Activation of Jun N-terminal kinase is a mediator of vincristine-induced apoptosis of melanoma cells

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The molecular changes involved in the induction of apoptosis by vincristine in melanoma have not yet been well defined. Two human melanoma cell lines showing moderate (Mel-RM) and high (IgR3) sensitivity to vincristine were selected from a panel of eight melanoma lines for analysis. Induction of apoptosis was caspase dependent, and was associated with increases in mitochondrial membrane permeability. Vincristine upregulated the expression of Bax, Bak, PUMA, Noxa, p53 and p21 proteins, and downregulated and/or phosphorylated the Bcl-2 protein. Inhibitors of the Jun N-terminal kinase (JNK), but not p38 mitogen-activated protein kinase, significantly inhibited vincristine-induced apoptosis in both IgR3 and Mel-RM cells. In addition, vincristine induced phosphorylation and reduction in Bcl-2 was prevented by an inhibitor of JNK. Downregulation of mRNA for p53, PUMA or Bim by RNA interference had little or no influence on vincristine-induced apoptosis in IgR3 cells. In addition, silencing Bim mRNA did not affect vincristine-induced apoptosis in Mel-RM cells. These results suggest that vincristine-induced apoptosis of at least some melanoma cell lines is dependent on the activation of JNK. The results are consistent with the phosphorylation of Bcl-2 protein, resulting in the activation of Bax/Bak, release of cytochrome *c* from the mitochondria and the resulting activation of caspases. *Anti-Cancer Drugs* 19:189–200

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Introduction

Vincristine is a naturally occurring substance extracted from the Madagascar periwinkle. It binds to tubulin, causing microtubule depolymerization, metaphase arrest and apoptosis in cells undergoing mitosis [1]. It has been widely used to treat patients with cancer, including melanoma [2–4], but the response rates of melanoma are low. Antimicrotubule-targeting drugs (taxols/vinca alkaloids) have been reported to induce apoptosis by the regulation and control of the Bcl-2 family – p53, p21, Fas/Fas ligand and c-Myc [5–7] – and/or by the degradation of IκBα and the inhibition of nuclear factor (NF)-κB activation [4,8,9]. The molecular basis for induction of apoptosis in melanoma by vincristine, however, remains unknown.

Four signaling pathways in the control of the growth and death of cells are present. The extracellular signal-regulated kinase (ERK) and phosphatidylinositol-3-kinase (PI3K)/Akt pathways contribute to the increased proliferative rates of cancer cells [10,11]. The [MEK: mitogen-activated protein (MAP)-extracellular signal-regulated kinase (ERK)-kinase] Ras–MEK–ERK pathway has been reported to be constitutively activated in human melanoma [12–15]. Mutation of *N-Ras* [16] and *BRAF* [17] is one of the factors involved in activation of the pathway and of tumor survival in melanoma. The PI3K/Akt pathway might also inhibit cell death by means of several mechanisms, including the activation of NF-κB transcription factor [18,19], phosphorylation of Bad [20,21] and inactivation of forkhead transcription factors. The latter upregulates Bim, procaspase-9 and Bad [22–24]. Activation of NF-κB by Akt also regulates the transcription of Bcl-x_L, and XIAP [25,26].

In contrast, the Jun N-terminal kinase (JNK)-signaling and p38-signaling pathways generally inhibit the growth of tumor cells [11]. In response to ultraviolet irradiation, heat stress, chemotherapy and inflammatory cytokines, JNKs and p38 MAP kinases (MAPKs) are activated to play an important role in apoptosis of cells [27–30]. Although the proapoptotic roles of activated JNK isoforms are not completely understood, active JNK can directly mediate apoptosis by phosphorylating and regulating the activity of transcription factors including c-Jun, ATF2, Elk-1, p53 and c-Myc [27,31–33], as well as of the nontranscription factors involving members of the Bcl-2 family, such as Bcl-2, Bcl-x_L, Bim

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and Bad [34–36]. Moreover, JNK stimulates apoptosis by the induction of the mitochondrial release of apoptotic factors and activation of Bax and Bak proteins [37–39].

Previous studies have shown that docetaxel-induced apoptosis in human melanoma is mediated by the activation of the JNK pathway, but is inhibited by the ERK pathway [40]. Two major protein families (caspase and Bcl-2 families) involved in apoptosis are regulated by the above pathways. Caspases are major executioners of apoptosis, which is regulated by the Bcl-2 family of proteins [41]. PUMA and Bim, for example, induce cytochrome *c* release by binding to antiapoptotic proteins; thereby they activate Bax/Bak, which increases permeability of mitochondria [42–44]. It is, however, not completely understood how vincristine initiates and activates the signal pathways involved in the induction of apoptosis. In this study, we report that vincristine activates the JNK pathway and that JNK phosphorylates and inhibits the antiapoptotic roles of Bcl-2. These activities were associated with the release of mitochondrial proteins, activation of caspases and apoptosis.

Materials and methods

Reagents and antibodies

Vincristine (MW923, 1 mg/25 ml in 0.9% sodium chloride solution for intravenous injection) was provided by Baxter Healthcare Pty. Ltd (Newcastle, Australia). All caspase inhibitors were purchased from Calbiochem. (Melbourne, Australia) as follows: caspase inhibitor I, z-VAD (oMe)-FMKa (cat no: 627610); caspase-2 inhibitor I, z-VD (oMe) VAD (oMe)-FMKa (cat no: 218744); caspase-3 inhibitor II, z-D (oMe) E (oMe) VD (oMe) FMKa (cat no: 264155); caspase-8 inhibitor II, z-IE (oMe) TD (oMe) FMKa (cat no: 218759) and caspase-9 inhibitor I, z-LE (oMe) HD (0Me)-FMKa (cat no: 218761). The propidium iodide (PI) was purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Antibodies used were as follows: the rabbit polyclonal antibodies against caspase-2, -3, -8 and -9; the mouse monoclonal antibodies (MAbs) against cytochrome *c*; poly(ADP ribose) polymerase (PARP) and inhibitor of caspase-activated DNase, p53, p21 and p27 were purchased from Pharmingen Technical (Bioclone, Marrickville, Australia); the rabbit polyclonal antibodies (Abs) against Bak (Ab-1) were purchased from Calbiochem (La Jolla, California, USA); the mouse MAbs against Bcl-2, Bcl-x, Mcl-1, Bax, Bad and phosphorylated ERK, and rabbit polyclonal Ab against Smac were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA) and the rabbit polyclonal Abs against ERK, PUMA, Bid and JNK and the MAbs against phosporylated JNK were purchased from Cell Signalling Technology (Beverly, Massachusetts, USA). The MAbs against Noxa and the polyclonal Ab against Bim were purchased from Imgenex (San Diego, California, USA); the mouse MAb against XIAP was purchased from Transduction Laboratories (Lexington Kentucky, USA); the rabbit polyclonal anti-Bax against amino acids 1 through 20 was purchased from Upstate Biotechnology (Lake Placid, New York, USA) and the MAb against glyceraldehyde-3-phosphate dehydrogenase was purchased from Ambion. Small interfering RNA (SiRNA) smart pools were purchased from Dharmacon (Melbourne, Australia), as follows: SiControl nontarget SiRNA pool (cat no: D-001206-13-20), Bim (cat no: M-004383-01-0010) and PUMA (M-004380-01). Clones containing human p53 short hairpin RNA (shRNA) and p53 negative shRNA (as a negative control) were kindly provided by Dr Helen Rizos (Westmead Institute for Cancer Research, University of Sydney, Sydney, Australia).

