

## **Docetaxel-Induced Apoptosis of Human Melanoma Is Mediated by Activation of c-Jun NH<sub>2</sub>-Terminal Kinase and Inhibited by the Mitogen-Activated Protein Kinase Extracellular Signal-Regulated Kinase 1/2 Pathway**

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**Abstract Purpose:** Our studies have shown variable sensitivity of cultured melanoma cells to docetaxel. To better understand this response, we studied the role of signal transduction pathways in modulating docetaxel-induced melanoma killing.

**Experimental Design:** Involvement of c-Jun NH<sub>2</sub>-terminal kinase (JNK), extracellular signal-regulated kinase 1/2 (ERK1/2), p38 mitogen-activated protein kinase, and Akt signaling was studied by evaluating their extent of activation in melanoma cells after treatment with docetaxel. The effect of their activation on docetaxel-induced apoptosis was assessed using biochemical inhibitors of the pathways and Western blot analysis of proteins involved.

**Results:** Docetaxel induced activation of both JNK and ERK1/2 but not p38 mitogen-activated protein kinase or Akt kinases. Apoptosis was dependent on activation of JNK and mediated through activation of caspase-2 and caspase-dependent changes in Bax and Bak. The levels of activated JNK in individual lines showed a close correlation with the levels of apoptosis. In contrast, activation of ERK1/2 by docetaxel inhibited apoptosis and the levels of activation in individual lines were inversely correlated to the degree of apoptosis. Studies on the Bcl-2 family proteins seemed to reflect changes induced by activation of JNK and ERK1/2 pathways. Docetaxel-induced JNK activation was required for Bcl-2 phosphorylation as well as caspase-2-dependent activation of Bax and Bak and subsequent mitochondrial release of apoptosis-inducing factor and cytochrome *c*. In contrast, activation of ERK1/2 resulted in degradation of BH3-only protein Bim and phosphorylation of Bad.

**Conclusions:** These studies provide further insights into sensitivity of melanoma cells to taxanes and provide a basis for the current rationale of combining taxanes with inhibitors of the Raf-ERK1/2 pathway.

Microtubules, the cytoskeletal structures formed by the polymerization of tubulin heterodimers, play a crucial role in many biological processes, including mitosis, cell-cell communication, intracellular transport, cell growth, and apoptosis. Given the pivotal importance of microtubules in cellular function, they have been the targets for anticancer drugs, such as taxanes and *Vinca* alkaloids. By interfering with the dynamics of microtubule assembly, microtubule-binding agents exert profound effects on cellular processes, including expression of

certain genes (1), cell cycle arrest, and apoptosis (2). The signal transduction pathways that mediate the cellular responses to these agents remain unclear.

The mitogen-activated protein kinases (MAPK) are a family of intracellular enzymes that constitute important mediators of signal transduction pathways and coordinate the cellular response to a variety of extracellular stimuli (3). This large superfamily of serine/threonine protein kinases consists of three major MAPK families: extracellular signal-regulated kinases (ERK1 and ERK2), c-Jun NH<sub>2</sub>-terminal kinases (JNK1, JNK2, and JNK3), and p38 MAPK (p38  $\alpha$ ,  $\beta$ ,  $\delta$ , and  $\gamma$ ; ref. 4). Recently, new members were added to the MAPK superfamily, including ERK3, ERK5, and ERK7 (5). Individual MAPKs are phosphorylated and activated by MAPK kinase [MAPK/ERK kinase (MEK) protein kinases that are usually specific for particular MAPKs] and control distinct cellular processes involved in cell division, differentiation, and survival (6).

The stress-activated kinases, JNKs, and p38 MAPKs have been implicated in apoptotic response of cells exposed to UV irradiation, heat shock, chemotherapy, and proinflammatory cytokines (7–10). The JNK-stimulated apoptosis involves mitochondrial release of apoptotic factors, and both Bax and Bak are essential for JNK-regulated apoptosis (11–13). The

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proapoptotic roles of activated JNK isoforms are not clearly defined, but the phosphorylation of transcription factors, such as c-Myc, p62<sup>TCF</sup>/Elk-1, c-Jun, ATF2, and ATF3 (1), as well as proapoptotic and antiapoptotic proteins, such as Bim, Bad, Mcl-1, and Bcl-2, has been suggested to be of importance (13–16).

In contrast to the induction of apoptosis by the stress-activated kinases, the Ras-MEK-ERK pathway is associated with suppression of apoptosis (17). ERK1/2 lies downstream of a group of kinases, including protein kinase C, Raf-1, and MEK1. On stimulation by extracellular signals, they are successively activated by phosphorylation (18). Constitutive activation of Ras/Raf/MEK/ERK signaling is a hallmark of many human cancers, such as breast, lung, and colorectal cancers as well as melanoma (19–22). In melanoma, mutations in either N-Ras (23) or BRAF (24) may be responsible for activation of the pathway but several autocrine and paracrine factors may also be responsible.

The phosphatidylinositol 3-kinase/Akt pathway may also promote tumor cell survival (25). It is constitutively activated in melanoma cells (26). The antiapoptotic effect of the Akt pathway is diverse and includes an activation of the nuclear factor- $\kappa$ B transcription factor (27, 28), phosphorylation of Bad (29), and inactivation of Forkhead transcription factors, which regulate several proapoptotic proteins, such as Bim, procaspase-9, and Bad (30–32). Activated Akt can also mediate the transcription of several antiapoptotic proteins, such as Bcl-XL, AI, and XIAP (33, 34).

