Involvement of BH3-only proapoptotic proteins in mitochondrial-dependent Phenoxodiol-induced apoptosis of human melanoma cells

Fu Yu, Ralph N. Watts, Xu Dong Zhang, Jodie M. Borrow and Peter Hersey

Phenoxodiol is a chemically modified analogue of the plant hormone isoflavone with antitumour activities. In the present study, we have examined its ability to induce apoptosis in human melanoma cells and the mechanisms involved. Apoptosis was observed in Phenoxodiol-treated cells by using annexin V/propidium iodide staining and determining mitochondrial membrane potential. To determine which caspase pathways were involved in Phenoxodiol-induced apoptosis, studies were performed using specific caspase inhibitors. Western studies were performed to ascertain which proteins of the apoptosis cascade were affected to cause Phenoxodiol-induced apoptosis. We found that induction of apoptosis by Phenoxodiol was maximal at 48 h with a range of apoptosis of 12 ± 4 to 48 ± 5% in different melanoma lines. This apoptosis was mainly dependent on activation of caspase-3 and caspase-9. Apoptosis was associated with induction of changes in mitochondrial membrane potential and was inhibited by over-expression of Bcl-2. Variation in sensitivity to Phenoxodiol appeared related to events upstream of the mitochondria and the degree of conformational change in Bax. The p53-regulated BH3-only proteins (Bad, PUMA and Noxa) were increased in the sensitive, but not in the resistant lines, whereas Bim was increased in all the lines tested. Bim appeared, however, to be partially involved because reduction of Bim by RNA interference resulted in decreased levels of apoptosis. Together, these studies suggest that Phenoxodiol induces apoptosis of melanoma cells by induction of p53-dependent BH3 proteins (Bad, PUMA and Noxa) and the p53-independent Bim protein, resulting in activation of Bax and its downstream events. *Anti-Cancer Drugs* 17:1151–1161 © 2006 Lippincott Williams & Wilkins.

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Introduction

Phenoxodiol, an isoflavone analogue, has been known to have effects on cell function, including regulation of apoptosis and cell cycle kinetics, and to exert different effects on human cancer cells, suggesting that this class of molecules may offer novel approaches to cancer therapy [1–3]. Preliminary studies had shown that Phenoxodiol inhibits cell proliferation and induces apoptosis in a wide range of human cancer cell lines, including leukaemia, breast cancer, prostate carcinomas and ovarian cancer [1,3,4].

Induction of apoptosis is believed to be critical for the activity of anticancer agents [5–7] and resistance to apoptosis may be the basis for much of the resistance of tumour cells to therapy [8–10]. Chemotherapeutic agents are believed to induce apoptosis through the intrinsic mitochondrial pathway via release of apoptogenic proteins such as cytochrome *c* and Smac/DIABLO [11–14]. Smac/DIABLO inhibits members of the inhibitor of apoptosis protein (IAP) family, which are bound to effector caspases such as caspase-3 [15]. Previous studies on the Bcl-2 family suggested that the antiapoptotic proteins Bcl-2, Bcl-X_L and Mcl-1 appear to preserve the integrity of the outer mitochondrial membrane by binding to mitochondrial porin channels [16,17]. These antiapoptotic members of the Bcl-2 family are also believed to be responsible for protecting cells against apoptosis by binding to proapoptotic BH3-only members, such as Bid, Bim, PUMA and Noxa [16–22]. In one model of apoptotic regulation, apoptotic stimuli such as damage to DNA or the cytoskeleton or interaction of cells with death-inducing ligands such as tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) induce production of proapoptotic BH3-only 'sensor' proteins of the Bcl-2 family such as Noxa, PUMA, Bim and Bid. The sensor proteins are believed to bind to the antiapoptotic Bcl-2 members, and thereby promote binding of the multi-

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domain proapoptotic Bax and Bak proteins to mitochondria, where they initiate changes in mitochondrial membrane potential (MMP) [16,21–23]. Certain chemotherapeutic agents may also induce apoptosis via the extrinsic death receptor pathway by p53-mediated up-regulation of TRAIL death receptors [3,9,10,24,25].

In the present study, we have examined the ability of Phenoxodiol to induce apoptosis in human melanoma cells and the mechanism involved in induction of apoptosis.

Materials and methods

Cell lines

Human melanoma cell lines Me4405, Mel-FH, Mel-RM, Mel-CV, MM200, RMu, Me1007, IgR3, Mel-AT, SK-Mel-110 and SK-Mel-28 have been described previously [26]. SK-Mel-110 was provided by Dr Albino (American Health Foundation, Valhalla, New York, USA). The cell lines were maintained in Dulbecco's modified Eagle's medium culture media containing 5% fetal calf serum (FCS) (Commonwealth Serum Laboratories, Melbourne, Australia) at 37°C in 5% CO_2.

Antibodies, recombinant proteins and other reagents

Phenoxodiol was kindly supplied by Marshall Edwards/Novogen (North Ryde, New South Wales, Australia). Recombinant human TRAIL was supplied by Immunex (Amgen, Seattle, Washington, USA). The preparation was supplied as leucine zipper fusion protein. The rabbit monoclonal antibody (MAb) against active caspase-3 (catalogue number 559565), the rabbit polyclonal Ab against Bid (catalogue number 559681), the mouse MAb against human poly(ADP-ribose) polymerase (PARP) (catalogue number 66391A) and the mouse anti-cytochrome *c* antibody (catalogue number 556433) were purchased from BD PharMingen (Bioclone, Marrickville, Australia). The rabbit antibody against inhibitor of caspase-activated deoxyribonuclease (ICAD) was purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA; catalogue number sc-9066). Rabbit anti-Smac/DIABLO (222–237) was purchased from Calbiochem (Adelaide, Australia; catalogue number 567365). Mouse anti-hILP/XIAP was purchased from BD Transduction Laboratories (BD Biosciences, San Jose, California, USA; catalogue number 610762). Mouse MAb against human Bcl-2 (C-2, recombine human 1–205), Bcl-x_L (H-5, the C-terminus of human Bcl-x_L), Bad (the full length of human Bad origin) and Mcl-1 [23] were purchased from Santa Cruz Biotechnology (catalogue numbers sc-7382, sc-8392, sc-8044 and sc-12756, respectively). Polyclonal rabbit anti-Bax NT antiserum was purchased from Upstate Biotechnology (Parkville, Australia; catalogue number 06-499). Polyclonal anti-Bim antibody (catalogue number IMG-171) and MAbs against human NOXA (catalogue number IMG-349) were purchased from Imgenex (San Diego, California, USA). p53 (clone Pab1801) mouse MAbs were purchased from Calbiochem (catalogue number OP09). Mouse monoclonal anti-p21 antibody was purchased from BD PharMingen (BD Bioscience; catalogue number 556430).

The cell-permeable pan-caspase inhibitor, Z-Val-Ala-Asp(OMe)-CH_2F (z-VAD-fmk), the caspase-3-specific inhibitor, Z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH_2F (z-DEVD-fmk), the caspase-9-specific inhibitor, Z-Leu-Glu(OMe)-His-Asp(OMe)-CH_2F (z-LEHD-fmk), the caspase-2-specific inhibitor, Z-Val-Asp(OMe)-Val-Ala-Asp(OMe)-CH_2F (z-VDVAD-fmk) and the caspase-8-specific inhibitor, Z-Ile-Glu(OMe)-Thr-Asp(OMe)-CH_2F (z-IETD-fmk) were purchased from Calbiochem (La Jolla, California, USA).