Cell culture

Human melanoma cells of IgR3, Mel-RM and other cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (Commonwealth Serum Laboratories, Melbourne, Australia) at 5% CO_2 in 37°C. Both IgR3 and Mel-RM cells contained wild-type p53.

Bcl-2 vector and its transfection

The stable Mel-RM cell line containing human Bcl-2 complementary DNA was kindly provided by Dr David Vaux (Walter and Eliza Hall Institute, Melbourne, Victoria, Australia). The protocol has been described previously [45].

Apoptosis

Apoptosis was measured by the propidium iodide (PI) method using a Becton Dickinson FACScan flow cytometer (Mountain View, California, USA). The protocol was performed as previously described by Zhang *et al.* [45].

Mitochondrial membrane potential

Tumor cells were cultured at 1×10^5 cells/well in 24-well plates and allowed to reach exponential growth for 24 h before treatment. JC-1 staining was performed according to the manufacturer's instructions (Molecular Probes, Engen, Oregon, USA). In brief, adherent and floated cells were collected with phosphate-buffered saline (PBS). Cells were then incubated with 10 μg/ml of JC-1 in warmed PBS at 37°C for 15 min. After washing with PBS, the cells were analyzed using a FACScan flow cytometer (Becton Dickinson, Sunnyvale, California, USA). The cells with polarized mitochondria presented in the upper right quadrant of the dot plot owing to the formation of JC-1 aggregates, which emit red fluorescence (590 nmol/l) when excited at 488 nmol/l. The cells with depolarized mitochondria emit green fluorescence (530 nmol/l). Untreated cells were used as controls.

Preparation of mitochondrial and cytosolic fractions

The protocol used for subcellular fractions was the same as that described previously [45].

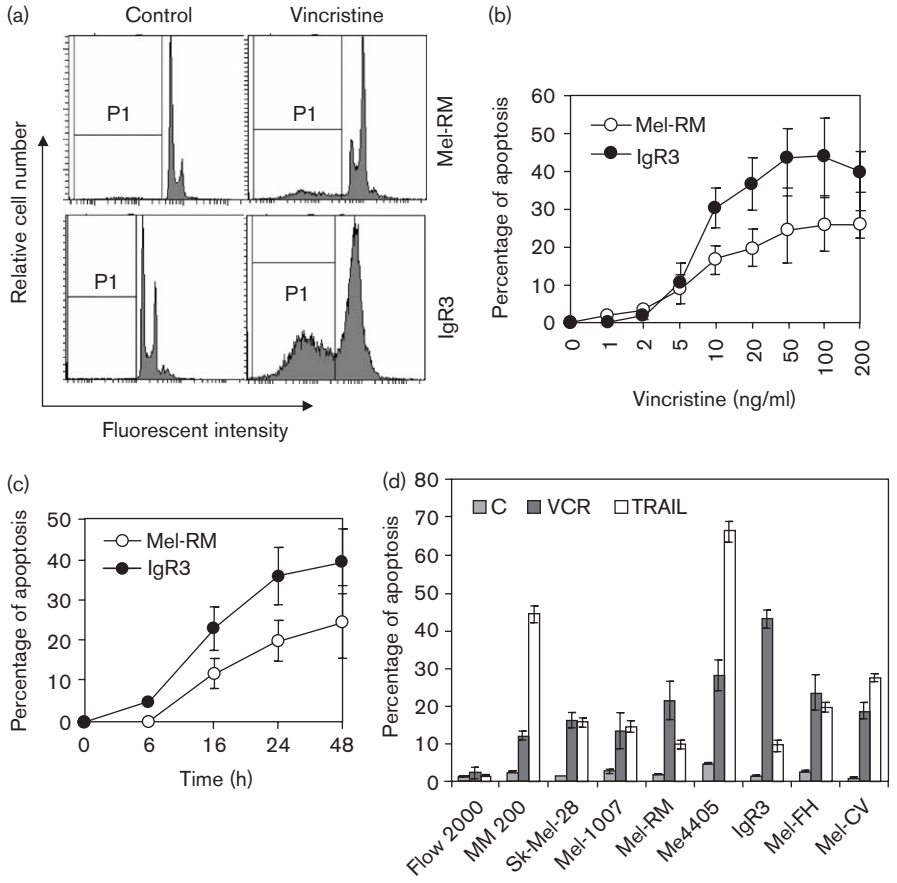
Western blots

The cells were washed with PBS, trypsinized, collected into tubes and centrifuged at 2500 rpm for 5 min. Cells were lysed in lysis buffer (10 mmol/l Tris–HCl, pH 7.4; 140 mmol/l NaCl; 0.5 mmol/l CaCl; 0.5 mmol/l MgCl; 0.02% NaN_3 and 1% NP-40) and supplemented with a cocktail of protease inhibitors. The protein content of cell extracts was determined by the Bradford assay (Bio-Rad, Regents Park, New South Wales, Australia). A total of 30 μg of protein was electrophoresed on 7.5–15% SDS–PAGE gels at 200 V for 35–46 min, followed by transfer to nitrocellulose membrane at 50 V for 1 h. Membranes were blocked and incubated with primary Abs at the appropriate concentrations, followed by incubation with horseradish peroxidase (HRP)-conjugated goat antirabbit or goat antimouse immunoglobulin (1 : 2500 dilution). Immunodetection was performed by using an Immune-Star HRP chemiluminescent kit (Bio-Rad) and images were captured.

Knockdown of mRNA for p53, Bim and PUMA by short hairpin RNA and small interfering RNA

Melanoma cells were seeded at 4×10^4 per well in 24-well plates. Twenty-four hours later, the number of cells reached to 60–70% confluence for transfection. In brief, 3 μl of SiRNA (50 nmol/l/SiRNA) and 5 μl of lipofectAMINE 2000 (Invitrogen, Carlsbad, California, USA) were incubated separately with 200 μl of Opti-MEM (Invitrogen) for 5 min, and then mixed together for 20 min at room temperature. Fifty microliters of mixing complex were applied to the cells plated in 300 μl of medium. The cells were incubated in the SiRNA transfection

Fig. 1



Vincristine induces apoptosis of melanoma. (a) Representative flow cytometry histograms of apoptosis assays by the propidium iodide (PI) method. IgR3 and Mel-RM cells were treated with vincristine (VCR) at 50 ng/ml for 48 h. (b) IgR3 and Mel-RM cells were treated with VCR at the indicated doses for 48 h before the assay of apoptosis by the PI method using flow cytometry. The data shown are mean ± SE of three independent experiments. (c) Kinetics of induction of apoptosis by vincristine. IgR3 and Mel-RM cell lines were exposed to vincristine at 50 ng/ml during the indicated time points before the assay of apoptosis by the PI method using flow cytometry. The data shown are representive of three independent experiments. (d) VCR-induced or tumor necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAIL)-induced apoptosis in a panel of melanoma cell lines. (C, control). Cells were treated with VCR or TRAIL at a concentration of 50 or 200 ng/ml for 48 h, and apoptosis was measured by the PI method using flow cytometry. The data shown are the mean ± SE from three independent experiments.

reagent mixture for 24 h before changing the medium afresh. Forty-eight hours after transfection, cells were treated with or without vincristine at a concentration of 50 ng/ml for another 48 h. The apoptosis was measured by the PI method using flow cytometry. Efficiency of SiRNA silencing was assessed by the Western blot method.