In the present study, we examined the role of JNK, ERK1/2, p38 MAPK, and Akt kinases in the induction of apoptosis by docetaxel in melanoma cells. We report here that docetaxel-induced activation of JNK and ERK1/2 kinases in melanoma cells seems to be key determinant of sensitivity of melanoma to docetaxel.

## Materials and Methods

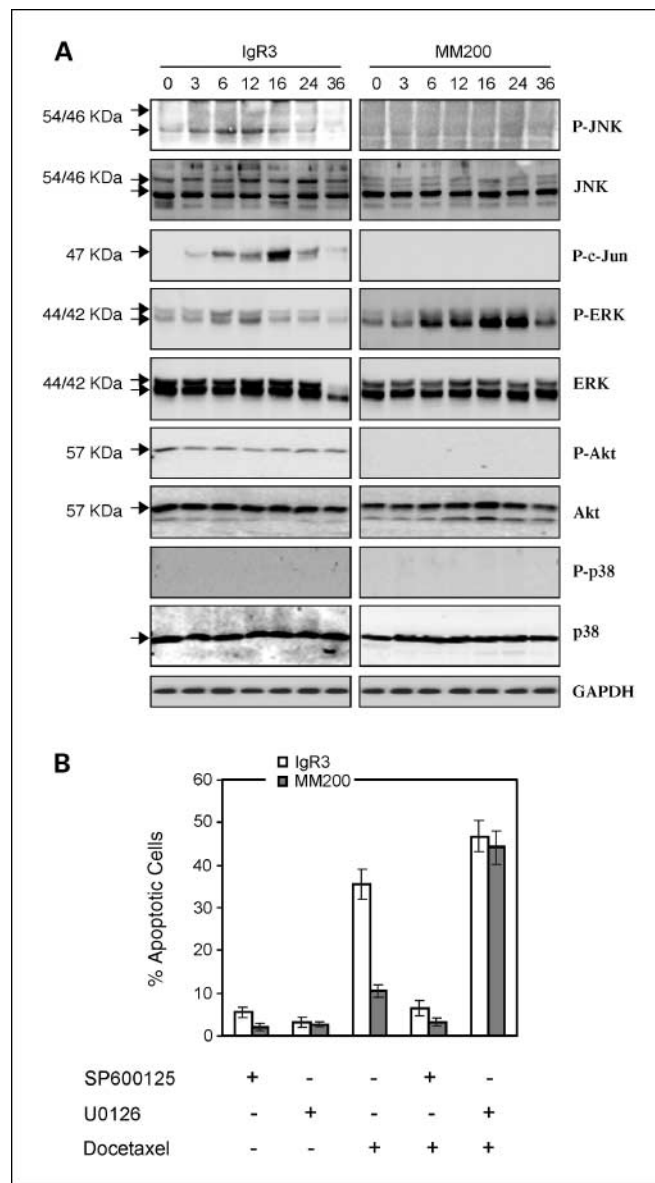
**Cell lines.** Human melanoma cell lines Me4405, Me1007, IgR3, Mel-FH, Mel-RM, Mel-CV, Mel-AT, SK-mel-28, SK-mel-110, and MM200 have been described previously (35). The cell lines were cultured in DMEM containing 5% FCS (Commonwealth Serum Laboratories, Melbourne, Victoria, Australia).

**Antibodies and other reagents.** Docetaxel (Taxotere), kindly provided by Aventis Pharma S.A (Paris, France), was stored as a 100 mmol/L solution in absolute ethanol at  $-80^{\circ}\text{C}$  and diluted with the medium before use. JNK inhibitor (SP600125), p38 MAPK inhibitor (SP203580), the caspase-2 substrate (Z-VDVAD-AFC) to measure caspase-2 activity, and the mouse anti-Bak Ab-1 antibody were purchased from Calbiochem (La Jolla, CA). The mouse monoclonal antibody against cytochrome *c* and the rabbit monoclonal antibody against the active form of caspase-3 were purchased from PharMingen (Bioclone, Marrickville, Australia). The mouse monoclonal antibodies against Bcl-2, Bcl-XL, Mcl-1, and phosphorylated ERK1/2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal antibodies against ERK1/2, phosphorylated Akt (Ser<sup>473</sup>), Akt, phosphorylated p38 MAPK (Thr<sup>180</sup>/Tyr<sup>182</sup>), p38 MAPK, and JNK/stress-activated protein kinase were purchased from Cell Signaling Technology (Beverly, MA). The rabbit polyclonal anti-Bax against amino acids 1 to 20 was purchased from Upstate Biotechnology (Lake Placid, NY). The propidium iodide was purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia). The ERK1/2 inhibitor (MEK inhibitor, U0126) was purchased from Promega Corp. (Sydney, New South Wales, Australia).