Apoptosis

Apoptotic cells were determined by the propidium iodide method [15,27,28], and by staining with phycoerythrin-conjugated annexin V and propidium iodide according to the manufacturer's instructions. In brief, melanoma cells were adhered overnight in a 24-well plate (Falcon 3047; Becton Dickinson, Lane Cove, Australia) at a density of 1×10^5 cells/well in media with 5% FCS. Cells in suspension were added on the day of the assay. Medium was removed and cells were treated for 1 h with different concentrations of Phenoxodiol, caspase inhibitors or kinase inhibitors in 500 µl of fresh media + 5% FCS before further treatment for 20 h at 37°C with the inhibitors in the presence of the reagents Phenoxodiol or TRAIL. The medium was removed, and adherent and suspended cells were washed once with phosphate-buffered saline (PBS). Subsequent procedures were as described previously [15,27,28].

Plasmid vector and transfection

Stable Mel-RM transfectants of Bcl-2 were established by electroporation of the PEF-puro vector carrying human Bcl-2 provided by Dr David Vaux (Walter and Eliza Hall Institute, Melbourne, Victoria, Australia) and described elsewhere [15].

Mitochondrial membrane potential ($\Delta\Psi_m$)

The methods used in this study were similar to those described previously [28]. Tumour cells were cultured in 24-well plates and were allowed to reach exponential growth for 24 h before treatment. MitoTracker Red CMXRos (Molecular Probes, Eugene, Oregon, USA) was added at 100 nmol/l during the last 30 min of treatment. The medium was removed into a 75-mm Falcon polystyrene tube (Becton Dickinson, Sunnyvale, California, USA), and the adherent cells were trypsinized and collected into the same tube. After washing with PBS, the cells were analysed using a FACScan flow cytometer (Becton Dickinson, Sunnyvale, California,

USA) for MitoTracker uptake. Untreated cells were used as controls.

Preparation of mitochondrial fractions

Methods used for preparation of subcellular fractions were similar to those described previously [15,29,30]. In brief, adherent cells were removed by trypsinization in 0.25% trypsin at 37°C for 5 min. When cells were pretreated with TRAIL, floating cells were also collected in the same tubes, after being washed once with ice-cold PBS, the cell pellet was suspended in five volumes of buffer A [20 mmol/l *N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid (HEPES)–KOH (pH 7.5), 10 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 1 mmol/l Na-ethylene diaminetetraacetic acid (EDTA), 1 mmol/l Na-ethyleneglycol-bis-(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mmol/l dithiothreitol and 0.1 mmol/l phenylmethylsulphonyl fluoride containing 250 mmol/l sucrose] supplemented with protease inhibitors [5 μg/ml pepstatin A, 10 μg/ml leupeptin, 2 μg/ml aprotinin and 25 μg/ml *N*-acetyl-leu-leu-norleucine (ALLN)]. After incubation on ice for 15 min, the cells were disrupted by passing them 15 times through a 22-gauge needle. After centrifugation twice at 750*g* for 10 min at 4°C, the supernatant was collected and centrifuged at 10 000*g* for 15 min at 4°C and the resulting mitochondrial pellets were resuspended in buffer A. The supernatants of the 10 000*g* spin were further centrifuged at 100 000*g* for 1 h at 4°C and the resulting supernatants were designated as the S-100 cytosolic fraction.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and Western blot analysis

Methods used were as described previously [15].

Detection of mRNA expression and semi-quantitative reverse transcription-polymerase chain reaction

Melanoma cells (1×10^6) were treated with Phenoxodiol (8 μmol/l) for 0 and 6 h. Total RNA was isolated and purified using the SV total RNA isolation system (Promega, Madison, Wisconsin, USA). First-strand cDNA was synthesized from purified total RNA samples (2 μg) using oligo$(dT)_{15}$ primer (Sigma-Genosys, Castle Hill, New South Wales, Australia) and reverse transcriptase M-MLV RT (200 U; Promega) in final 25-μl volume. The first-strand cDNA samples (1 μl each) were then amplified by polymerase chain reaction (PCR) using Taq enzyme (5 U/reaction; Promega). All PCR reactions were processed on a GeneAmp PCR system 9600 (Perkin-Elmer, Boston, Massachusetts, USA) at 94°C for 5-min denaturing, followed by processing at 94°C for 30 s, 58°C for 30 s and 72°C for 1 min. PCR products were detected on 2% agarose gel containing ethidium bromide (0.5 μg/ml). Densities of the bands on gel were quantitated using the public domain NIH image program as above (http://rsb.info.nih.gov/nih-image).

Primers for Bim and β-actin are as follows:

Primers		PCR cycle numbers
Bim	Forward 5′-GCCCCTACCTCCCTACAGAC-3′	35 cycles
	Reverse 5′-ATGGTGGTGGCCATACAAAT-3′	
Noxa	Forward 5′-AGATGCCTGGGAAGAAG-3′	35 cycles
	Reverse 5′-AGTCCCCTCATGCAAGT-3′	
PUMA	Forward 5′-GCCCAGACTGTGAATCCTGT-3′	38 cycles
	Reverse 5′-TCCTCCCTCTTCCGAGATTT-3′	
β-Actin	Forward 5′-GGACTTCGAGCAAGAGATGG-3′	25 cycles
	Reverse 5′-AGCACTGTGTTGGCGTACAG-3′	

Small RNA interference

In order to confirm functions of the proapoptotic protein Bim in Phenoxodiol-induced apoptosis, DNA–RNA hybrid small interfering RNAs (siRNAs), were used to knockdown Bim protein expression in melanoma cells. Sequences of siRNA used for Bim RNA interference were Bim sense 5′-CAAUUGUCUACCUUCUCGG(dTdT)-3′ and Bim antisense 5′-CCGAGAAGGUAGACAAU UG(dTdT)-3′ synthesized by Qiagen (Doncaster, Victoria, Australia). Methods for transfection procedures were completed according to the instructions in the QIAgen Start kit (Qiagen).

Results

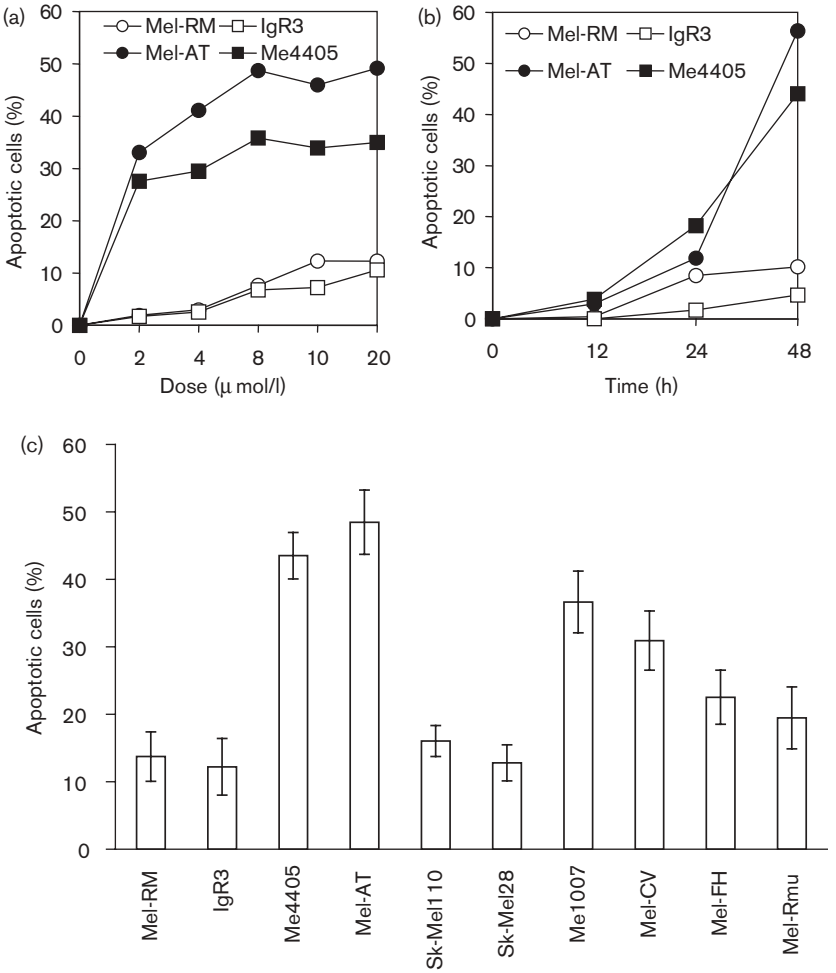
Phenoxodiol induces apoptosis in melanoma cells

