effects of prior versus subsequent administration of MEK inhibitors on paclitaxel-induced lethality.

Assessment of Apoptosis. After drug exposures, cytocentrifuge preparations were stained with Wright-Giemsa stain and viewed by light microscopy to evaluate features of cellular differentiation as well as apoptosis (i.e., cell shrinkage, nuclear condensation, formation of apoptotic bodies, etc.,) as described previously (Wang et al., 1998). For the latter studies, the percentage of apoptotic cells was determined by evaluating ≥ 500 cells/condition in triplicate. We have previously reported that the incidence of apoptosis as determined by these morphological criteria correlates very closely with the degree of low molecular weight DNA fragmentation assayed quantitatively by spectrofluorometry, and qualitatively with the amount of internucleosomal DNA fragmentation determined by agarose gel electrophoresis (Wang et al., 1998).

Determination of Mitochrondrial Membrane Potential($\Delta\psi_m$). Mitochondrial membrane potential was monitored using 3,3-dihexyloxacarbocyanine iodide (Calbiochem, San Diego, CA; Wang et al., 1999b). For each condition, 4×10^5 cells were incubated for 15 min at 37°C in 1 ml of 40 nM 3,3-dihexyloxacarbocyanine iodide and subsequently analyzed using a FACScan cytofluorometer (Becton-Dickinson, San Jose, CA) with excitation and emission settings of 488 and 525 nm, respectively. Control experiments documenting the loss of $\Delta\psi_m$ were performed by exposing cells to 5 μM carbamoyl cyanide *m*-chlorophenylhydrazone (Sigma) (15 min, 37°C), an uncoupling agent that abolishes the mitochondrial membrane potential.

Preparation of S-100 Fractions and Assessment of Cytochrome *c* Release. U937 cells were harvested after drug treatment by centrifugation at 600*g* for 10 min at 4°C. The cytosolic S-100 fraction was prepared as described previously (Wang et al., 1999b), with minor modifications. Cell pellets were washed once with ice-cold phosphate-buffered saline (PBS) and resuspended in 5 volumes of buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreotol, and

0.1 mM phenylmethylsulfonyl fluoride, 250 mM sucrose). After chilling for 30 min on ice, the cells were disrupted by 15 strokes of a glass homogenizer. The homogenate was centrifuged twice to remove unbroken cells and nuclei (750*g*, 10 min, 4°C). S-100 fractions (supernatants) were then obtained by centrifugation at 100,000*g*, 60 min at 4°C. All steps were performed on ice or 4°C. Cytochrome *c* release into the S-100 fraction for each condition was assessed by Western blot analysis of the resulting fractions as detailed below.

7-AAD. Assessment of cell viability was determined by monitoring cellular accumulation of 7-amino actinomycin D (Sigma) by flow cytometry, as described previously (Philpott et al., 1996).

Western Analysis. A minor modification of a method described previously was employed (Wang et al., 1997a). After treatment, whole-cell pellets (1×10^7 cells/condition) were washed twice in PBS, resuspended in 50 μl of PBS, lysed by the addition of 50 μl of 2× Laemmli buffer (1×, 30 mM Tris-base, pH 6.8, 2% SDS, 2.88 mM β-mercaptoethanol, 10% glycerol), and briefly sonicated. Homogenates were quantified using Coomassie protein assay reagent (Pierce, Rockford, IL). Equal amounts of protein (20 μg) were boiled for 10 min, separated by SDS-PAGE (5% stacker and 10% resolving) and electroblotted to nitrocellulose. The blots were stained in 0.1% amido black and destained in 5% acetic acid to ensure transfer and equal loading. After blocking in PBS-Tween (PBS-T; 0.05%) and 5% milk for 1 h at 22°C, the blots were incubated in fresh blocking solution with an appropriate dilution of primary antibody for 4 h at 22°C. The source and dilution of antibodies are were as follows: Bcl-2 1:2000, mouse monoclonal, DAKO (Carpinteria, CA); Bax N20, 1:2000, rabbit polyclonal, Santa Cruz Biotechnology (Santa Cruz, CA); Bcl-x_L 1:500, rabbit polyclonal, Santa Cruz; XIAP 1:500, rabbit polyclonal, R & D Systems (Minneapolis, MN); Mcl-1 1:1000, mouse monoclonal Pharmingen (San Diego, CA); ERK 1/2 1:1000, rabbit polyclonal, Cell Signaling Technology (Beverly, MA); phospho-ERK 1/2 (thr202/tyr204) 1:1000, rabbit polyclonal, Cell Signaling Technology; JNK 1:1000, rabbit polyclonal, Santa Cruz; phospho-JNK, 1:1000, mouse monoclonal, Santa Cruz; phospho p38 MAPK, 1:1000, rabbit polyclonal, Cell Signaling Technology; phospho-cdc2 1:1000, rabbit polyclonal, Cell Signaling Technology; procaspase-3, 1:1000, mouse monoclonal, Transduction Laboratories (Lexington, KY); PARP (C-2–10), 1:3000, mouse monoclonal, BioMol Research Laboratories (Plymouth, MA); cytochrome *c* 1:500, mouse monoclonal, Pharmingen; α-tubulin 1:2000; Calbiochem; actin 1:1000, mouse monoclonal, Sigma). Blots were washed three times for 5 min each in PBS-T and then incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, Hercules, CA) for 1 h at 22°C. Blots were again washed three times for 5 min each in PBS-T and then developed by enhanced chemiluminescence (Pierce).

Determination of Free Bax Levels. A minor modification of a technique described previously (Wang et al., 1998) was employed to quantify free Bax levels in supernatants immunodepleted of Bcl-2- and Bcl-xL-Bax heterodimers. After treatment, cells were washed twice with cold PBS and lysed in 0.2% NP-40 isotonic lysis buffer containing protease inhibitors (142.5 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES, pH 7.2, 1 mM EGTA, 0.2% NP-40, 0.2 mM phenylmethylsulfonyl fluoride, 0.1% aprotinin, and 0.7 μg/ml leupeptin) by incubation for 30 min. Nuclei and unlysed cellular debris were removed by centrifugation at 15000*g* for 10 min. Supernatant protein (200 μg per condition) was incubated with 1 μg monoclonal Bcl-2 antibody (DAKO A/S, Glostrup, Denmark) and polyclonal Bcl-xL antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 4 h at 4°C. Immunoprecipitates were then captured using Dynabeads (Dynal A.S., Oslo, Norway) for another 4 h according to the manufacturer's instructions. The heterodimer-depleted supernatants were then exposed to anti-Bax polyclonal antibody (1 ìg; Santa Cruz), and the resulting immunoprecipitates containing free Bax were washed three times in 0.2% NP-40 lysis buffer. After solubilization in SDS-PAGE gel electrophoresis sample buffer, electrophoresis of samples was carried out on 12% SDS-PAGE gels as described above. Free Bax levels were detected by immunoblotting with primary Bax antibody and quantification of ECL-developed blots performed using a PhosphorImager (Molecular Dynamics).

Flow Cytometry Cell Cycle Analysis. After treatment, cells were pelleted at 500*g* and resuspended in 70% ethanol. The cell pellets were incubated on ice for 1 h, and resuspended in 1 ml of cell cycle buffer (0.38 mM sodium citrate, 0.5 mg/ml RNase A, and 0.01 mg/ml propidium iodide; all Sigma) at a concentration of 10^6 cells/ml. Samples were stored in the dark before analysis at 4°C (usually within 24 h), and analyzed on a Becton-Dickinson FACScan flow cytometer (Cambridge, MA) using a commercially available software program (ModFit LT 2.0; Verity Software, Topsham, ME) (Wang et al., 1999b).