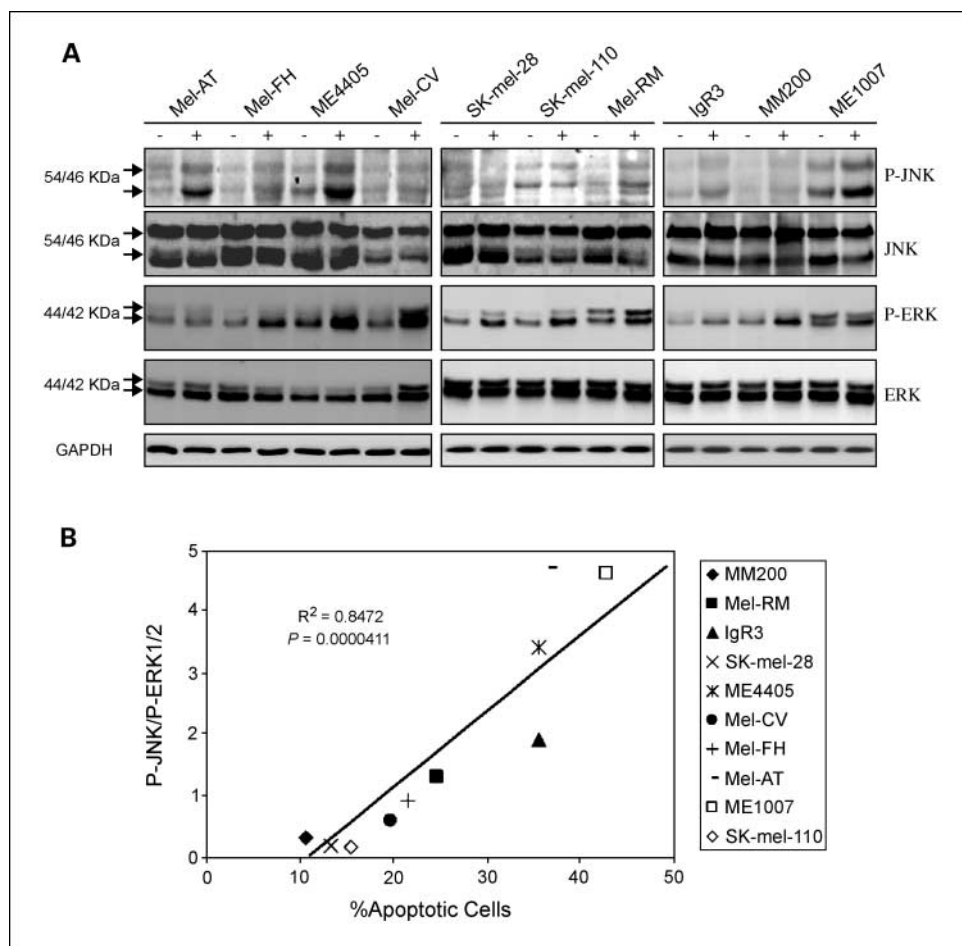
**Apoptosis.** Quantitation of apoptotic cells by measurement of sub-G<sub>1</sub> DNA content using the propidium iodide method was carried out as described elsewhere (36).

**Flow cytometry.** Immunostaining on intact and permeabilized cells was carried out as described previously (37). Analysis was carried out using a Becton Dickinson (Mountain View, CA) FACScan flow cytometer. The percentage of antigen-positive cells was calculated as the difference in positive area between the positive and negative control histograms. The positive area was that to the right of the intersection of the two curves (36).

**Mitochondrial membrane potential ( $\Delta\Psi$ m).** Tumor cells were seeded at  $1 \times 10^5$  per well in 24-well plates and allowed to reach exponential growth for 24 h before treatment. JC-1 staining was done according to the manufacturer's instructions (Molecular Probes,



**Fig. 1.** Involvement of different signal transduction pathways in docetaxel-induced apoptosis. **A**, effects of docetaxel on stress and survival signaling pathways. IgR3 and MM200 cells were treated with docetaxel at 20 nmol/L for the indicated times. Whole-cell lysates were subjected to Western blot analysis. Western blot analysis of GAPDH levels was included to show that equivalent amounts of protein were loaded in each lane. Data are representative of two individual experiments. **B**, docetaxel-induced apoptosis is regulated by JNK and ERK1/2 signaling pathways. IgR3 and MM200 cells were treated with inhibitors of JNK (SP600125) and ERK1/2 (U0126) 1 h before adding docetaxel for another 48 h. Apoptosis was measured by the propidium iodide method using flow cytometry. Columns, mean of three individual experiments; bars, SE.



**Fig. 2.** Docetaxel-induced apoptosis is correlated with activation of JNK and ERK1/2. **A**, docetaxel-induced activation of JNK and ERK1/2 in a panel of melanoma cell lines. Cells were treated with or without docetaxel at 20 nmol/L for 12 h before harvest. Whole-cell lysates were subjected to Western blot analysis. Western blot analysis of GAPDH levels was included to show that equivalent amounts of protein were loaded in each lane. Data are representative of two individual experiments. **B**, correlation of the ratio of activated JNK to the activated ERK1/2 and the levels of docetaxel-induced apoptosis in a panel of melanoma cell lines. The relative expression of activated protein was determined by dividing the densitometric value of the protein by that of the GAPDH control (the intensity of the bands was quantitated with the Bio-Rad VersaDoc image system). The ratio of activated JNK to the activated ERK1/2 was calculated by dividing the relative expression of phosphorylated JNK ( $P$ -JNK) to the relative expression of phosphorylated ERK1/2 ( $P$ -ERK1/2). Regression analyses were carried out in a Macintosh computer using the StatView software.