We assessed the relative degrees of apoptosis and necrosis of melanoma after treatment with Phenoxodiol for 24 h by staining with annexin V and propidium iodide, and analysed by flow cytometry. The results indicated that the cells were initially annexin V-positive and then became propidium iodide-positive, indicating that the mode of death was predominately via apoptosis [27,26]. Phenoxodiol-induced apoptosis was further confirmed by visualization of DNA condensation and fragmentation using diamidine phenyl indole to stain cellular DNA. Nuclear condensation and fragmentation was seen in the Phenoxodiol-treated, but not the control cells (data not shown). The effects were dose dependent and were more apparent in studies on Me4405 than in those on Mel-RM (Fig. 1a). The maximum percentage of Phenoxodiol-induced apoptosis was observed at a concentration of 8 μmol/l after 48 h of treatment (Fig. 1b). Studies on a panel of melanoma cell lines (Fig. 1c) showed wide variation between lines in sensitivity to Phenoxodiol, e.g. Me4405, Me1007 and Mel-AT were sensitive to the drug, whereas other lines such as MM200, SK-Mel-28, Mel-RM and IgR3 were relatively resistant.

Phenoxodiol-induced apoptosis is caspase dependent

To examine whether Phenoxodiol-induced apoptosis was dependent on the caspase cascade, Mel-RM, IgR3, Me4405 and Mel-AT cells were treated with the pan-caspase inhibitor, z-VAD-fmk, the caspase-3 inhibitor, z-DEVD-fmk, the caspase-9 inhibitor, z-LEHD-fmk, and the caspase-2 inhibitor, z-VDVAD-fmk, 1 h before exposure to Phenoxodiol for 48 h. The pan-caspase

Fig. 1



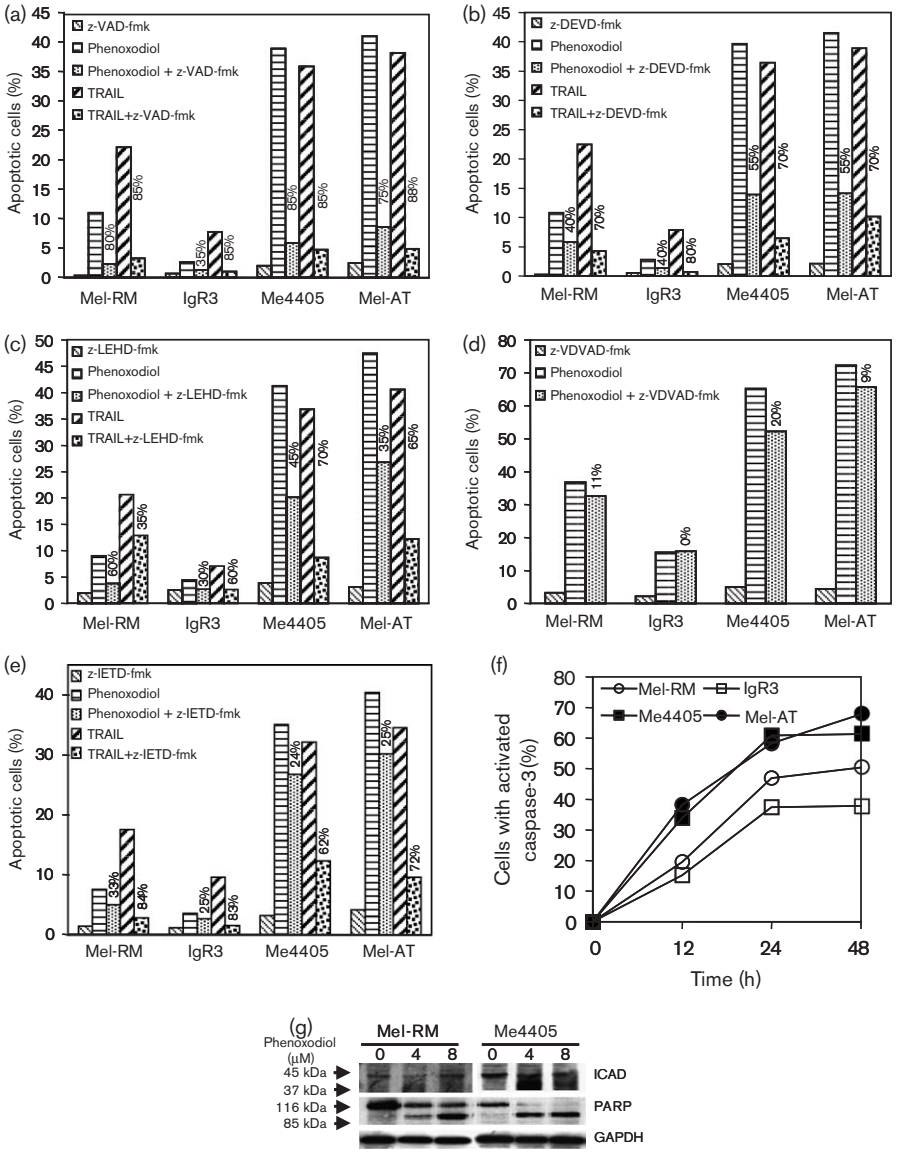
Phenoxodiol induces apoptosis in melanoma cells, but not in melanocytes. (a and b) Time and dose dependency of apoptosis induced by Phenoxodiol. The optimal conditions for induction of apoptosis of cultured melanoma cells were 8 μmol/l for 48 h. Results shown are representative of two similar experiments. Percentage of Phenoxodiol-induced apoptosis (8 μmol/l for 48 h) was measured in melanocytes and a panel of melanoma cell lines. Results shown are mean ± 1 SE of three experiments.

inhibitor markedly inhibited Phenoxodiol-induced apoptosis; however, the caspase-3 inhibitor only partially inhibited Phenoxodiol-induced apoptosis (Fig. 2a and b). The caspase-9 inhibitor was found to inhibit Phenoxodiol-induced apoptosis, but not to the same extent as the inhibition of TRAIL-induced apoptosis (Fig. 2c). Inhibition of caspase-2 with z-VDVAD-fmk did not appreciably decrease apoptosis in any of the sensitive or non-sensitive cell lines (Fig. 2d). The caspase-8 inhibitor, z-IETD-fmk, however, produced only low levels of inhibition in Phenoxodiol-induced apoptosis compared with that seen with TRAIL, suggesting that the extrinsic pathway may not be primarily involved (Fig. 2e). Studies with TRAIL were included as a positive control [15].

Activation of caspase-3 in melanoma cells after exposure to Phenoxodiol was studied using a MAb that recognizes proteolytically cleaved caspase-3. As shown in Fig. 2 (f), the degree of activation was related to sensitivity to apoptosis, in that there were higher levels of activation in the two sensitive cell lines, Me4405 and Mel-AT, than in the resistant cell lines, Mel-RM and IgR3. The study is representative of two similar studies.

To determine whether the activated caspase-3 was proteolytically active, we examined the breakdown of two of its substrates, PARP and ICAD [31]. Cleavage of ICAD and PARP was detected in extracts of melanoma cells after exposure to Phenoxodiol at 4 μmol/l as early as 24 h (Fig. 2g), consistent with the kinetics of activation of caspase-3. The degree of PARP and/or ICAD cleavage was associated with sensitivity to apoptosis in that there were higher levels of PARP and/or ICAD in the sensitive cell line Me4405 than in the resistant cell line Mel-RM.