Clonogenic Assays and Cloning Studies. A method described previously was employed (Wang et al., 1998). Briefly, after drug treatment, cells were washed three times in drug-free RPMI medium containing 5% FCS and cell counts determined using a Coulter counter (model ZBI; Beckman Coulter, Fullerton, CA). After normalization, cells were plated in 18-mm, 12-well plates (Costar, Cambridge, MA). Each well contained 1 ml of supplemented RPMI 1640 medium, 20% FCS, 0.3% Bacto agar (Difco, Detroit, MI), and 4×10^2 cells/condition. The plates were placed in a fully humidified, 37°, 5% CO_2 incubator for 12 days, after which colonies, consisting of groups of $\geq$ 50 cells, were scored with the aid of an Olympus Model CK inverted microscope. The total number of colonies for each condition was calculated by multiplying the cell number at the end of the initial incubation period by the cloning efficiency. Values for each condition were expressed as a percentage relative to untreated controls.

Statistical Analysis. The significance of differences between experimental conditions was determined using the two-tailed Student's *t* test.

Results

To assess sequence-dependent interactions between paclitaxel and PD98059, logarithmically growing U937 cells were treated with 250 nM paclitaxel for 6 h before a 15-h exposure to PD98059 (40 μM); alternatively, cells were preincubated with PD98059 for 15 h before the 6-h paclitaxel exposure, after which cells were washed, resuspended in fresh medium, and mitochondrial function and apoptosis monitored over the ensuing 15 h (Fig. 1). With this protocol, paclitaxel by itself induced apoptosis in 27% of cells, whereas administration of PD98059 alone exerted negligible effects on cell viability. Whereas pretreatment with PD98059 reduced the susceptibility of cells to paclitaxel-induced apoptosis by ~ 20% ($P \leq 0.05$ versus paclitaxel alone), subsequent exposure of paclitaxel-treated cells to PD98059 significantly increased the extent of apoptosis (e.g., by ~65%; $P \leq 0.002$ versus paclitaxel alone) (Fig. 1A). The solid bars reflect the minor contribution of PD98059 by itself to the increase in apoptosis. The extent of apoptosis in cells exposed to the sequence paclitaxel → PD98059 was also very significantly greater than that observed in cells exposed to the sequence PD98059

→ paclitaxel (e.g, 40.3 ± 3.2 versus 21.2 ± 2.8; $P \leq 0.001$). Lastly, similar results were obtained when apoptosis was monitored at later intervals (e.g., 36–48 h after initial drug exposure; data not shown).

Parallel results were obtained when loss of mitochondrial membrane potential ($\Delta\psi_m$; Fig. 1B) or viability, determined by 7-AAD uptake (Fig. 1C) were examined. Thus, prior exposure of cells to PD98059, which by itself exerted minimal effects on mitochondrial function or viability, significantly reduced the susceptibility of cells to paclitaxel-related lethality, whereas subsequent exposure enhanced toxicity.

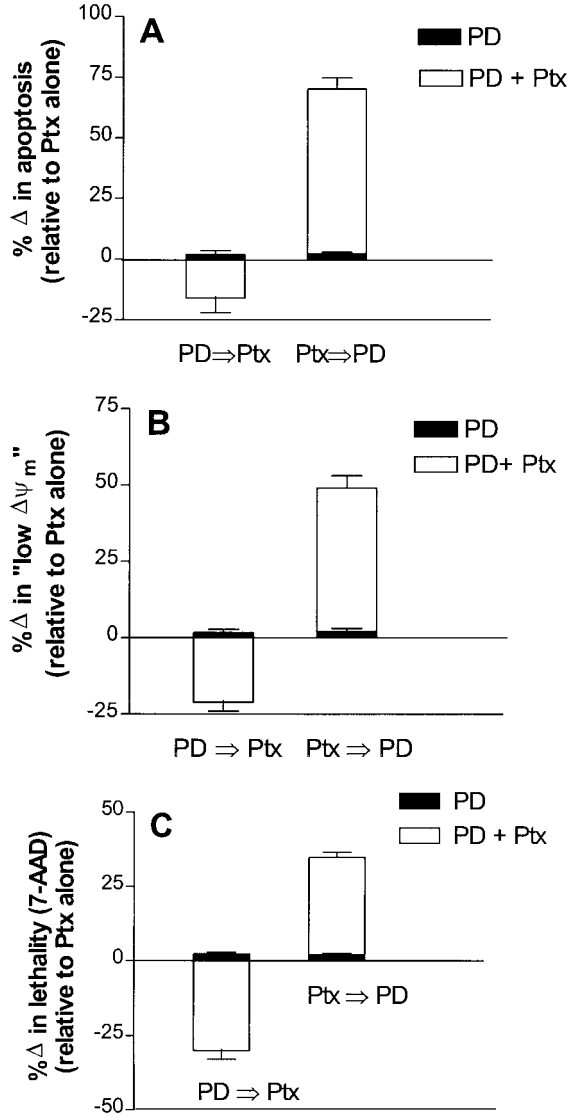

Fig. 1. Logarithmically growing U937 cells were exposed to paclitaxel (Ptx; 250 nM; 6 h), washed free of drug, and incubated for an additional 15 h in the presence or absence of 40 μM PD98059. Alternatively, cells were treated with PD98059 before paclitaxel exposure, after which they were washed and resuspended in drug-free medium for 15 h as above. A, at the end of the incubation period, the percentage of apoptotic cells was determined by examining Wright Giemsa-stained cytocentrifuge preparations and scoring 10 randomly selected fields containing at least 500 cells. Values are expressed as the change in paclitaxel-mediated apoptosis induced by prior or subsequent PD98059 exposure relative to values obtained for paclitaxel alone (mean value, 27.6 ± 2.7%). The dark areas within the bar correspond to the contribution of PD98059 administered alone. Similarly, the effects of PD98059 on the percentage of cells exhibiting a low mitochondrial membrane potential compared with paclitaxel by itself ($\Delta\psi_m$; 1B; mean value for paclitaxel alone, 30.1 ± 3.1%) or an increase in 7-AAD uptake (1C; mean value for paclitaxel alone, 22.4 ± 3.4%) were determined as described under *Materials and Methods*. In each case, values are expressed as the means for three separate experiments performed in triplicate ± 1 S.D.

Similar interactions were observed when other MEK inhibitors were employed. These included U0126, an agent approximately 100-fold more potent than PD98059 as an inhibitor of the MEK ATP binding site (Favata et al., 1998), and PD184352, which inhibits MEK/MAPK in vivo (Sebolt-Leopold et al., 1999). Pretreatment of cells with U0126 (20 μM) or PD184352 (10 μM) protected cells from paclitaxel-mediated mitochondrial damage and apoptosis to an even greater extent than PD98059, whereas subsequent exposure increased paclitaxel-related lethality to a comparable degree (Fig. 2A-C). As in the case of PD98059, administration of U0126 or PD184352 alone exerted minimal toxicity (solid bars). Consistent with previous findings, sequential administration of paclitaxel followed by U0126 or PD184352 induced significantly more mitochondrial damage and loss of viability than the opposite sequences ($P \leq 0.005$ in each instance).

Sequence-dependent effects were also noted when effects on procaspase-3, PARP degradation, and cytochrome *c* release were monitored (Fig. 3). Thus, pretreatment of cells with PD98059 exerted little effect on, or slightly reduced, cleavage/activation of procaspase-3 and degradation of PARP to an 85 kDa species, whereas subsequent exposure of paclitaxel-treated cells to PD98059 increased the extent of procaspase-3 and PARP cleavage (Fig. 3). Similarly, prior exposure to PD98059 reduced, whereas subsequent exposure increased cytochrome *c* release in paclitaxel-treated cells (Fig. 3). Comparable results were obtained when U0126 or PD184352 were used (data not shown).

In other studies, it was found that shorter PD98059 incubation intervals (e.g., 6 and 3 h) reduced paclitaxel lethality by 24.7 ± 4.7 and 17.2 ± 3.7%, respectively (data not shown), values that were significantly less than those obtained for the 15-h preincubation interval ($P \leq 0.01$ in each case). In addition, cells exposed simultaneously to paclitaxel and PD98059 for 6 h, washed, and incubated for an additional 15 h in drug-free medium did not exhibit a significant increase in apoptosis relative to cells exposed to paclitaxel alone (data not shown). These findings indicate that a prolonged PD98059 pre-exposure interval is necessary for maximal attenuation of paclitaxel lethality and that sustained exposure to PD98059 after paclitaxel treatment is required to potentiate cell death.