Statistical analysis

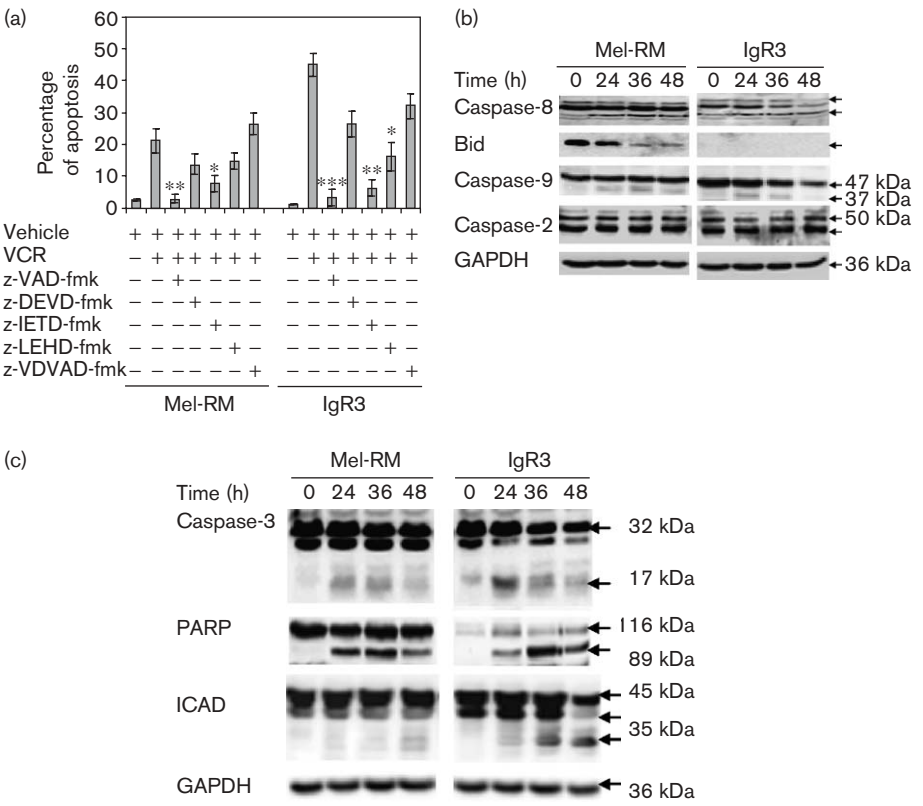
Data are described as mean ± SE. The difference between groups was analyzed using the Student's *t*-test. *P* values of less than 0.05 were considered statistically significant.

Results

Vincristine-induced apoptosis of melanoma

The concentration and kinetics of apoptosis induced by vincristine in melanoma cells were studied by flow cytometric detection of the sub-G_1 DNA fraction, as shown in Fig. 1a. Vincristine-induced apoptosis of Mel-RM and IgR3 melanoma cells at 48 h was detected at 5 ng/ml and was maximal at 50 ng/ml (Fig. 1b). Apoptosis was detected at 6 h in the sensitive IgR3 cells and was maximal at 48 h (Fig. 1c). The sensitivity of a panel of melanoma cell lines to vincristine-induced apoptosis is shown in Fig. 1d. The IgR3 cell line was the most sensitive cell line to vincristine with 45% apoptosis, followed by 4405, Mel-FH, Mel-RM, CV, Sk-Mel-28, Mel-1007 and MM200. Apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAIL) as described previously [45] was used as a positive control (Fig. 1d). Neither vincristine nor TRAIL induced apoptosis in fibroblast cells (Flow 2000; Flow Laboratories, McLean, Virginia, USA) (Fig. 1d). Mel-RM and IgR3 cells were resistant to TRAIL treatment; IgR3 cells were the most sensitive cells to vincristine. Therefore, Mel-RM and IgR3 cells were chosen for following study.

Fig. 2



Induction of apoptosis by vincristine (VCR) is caspase dependent. (a) The effect of the inhibition of caspases on VCR-induced apoptosis of melanoma. IgR3 and Mel-RM cells were treated with the vehicle, dimethyl sulfoxide (2 μl); the pan-caspase inhibitor, z-VAD-fmk (20 μmol/l) and the specific inhibitors against caspase-3, z-DEVD-fmk (20 μmol/l); caspase-8, z-IETD (20 μmol/l); caspase-9, z-LEHD (20 μmol/l) and caspase-2, z-VDVAD-fmk (50 μmol/l); 1 h before adding VCR (50 ng/ml) for another 48 h. Apoptosis was assayed by the propidium iodide (PI) method using flow cytometry. The results shown are the means ± SE from three independent experiments. *, ** and *** indicate $P<0.01$–0.001, determined using *t*-test. (b) VCR activates caspase-8 and caspase-9, but not caspase-2. Both cell lines were treated with VCR (50 ng/ml) for the indicated time frames. Whole-cell lysates were subjected to Western blot analysis. Western blot analysis of β-glyceraldehyde-3-phosphate dehydrogenase levels was included to show that an equivalent amount of proteins was loaded in each lane. (c) VCR induces the activation of caspase-3 and the cleavage of its substrates poly(ADP ribose) polymerase (PARP) and inhibitor of caspase-activated DNAse (ICAD). These cell lines were treated with VCR (50 ng/ml) for the indicated time periods. Whole-cell lysates were subjected to Western blot analysis. The data shown are the results from three independent experiments.