Fig. 2

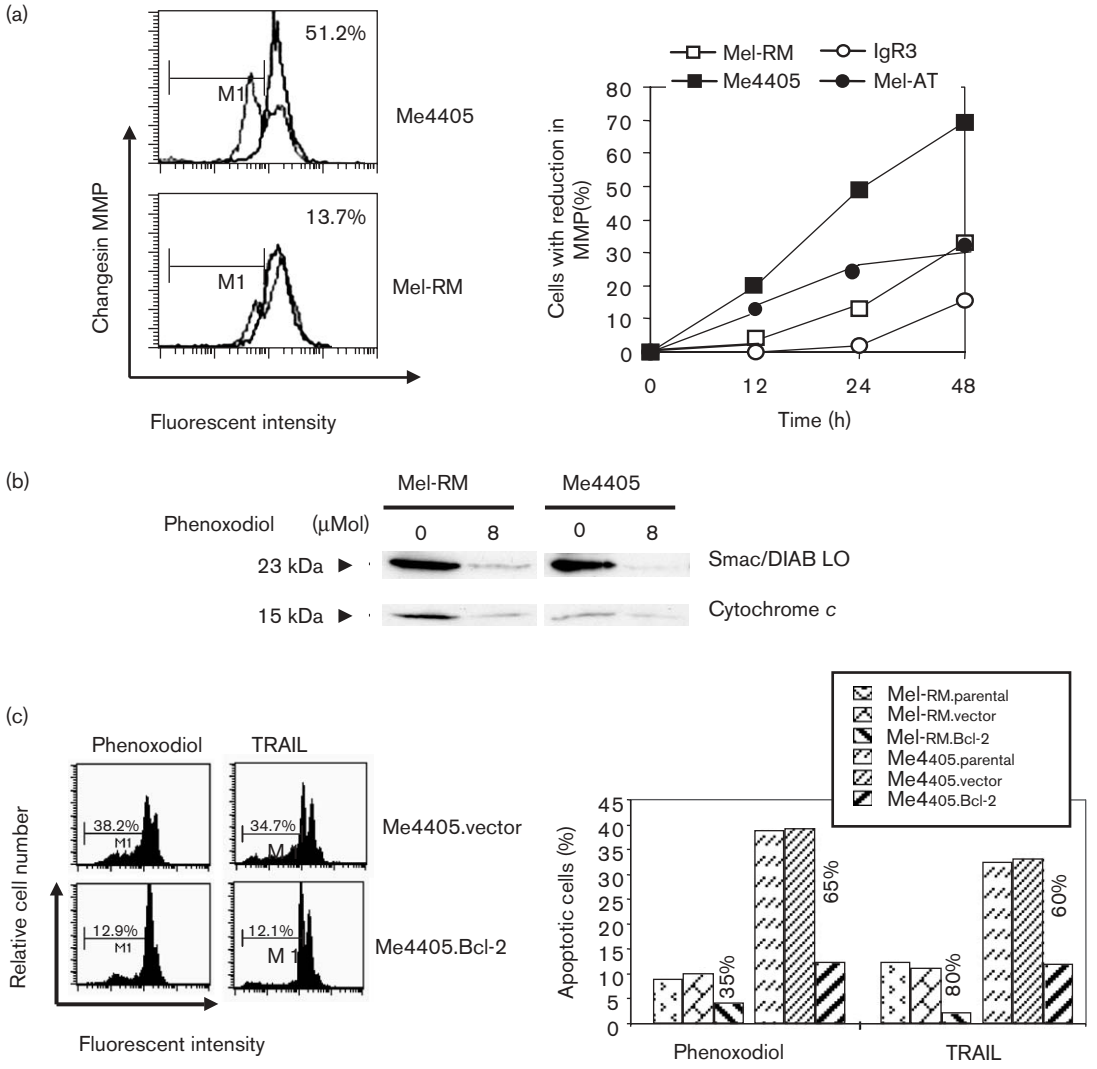


Phenoxodiol-induced apoptosis is caspase dependent. (a) Inhibition of Phenoxodiol-induced apoptosis of melanoma by the pan-caspase inhibitor (z-VAD-fmk) at 20 μmol/l. Phenoxodiol was added at 8 μmol/l for 48 h in both Me4405 and Mel-RM cell lines. The inhibition of caspases in tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis was used as a control in this and the following experiments. The percentages next to the bars indicate percentage inhibition. Results shown are mean values of two experiments. Standard errors were less than 5%. Inhibition of Phenoxodiol-induced apoptosis by z-VAD-fmk was statistically significant by *t*-tests of the data ($P<0.02$) except for studies on IgR3 ($P<0.2$). (b–e) Partial inhibition of Phenoxodiol-induced apoptosis of melanoma by the caspase-3 inhibitor (z-DEVD-fmk) at 30 μmol/l (b) and the caspase-9 inhibitor (z-LEHD-fmk) at 30 μmol/l (c), and no significant inhibition with the caspase-2 inhibitor (z-VDVAD-fmk) at 50 μmol/l (d) or the caspase-8 inhibitor (z-IETD-fmk) at 30 μmol/l, respectively. Results shown are mean values of two experiments. Standard errors were less than 5%. *P* values by *t*-test <0.02 for inhibition of apoptosis by the inhibitors of caspase-3 and -9 except for studies on IgR3 ($P<0.2$). (f) Kinetic studies of caspase-3 activation indicated that activated caspase-3 could be detected in melanoma cells at least by 12 h, and the levels of activation were higher in the sensitive cell lines Me4405 and Mel-AT than in the resistant cell lines Mel-RM and IgR3. (g) Activation of caspase-3 was confirmed by detection of the cleavage of inhibitor of caspase-activated deoxyribonuclease (ICAD) and poly(ADP-ribose) polymerase (PARP) by sodium dodecyl sulphate–polyacrylamide gel electrophoresis of whole-cell lysates taken 24 h after treatment with Phenoxodiol. Results shown are representative of two similar studies.

Involvement of the mitochondrial pathway in Phenoxodiol-induced apoptosis

Changes in MMP were studied using MitoTracker red, a fluorescent dye that is sequestered by mitochondria but lost once the mitochondria undergo a reduction in membrane potential ($\Delta\Psi$). As shown in Fig. 3(a), there was a reduction in MMP in both Mel-RM and Me4405 cells as early as 12 h after exposure to Phenoxodiol. The study shown is representative of two similar studies.