To investigate the hierarchy of apoptotic events triggered by exposure of cells to paclitaxel followed by PD98059, cells were first treated with paclitaxel as above and subsequently incubated with PD98059 in the presence or absence of the broad caspase inhibitor ZVAD-fmk (Fig. 4). Coexposure to ZVAD-fmk essentially abrogated the increase in morphological evidence of apoptosis (Fig. 4A) and loss of $\Delta\psi_m$ (Fig. 4B) in paclitaxel-treated cells that accompanied subsequent PD98059 treatment. In contrast, ZVAD-fmk failed to prevent the increase in paclitaxel-induced cytochrome *c* release induced by PD98059, although it did antagonize degradation of procaspase-3 (Fig. 4B). These findings suggest that increased cytochrome *c* release represents a primary consequence of combined drug exposure, whereas the loss of $\Delta\psi_m$ stems, at least in part, from caspase activation.

As shown in Fig. 5A, subsequent treatment with PD98059

(40 μM) increased apoptosis in cells exposed to a range of paclitaxel concentrations (e.g., 50–250 nM), although effects were somewhat more pronounced at the upper end of this range. Potentiation of paclitaxel lethality became apparent at a PD98059 concentration of 30 μM and was more even more marked at concentrations of 40 to 50 μM (Fig. 5B). Lastly, in view of evidence that paclitaxel lethality may be related to activation of the TNF pathway (Ding et al., 1990), cells were treated with paclitaxel followed by PD98059 in the presence or absence of the caspase-8 inhibitor IETD-fmk (Fig. 5C). Whereas apoptosis induced by TNF + cycloheximide was abrogated by IETD-fmk, no protection was observed in cells exposed to the paclitaxel/PD98059 combination. Similar results were obtained in U937 cells ectopically expressing the caspase 8 inhibitor CrmA (data not shown). These findings argue against the possibility that PD98059 enhances paclitaxel lethality by promoting TNF release.

Sequence-dependent effects of PD98059 were then examined in relation to MAPK activation. Induction of phosphorylated MAPK (reflected by increased expression of phospho-ERK on Western blots) was noted 2 h after initial paclitaxel exposure and increased further at the 3 and 6 h intervals (Fig. 6A). In cells first exposed to PD98059, washed, and subsequently treated with paclitaxel, MAPK activation was very apparent 3 h after the end of the paclitaxel exposure (Fig. 6B). In fact, activation was even more pronounced than at the end of the 6-h exposure interval. However, when cells were first exposed to paclitaxel, washed, and subsequently treated with PD98059, induction of phospho-ERK at this (as well as at later intervals; Fig. 10) was essentially abrogated (Fig. 6C). Thus, subsequent, but not prior, treatment of cells with PD98059 opposed paclitaxel-induced activation of the MEK/MAPK pathway.

Previous studies have related paclitaxel lethality to activation of $p34^{cdc2}$ (Shen et al., 1998). To determine whether subsequent exposure to PD98059 might increase paclitaxel-mediated $p34^{cdc2}$ activation, Western analysis was carried out using an antibody that recognizes $p34^{cdc2}$ phosphorylated on the tyr_{15} residue (Fig. 7A). Consistent with previous findings (Shen et al., 1998), treatment of cells with paclitaxel alone reduced levels of tyr_{15} phospho-$p34^{cdc2}$. However, sequential exposure of cells to paclitaxel followed by PD98059 did not result in further dephosphorylation of $p34^{cdc2}$. For all conditions, levels of total $p34^{cdc2}$ protein were equivalent (data not shown). These findings argue against the possibility that PD98059 increases paclitaxel lethality by enhancing $p34^{cdc2}$ activation. In separate studies, the impact of PD98059 on paclitaxel-associated perturbations in the mitotic index was examined (Fig. 7B). Although PD by itself did not modify the percentage of mitotic cells (or the G_2M fraction; data not shown), it significantly reduced the mitotic index in cells exposed to paclitaxel. It should be noted that the magnitude of this decline approximated the observed increase in apoptosis.

MAPK has recently been implicated in modulating phosphorylation of the Bcl-2 protein (Deng et al., 2000). Consistent with previous reports (Yamamoto et al., 1999), exposure of cells to paclitaxel resulted in the appearance of a slowly

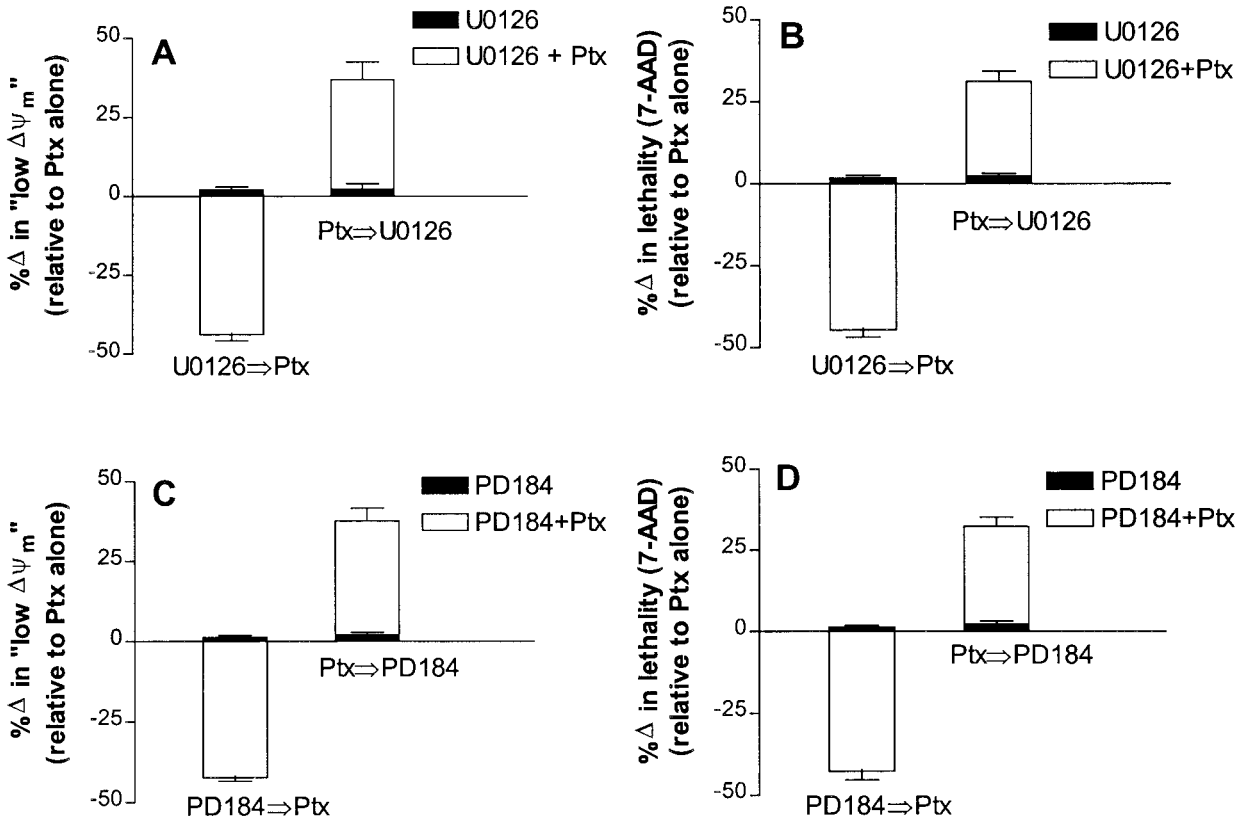

Fig. 2. U937 cells were treated with paclitaxel (250 nM; 6 h) either before or after a 15-h exposure to 20 μM U0126 or 10 μM PD184352, after which changes in loss of $\Delta\psi_m$ (UO126–2A; PD184352–2C) or increase in 7-AAD uptake (U0126–2B; PD184352–2D) determined as described under *Materials and Methods*. Changes in mitochondrial function and viability were then expressed in relation to values obtained for cells treated with paclitaxel alone. Values represent the means for three separate experiments performed in triplicate ± S.D.