Vincristine-induced apoptosis is caspase dependent

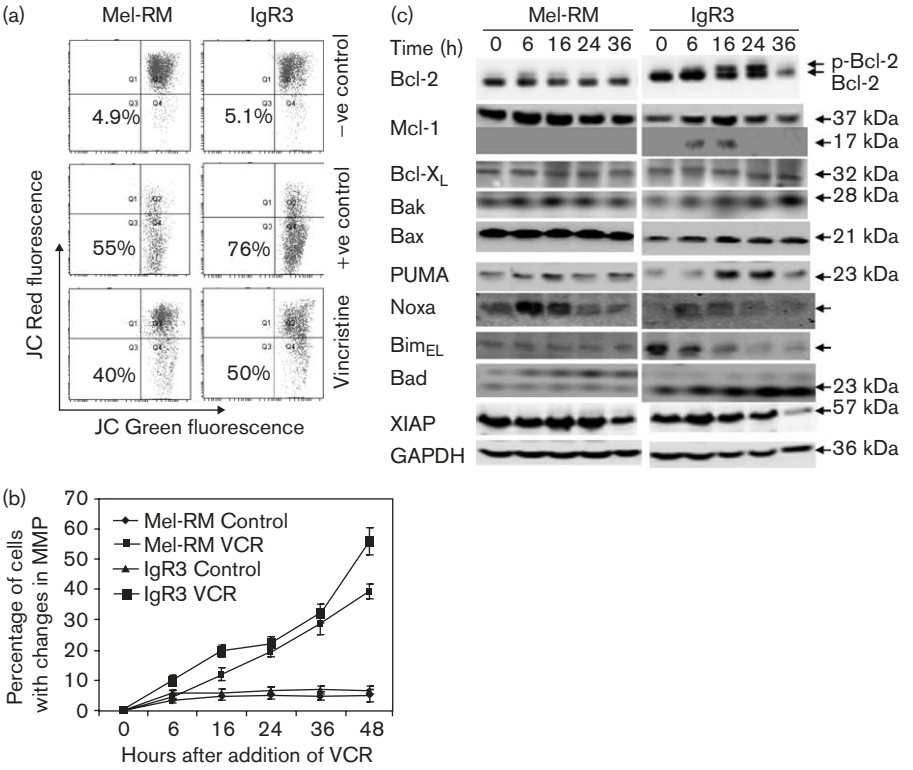
To determine whether vincristine-induced apoptosis was caspase dependent, IgR3 and Mel-RM cells were pretreated for 1 h with a pan-caspase inhibitor and inhibitors that were specific for caspases -8, -9, -3 and -2. As shown in Fig. 2, the pan-caspase inhibitor completely inhibited apoptosis of both cell lines. Marked inhibition of apoptosis with the inhibitor of caspase-9 and various degrees of inhibition with the inhibitors of caspases -3 and -8 were also found. Inhibition of caspase-2 had minimal effects on vincristine-induced apoptosis (Fig. 2a).

The kinetic analysis of changes in the caspase proteins in the two cell lines before and after the treatment with vincristine is shown in Figs. 2b and c. The processed fraction of caspase-8 (with molecular weight of 43/36 kDa) increased strongly at 24, 36 and 48 h after the treatment with vincristine. Similarly, the processed fraction of caspase-9 (with molecular weight of 37 kDa) was detected within 24 h of treatment with vincristine. Activation products of caspase-3 were also observed in both cell types, but a higher level was found in IgR3 than in Mel-RM cells. No evidence for activation of caspase-2 in either cell type was found (Fig. 2c). Furthermore, the activation of caspase-3 was demonstrated by the appearance of processed fractions of inhibitor of caspase-activated DNAse and PARP in both lines. These results clearly indicated that vincristine-induced apoptosis involved the activation of caspases.

Vincristine activates the mitochondrial apoptotic pathway

To determine whether the apoptotic pathway activated by vincristine could occur via the mitochondria, we measured the mitochondrial membrane potential (MMP) in melanoma cells treated with vincristine at different time points. Changes in MMP were studied using a fluorescent cationic dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolocarbocycnian iodide, also known as JC-1. In

Fig. 3



Changes in mitochondrial membrane potential (MMP) and in proteins of the Bcl-2 family in vincristine (VCR)-induced apoptosis of melanoma cells. (a) Representative flow cytometry histograms of MMP change assays by JC-1 method. Both IgR3 and Mel-RM cells were treated with VCR at 50 ng/ml for 48 h (data were from one experiment). (b) Induction of changes in the mitochondrial membrane potential (MMP) ($\Delta\Psi_m$, and also referred to as DC_m) by VCR in both cell lines. Cells were treated with VCR (50 ng/ml) during the indicated time points. The DC_m was measured by the JC-1 kit, using flow cytometry. Data shown are the results from three independent experiments. 'a–g' is indicated as $P<0.05$–0.001 at different time points in Mel-RM or IgR3 cells. ** indicates $P<0.01$ at 48 h between Mel-RM and IgR3 cells. (c) Regulation of the expression and/or phosphorylation of Bcl-2 family members and XIAP in melanoma cells. Whole-cell lysates from both cell lines with or without the treatment with VCR (50 ng/ml) during the indicated times were subjected to Western blot analyses. The data shown are the results from two independent experiments.

normal cells, JC-1 appears as a monomer in the cytosol (FL1-positive; green) and accumulates as aggregates in the mitochondria (FL2-positive; red). In apoptotic cells, JC-1 aggregates change to the monomeric form and produces a green cytosolic signal. As shown in Fig. 3b, vincristine induced mitochondrial depolarization that was detected within 6 h, and was maximal ($P < 0.001$) at 48 h. Reduction in MMP was significantly ($P < 0.01$) lower in Mel-RM cells than in IgR3 cells at 48 h, as shown in Fig. 3b.

Vincristine induced changes in the Bcl-2 family of proteins and in inhibitor-of-apoptosis proteins

The expression profiles of the Bcl-2 family of proteins in IgR3 and Mel-RM cells were examined by Western blots. As shown in Fig. 3c, the proapoptotic protein, Noxa was upregulated within 6 h, particularly in Mel-RM, and it decreased within 24 h. PUMA was increased at 16 h in both cell lines and became reduced thereafter. BimEL was constitutively present in both cell lines, particularly in IgR3, but decreased within 6 h in both cell types. Bad levels were increased within 6 h and peaked at 24 h in IgR3 cells. In Mel-RM cells, a fraction of Bad with a higher molecular weight was observed from 6 to 36 h.

Phosphorylated Bcl-2 was detected within 6 h in both cell lines, but peaked at 24 h in the sensitive IgR3 cells. Mcl-1 level increased at 6 and 16 h in both cell lines. In addition, a small-molecular-weight fraction of Mcl-1 was detected at 6 and 16 h in IgR3 cells, which might have been a cleavage product resulting from the activation of caspase-3 (Fig. 3c).

Levels of the inhibitor-of-apoptosis proteins were not changed before and after the treatment with vincristine (Fig. 3c). The expression level, however, was much higher in Mel-RM than in IgR3 cells.

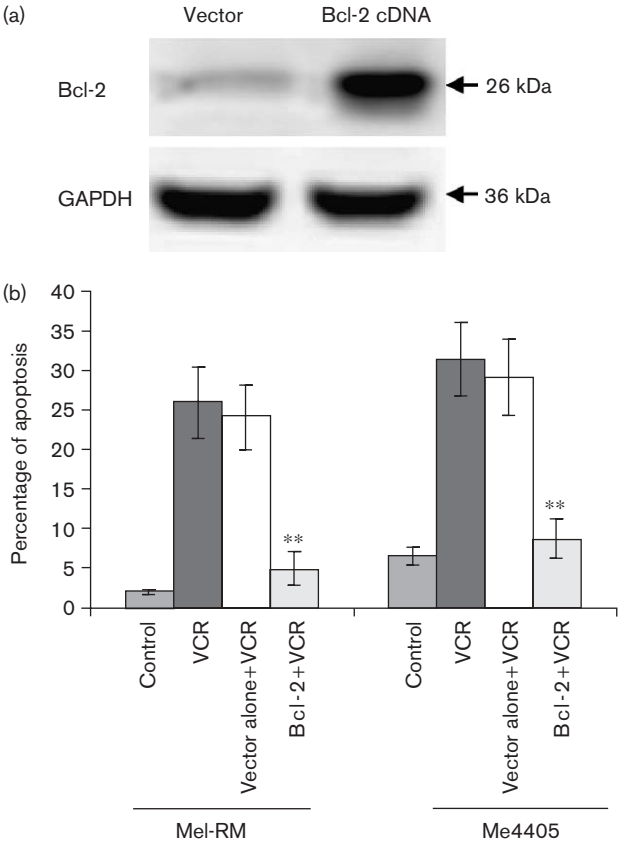
Overexpression of Bcl-2 inhibits vincristine-induced apoptosis

Bcl-2 has been reported to regulate mitochondrial activity by binding the proapoptotic proteins Bid and Bim, thereby preventing the activation of Bax and Bak, and inhibiting the release of cytochrome *c* from the mitochondria [43,46,47]. Mel-RM was chosen for this experiment because it contained a lower Bcl-2 level, compared with IgR3. As shown in Fig. 4, overexpression of Bcl-2 significantly ($P < 0.001$) reduced vincristine-induced apoptosis in Mel-RM cells. These results provide further evidence that vincristine-induced apoptosis involved the mitochondria resulting from changes in the Bcl-2 family of proteins.