Fig. 3



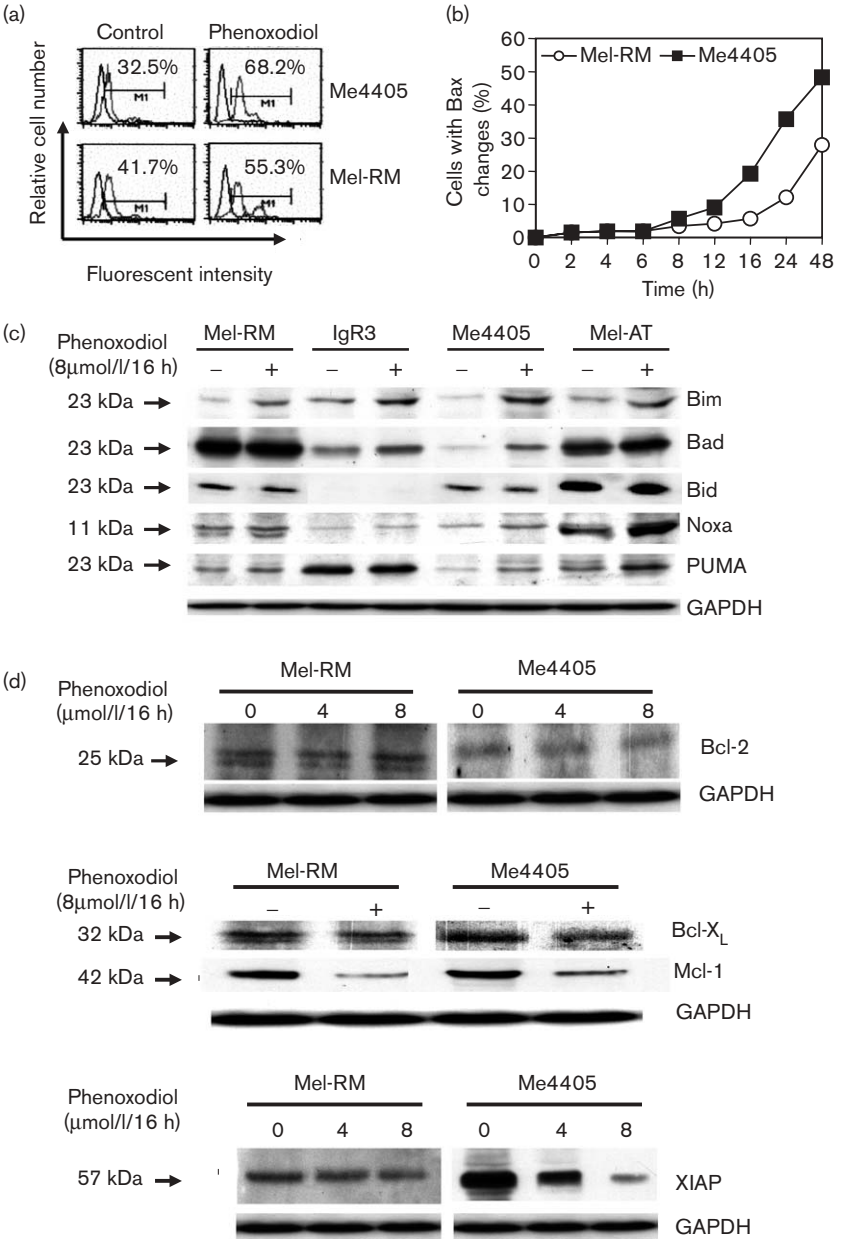
Phenoxodiol induces changes in mitochondria that are inhibitable by over-expression of Bcl-2. (a) Flow cytometric histograms showing changes in mitochondrial membrane potential (MMP) in melanoma cells after treatment with Phenoxodiol. Changes in MMP were more marked in the sensitive Me4405 cells than in the resistant Mel-RM cells (Phenoxodiol at 8 μmol/l was added for indicated time periods). (b) Changes of MMP after treatment with Phenoxodiol were confirmed by loss of Smac/DIABLO and cytochrome *c* from mitochondria, 24 h after treatment with Phenoxodiol (8 μmol/l). Western blot analysis of COXIV levels was included to show relative purity of the mitochondrial fractions. Results shown are representative of two similar studies. (c) Over-expression of Bcl-2 inhibited apoptosis induced by Phenoxodiol. Mel-RM and Me4405 cells were transfected with c-DNA for Bcl-2 or vector alone. The left panel shows expression levels of Bcl-2 in the transfected cells. These cells were treated with Phenoxodiol (8 μmol/l for 48 h) and the fraction detected by the propidium iodide method using flow cytometry (right panel). Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in these cells was measured in parallel as a positive control. Results shown are representative of two similar experiments. Standard errors were less than 5%. $P < 0.05$ for differences in apoptosis between vector-alone and Bcl-2-transfected cells.

Smac/DIABLO and cytochrome *c* were seen in mitochondrial fractions before treatment with Phenoxodiol (Fig. 3b), but after exposure to Phenoxodiol at 8 μmol/l for 24 h, both proteins were barely detected in the mitochondrial fractions (Fig. 3b).

Over-expression of Bcl-2 inhibits Phenoxodiol-induced apoptosis

Over-expression of Bcl-2 was shown in previous studies to inhibit apoptosis induced via the mitochondrial pathway [28,32]. We therefore over-expressed Bcl-2 in Me4405 and Mel-RM cells to examine the role of mitochondria in Phenoxodiol-induced apoptosis. Representative flow cytometric histograms in Fig. 3(c) show that the sub-G_1 population was markedly reduced in Me4405 cells transfected with Bcl-2 compared with the vector-alone transfected cells. These results, together with those from studies on Mel-RM cells, are summarized in Fig. 3(c). Studies with TRAIL are included as a positive control.

Fig. 4



Phenoxodiol induces conformational changes in the protein Bax and up-regulates proapoptotic BH3 proteins. Representative flow cytometric histogram showing conformational change in Bax before (dark lines) and after (light lines) treatment with Phenoxodiol (8 μmol/l for 24 h). Results show that conformational changes in Bax were greater in the sensitive Me4405 cell line than in the less-sensitive Mel-RM line. Kinetic studies showed conformational changes in Bax in the sensitive Me4405 cells by 8 h. These changes were less in the resistant cell line Mel-RM following treatment with Phenoxodiol (8 μmol/l). The results shown are representative of two such studies. Representative Western blots of the proapoptotic proteins Bim, Bad, Bid, PUMA and Noxa expression in melanoma cell lines before and after treatment with Phenoxodiol (8 μmol/l for 16 h). Bim expression was increased in both sensitive (Me4405 and Mel-AT) and resistant melanoma cell lines (Mel-RM and IgR3). The proapoptotic proteins, Bad, PUMA and Noxa, were increased in the sensitive cell lines, Me4405 and Mel-AT, but not in the resistant cell lines, Mel-RM and IgR3. Expression of Bid was unchanged between treated and non-treated samples in all cell types except IgR3, in which there was little expression. PUMA was constitutively expressed in IgR3 and Noxa in Mel-RM. Bad was also expressed at high levels before treatment in Mel-RM and Mel-AT.
(d) Representative Western blots showing expression of antiapoptotic Bcl-2, Bcl-x_L and Mcl-1 in both sensitive Me4405 and resistant Mel-RM cell lines before and after treatment with Phenoxodiol at 4 and 8 μmol/l for 16 h. No major changes were seen in Bcl-2, but Bcl-x_L and Mcl-1 expression was decreased after treatment with Phenoxodiol in both cell lines. X-linked inhibitor of apoptosis protein (XIAP) expression was decreased markedly in the sensitive melanoma cell line Me4405 but only slightly decreased in the resistant cell line Mel-RM. Results shown are representative of three similar studies.

Phenoxodiol induces conformational changes in Bax

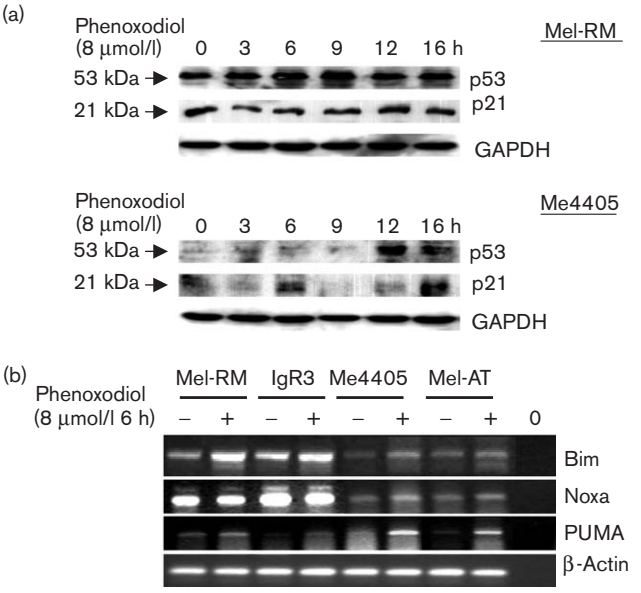
Activation of the proapoptotic protein Bax involves a conformational change that exposes the NH_2 terminus and hydrophobic (-COOH) terminus, which targets mitochondria [33,34]. The conformational status of Bax in melanoma cells was assessed using a specific antibody directly against the NH_2-terminal region of Bax and measured by flow cytometry. Flow cytometric histograms (representative of two experiments) show that conformational changes in Bax were observed in both Mel-RM and Me4405 melanoma cells after exposure to Phenoxodiol for 16 h (Fig. 4a). This was more marked in the melanoma cell line Me4405, which was sensitive to Phenoxodiol-induced apoptosis, and was less marked in the resistant cell line Mel-RM (Fig. 4b).