Eugene, OR). Briefly, adherent and nonadherent cells were collected and washed with PBS. Cells were then incubated with 10  $\mu$ g/mL JC-1 in warm PBS at 37°C for 15 min. After washing with PBS, the cells were analyzed using a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA). Cells with polarized mitochondria presented in the upper right quadrant of the dot plot due to the formation of JC-1 aggregates, which emit orange fluorescence (590 nmol/L) when excited at 488 nmol/L. Cells with depolarized mitochondria emit green fluorescence (530 nmol/L) and are visualized in the lower right quadrant in the dot plot (38).

**Western blot and protein expression analysis.** The protein content of cell extracts was determined by the Bradford assay (Bio-Rad, Regents Park, New South Wales, Australia). A total of 20 to 30  $\mu$ g of protein was electrophoresed on 10% to 15% SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked, incubated with primary antibodies at the appropriate concentration, and subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:3,000 dilution; Bio-Rad). Labeled bands were detected by Immuno-Star horseradish peroxidase chemiluminescent kit, and images were captured. The intensity of the bands was quantitated with the Bio-Rad VersaDoc image system (Bio-Rad). The relative expression of certain protein was determined by dividing the densitometric value of the test protein by that of the control [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)].

**Preparation of mitochondrial and cytosolic fractions.** Methods used for subcellular fraction were similar to the methods described previously (36).

**Small interfering RNA-mediated silencing of Bim.** Cells were transfected in six-well plates with Bim-specific or nontarget control small interfering RNA (siRNA; SMARTpool, Dharmacon, Lafayette, CO)

for 24 h. Cells were transfected with 100 nmol/L of the above siRNA using Oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Following silencing, cells were treated for 24 h with docetaxel at 20 nmol/L and apoptotic cells were quantified by the propidium iodide method in flow cytometry. Efficiency of RNA interference was assessed by immunoblotting.

**Caspase activity assay.** A method used to measure the activity of caspase-2 was described previously (39).

**Statistical analysis.** Data are expressed as mean  $\pm$  SE. Statistical analyses were carried out with statistical package StatView 4.5. Regression analysis was used to examine whether there was a correlation between phosphorylated JNK or phosphorylated ERK1/2 expression and docetaxel-induced apoptosis in studies on melanoma cell lines. The statistical significance of intergroup differences in normally distributed continuous variables was determined using Student's *t* test. Tests were considered significant when *P* values were <0.05.

## Results

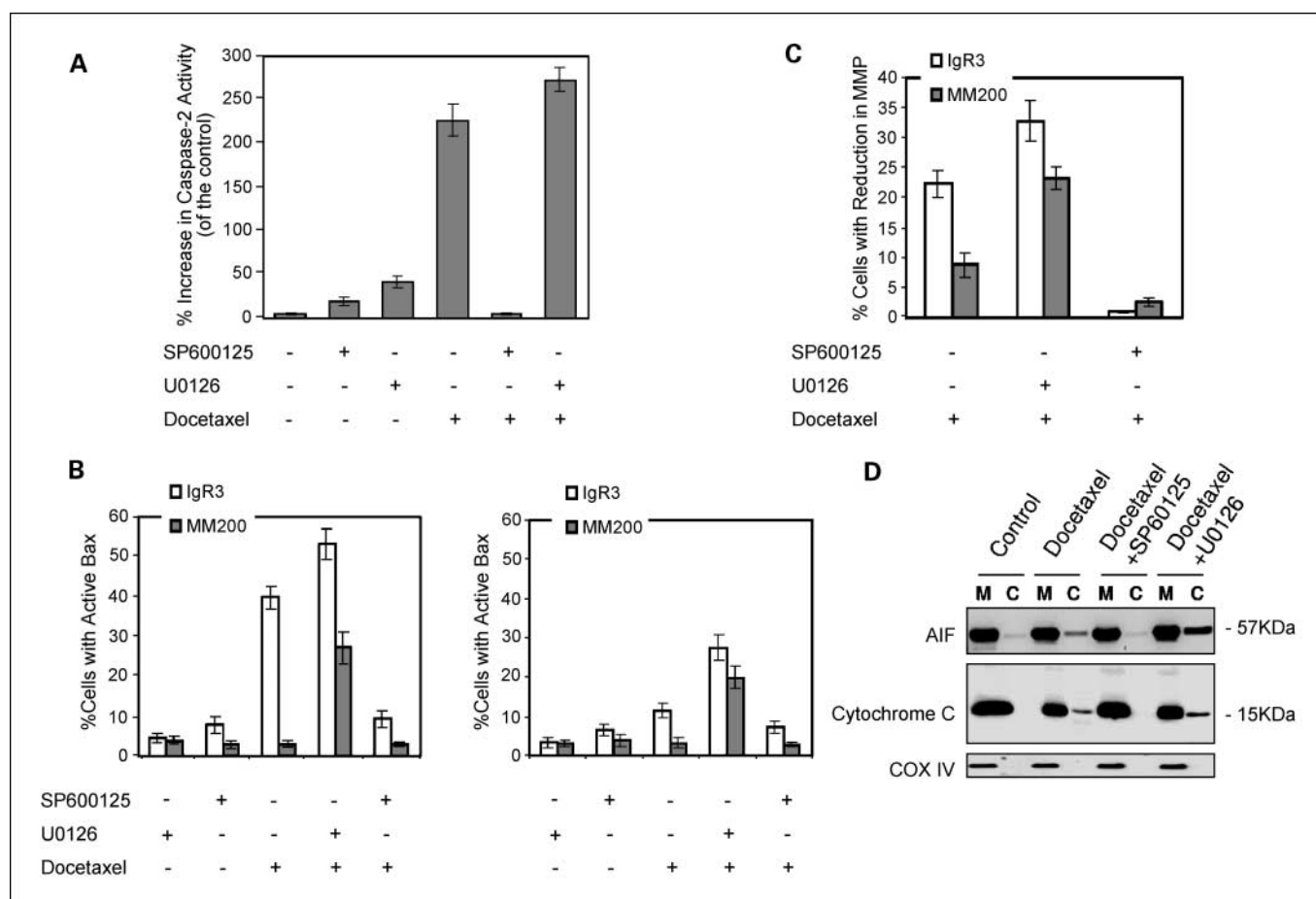
**Docetaxel elicits differential effects on survival and stress signaling pathways.** We have shown that docetaxel induces caspase-dependent apoptosis in melanoma cells, but not in normal fibroblasts, and that caspase-2 seemed to be the initiating caspase. Docetaxel induced variable degrees of apoptosis among different cell lines (39). To better understand the varying sensitivity of melanoma cells to docetaxel, we studied the role of signal transduction pathways in modulating docetaxel-induced apoptosis. The kinetics of activation of JNK,