Vincristine induces apoptosis in Mel-RM and IgR3 cells by the activation of the Jun N-terminal kinase-signaling pathway

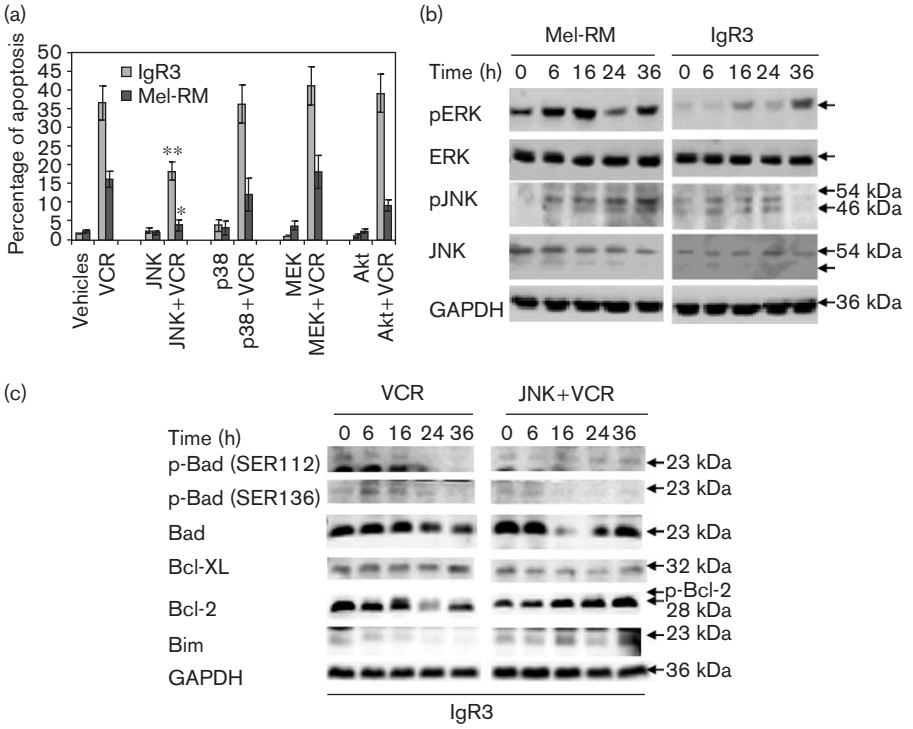
We examined the effect of vincristine on the JNK and/or MAPK p38-signaling pathway and on the ERK and/or PI3K/Akt pathways. The inhibitor (SP600125) of JNK significantly ($P < 0.01$) reduced vincristine-induced apoptosis, but inhibitors of MAPK p38, MEK and Akt did not affect the apoptosis induced by vincristine in either the IgR3 or the Mel-RM cells (Fig. 5a). The p-JNK fraction was upregulated within 16 h and was maximal within 36 h in Mel-RM cells (Fig. 5b). In IgR3 cells, p-JNK protein was increased maximally within 6 h and maintained at this level until 24 h (Fig. 5b). We examined the effect of the inhibitors on MM200, Mel-FH, Sk-Mel-28, Mel-1007 and Me4405 cells. The results shown in Table 1 indicate that the JNK inhibitor strongly inhibited vincristine-induced apoptosis of Mel-FH, but had no effect on vincristine-induced apoptosis in MM200 or Sk-Mel-28 cells. An increase in

Fig. 4



Influence of Bcl-2 and the accumulation of p53 in vincristine (VCR)-induced apoptosis. (a) Overexpression of Bcl-2 in Mel-RM cell. Whole-cell lysate was subjected to Western blot analysis, 48 h after the transfection of Bcl-2 complementary DNA. (b) The effect of overexpression of Bcl-2 on VCR-induced apoptosis in Mel-RM and Me4405 cell lines. Overexpression of Bcl-2 significantly inhibited the apoptosis induced by VCR. Mel-RM cells transfected either with Bcl-2 cDNA or vector alone or without them were treated with VCR (50 ng/ml) for 48 h before the assay of the apoptosis by the propidium iodide method using flow cytometry. Columns, means from three independent experiments; bars, SE. *** indicates $P<0.001$, determined using *t*-test.

Fig. 5



Vincristine (VCR) induces the activation of the Jun N-terminal kinase (JNK). (a) Inhibition of JNK attenuates VCR-inducing apoptosis in IgR3 and Mel-RM. JNK, JNK inhibitor (SP600125); p38, p38 inhibitor (SB203580); extracellular signal-regulated kinase (ERK), ERK inhibitor (U0126); Akt, Akt inhibitor (LY294002); IgR3 and Mel-RM cells were treated with vehicle [dimethyl sulfoxide (DMSO), 2 μl] or VCR (50 ng/ml) in the presence or absence of the JNK inhibitor (25 μmol/l), the p38 inhibitor (10 μmol/l), the MEK inhibitor (20 μmol/l) and the Akt inhibitor (20 μmol/l). Apoptosis was measured by the propidium iodide method using flow cytometry. The data shown are the means ± SE from three independent experiments. **indicates $P < 0.01$, determined using the *t*-test. (b) VCR activates JNK and ERK. Whole-cell lysates from both cell lines with or without treatment with VCR (50 ng/ml) during the indicated time points were subjected to Western blot analyses. The data shown are the results from two independent experiments. (c) The effect of the JNK inhibitor on VCR-induced changes in the Bcl-2 family of proteins in IgR3 cells. VCR and JNK + VCR are the same means as (a). IgR3 cells were pretreated with the JNK inhibitor (SP600125) at a concentration of 25 μmol/l for 1 h. Later, they were treated with or without VCR (50 ng/ml) alone or VCR (50 ng/ml) in addition to the JNK inhibitor (25 μmol/l) at the indicated time points. After that, whole-cell lysates were collected for Western blot analysis. The data shown are representative of three independent experiments.