Changes in proapoptotic and antiapoptotic proteins induced by Phenoxodiol

The changes in Bax suggested that proapoptotic BH3-only proteins may have been activated. This was examined by Western blots, as shown in Fig. 4(c). Expression of Bim was increased in both sensitive and resistant cell lines, but was more evident in Me4405 and Mel-AT, the sensitive cell lines (Fig. 4c). Expression of Noxa and PUMA was unchanged in the resistant Mel-RM and IgR3 cell lines after treatment with Phenoxodiol but were increased in the sensitive cell lines, while Bid expression levels remained the same in all cell types after treatment with Phenoxodiol with very little expression found in the resistant cell line IgR3. It was of interest to note that PUMA was constitutively expressed at relatively high levels in the IgR3 cell line. Similarly, Noxa was constitutively expressed in Mel-RM and Mel-AT. Bad was also expressed at high levels in Mel-RM and Mel-AT in the absence of Phenoxodiol. The results shown are representative of two similar studies.

Expression of the antiapoptotic Bcl-2 protein in whole cell lysates did not change before and after treatment with Phenoxodiol (Fig. 4d); however, the level of the antiapoptotic proteins Mcl-1 and to a less extent Bcl-x_L decreased in both Me4405 and Mel-RM (Fig. 4d). X-linked inhibitor of apoptosis protein (XIAP) is a member of antiapoptotic proteins that confers protection from death-inducing stimuli by directly blocking activated caspase-3 or the activation of caspase-9 [34]. After treatment with Phenoxodiol for 16 h, XIAP levels decreased in Me4405, the melanoma cell line sensitive to Phenoxodiol-induced apoptosis, but was only slightly decreased in Mel-RM, the melanoma cell line resistant to Phenoxodiol-induced apoptosis (Fig. 4d). The decrease in XIAP was not seen in cells pretreated with the pan-caspase inhibitor, suggesting that it was due to activation of caspases (data not shown).

Fig. 5



Representative Western blots of the p53 and p21 expression in melanoma cells lines Mel-RM and Me4405 following 0, 3, 6, 9, 12 and 16 h of treatment with Phenoxodiol (8 μmol/l). GAPDH expression on the same nitrocellular membranes was used as loading controls. Results shown are representative of three similar studies.
(b) Phenoxodiol up-regulates mRNA expression of Bim, Noxa and PUMA mRNA expression of Bim, Noxa and PUMA was measured by semi-quantitative reverse-transcription polymerase chain reaction (PCR) in four melanoma cell lines before and after Phenoxodiol treatment for 6 h. Endogenous mRNA level for β-actin was used as loading control. Representative agarose test-gels showed that mRNA for Bim was up-regulated at 6 h in the four cell lines. Noxa mRNA levels were high before and after in the resistant cell lines, Mel-RM and IgR3, but were increased from low levels in the sensitive cell lines, Me4405 and Mel-AT. PUMA mRNA was at low levels before Phenoxodiol treatment and increased in all cell lines, but more so in the sensitive Me4405 and Mel-AT lines. The results shown are representative of three similar studies, some with different PCR cycle numbers.

Phenoxodiol increases p53 levels in sensitive melanoma lines

Expression of p53 and p21 was examined in melanoma cells lines after treatment with Phenoxodiol (8 μmol/l) for 0, 3, 6, 9, 12 and 16 h, to identify whether p53 may play a role in Phenoxodiol-induced apoptosis (Fig. 5a). p53 expression was markedly increased at 12 h in Me4405 after treatment with Phenoxodiol. In Mel-RM, p53 was detected in untreated cells, but there were no major changes in levels after treatment with Phenoxodiol. p21 was increased at 16 h in Me4405, but there were no changes in the pretreatment levels in Mel-RM cells.

Phenoxodiol increases mRNA levels of Bim, Noxa and PUMA

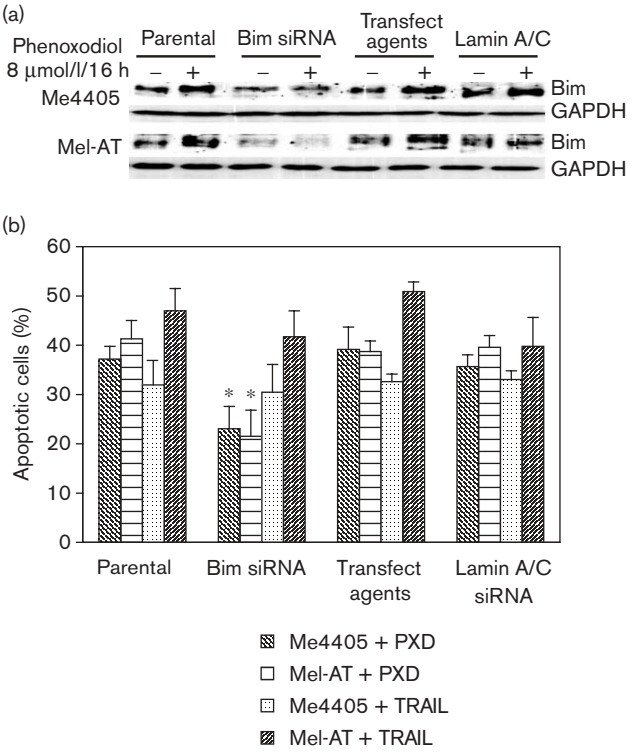
mRNA levels of Bim, Noxa and PUMA were assessed by reverse transcription-PCR, before and after Phenoxodiol treatment for 5 h. As shown in Fig. 5(b), mRNA levels of Bim were increased in both sensitive and resistant

melanoma cells. mRNA for PUMA was increased in the sensitive cell lines, and to a lesser extent in the resistant Mel-RM and IgR3 lines. Noxa mRNA levels were increased only in the sensitive melanoma cell lines, Me4405 and Mel-AT, but not in the resistant cell lines, Mel-RM and IgR3. The pretreatment mRNA levels in these lines were, however, high and may have obscured small increases. This was consistent with the results in the Western blotting studies shown in Fig. 4(c). Endogenous mRNA levels were assessed by measuring mRNA levels of β-actin.

Bim is involved in induction of apoptosis by Phenoxodiol

To investigate the possible involvement of Bim in Phenoxodiol-induced apoptosis, Bim levels in Me4405 and Mel-AT were reduced by RNA interference. Importantly, exposure to Phenoxodiol did not increase Bim levels in the cell lines transfected with siRNA for Bim whereas Phenoxodiol increased Bim levels in the parental cells and the control transfected cells (Fig. 6a). As shown in Fig. 6(b), reduction of Bim protein expression was associated with clear inhibition (40% reduction) of Phenoxodiol-induced apoptosis in both Me4405 and Mel-AT. TRAIL-induced apoptosis was not manifestly different from that seen in the parental or controls treated with the transfection agents alone or for Lamin.

Fig. 6



Down-regulation of Bim by small interfering RNA (siRNA) inhibits Phenoxodiol-induced apoptosis. (a) Representative Western blots showing up-regulation of Bim expression in Me4405 and Mel-AT after treatment with Phenoxodiol at 8 μmol/l for 16 h, but not in these cell lines treated by siRNA to silence Bim expression. Control parental cells showing up-regulation of Bim after treatment with Phenoxodiol. Bim siRNA-treated cells showing no increase after treatment with Phenoxodiol. Cells treated with transfection agent alone. Control cells treated with Lamin A/C siRNA, unrelated to Bim siRNA. Results are representative of two experiments. (b) Apoptosis was markedly reduced in the cells transfected with Bim siRNA, but not in the controls. TRAIL-induced apoptosis was not appreciably reduced in the cells transfected with Bim siRNA. Results shown are means ±1 SE of three experiments.