migrating, putatively phosphorylated Bcl-2 species (Fig. 8). However, expression of this species was not altered in cells subsequently exposed to PD98059, despite the observed potentiation of apoptosis. In addition, MAPK inhibition has been reported to reduce expression of the antiapoptotic Mcl-1 protein in human ML-1 cells and to increase their susceptibility to the antimitotic agent VP-16 (Townsend et al., 1998). However, in contrast to the effects of VP-16 in ML-1 cells, Mcl-1 levels declined in U937 cells treated with paclitaxel, and this phenomenon was even more pronounced in cells exposed to paclitaxel + PD98059 (Fig. 8). PD treatment did not modify expression or mobility of the antiapoptotic proteins Bcl-x_L or XIAP in paclitaxel-treated U937 cells. Lastly, PD98059 did not alter total Bax levels in paclitaxel-treated cells. Moreover, in cell extracts immunodepleted of Bcl-2/Bax and Bcl-xL/Bax heterodimers, levels of free Bax were diminished after paclitaxel-treatment, consistent with previous reports (Wang et al., 1998), but this effect was not enhanced by subsequent exposure of cells to PD98059 (Fig. 8). Together, these findings argue against the possibility that PD98059 enhances paclitaxel-mediated lethality by altering Bcl-2 phosphorylation status or free Bax levels, although the contribution of reduced Mcl-1 expression cannot be excluded.

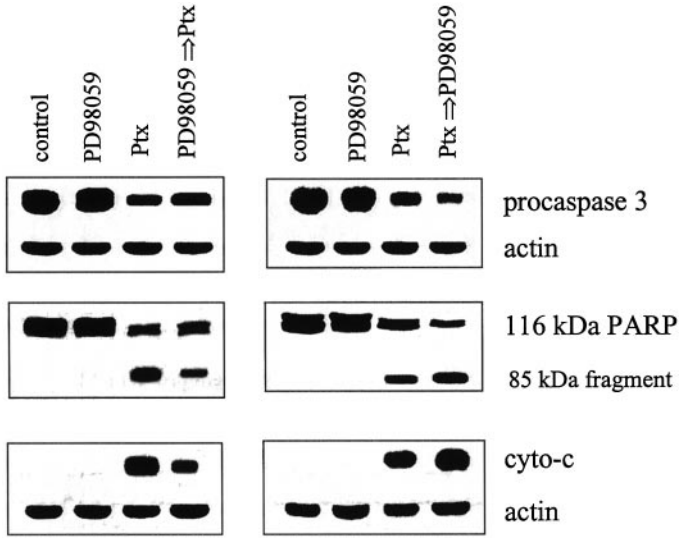

Fig. 3. U937 cells were exposed to paclitaxel (250 nM; 6 h), washed, and incubated for 15 h in the presence or absence of 40 μM PD98059, after which cell lysates were obtained, separated by PAGE-SDS, and probed with antibodies directed against procaspase-3 or PARP. Alternatively, S-100 cytoplasmic fractions were obtained as described under *Materials and Methods*, and Western analysis was performed to assess cytochrome *c* release. Parallel studies were performed in which cells were treated with PD98059 before paclitaxel exposure. Each lane was loaded with 25 μg protein; blots were stripped and reprobed with antibodies to actin to ensure equal loading and transfer of proteins. The results of a representative experiment are shown; two additional studies yielded equivalent results.

The impact of sequential exposure of cells to paclitaxel and PD98059 on the stress-related JNK and p38 pathways was examined next (Fig. 9). Treatment of cells with paclitaxel alone (250 nM) resulted in increased expression of phospho-JNK that persisted for 6 h after paclitaxel exposure, and this effect was increased slightly by subsequent exposure of cells to PD98059 (Fig. 9A). As shown in Fig. 9B, paclitaxel exposure increased phospho-JNK expression in a dose-dependent manner, and treatment with PD98059 enhanced JNK activation modestly at each paclitaxel dose. Similar effects were noted at other time points throughout the 15-h postpaclitaxel exposure interval (data not shown). PD98059 also increased paclitaxel-induced apoptosis in cells expressing the c-Jun transactivation domain-deficient mutant protein TAM67 (data not shown). However, although paclitaxel by itself failed to increase expression of phospho-p38 MAPK (Fig. 9B, bottom), subsequent exposure of cells to PD98059 resulted in a very striking increase in p38 MAPK activation. As shown in

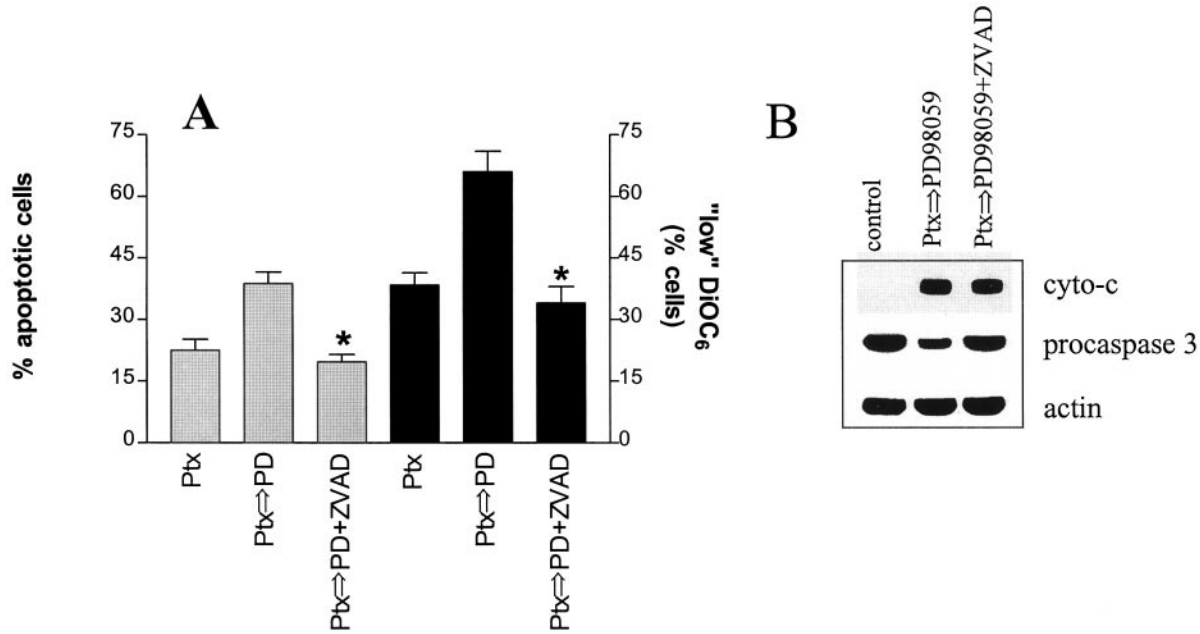

Fig. 4. A, U937 cells were exposed to 250 nM paclitaxel for 6 h, washed, and incubated with 40 μM PD98059 ± 25 μM ZVAD-fmk for an additional 15 h, after which the percentage of apoptotic cells (left axis; ▨) or the loss of $\Delta\psi_m$ (right axis; ■) determined as described under *Materials and Methods*. *, not significantly different from values for paclitaxel alone; $P \geq 0.05$. B, cells were treated as described above, after which lysates were obtained and cytosolic cytochrome *c* or procaspase-3 levels determined as described above. Each lane was loaded with 25 μM protein. The results of a representative experiment are shown; two others yielded equivalent results.