Table 1 The effect of inhibitors on vincristine-induced apoptosis in several melanoma cell lines

Cell lines	Vehicles	VCR	JNK	JNK + VCR	p38	p38 + VCR	MEK	MEK + VCR	Akt	Akt + VCR
Mel-FH	2.2 ± 0.6	24.1 ± 5.2	6.4 ± 0.1	12.0 ± 1.6	2.4 ± 0.4	34.3 ± 6.2	9.9 ± 1.2	43.4 ± 2.0	4.7 ± 0.0	36.2 ± 8.1
MM200	1.9 ± 0.8	7.1 ± 1.2	1.4 ± 0.2	6.2 ± 0.5	0.9 ± 0.2	31.1 ± 0.7	4.6 ± 0.2	66.4 ± 3.8	1.2 ± 0.0	39.3 ± 3.3
Sk-Mel-28	1.2 ± 0.2	17.6 ± 2.4	1.3 ± 0.2	17.6 ± 0.2	1.1 ± 0.2	31.0 ± 2.4	4.0 ± 1.7	26.9 ± 1.0	2.7 ± 0.3	19.9 ± 1.9
Me4405	4.8 ± 0.5	48.2 ± 2.1	7.1 ± 2.8	34.1 ± 1.7	4.9 ± 0.4	48.9 ± 3.1	12.2 ± 1.1	53.4 ± 5.4	11.9 ± 1.4	57.2 ± 2.2
Mel-1007	1.3 ± 0.2	24.1 ± 0.6	2.9 ± 0.4	20.4 ± 2.0	1.4 ± 0.1	16.1 ± 1.9	5.3 ± 1.4	53.2 ± 2.9	1.5 ± 0.1	27.9 ± 1.1

Vehicles [vincristine (VCR), Jun N-terminal kinase inhibitor (JNK), p38 inhibitor (p38), MEK inhibitor (MEK) and Akt inhibitor (Akt)], concentrations of these inhibitors and the protocols used are the same as those in Fig. 5a.The data shown are the means ± SE from three independent experiments.

the Me4405 cells and Mel-1007 cells was found. A marked increase was observed in the apoptosis of MM200 and Mel-FH, in the presence of the inhibition of the MAPK p38, MEK and PI3K inhibitors. This also applied to the Me4405, Sk-Mel-28 and Mel-1007 cells for inhibition of MEK and inhibition of Akt for Me4405. It is of note that these cell lines were the same as those in which MEK had been upregulated by docetaxel [40].

Downstream targets of JNK, such as the Bcl-2 family of proteins, were examined. As shown in Fig. 5c, vincristine induced phosphorylation and reduction of Bcl-2 proteins in IgR3 cells, which was prevented by the inhibition of JNK. The levels of Bcl-X_L proteins were unchanged in the presence of the JNK inhibitor. Furthermore, the JNK inhibitor reduced the expression of vincristine-induced Bad and p-Bad (SER136, but not SER112) proteins.

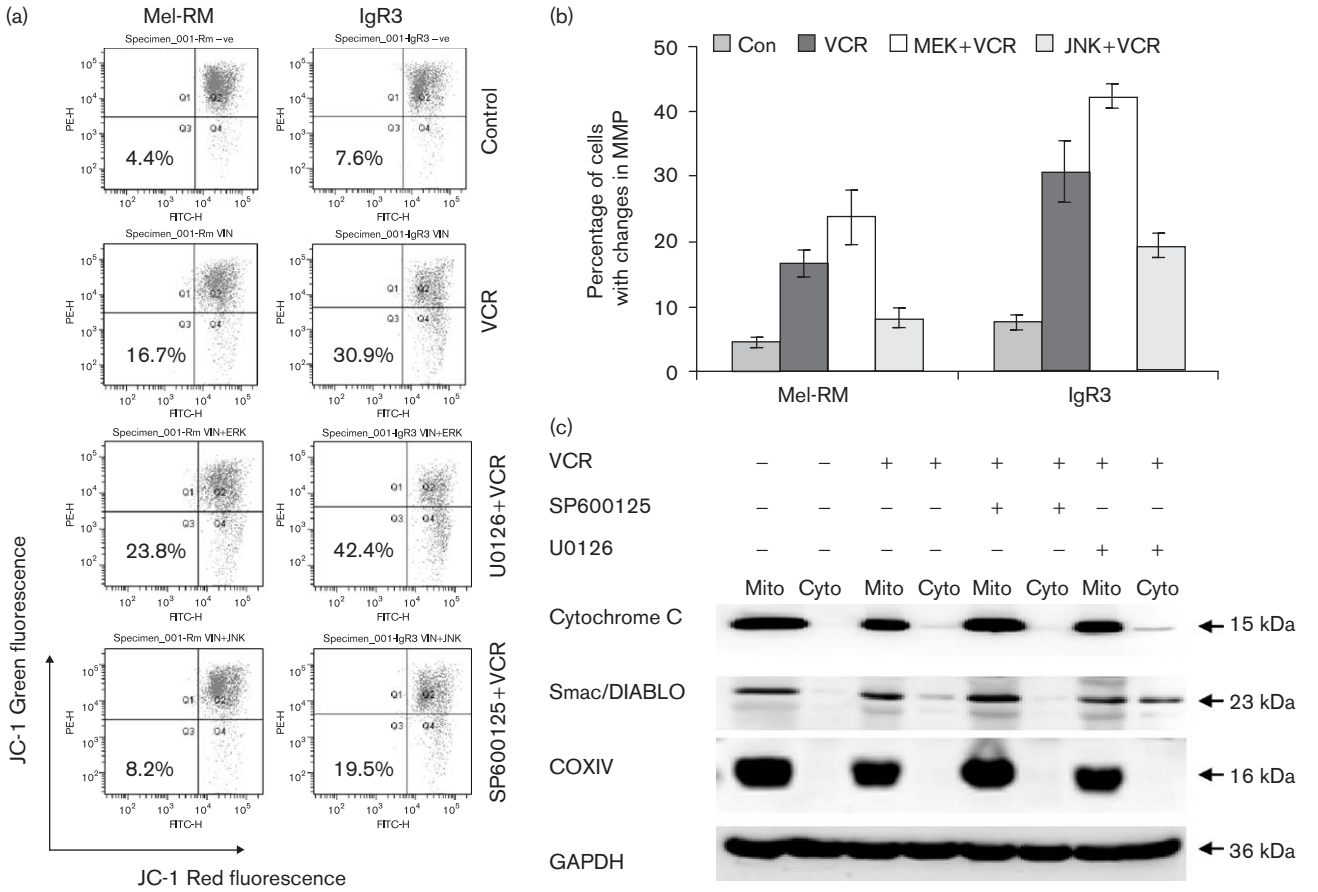
The JNK inhibitor significantly ($P < 0.01$) reduced the MMP (Fig. 6a and b) and the release of Smac/DIABLO and cytochrome C from the mitochondria to the cytosol (Fig. 6c), in both IgR3 and Mel-RM cells. Conversely, the MEK inhibitor significantly ($P < 0.01$) increased the MMP (Fig. 6b) and the release of cytochrome *c* and Smac/DIABLO from the mitochondria to the cytosol (Fig. 6c).

Vincristine upregulated the expression of p53, but vincristine-induced apoptosis was independent of the p53-signaling pathway

As shown in Fig. 7a, vincristine increased the amount of the p53 protein within 16, 24 and 36 h in Mel-RM and IgR3 cells. In addition, vincristine upregulated the expression of p21 and p27 within 16–36 h, and of Noxa and PUMA within 6 and 16 h, respectively, in Mel-RM cells. In IgR3 cells, p21 and PUMA were upregulated within 16–36 h and Noxa within 6–16 h.