Discussion

In the present study, we have examined the effects of Phenoxodiol on human melanoma cells and show considerable levels of apoptosis in over half the cell lines tested. Apoptosis was shown by annexin V and propidium iodide staining, degradation of DNA and nuclear condensation. Apoptosis induction appeared dependent on activation of caspases and was mediated through the mitochondrial pathway, as shown by inhibition of apoptosis in melanoma cells following over-expression of Bcl-2. Evidence for the importance of caspase activation came from studies showing inhibition of apoptosis with a pan-caspase inhibitor and inhibitors of caspase-8, caspase-9 and caspase-3. Caspase-dependent induction of apoptosis by Phenoxodiol was also reported in cell lines from ovarian [3,4,35], and head and neck [36] cancers.

A marked variation was observed in sensitivity of the melanoma cell lines to Phenoxodiol. The available evidence suggests that regulation of sensitivity was at the level of mitochondria or upstream of mitochondria. For instance, changes in conformation of Bax, changes in MMP and activation of caspase-3 showed a general correlation with sensitivity to Phenoxodiol-induced apoptosis. This suggested that events determining the level of activation of Bax determined the overall sensitivity to Phenoxodiol.

To better understand the mechanism involved in induction of apoptosis induced by Phenoxodiol, we studied key proteins involved in apoptosis mediated via the mitochondrial pathway. This revealed up-regulation of the (p53-dependent) proapoptotic BH3 proteins Noxa and PUMA in the sensitive melanoma cell lines Me4405 and Mel-AT, but not in the resistant melanoma cell lines Mel-RM and IgR3. Down-regulation of the antiapoptotic proteins Mcl-1, Bcl-x_L and XIAP, but not Bcl-2, was also found. This array of changes was similar to those induced by treatment of melanoma cells with a histone deacetylase inhibitor [37]. Phenoxodiol at 8 and 80 μmol/l over 6 h, however, did not induce hyperacetylation of H4 histone in extracts from Me4405 or Mel-RM (data not shown). The antiapoptotic protein XIAP is an inhibitor of activated caspase-3 and inhibits activation of caspase-9 [15,38,39]. It was detected at high levels in the

melanoma cells and treatment with Phenoxodiol induced a considerable decrease in XIAP levels, particularly in the Me4405-sensitive cell lines similar to that reported in ovarian carcinoma cells [3,4]. Pretreatment with a pan-caspase inhibitor, however, blocked the down-regulation of XIAP by Phenoxodiol, suggesting that the changes in XIAP were secondary to activation of caspase-3, as reported in previous studies [15].

Detection of changes in the proapoptotic BH3-only proteins was of particular interest in that they are believed to be key initiators of apoptosis in response to cell damage. Noxa, PUMA and Bad are believed to be transcriptionally regulated by the transcriptional factor p53 [26,40]. No evidence for mutation of p53 in the four cell lines was found by genomic sequencing of p53 (data not shown). Western blotting analysis of p53 expression was carried out on extracts from Mel-RM and Me4405. Clear up-regulation of p53 was observed in the sensitive cell line Me4405 after 12 h of treatment with Phenoxodiol, but not in the resistant cell line Mel-RM. This preceded an increase in p21 expression in the sensitive cell line Me4405, with no observable G_1–S arrest. In Phenoxodiol-resistant Mel-RM cells, p21 expression remained unchanged. This finding is in contrast to that of Aguero *et al.* [36], who found that Phenoxodiol caused a G_1–S arrest by p21 in a p53-independent manner. A number of different variables, however, exist between the two studies, including different cell types and different Phenoxodiol concentrations, which may be the cause of the differences [36].

We showed that Phenoxodiol increased the levels of all three proteins, p53, PUMA and Noxa, in the sensitive lines (Me4405 and Mel-AT), but not in the resistant lines (Mel-RM and IgR3). Similarly, increases in mRNA for the BH3-only proteins, Noxa and PUMA, were shown by reverse transcription-PCR after treatment of the sensitive but not the resistant cell lines consistent with involvement of p53 in Phenoxodiol-induced apoptosis. It is not clear why non-mutated p53 in Mel-RM and IgR3 appeared silenced in function. Apparent non-function of p53 has, however, been reported by others in melanoma cells [41] and is the subject of ongoing studies in our laboratory.

Phenoxodiol also increased the levels of the proapoptotic p53-independent BH3-only protein Bim. Bim is bound to the dynein motor complex in microtubules and may be released by agents that act on microtubules such as taxanes [42]. It is also transcriptionally regulated by the FoxO3a transcription factor that is normally located in the cytosol but enters the nucleus in response to signal pathways such as the JNK pathway [42]. Up-regulation of Bim by Phenoxodiol was seen in all the cell lines, particularly in the two sensitive cell lines Me4405 and Mel-AT, after exposure to Phenoxodiol. Down-regulation of Bim by RNA interference resulted in appreciable inhibition of Phenoxodiol-induced apoptosis in the Me4405 melanoma cell lines, suggesting that up-regulation of Bim expression may be at least one of the factors involved in Phenoxodiol-induced apoptosis. Whether this reflects direct effects on microtubules or transcriptional effects due to entry of FoxO3a into the nucleus is not known. Involvement of dual pathways in induction of apoptosis was reported elsewhere [43].

It is of note that among the melanoma cell lines used in this study, those that harbour the activating B-Raf mutation (^{V600E}B-Raf), MM200, Mel-RMu and SK-Mel-28, were relatively resistant to Phenoxodiol-induced apoptosis. It is known that the Ras–Raf–MEK–ERK pathway plays an important role in regulation of the expression and/or activation of multiple Bcl-2 family members including the major isoforms of Bim, BimEL and Bad. The former can be phosphorylated by activated ERK1/2, which promotes proteosomal degradation, and the latter can also be phosphorylated by ERK1/2, which leads to its cytosolic sequestration. Given the role of Bim and Bad in Phenoxodiol-induced apoptosis of melanoma as demonstrated in this study, it is conceivable that activation of ERK1/2 by activating mutations of B-Raf may play a critical role in rendering melanoma cells resistant to apoptosis induced by Phenoxodiol.

In a recent study by Alvero *et al.* [4], they showed that Phenoxodiol caused apoptosis in epithelial ovarian carcinoma cells via activation of caspase-2, an increase in Bid activity and a decrease in XIAP levels. This was observed using the caspase-2 inhibitor, z-VDVAD-fmk, which inhibited the caspase-2 activation of Bid, subsequently decreasing apoptosis [4]. Our results show that Phenoxodiol causes apoptosis through a different pathway, as the caspase-2 inhibitor did not appreciably decrease apoptosis and Bid levels remained the same in both treated and untreated samples. Our findings do concur with those of Alvero *et al.* in that there is a decrease in XIAP levels. Another significant difference between the two studies is that our maximum concentration of Phenoxodiol to cause maximal apoptosis was 8 μmol/l, while Alvero *et al.* used approximately 40 μmol/l, a five-fold increase in concentration. These differences highlight the fact that medicines can act through varying pathways in different cell types, but can produce similar results.

In summary, the evidence from these studies suggests that Phenoxodiol induces apoptosis via mitochondrial pathways and that this is due to up-regulation of both p53 dependent (Bad, PUMA and Noxa) and independent (Bim) pathways. It is not clear how Phenoxodiol induces up-regulation of p53 and why some melanoma cell lines are particularly resistant to Phenoxodiol-induced apoptosis. Further studies on the aspects may help define

melanomas that are sensitive to treatment with Phenoxodiol.

References

1 Constantinou A, Husband A. Phenoxodiol (2H-1-benzopyran-7-0,1,3-4-hydroxyphenyl), a novel isoflavone derivative, inhibits DNA topoisomerase II by stabilizing the cleavable complex. *Anticancer Res* 2002; **22**:2581–2585.