Fig. 9B, lower, this effect was also observed in cells exposed to 150 nM but only marginally at 50 nM paclitaxel. Furthermore, activation of p38 MAPK was not observed in cells treated with PD98059 before paclitaxel (data not shown). To assess the functional significance of enhanced p38 MAPK activation in cells exposed to paclitaxel followed by PD98059, experiments were performed in the presence of the p38 MAPK inhibitor SB203580, and caspase activation and apoptosis monitored (Fig. 8, C and D). It can be seen that both the potentiation of paclitaxel-induced procaspase-3 degradation (Fig. 9C) and apoptotic morphology (Fig. 9D) by PD98059 were essentially abrogated by SB203580. Similarly, the p38 MAPK inhibitor SB202190 also attenuated paclitaxel/PD98059-induced apoptosis (Fig. 9D). Together, these findings indicate that antagonism of paclitaxel-induced MAPK phosphorylation by the MEK/MAPK inhibitor PD98059 in U937 cells is accompanied by a reciprocal increase in p38 MAPK activation and raise the possibility that this phenomenon contributes to potentiation of paclitaxel-induced lethality.

The temporal relationship between p42/44 and p38 MAPK activation in cells exposed to paclitaxel ± PD98059 was examined further (Fig. 10). As noted previously (Fig. 6), p42/44 MAPK activation was noted 3 h after paclitaxel treatment; although the extent of activation subsequently declined, it was maintained over the 15-h exposure interval. However, administration of PD98059 effectively abrogated p42/44 MAPK activation throughout this period. Phosphorylated p38 MAPK was not observed in paclitaxel-treated cells at the 3-h interval, but could be very faintly discerned at later time points. However, in paclitaxel-treated cells subsequently exposed to PD98059, activation of p38 MAPK was clearly present at 3 h, very apparent at 6 h, and even more pronounced at the end of the incubation interval. Thus, in paclitaxel-treated cells, inhibition of sustained activation of

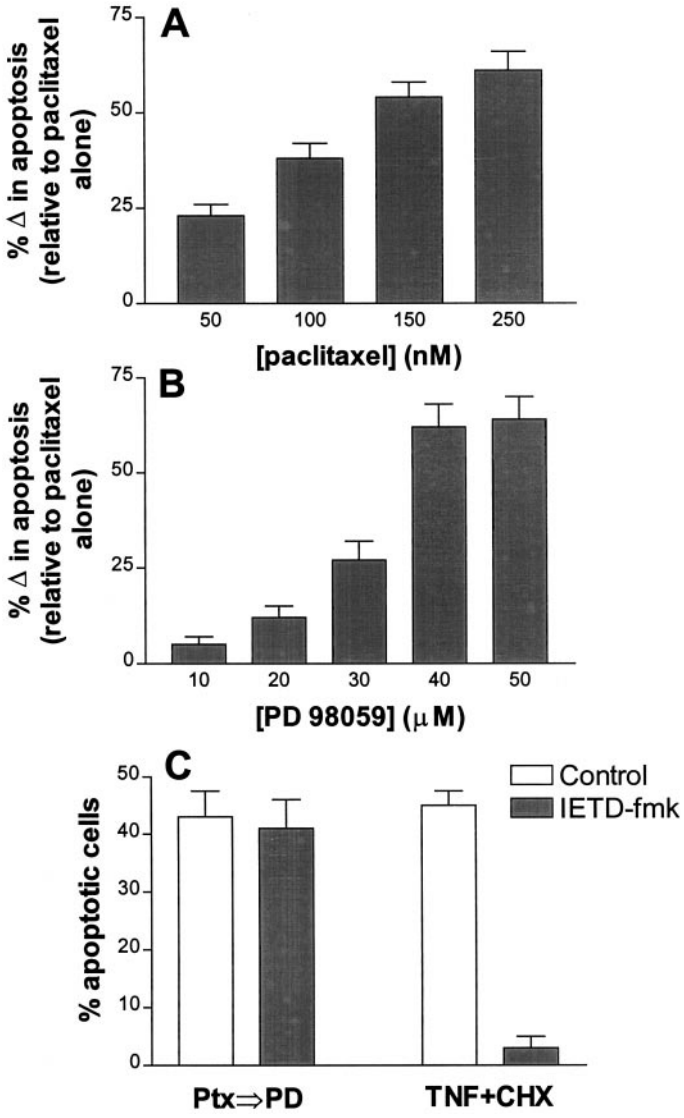

Fig. 5. A, U937 cells were treated with the designated concentration of paclitaxel, washed, and incubated with 40 μM PD98059 for 15 h, after which increases in the percentage of apoptotic cells (relative to cells treated with paclitaxel alone) determined as described above. B, U937 cells were treated with 250 nM paclitaxel for 6 h, washed, and exposed to the designated concentration of PD98059 for 15 h, after which potentiation of apoptosis was determined as above. C, cells were exposed to paclitaxel (250 nM; 6 h) followed by 40 μM PD98059 (15 h) or TNFα (5 ng/ml) + cycloheximide (1 μM) in the presence or absence of the caspase-8 inhibitor IETD-fmk (8 μM), after which the extent of apoptosis was determined as above. In each case, values represent the means for three separate experiments performed in triplicate ± S.D.

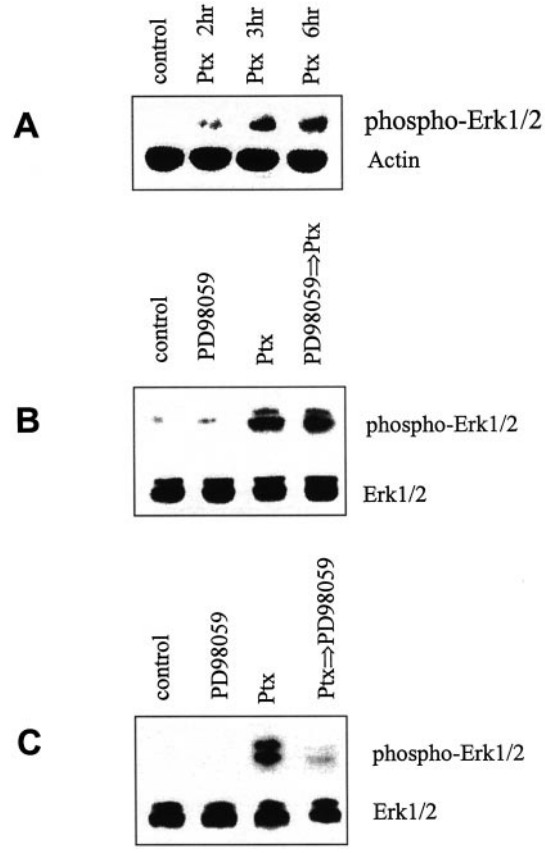

Fig. 6. A, U937 cells were exposed to 250 nM paclitaxel for the designated intervals, after which cell lysates were obtained as described under *Materials and Methods*. B, U937 cells were exposed to 250 nM paclitaxel for 6 h after a 15-h incubation with 40 μM PD98059; 3 h after removal of the paclitaxel, cells were lysed. C, alternatively, cells were exposed to paclitaxel for 6 h, washed, and treated with PD98059 as above. In each case, expression of phospho-Erk 1/2 determined by Western analysis using antibodies directed against phospho-thr202 and -tyr204 sites as described under *Materials and Methods*. Proteins were also probed with an antibodies directed against actin or total Erk 1/2. Each lane was loaded with 25 μg of protein. The results of a representative experiment are shown; two additional studies yielded equivalent results.

p42/44 MAPK was accompanied by a reciprocal increase in expression of phosphorylated p38 MAPK.