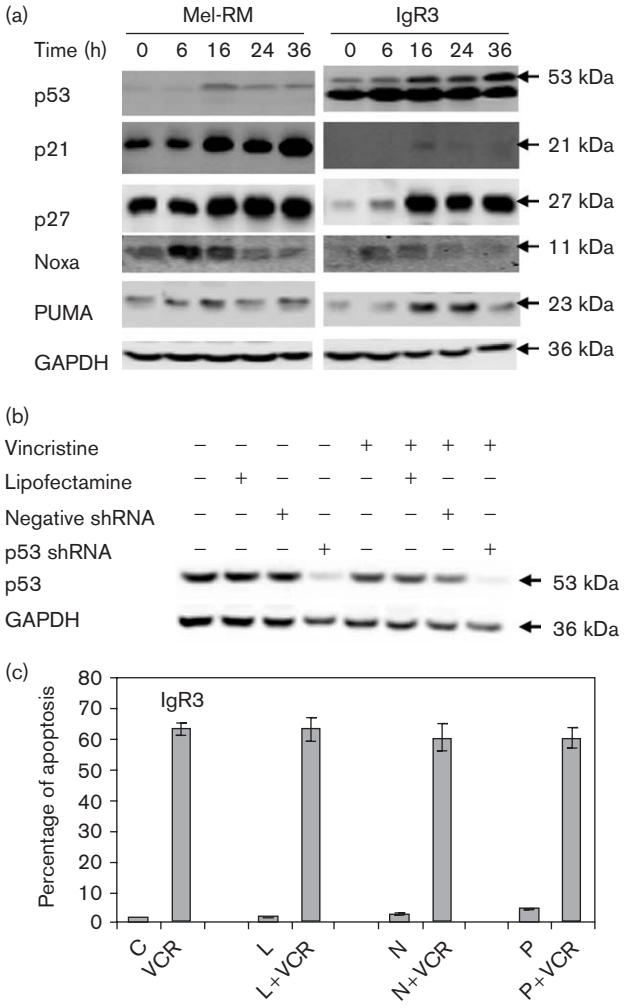
The role of p53 in apoptosis induced by vincristine in IgR3 cells was examined by the shRNA knockdown of p53. The results shown in Figs. 7b and c showed that knockdown of p53 did not reduce vincristine-induced apoptosis in IgR3 cells. Although knockdown of p53 mRNA by its shRNA slightly increased apoptosis in the IgR3 cells, there was no significant difference between the control and experimental groups (Fig. 7c). Therefore, vincristine-induced apoptosis seemed to be independent of the p53-signaling pathway even though p53 was increased in the IgR3 cells.

Fig. 6



The effect of Jun N-terminal kinase (JNK) inhibitors on vincristine (VCR)-induced changes in mitochondrial membrane potential (MMP) (a) and (b). Inhibition of extracellular signal-regulated kinase (ERK)1/2 promotes, but inhibition of JNK inhibits VCR-induced reduction in MMP. Both cell lines were treated with VCR (50 ng/ml) in the presence or absence of MEK inhibitor, U0126 (20 μmol/l), or the JNK inhibitor, SP600125 (25 μmol/l), for 24 h before measurement of MMP using JC-1 by flow cytometry. The number in the lower quadrant plot represents the percentage of cells with reduction in MMP shown in the lower quadrant. The data shown are the results from three independent experiments. Con, control; MEK, MEK inhibitor U0126; JNK, JNK inhibitor. * and ** indicate as $P < 0.05$–0.01. (c) Inhibition of JNK attenuates VCR-mediated release of Smac/DIABLO from mitochondria into the cytosol. VCR-induced release of cytochrome *c* and Smac/DIABLO from the mitochondria into the cytosol in IgR3 cells were promoted or attenuated respectively by the MEK inhibitor, U0126 (20 μmol/l), or the JNK inhibitor for 24 h. Mitochondrial (Mito) and cytosolic (Cyto) fractions were subjected to Western blot analyses. Cyclo-oxygenase enzyme IV was used show the relative purity of the mitochondrial fractions. The data shown are the results from two independent experiments.

Fig. 7



Vincristine (VCR) increased the expression of the p53 protein, but VCR-induced apoptosis was independent of the p53 pathway. (a) Accumulation of p53, p21 and p21WAF1 in VCR-treated cells. Mel-RM and IgR3 cells were treated with VCR (50 ng/ml) at the indicated times and subjected to Western blot analysis. The data shown are representative of the results from three independent experiments. (b) Knockdown of p53 by short hairpin RNA (shRNA) in IgR3 cells. The IgR3 cell was transfected with the control and p53 shRNA. Twenty-four hours later, the media were changed into fresh media containing 5% fetal calf serum, and the cells were cultured for 24 h. After that, VCR at a concentration of 50 ng/ml was added and the cells were cultured for another 24 h. Following that, whole-cell lysates were subjected to Western blot analysis. The data shown are representative of three independent experiments. (c) Reduction of the p53 protein has no effect on VCR-induced apoptosis of IgR3 cells. C, control; L, lipofectamine; N, negative shRNA; N + VCR, negative control (shRNA + vincristine); P, p53 shRNA.

PUMA and Bim do not seem to be involved in vincristine-induced apoptosis

PUMA was reported to be upregulated by the p53 independent pathway [48]. We found that PUMA was constitutively expressed in most of the melanoma cell lines examined but not in the melanocytes (data not shown). The role of PUMA in the induction of apoptosis by vincristine was examined by SiRNA knockdown of PUMA. This resulted in a small reduction in vincristine-induced apoptosis in IgR3 cells (Fig. 8a and b), but not in Mel-RM cells (Figs. 8c and d). These findings indicate that PUMA has minimal involvement in vincristine-induced apoptosis of IgR3 cells.

Similarly, we examined the role of Bim by SiRNA knockdown of Bim. As shown in Fig. 8e and f, there was no evidence to show that Bim was involved in the vincristine-induced apoptosis in IgR3 cells. In addition, knockdown of Bim mRNA by Bim SiRNA did not affect vincristine-induced apoptosis in Mel-RM cells (data not shown).