2 Constantinou AI, Mehta R, Husband A. Phenoxodiol, a novel isoflavone derivative, inhibits dimethylbenz[a]anthracene (DMBA)-induced mammary carcinogenesis in female Sprague-Dawley rats. *Eur J Cancer* 2003; **39**:1012–1018.

3 Kamsteeg M, Rutherford T, Sapi E, Hanczaruk B, Shahabi S, Flick M, *et al.* Phenoxodiol – an isoflavone analog – induces apoptosis in chemoresistant ovarian cancer cells. *Oncogene* 2003; **22**:2611–2620.

4 Alvero AB, O'Malley D, Brown D, Kelly G, Garg M, Chen W, *et al.* Molecular mechanism of Phenoxodiol-induced apoptosis in ovarian carcinoma cells. *Cancer* 2006; **106**:599–608.

5 Schmitt CA, Lowe SW. Apoptosis is critical for drug response *in vivo*. *Drug Resist Update* 2001; **4**:132–134.

6 Ferreira CG, Epping M, Kruyt FAE, Giaccone G. Apoptosis: target of cancer therapy. *Clin Cancer Res* 2002; **8**:2024–2034.

7 Soengas MS, Lowe SW. Apoptosis and melanoma chemoresistance. *Oncogene* 2003; **22**:3138–3151.

8 Green DR, Evan GI. A matter of life and death. *Cancer Cell* 2002; **1**:19–30.

9 Hersey P, Zhang XD. How melanoma cells evade TRAIL-induced apoptosis. *Nat Rev* 2001; **1**:142–150.

10 Hersey P, Zhang XD. Overcoming resistance of cancer cells to apoptosis. *J Cell Physiol* 2003; **196**:9–18.

11 Earnshaw WC. Apoptosis. A cellular poison cupboard. *Nature* 1999; **397**:387–389.

12 Coultas L, Strasser A. The molecular control of DNA damage-induced cell death. *Apoptosis* 2000; **5**:491–507.

13 Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, *et al.* Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 2000; **102**:43–53.

14 Green DR. Apoptotic pathways: paper wraps stone blunts scissors. *Cell* 2000; **102**:1–4.

15 Zhang XD, Zhang XY, Gray CP, Nguyen T, Hersey P. Tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis of human melanoma is regulated by Smac/DIABLO release from mitochondria. *Cancer Res* 2001; **61**:7339–7348.

16 Shimizu S, Narita M, Tsujimoto Y. Bcl-2 family proteins regulate the release of apoptogenic cytochrome *c* by the mitochondrial channel VDAC. *Nature* 1999; **399**:483–487.

17 Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* 2002; **2**:647–656.

18 O'Connor L, Strasser A, O'Reilly LA, Hausmann G, Adams JM, Cory S, *et al.* Bim: a novel member of the Bcl-2 family that promotes apoptosis. *EMBO J* 1998; **17**:384–395.

19 Korsmeyer SJ, Wei MC, Saito M, Weiler S, Oh KJ, Schlesinger PH. Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome *c*. *Cell Death Differ* 2000; **7**:1166–1173.

20 Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamasita T, *et al.* Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* 2000; **288**:1053–1058.

21 Cheng EH, Wei MC, Weiler S, Flavell RA, Mak TW, Lindsten T, *et al.* BCL-2, BCL-X_L sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol Cell* 2001; **8**:705–711.

22 Puthalakath H, Strasser A. Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. *Cell Death Differ* 2002; **9**:505–512.

23 Bouillet P, Strasser A. BH3-only proteins-evolutionarily conserved proapoptotic Bcl-2 family members essential for initiating programmed cell death. *J Cell Sci* 2002; **115**:1567–1574.

24 Sheikh MS, Burns TF, Huang Y, Wu GS, Amundson S, Brooks KS, *et al.* p53-dependent and -independent regulation of the death receptor KILLER/DR5 gene expression in response to genotoxic stress and tumor necrosis factor alpha. *Cancer Res* 1998; **58**:1593–1598.

25 Wang S, el-Deiry WS. TRAIL and apoptosis induction by TNF-family death receptor. *Oncogene* 2003; **22**:8628–8633.

26 Zhang XD, Franco A, Myers K, Gray C, Nguyen T, Hersey P. Relation of TNF-related apoptosis-inducing ligand (TRAIL) receptor and FLICE-inhibitory protein expression to TRAIL-induced apoptosis of melanoma. *Cancer Res* 1999; **59**:2747–2753.

27 Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* 1991; **139**:271–279.

28 Thomas WD, Zhang XD, Franco AV, Nguyen T, Hersey P. TNF-related apoptosis-inducing ligand-induced apoptosis of melanoma is associated with changes in mitochondrial membrane potential and perinuclear clustering of mitochondria. *J Immunol* 2000; **165**:5612–5620.

29 Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. *Cell* 1996; **86**:145–157.

30 Zhang XD, Franco AV, Nguyen T, Gray CP, Hersey P. Differential localization and regulation of death and decoy receptors for TNF-related apoptosis-inducing ligand (TRAIL) in human melanoma cells. *J Immunol* 2000; **164**:3961–3970.

31 Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* 1998; **391**:43–50.

32 Walczak H, Krammer PH. The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. *Exp Cell Res* 2000; **256**:58–66.

33 Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, *et al.* Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* 2001; **292**:624–626.

34 Hussein MR, Haemel AK, Wood GS. Apoptosis and melanoma: molecular mechanisms. *J Pathol* 2003; **199**:275–288.

35 Sapi E, Alvero AB, Chen W, O'Malley D, Hao XY, Dwipoyono B, *et al.* Resistance of ovarian carcinoma cells to docetaxel is XIAP dependent and reversible by phenoxodiol. *Oncol Res* 2004; **14**:567–578.

36 Aguero MF, Facchinetti MM, Sheleg Z, Senderowicz AM. Phenoxodiol, a novel isoflavone, induces G_1 arrest by specific loss in cyclin-dependent kinase 2 activity by p53-independent induction of $p21^{WAF1/CIP1}$. *Cancer Res* 2005; **65**:3364–3373.

37 Zhang XD, Borrow JM, Zhang XY, Nguyen T, Hersey P. Activation of ERK1/2 protects melanoma cells from TRAIL-induced apoptosis by inhibiting Smac/DIABLO release from mitochondria. *Oncogene* 2003; **22**:2869–2881.

38 Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM, *et al.* IAPs block apoptotic events induced by caspase-8 and cytochrome *c* by direct inhibition of distinct caspases. *EMBO J* 1998; **17**:2215–2223.

39 Deveraux QL, Reed JC. IAP family proteins – suppressors of apoptosis. *Genes Dev* 1999; **13**:239–252.

40 Demidenko ZN, Blagosklonny MV. Flavopiridol induces p53 via initial inhibition of mdm2 and p21 and, independently of p53, sensitizes apoptosis -reluctant cells to tumor necrosis factor. *Cancer Res* 2004; **64**: 3653–3660.

41 Satyamoorthy K, Chehab NH, Waterman MJ, Lien MC, El-Deiry WS, Herlyn M, *et al.* Aberrant regulation and function of wild-type p53 in radioresistant melanoma cells. *Cell Growth Differ* 2000; **11**:467–474.

42 Sunters A, Fernandez De Mattos S, Stahl M, Brosens JJ, Zoumpoulidou G, Saunders CA, *et al.* Fox03a transcriptional regulation of Bim controls apoptosis in paclitaxel-treated breast cancer cell lines. *J Biol Chem* 2003; **278**:49795–49805.

43 Wong HK, Fricker M, Wyttenbach A, Villunger A, Michalak EM, Strasser A, *et al.* Mutually exclusive subsets of BH3-only proteins are activated by the p53 and c-Jun N-terminal kinase/c-Jun signaling pathways during cortical neuron apoptosis induced by arsenite. *Mol Cell Biol* 2005; **25**: 8732–8747.