ERK1/2, p38 MAPK, and Akt kinases in Igr3 and MM200 cells treated with docetaxel at different times is shown in Fig. 1A. Activation of JNK in Igr3 cells was evident at 3 h following docetaxel treatment and continued to increase up to 12 h. However, activated JNK was barely detectable in MM200 cells. Activation of JNK pathway was also shown by the activation of c-Jun, which paralleled JNK activation. The phosphorylated form of c-Jun was not detected in MM200 cells. In contrast to JNK, docetaxel induced marked activation of ERK1/2 in MM200 cells and only weak activation in Igr3 cells. The phosphorylated form of Akt was detected in the untreated Igr3 cells but did not seem to be influenced by docetaxel. Phosphorylated Akt was not seen in MM200 cells before and after treatment with docetaxel. Activation of p38 MAPK was not detected in either cell line.

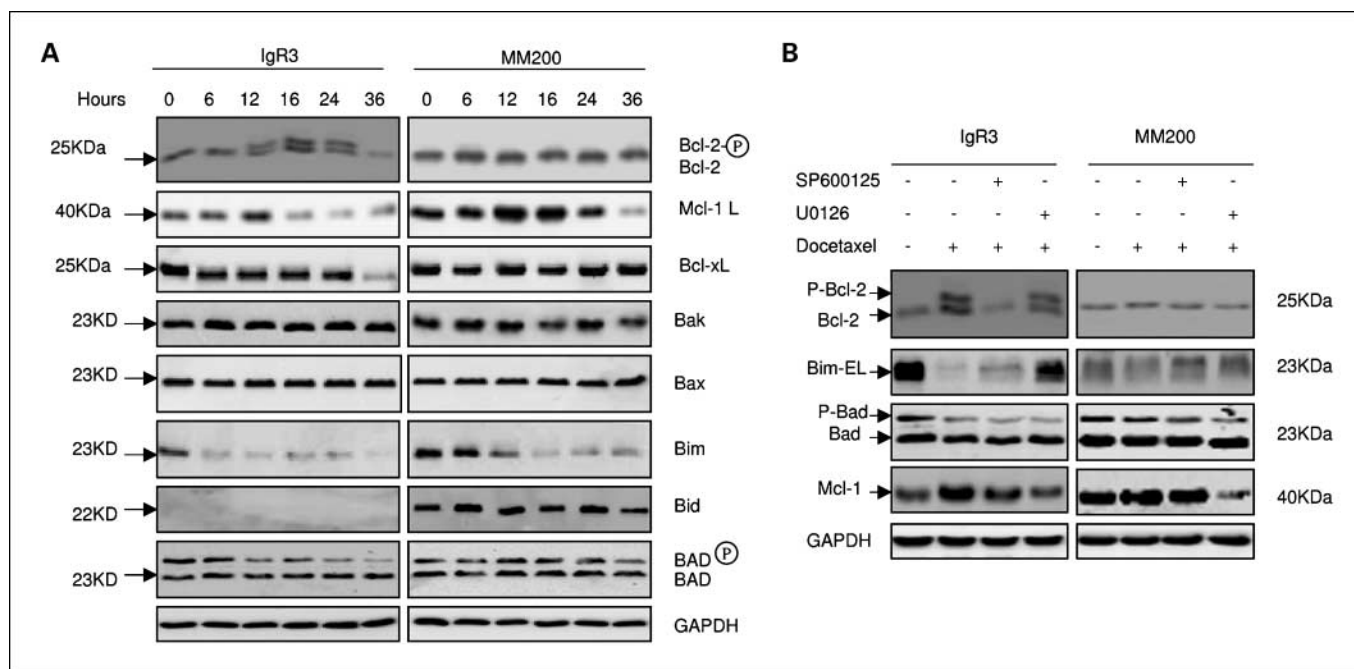
To determine the role of the above signal pathways in docetaxel-induced apoptosis of melanoma, Igr3 and MM200 cells were treated with inhibitors of JNK (SP600125) and

ERK1/2 (U0126) 1 h before adding docetaxel for another 48 h. As shown in Fig. 1B, inhibition of the JNK pathway significantly blocked docetaxel-induced apoptosis, whereas inhibition of ERK1/2 pathway enhanced killing of cells by docetaxel. The latter was particularly evident in the MM200 cell line.