To investigate the biological consequences of PD98059-mediated potentiation of paclitaxel-induced apoptosis by PD98059, clonogenic assays were performed (Fig. 11). PD98059 by itself did not significantly reduce colony formation. While pretreatment with PD98059 only modestly protected clonogenic cells from paclitaxel-associated lethality, this effect was nevertheless significant ($P \leq 0.05$). Moreover, subsequent exposure of paclitaxel-pretreated cells to PD98059 resulted in a significant loss of colony forming potential ($P \leq 0.01$ versus paclitaxel alone). In addition, the reduction in colony formation for the sequence paclitaxel → PD98059 was very significantly greater than that for the sequence PD98059 → paclitaxel ($P \leq 0.005$). These findings indicate that subsequent exposure of paclitaxel-pretreated cells to the MEK/MAPK inhibitor PD98059 enhances lethality in self-renewing leukemic cells, and raises the possibility that an increase in apoptosis may be involved in this phenomenon.

Discussion

The present studies demonstrate that in U937 cells, subsequent, but not prior exposure of paclitaxel-treated cells to pharmacologic MEK/MAPK inhibitors potentiates mitochondrial damage and apoptosis. The relationship between p42/44 MAPK activity and paclitaxel exposure is complex and may be cell-type–specific. Thus, treatment with paclitaxel has led to an increase (Huang et al., 1999) or no change in MCF-7 cells (Shtil et al., 1999), or a decline in MAPK activity in KB-3 carcinoma cells (Stone and Chambers, 2000). Furthermore, PD98059 exerts either no effect on paclitaxel toxicity (Huang et al., 1999), or attenuates its lethal actions (Lieu et al., 1998). In the latter study, pretreatment combined with concurrent (24-h) exposure of U937 cells to PD98059 (80 μM) decreased the paclitaxel cytotoxicity (Lieu et al., 1998). Consistent with these findings, we also found that pretreatment of U937 cells with PD98059 (e.g., 40 μM), or other pharmacologic MEK inhibitors (e.g., U0126 and PD184352), attenuated paclitaxel-mediated mitochondrial damage (i.e., cytochrome *c* release and loss of $\Delta\psi_m$), caspase activation, and apoptosis. However, in contrast to the results of Lieu et al., subsequent exposure of paclitaxel-treated U937 cells to multiple MEK/MAPK inhibitors significantly increased paclitaxel-mediated lethality. The latter findings are consistent with those of MacKeigan et al., who recently demonstrated that coadministration of PD98059 or U0126 with paclitaxel for 24 h led to a marked increase in cytotoxicity in lung (H157) and ovarian (OVCA194) cancer cells (MacKeigan et al., 2000). Together, these findings suggest that scheduling and cell type may play a key role in determining the net effect of MEK/MAPK inhibition on paclitaxel toxicity.

Activation of MEK/MAPK is generally, although not invariably, associated with cytoprotective actions (Kang et al., 2000). For example, the relative outputs of the JNK and ERK

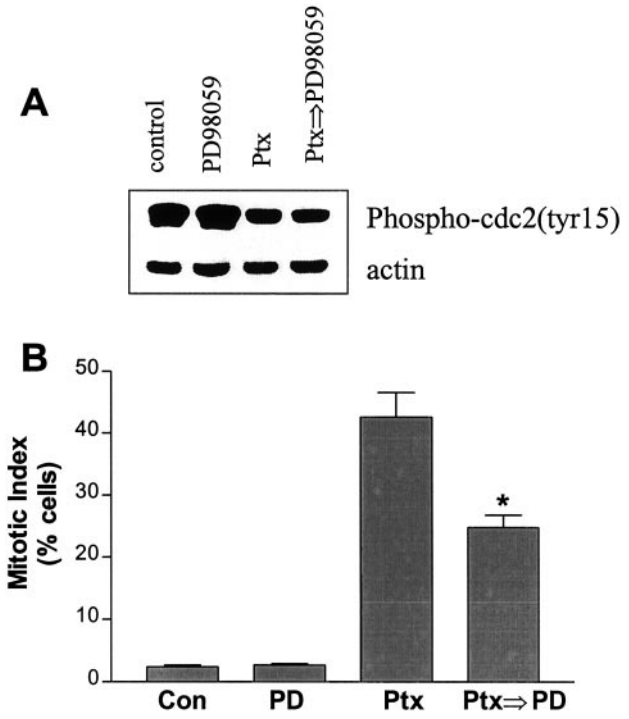

Fig. 7. A, U937 cells were treated with paclitaxel (250 nM; 6 h) washed, and subsequently incubated for 15 h in the presence or absence of 40 μM PD98059, after which cells were lysed, the proteins separated by PAGE, and probed with an antibody directed against phospho-Tyr_{15} p34cdc2 as described in the text. Each lane contained 25 μg of protein. Blots were stripped and reprobed with antibodies directed against actin to ensure equal loading and transfer of proteins. The results of a representative experiment are shown; two additional studies yielded equivalent results. B, cells were treated as above after which cytospin preparations were obtained, stained with Wright-Giemsa, and viewed under light microscopy. The mitotic index was determined by scoring the number of cells exhibiting typical mitotic features after viewing 20 randomly selected fields encompassing at least 1000 cells/condition. Values represent the means ± S.D. for triplicate determinations obtained from three separate experiments. *, significantly less than values for paclitaxel alone; $P \leq 0.02$.

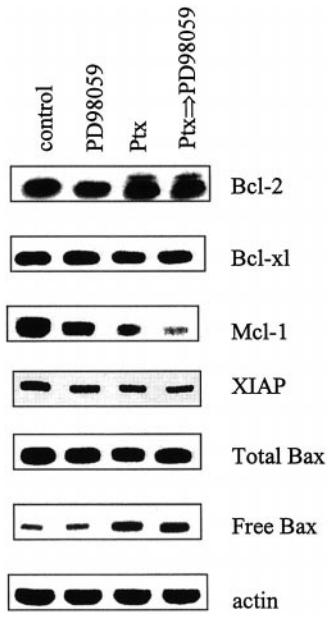

Fig. 8. U937 cells were exposed to 250 nM paclitaxel for 6 h, washed, and incubated in the presence or absence of 40 μM PD98059 for 15 h, after which cells were lysed, the protein separated by PAGE-SDS, and probed with antibodies directed against Bcl-2, Bcl-x_L, Mcl-1, XIAP, and Bax, as described under *Materials and Methods*. Alternatively, lysates were immunodepleted of Bcl-2- and Bcl-x_L associated heterodimers, and levels of free Bax determined as above. Blots were subsequently stripped and reprobed with antibodies directed against actin to ensure equivalent loading and transfer of protein. Each lane was loaded with 25 μg of protein. The results of a representative study are shown; two other experiments yielded equivalent results.

pathways regulate survival in PC12 pheochromocytoma cells subjected to growth factor deprivation (Xia et al., 1995). In HL-60 cells, potentiation of ara-C-mediated lethality (e.g., by the PKC down-regulator bryostatin) has been attributed to inhibition of MAPK rather than JNK activation (Jarvis et al., 1998). Analogously, subsequent (but not prior) exposure of paclitaxel-treated U937 cells to bryostatin, like PD98059, potentiated apoptosis (Wang et al., 1998). These findings raise the possibility that exposure of leukemic cells to cytotoxic agents elicits a cytoprotective MAPK response and that interruption of this process (e.g., either upstream at the level of PKC or downstream at the level of MEK/MAPK) promotes lethality. The observation that only subsequent exposure of paclitaxel-treated cells to MEK inhibitors prevented MAPK activation suggests that the actions of the latter agents are relatively short-lived. It is worth noting that in HL-60 cells, paclitaxel exposure failed to induce MAPK activation, and PD98059 did not modify paclitaxel-mediated apoptosis (Blagosklonny et al., 1999). Aside from the fact that the latter study employed a simultaneous rather than sequential schedule, it is possible that MEK inhibitors only enhance paclitaxel lethality in cells in which MAPK is activated. It should be noted that the failure of simultaneous (6-h) administration of PD98059 to potentiate paclitaxel-induced apoptosis suggests that other factors (e.g., cell cycle-related) are involved in this phenomenon.