Discussion

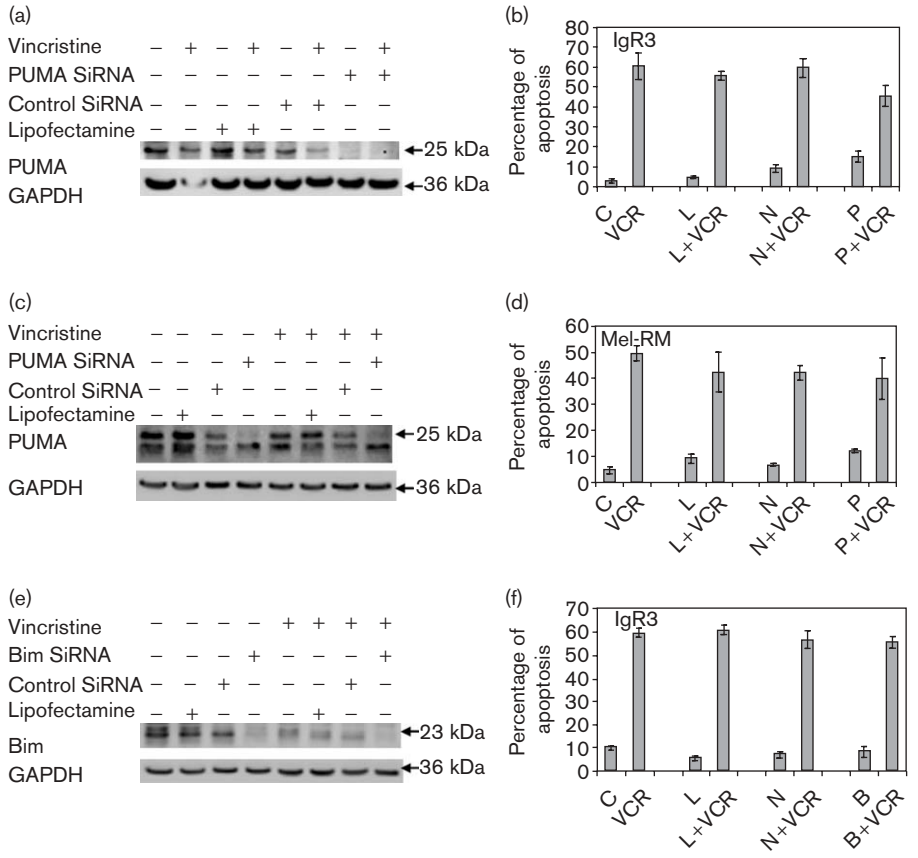
The above results show that vincristine induced various levels of apoptosis in a panel of eight cultured melanoma cells. Apoptosis was caspase dependent and involved the mitochondrial pathway, as shown by a reduction in the MMP associated with the release of cytochrome *c*, activation of caspase-9 and caspase-3 and the cleavage of PARP. These changes were inhibitable by the over-expression of Bcl-2.

Microtubule inhibitors (e.g. paclitaxel, docetaxel, vinblastine and vincristine) have been reported to induce apoptosis in different types of cancer cells including human melanoma cells via the activation of the JNK and p38 pathways [7,34]. The level of apoptosis induced by docetaxel was also influenced by the concomitant activation of the ERK pathways, which inhibited apoptosis in melanoma cells [40]. Earlier studies show that the JNK pathway was required for the induction of apoptosis by vincristine in three of the seven lines tested. Inhibition of MEK resulted in a marked increase in vincristine-induced apoptosis in four of the seven lines, and seemed to be a major factor in the resistance to vincristine-induced apoptosis in these four lines. Inhibition of PI3K had similar but less marked effects on resistance to vincristine.

Activation of JNK was reported to mediate apoptosis by phosphorylating and regulating the activity of transcription factors including c-Jun, ATF2, Elk-1, p53 and c-Myc [27,31–33] and members of the Bcl-2 family, such as Bcl-2, Bcl-x_L, Bim and Bad [34–36]. We show here that the inhibition of JNK (SP600125) inhibited the release of cytochrome *c* and Smac/DIABO from the mitochondria to the cytosol. A similar finding was reported by Aoki *et al.* [49]. In addition, activated JNK can inhibit the antiapoptotic role of Bcl-2 by the phosphorylation of Bcl-2 [7,34]. This study showed that inhibition of JNK reduced the vincristine-induced phosphorylation of Bcl-2.

JNK was reported to mediate apoptosis by phosphorylating Bim, Bmf [38,50] and Bad [51,52]. In this study,

Fig. 8



Small interfering RNA (SiRNA) knockdown of PUMA or Bim had little or no effect on the induction of apoptosis by vincristine (VCR). (a) Knockdown of PUMA mRNA by SiRNA in IgR3 cells was carried out as described for studies on p53 above (7b). (b) The effect of silencing PUMA on VCR-induced apoptosis in IgR3 cells. C, control; L, lipofectamine; N, negative control SiRNA; P, PUMA SiRNA. Vincristine at a concentration of 50 ng/ml was added into media and the cells were cultured for another 48 h. Following that, the cells were collected for analysis of apoptosis using flow cytometry. The data shown are the means ± SE from three independent experiments. (c) Knockdown of PUMA mRNA by SiRNA in Mel-RM cells. Methods were as for (a) and (d) The effect of silencing PUMA mRNA on VCR-induced apoptosis in Mel-RM cells. The protocol was the same as for (b). (e) Knockdown of Bim mRNA by Bim SiRNA was confirmed by the Western blot method. The protocol is the same as for (a, c). (f) Silencing of Bim mRNA did not affect VCR-induced apoptosis in IgR3 cells. The protocol was the same as for (b). B, Bim SiRNA.

SP600125 inhibited the phosphorylation of p-Bad (SER136) but not p-Bad (SER112), and also down-regulated the expression of Bad protein, but had no effect on the expression of Bim. Furthermore, knockdown of Bim by SiRNA did not reduce vincristine-induced apoptosis. This contrasts with other studies that reported that Bim plays a very important role in the induction of apoptosis by other drugs such as glucocorticoids, suberic bishydroxamate and TRAIL [53–56]. This difference might be related to the use of different cell types.

Activation of JNK has been reported by others to increase the p53 proteins [57,58]. Similar results were found in the current study. In addition, p53 can increase the expression of the proapoptotic PUMA protein, but downregulation of p53 by shRNA or PUMA by siRNA had no or very little effect on vincristine-induced apoptosis in IgR3 cells. Similar findings were reported in other tumors such as lymphoma, breast [57] and renal cancer [59]. These findings suggest that the upregulation of p53 is not a dominant factor in apoptosis induced by vincristine and is independent of the p53-signaling pathway. Our findings, however, differ from one study that showed that vincristine-induced apoptosis in melanoma cells was p53 dependent [60]. This difference might be due to the different cell lines studied.

The results from this study are similar to those reported from studies on docetaxel, in that the levels of apoptosis induced by vincristine seem to be determined by the balance between the activations of the proapoptotic JNK pathway and the antiapoptotic MEK/ERK1/2 and Akt pathways. Both drugs activate the ERK1/2 pathway and both activate JNK. It is not clear what determines

the activation patterns between melanoma lines, but in the case of docetaxel, this seemed to be related to the variable protein kinase C (PKC) isotype expression between lines [61]. Cell lines high in PKCε seemed to preferentially activate the MEK/ERK1/2 pathway, whereas those with relatively high levels of PKCδ isoforms strongly activated the JNK pathway. We expect that these findings are relevant to the current study. Despite these similarities, vincristine did not activate caspase-2, whereas this was a prominent effect of docetaxel [62].

In summary, these results are consistent with an apoptotic mechanism mediated by JNK, in which the phosphorylation of Bcl-2 results in the release of proapoptotic Bcl-2 proteins, the activation of Bax/Bak and the triggering of the mitochondrial pathway. The actual level of apoptosis seems to be determined by the level of activation of JNK, and in some melanoma cells, by the strength of the activation of the antiapoptotic MEK/ERK1/2 and Akt pathways. Further studies are needed on the upstream events that determine the activation of these pathways and to explain the variation in the sensitivities of melanoma cells to vincristine-induced apoptosis.

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