**Docetaxel-induced apoptosis is correlated with activation of JNK and ERK1/2 pathways.** The above results indicated that docetaxel-induced activation of JNK was necessary for apoptosis and that activation of ERK1/2 inhibited apoptosis. Figure 2A shows the status of JNK and ERK1/2 and their activated forms in a panel of melanoma cells before and after addition of docetaxel. The results indicate that there was a correlation between the increase in phosphorylated JNK and apoptosis, whereas activation of ERK1/2 was associated with decreased levels of docetaxel-induced apoptosis. In some cell lines (e.g., ME4405), docetaxel induced activation of both JNK and ERK1/2. To take this into account, the relative activation of JNK and ERK1/2 was calculated by dividing the densitometric value of activated JNK by that of



**Fig. 3.** Activated JNK and ERK1/2 are oppositely regulating docetaxel-induced apoptosis. **A**, JNK is required for caspase-2 activation. Igr3 cells were treated with JNK (SP600125) or ERK1/2 (U0126) inhibitors for 1 h before adding docetaxel at 20 nmol/L for another 16 h. The cell lysate then was analyzed for caspase-2 activation using caspase-2–specific fluorescent AFC substrate. The percentage of increase of caspase-2 activity was calculated as the increase in AFC fluorescence with docetaxel treatment minus background fluorescence divided by AFC fluorescence without docetaxel treatment minus the background. Columns, mean of three individual experiments; bars, SE. **B**, effect of JNK or ERK1/2 inhibition on docetaxel-induced Bax and Bak activation. Igr3 and MM200 cells were treated with JNK (SP600125) or ERK1/2 (U0126) inhibitors for 1 h before adding docetaxel at 20 nmol/L for another 24 h. Activated Bax and Bak were measured in permeabilized cells in flow cytometry using either a Bax NH<sub>2</sub>-terminal epitope-specific antibody or anti-Bak Ab-1 that are specifically recognized activated forms of Bax or Bak, respectively. Columns, mean of three individual experiments; bars, SE. **C**, effects of JNK or ERK1/2 inhibition on docetaxel-induced matrix metalloproteinase (MMP) changes. Igr3 and MM200 cells were treated with JNK (SP600125) or ERK1/2 (U0126) inhibitors for 1 h before adding docetaxel at 20 nmol/L for another 24 h followed by measurement of  $\Delta\Psi_m$  using JC-1 in flow cytometry. Columns, mean of three individual experiments; bars, SE. **D**, effects of JNK or ERK1/2 inhibition on docetaxel-induced release of apoptosis-inducing factor (AIF) and cytochrome c. Igr3 cells were treated with docetaxel at 20 nmol/L for 16 h before harvest. Mitochondrial and cytosolic fractions were subjected to Western blot analysis. Western blot analysis of cytochrome c oxidase IV (COX IV) levels was included to show relative purity of mitochondrial fractions. Data are representative of two individual experiments.



**Fig. 4.** Involvement of Bcl-2 family proteins in docetaxel-induced apoptosis. **A**, IgR3 and MM200 cells were treated with docetaxel at 20 nmol/L for the indicated times. Whole-cell lysates were subjected to Western blot analysis. Western blot analysis of GAPDH levels was included to show that equivalent amounts of protein were loaded in each lane. Data are representative of two individual experiments. **B**, docetaxel-induced changes in Bcl-2 family members are regulated by JNK and ERK1/2 signaling. IgR3 and MM200 cells were treated with either JNK (SP600125) or ERK1/2 (U0126) inhibitors for 1 h before adding docetaxel at 20 nmol/L for another 6 or 24 h. Whole-cell lysates were subjected to Western blot analysis. Western blot analysis of GAPDH levels was included to show that equivalent amounts of protein were loaded in each lane. Data are representative of two individual experiments.

activated ERK1/2. Figure 2B indicates a direct correlation between this ratio and docetaxel-induced apoptosis.

**Docetaxel-induced activation of caspase-2, Bax, and Bak is regulated by activation of JNK and ERK1/2.** Docetaxel-induced apoptosis of melanoma cells was shown to be dependent on activation of caspase-2 (39). To investigate whether activation of caspase-2 may be regulated by JNK and ERK1/2 signaling, we used inhibitors of JNK (SP600125) and ERK1/2 (U0126) in the assays. As shown in Fig. 3A, inhibition of JNK in IgR3 cells resulted in complete inhibition of caspase-2 in the fluorogenic assay, whereas inhibition of ERK1/2 did not affect caspase-2 activity. This placed activation of JNK upstream of activation of caspase-2. Given that activation of JNK and caspase-2 seems important in induction of apoptosis by docetaxel, it is possible that the two events are linked. This was examined by studying activation of JNK in the presence of inhibitor of caspase-2. The caspase-2 inhibitor was unable to inhibit docetaxel-induced activation of JNK (data not shown). Inhibition of JNK inhibited docetaxel-induced activation of Bax and Bak, whereas inhibition of ERK1/2 resulted in an increase in their activity (Fig. 3B). Events downstream of Bax and Bak, including changes in matrix metalloproteinase and the release of cytochrome *c* and apoptosis-inducing factor, were similarly affected by the pathway inhibitors (Fig. 3C and D).