The present results indicate that the proximal cause of enhanced apoptosis in cells exposed to paclitaxel and MEK inhibitors is cytochrome *c* release. Although loss of the $\Delta\psi_m$ represents the central executioner of apoptosis in some cells (Marchetti et al., 1996), evidence that 1) cytochrome *c* release often precedes loss of $\Delta\psi_m$ (Goldstein et al., 2000) and 2) caspase activation may occur in the absence of $\Delta\psi_m$ changes (Li et al., 2000) suggests that the cytochrome *c* redistribution

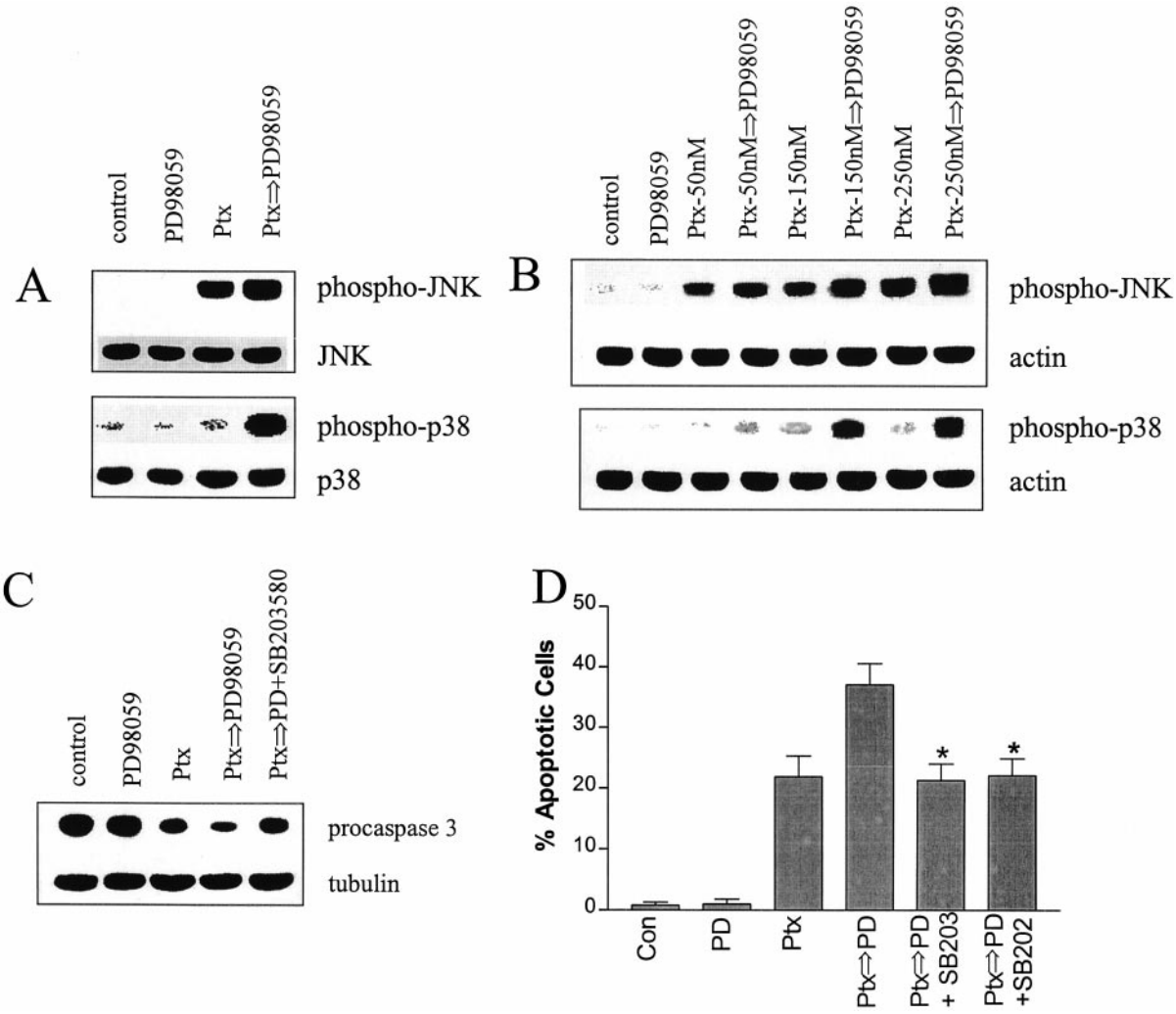

Fig. 9. A, U937 cells were treated with paclitaxel (250 nM; 6 h), washed, and incubated in the presence or absence of 40 μM PD98059 for 6 h, after which cells were lysed, protein separated by PAGE-SDS, and probed for phospho-JNK and phospho-p38 MAPK as described under *Materials and Methods*. B, cells were treated with the designated concentration of paclitaxel followed by PD98059 for 6 h as above, after which expression of phospho-JNK and phospho-p38 MAPK determined as in A. C, cells were treated paclitaxel (250 nM) as above, after which they were incubated with PD98059 (40 μM) in the presence or absence of SB203580 (5 μM) for an additional 15 h. Cells were then lysed and expression of full-length (32 kDa) procaspase-3 determined by Western analysis as described previously. For these studies, each lane was loaded with 25 μg of protein; blots were stripped and probed for expression of actin or tubulin to ensure equal loading and transfer of protein. D, cells were treated with paclitaxel followed by PD98059 ± SB203580 (5 μM) or SB202190 (10 μM) for 15 h as above, after which the percentage of apoptotic cells was determined as described previously. Values represent the means ± S.D. for three separate experiments performed in triplicate. *, not significantly different from paclitaxel alone; $P \geq 0.05$.

represents the primary cell death trigger. Consistent with this concept, ZVAD-fmk blocked enhanced caspase activation and apoptosis in paclitaxel-treated cells subsequently exposed to PD98059, but not cytochrome *c* release. These findings argue that MEK inhibitors lower the threshold for cytochrome *c* release in paclitaxel-pretreated cells and that loss of $\Delta\psi_m$ represents a consequence of caspase activation.

In view of evidence linking paclitaxel toxicity to cell cycle dysregulation, it seems plausible that MEK/MAPK inhibitors might promote the latter action. For example, exposure of cells to paclitaxel induces dephosphorylation of the cyclin-dependent kinase $p34^{cdc2}$ (Shen et al., 1998), unscheduled activation of which is associated with apoptosis (Meikrantz et al., 1994). Furthermore, MAPK has been implicated in the G_2-M transition, normal microtubular function, and the mitotic spindle checkpoint (Wang et al., 1997b; Cross and Smythe, 1998). However, subsequent exposure of paclitaxel-treated cells to PD98059 did not lead to further dephosphorylation/activation of $p34^{cdc2}$, although it did reduce the mitotic index while reciprocally increasing apoptosis. These findings raise the possibility that disruption of the mitotic spindle assembly apparatus by MEK/MAPK inhibitors may amplify the lethal consequences of microtubule stabilization induced by prior paclitaxel treatment. Conversely, interference with the G_2-M transition (e.g., by prior MEK inhibitor exposure) might attenuate the lethal effects of subsequently administered paclitaxel. This concept is compatible with recent findings indicating that MEK inhibitors (e.g., PD98059 and U0126) block the transition from G_2 to mitosis induced by nocodazole (Hayne et al., 2000).

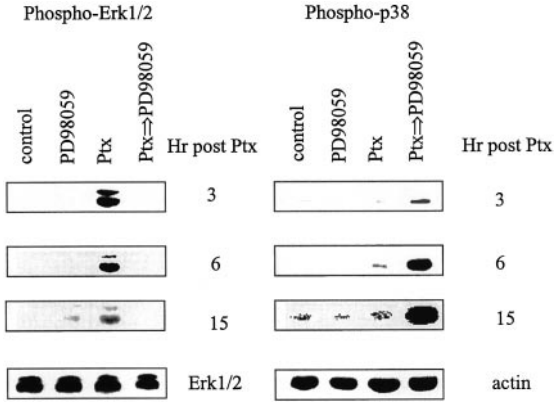

Fig. 10. U937 cells were treated with 250 nM paclitaxel for 6 h, washed, and incubated in the presence or absence of 40 μM PD98059 for the designated intervals, after which cells were lysed, the proteins separated by PAGE-SDS, and probed for expression of phospho-ERK 1/2 and phospho-p38 MAPK as described above. Each lane was loaded with 25 μg of protein; blots were subsequently stripped and reprobed with antibodies directed against total ERK 1/2 or actin to ensure equivalent loading and transfer. The results of a representative study are shown; two additional experiments yielded equivalent results.

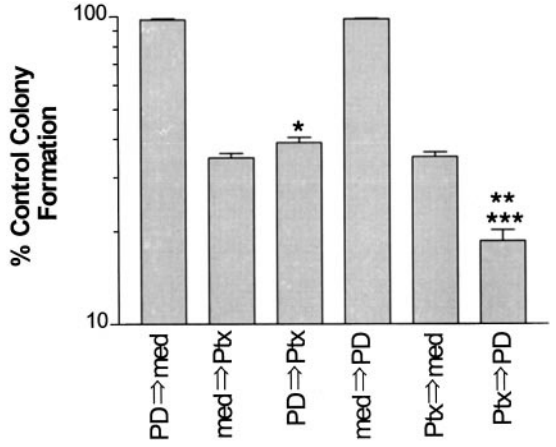

Fig. 11. Logarithmically growing U937 cells were treated with 250 nM paclitaxel (6 h), washed, and subsequently incubated in either drug-free medium (med) or medium containing PD98059 (40 μM) for 15 h. Alternatively, cells were exposed to these drugs in the opposite sequence (i.e., PD98059 followed by paclitaxel). In both cases, cells were plated in soft agar 15 h after the end of the paclitaxel exposure interval as described under *Materials and Methods* and colonies, consisting of groups of $\geq$ 50 cells, scored at day 12 using an Olympus inverted microscope. Values are expressed as a percentage relative to untreated control cells and represent the means $\pm$ S.D. for three separate experiments performed in triplicate. *, not significantly different from values for paclitaxel alone; $P \geq 0.05$. **, significantly less than values for paclitaxel alone; $P \leq 0.02$. ***, significantly less than values for the sequence PD98059 followed by paclitaxel; $P \leq 0.01$.

Interruption of signaling cascades may influence apoptosis by modulating the expression of apoptotic regulatory proteins. In this regard, paclitaxel-induced cell death has been associated with Bcl-2 phosphorylation (Blagosklonny et al., 1997; Yamamoto et al., 1999). Conversely, in IL-3-dependent murine myeloid cells subjected to growth factor deprivation, Bcl-2 phosphorylation exerted an antiapoptotic effect that was blocked by PD98059 (Deng et al., 2000). However, although exposure of cells to paclitaxel led, as reported previously (Blagosklonny et al., 1999), to the appearance of a phosphorylated Bcl-2 species, this effect was not appreciably modified by PD98059 treatment. Nevertheless, the possibility that PD98059 induces qualitative alterations in Bcl-2 phosphorylation (Hu et al., 1998) cannot be excluded. Interestingly, exposure of U937 cells to PD98059 did induce down-regulation of the antiapoptotic protein Mcl-1, consistent with findings in human ML-1 leukemia cells treated with VP-16 (Townsend et al., 1998). In this report, cotreatment with PD98059 blocked up-regulation of Mcl-1 induced by VP-16. In contrast, paclitaxel by itself reduced Mcl-1 expression in U937 cells, an effect that was enhanced by subsequent exposure to PD98059. Thus, the present findings raise the possibility that down-regulation of Mcl-1 by MEK inhibitors contributes to enhanced paclitaxel toxicity.

Earlier studies have implicated JNK activation in paclitaxel-mediated lethality (Lee et al., 1998), a phenomenon linked to Bcl-2 phosphorylation/inactivation (Yamamoto et al., 1999). Moreover, the concept that perturbations in stress and cytoprotective signaling pathways contribute to the lethal actions of other microtubule-active agents (e.g., vinblastine) has been proposed (Fan et al., 2000a,b). However, the observations that subsequent exposure of paclitaxel-treated cells to PD98059 resulted in only a modest increase in phospho-JNK expression, and the ability of PD98059 to increase paclitaxel toxicity in TAM67-expressing cells argues against a primary role for JNK in enhanced cell killing. The relationship between p38 MAPK activation and paclitaxel lethality, as in the case of the other MAPKs, seems to vary with cell type. For example, paclitaxel activated p38 MAPK in MCF-7 cells (Huang et al.,1999), reduced basal activity in KB-3 carcinoma cells (Stone and Chambers, 2000), and exerted no effect in HL-60 cells (Blagosklonny et al., 1999). However, in contrast to JNK activation, exposure of paclitaxel-pretreated

U937 cells to PD98059 produced a very dramatic increase in p38 MAPK activation. Furthermore, the ability of pharmacologic p38 MAPK inhibitors (e.g., SB203580 and SB202190) to block potentiation of apoptosis suggests a functional role for this MAPK in the cell death process. One model that might explain these findings, summarized in Fig. 12, is that treatment of U937 cells with paclitaxel elicits a stress-related JNK and a cytoprotective p42/44 MAPK response, the relative outputs of which determine the extent of apoptosis. Subsequent exposure of paclitaxel-treated cells to MEK/MAPK inhibitors opposes p42/44 MAPK activation, triggering a reciprocal induction of p38 MAPK. The shift away from cytoprotective and toward stress-associated MAPK signaling may contribute to potentiation of cell death. However, the finding that simultaneous exposure of cells to PD98059 and paclitaxel did not lead to enhanced lethality strongly suggests that other factors (e.g., cytokinetic) are also involved in this phenomenon, as discussed previously. Finally, the possibility that interactions between paclitaxel and MEK/MAPK inhibitors involve perturbations in both signal transduction and cell cycle events cannot be excluded.

In summary, the present findings demonstrate that subsequent but not prior exposure of paclitaxel-treated U937 to pharmacologic MEK/MAPK inhibitors results in a significant increase in mitochondrial injury, and cell death. Furthermore, these events are associated with inhibition of paclitaxel-induced p42/44 MAPK activation, down-regulation of Mcl-1, and a dramatic increase in p38 MAPK activation. In view of the development of MEK inhibitors with in vivo activity (Sebolt-Leopold et al., 1999), these studies could have implications for future attempts to use such agents to enhance the activity of paclitaxel and possibly other taxanes. Accordingly, efforts to extend these findings to other malignant hematopoietic and nonhematopoietic cell types, including those that, unlike U937 cells, express wild-type -53, are underway.

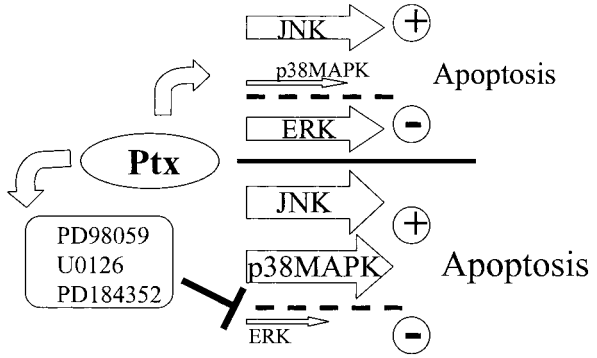

Fig. 12. Hypothetical model of signaling interactions in cells exposed to paclitaxel and MEK/MAPK inhibitors. In U937 cells, exposure to paclitaxel leads to activation of JNK and ERK1/2, but not p38 MAPK. The relative outputs of these pathways may influence the cell death response to this agent. Exposure of paclitaxel-pretreated cells to pharmacologic MEK/MAPK inhibitors opposes the sustained MAPK response, modestly increases JNK activation, and dramatically increases expression of phospho-p38 MAPK. Thus, the shift toward stress (e.g., JNK, p38 MAPK) and away from cytoprotective (e.g., ERK 1/2) signaling pathways, particularly when combined with as yet to be defined perturbations in cell cycle progression and/or mitotic events, may contribute to the observed potentiation of paclitaxel lethality.

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