**Docetaxel induced changes in Bcl-2 family members.** To determine whether the JNK pathway acting via caspase-2 may be influencing other Bcl-2 family members, we examined the expression of the antiapoptotic proteins Bcl-XL, Bcl-2, and Mcl-1 together with the proapoptotic proteins Bax, Bak, and the BH3-only proteins Bad and Bim in IgR3 and MM200 cell lines after treatment with docetaxel at 20 nmol/L at different times.

As shown in Fig. 4A, docetaxel markedly induced Bcl-2 phosphorylation at 12 to 24 h in the sensitive IgR3 cells in comparison with the resistant MM200 cells. There was also an increase in Mcl-1 level from 12 to 16 h followed by reduction at 24 and 36 h. The expression of Bcl-XL was not affected in both cell lines until 36 h following treatment with docetaxel in IgR3. No changes were detected in the expression levels of the Bax and Bak proteins. Bim was degraded by 6 h in the IgR3 cells and by 12 h in the MM200 cells following exposure to docetaxel.

The IgR3 cell line did not express the proapoptotic protein Bid, and Bid levels were not changed in MM200 before 36 h. The BH3-only protein Bad levels were not changed in both cell lines before and after treatment with docetaxel; however, a continuous decrease in the levels of phosphorylated form was seen in the sensitive IgR3 cells. In contrast, the phosphorylated form of Bad was persistent in MM200 until 36 h following the treatment.

To determine which changes in Bcl-2 proteins were due to activation of JNK or ERK1/2, IgR3 and MM200 cells were treated with or without SP600125 or U0126 for 1 h before adding docetaxel for another 6 or 24 h. As shown in Fig. 4B, inhibition of the JNK pathway led to inhibition of docetaxel-induced phosphorylation of Bcl-2 and partially prevented loss of Bim-EL. Inhibition of ERK1/2 resulted in partial reduction of phosphorylated Bcl-2 levels, restoration of Bim-EL levels, and enhanced dephosphorylation of the BH3-only protein Bad. The latter was to a higher extent in MM200. Moreover, inhibition of ERK1/2 prevented the increase in Mcl-1 and also decreased its basal level of expression, notably in MM200 cells.

Previous studies have suggested that Bim is a mediator of taxanes-induced apoptosis (40). To investigate its role in



docetaxel-induced apoptosis of melanoma, we knocked down Bim-EL levels by introducing siRNA for Bim. Figure 5A shows that, 24 h after transfection with Bim siRNA, the level of endogenous Bim-EL in IgR3 cells was reduced to <12% of that in control cells. The transfected cells were then cultured in the presence or absence of docetaxel to measure apoptosis (Fig. 5B). The results show that down-regulation of Bim-EL by siRNA had no effect on the extent of docetaxel-induced apoptosis. Docetaxel-induced Bax and Bak activation and matrix metalloproteinase changes were not affected by down-regulation of Bim-EL (data not shown).

## Discussion

We have reported that docetaxel induces caspase-2-dependent apoptosis of melanoma via the mitochondrial pathway (39). In the present study, we show for the first time that docetaxel-induced activation of JNK is responsible for activation of caspase-2 and its downstream events, whereas activation of ERK1/2 pathway by docetaxel inhibited apoptosis. The p38 pathway did not seem to play any role in docetaxel-induced apoptosis of melanoma in that its inhibition did not protect melanoma cells against docetaxel-induced apoptosis and its activated form was not detected before and after exposure to docetaxel. The critical role of the JNK pathway was

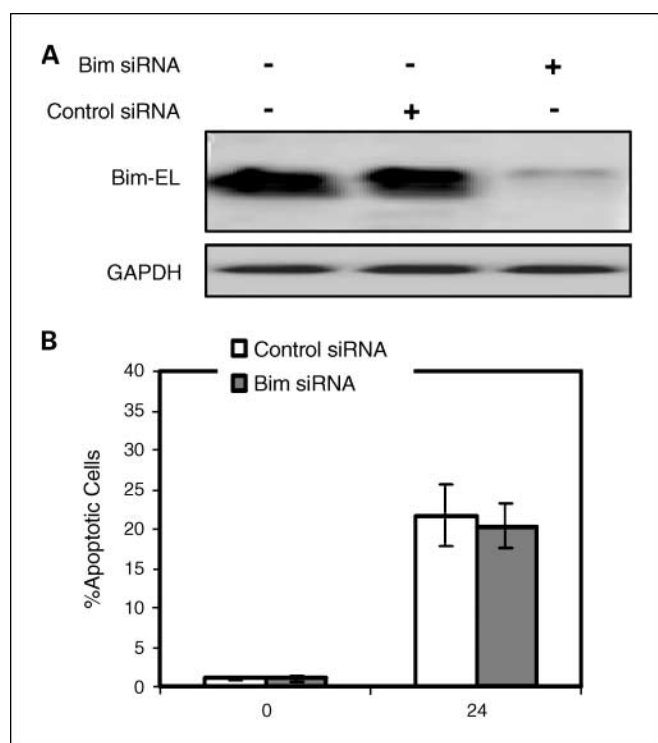
shown by showing a direct correlation between the ratio of activated JNK to that of ERK1/2 and the level of apoptosis induced by docetaxel. Moreover, inhibition of JNK completely inhibited apoptosis induced by docetaxel.

Activation of the JNK pathway is a common phenomenon in stress-induced apoptosis in response to stress stimuli, such as UV light, heat shock, and ionizing radiation (41). In addition, the JNK pathway is activated by intracellular stresses, such as inhibition of protein synthesis, DNA damage, and treatment with anticancer agents, such as antimetabolites (1, 42), and by several antimicrotubule agents (41, 43, 44). The role of activated JNK in taxanes-induced apoptosis, however, remains controversial (45, 46), and no clear correlation has been made between docetaxel-induced apoptosis and the degree of JNK activation. JNK activation was essential for paclitaxel-induced apoptosis in human KB-3 carcinoma cells, ovarian cancer cells, and MCF-7 breast carcinoma cells (44, 45, 47). The present studies extend these findings to human melanoma cells and are the first to show that the levels of apoptosis are governed by activation of two opposing signal pathways, the JNK and ERK1/2 pathways. They show that JNK activation was upstream of caspase activation in that inhibition of JNK resulted in complete inhibition of caspase-2 activation, whereas the caspase-2 inhibitor was unable to inhibit the kinase activity of JNK. Our previous studies have shown that docetaxel-induced apoptosis was independent of p53 and was associated with activation of Bax (39).

Activation of JNK by docetaxel also resulted in several changes in Bcl-2 family proteins that may promote apoptosis. This included phosphorylation and inactivation of Bcl-2 that was mediated by JNK. This may have contributed, at least in part, to docetaxel-induced apoptosis. The antiapoptotic member Mcl-1 was previously shown to be a target for JNK-mediated phosphorylation (13). In the present study, Mcl-1 levels increased initially at 6 to 12 h and were then down-regulated at 16 h in the sensitive IgR3 melanoma cells. Inhibition of JNK did not alter the changes of Mcl-1 induced by docetaxel and kinetic studies of ERK1/2 activation and changes in Mcl-1 expression made it more likely that these changes were due to ERK1/2 kinase activity. Similarly, Bim levels were increased in the presence of the JNK inhibitor but knockdown of Bim using siRNA suggested that Bim played no role in the docetaxel-induced apoptosis.

Activation of the ERK1/2 pathway by taxanes is well described (48), and activation of this pathway is known to inhibit apoptosis. Nevertheless, the mechanism by which this pathway inhibits apoptosis is not well established. In the current study, activation of ERK1/2 by docetaxel seemed upstream of Bax and an inhibitor of ERK1/2 resulted in increased relocation of Bax to mitochondria and reduced mitochondrial membrane potential particularly in the resistant MM200 melanoma cells. Activation of ERK1/2 by docetaxel was not as rapid as that seen after exposure to tumor necrosis factor-related apoptosis-inducing ligand (35) and occurred by 6 to 12 h compared with 30 min for tumor necrosis factor-related apoptosis-inducing ligand. The mechanism of activation of the ERK1/2 pathway by taxanes is not clear but is believed to reflect changes in ERK1/2 bound to microtubules (49).

In summary, our results showed that docetaxel induced activation of JNK and ERK1/2 pathways and their relative degrees of activation seemed to be a key determinant of



**Fig. 5.** Docetaxel induces apoptosis independently of Bim. *A*, immunoblotting for Bim-EL was done in untransfected, nontarget (control) siRNA-transfected, and Bim-EL siRNA-transfected IgR3 cells treated with or without docetaxel at 20 nmol/L for 24 h. Western blot analysis of GAPDH levels was included to show that equivalent amounts of protein were loaded in each lane. Data are representative of two individual experiments. *B*, inhibiting Bim expression by siRNA does not affect docetaxel-induced apoptosis. Untransfected, nontarget (control) siRNA-transfected, and Bim-EL siRNA-transfected IgR3 cells were treated with or without docetaxel at 20 nmol/L for 24 h, and apoptotic cells were measured by the propidium iodide method in flow cytometry. Columns, mean of three individual experiments; bars, SE.

sensitivity to docetaxel. JNK activation was upstream of caspase-2 and mitochondria and facilitated the caspase-2-dependent mitochondrial release of apoptosis-inducing factor and cytochrome *c*. These results seem to have important implications for the treatment of melanoma with taxanes in that they suggest that melanoma cells in which the ERK1/2 pathway is strongly activated by taxanes may be made sensitive to killing by concomitant treatment with inhibitors of the Ras-Raf-MEK-ERK pathway, such as sorafenib (Nexavar). It is of particular interest that high durable response rates have been reported in patients

treated with sorafenib and paclitaxel plus carboplatin by Flaherty et al. (50). The results of randomized phase II study to test the role of sorafenib in these responses are awaited with much interest. Should this study show that sorafenib is required to achieve the high response rates, the present studies provide an explanation for its role and provide a basis for further evaluation of taxanes in combination with inhibitors of this MAPK pathway. The mechanism(s) by which JNK regulates caspase-2 activity and the role of ERK1/2 in Mcl-1 expression and Bax sequestration are the subject of further studies.

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