

Phenoxodiol – an isoflavone analog – induces apoptosis in chemoresistant ovarian cancer cells

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Interference with the innate apoptotic activity is a hallmark of neoplastic transformation and tumor formation. In this study we characterize the cytotoxic effect of phenoxodiol, a synthetic anticancer drug analog of genestein, and demonstrate the mechanism of action by which phenoxodiol affects the components of the Fas apoptotic pathway on ovarian cancer cells. Primary ovarian cancer cells, isolated from ascitic fluids of ovarian cancer patients, resistant to conventional chemotherapy, undergo apoptosis following phenoxodiol treatment. This effect is dependent upon the activation of the caspase system, inhibiting XIAP, an inhibitor of apoptosis, and disrupting FLICE inhibitory protein (FLIP) expression through the Akt signal transduction pathway. We suggest that phenoxodiol is an efficient inducer of cell death in ovarian cancer cells and sensitizes the cancer cells to Fas-mediated apoptosis. We identified FLIP and XIAP signalling pathways as key factors regulating the survival of ovarian cancer cells. These findings demonstrate a novel nontoxic drug that controls FLIP/XIAP function and has the potential to eliminate tumor cells through Fas-mediated apoptosis.

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Introduction

Flavonoids are one of the principal plant hormones and are known to possess diverse functional roles including the regulation of plant apoptosis and cell cycle kinetics. A number of plant flavonoids show similar functional effects in animals and may be effective in inducing mitotic arrest and apoptosis. In particular, genistein, quercetin and flavopiridol have been shown to exert different effects on human cancer cells (Lamartiniere *et al.*, 1998; Weber *et al.*, 2000), suggesting that this class

of molecules may offer novel approaches to cancer therapy. Preliminary studies involving a number of flavonoid derivatives showed that phenoxodiol (2H-1-benzopyron-7-0,3-(hydroxy phenyl)) inhibits cell proliferation of a wide range of human cancer cell lines including leukemia, breast and prostate carcinomas, and is five to 20 times more potent than genistein (Constantinou and Husband, 2002).

Ovarian cancer is the most lethal gynecological malignancy and is the fifth leading cause of all cancer deaths in women (Berchuck *et al.*, 1994). Due to its insidious onset, ovarian cancer is often discovered in advanced stages, resulting in a 20% 5-year survival rate. Current treatment of ovarian cancer includes cytoreductive surgery and combination chemotherapy using a taxane and platinum regimen. Although the initial response to chemotherapy is greater than 80%, the majority of ovarian cancer ultimately recurs due to resistance to chemotherapy (Green *et al.*, 1984).

The Fas/FasL system has been recognized as an important pathway not only in tissue remodelling but also in drug-induced apoptosis and its deficient activation has been implicated in the development of drug resistance (Muller *et al.*, 1998). We and others have shown that ovarian cancer cells are resistant to Fas activation, despite the expression of the Fas protein (Xerri *et al.*, 1997; Mor *et al.*, 2002). The resistance or sensitivity to Fas-mediated apoptosis is dependent on the normal expression and function of antiapoptotic and proapoptotic molecules (Johnstone *et al.*, 2002; Mor *et al.*, 2002). Without a doubt, the ability of cancer cells to evade apoptosis is an essential ‘hallmark of cancer’ (Hanahan and Weinberg, 2000). A key objective in cancer therapy is to restore sensitivity to apoptosis.

In this study, we evaluated the effect of phenoxodiol on a panel of 20 primary human ovarian cancer cell lines, isolated from the ascitic fluid of patients with ovarian cancer, and two established cell lines CP-70 and Hey cells. We demonstrated heterogeneity in the blocking elements present in ovarian cancer cells, having FLICE inhibitory protein (FLIP) and X-linked inhibitor of apoptosis (XIAP) as the main antagonists responding to signals emerging from the Akt survival pathway. We also showed that phenoxodiol induces apoptosis in ovarian cancer cells by regulating these blocking elements, suggesting that phenoxodiol may be a potential new drug in the treatment of ovarian cancer.

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Results

Phenoxodiol induces cell death in primary ovarian cancer cells

Any successful cancer treatment involves an agent that induces apoptosis in the cancer cell. Therefore, we evaluated whether phenoxodiol has an effect on the viability of ovarian cancer cells. Primary ovarian cancer cell cultures, isolated from ascites of ovarian cancer patients, and the established cell lines CP-70 and Hey were treated with phenoxodiol (NV06) and three other derivatives of genestein (NV03, NV05 and NV07), and cell viability was determined using the CellTiter assay. Phenoxodiol treatment, but not NV03, NV05 or NV07, induced cell death of all the tested ovarian cancer cells ($n=22$) in a dose-dependent manner (shown for a selection in Figure 1a). After phenoxodiol treatment, a significant decrease in cell viability in all the ovarian cancer cell cultures was observed at a concentration of $10 \mu\text{g/ml}$ ($41.6 \mu\text{M}$). However, phenoxodiol did not affect ovarian surface epithelial (OSE) cells' viability (Figure 1a). Some of the ovarian cancer cells showed an increase in the number of cells after incubation with NV-05 and NV-07, which may be because of an estrogenic effect of these compounds (data not shown).

When we compared the effect of phenoxodiol and genestein on cell viability, phenoxodiol was 30 times more effective. Thus, in CP70 cells, the IC_{50} for phenoxodiol was $1.35 \mu\text{M}$, while for genestein it was $38.95 \mu\text{M}$.

We then evaluated the effect of phenoxodiol on colony formation of the ovarian cancer cells. As shown in Figure 1b, 4-h treatment with phenoxodiol significantly reduced the number of colonies observed after 10 days in culture.

Phenoxodiol induces apoptosis in ovarian cancer cells

To test whether the decrease in cell viability after phenoxodiol treatment was because of apoptosis, the treated cells were stained with Hoechst 33342 dye that stains condensed chromatin of apoptotic cells more brightly than the chromatin of normal cells. The chromatin was stained in the cells treated with phenoxodiol, but not in the controls (Figure 2a). This staining correlates with the presence of cells with typical apoptotic nuclear morphology (nuclear shrinkage, DNA condensation and fragmentation). Also, phenoxodiol treatment resulted in a twofold increase in caspase-3 activity (Figure 2b), indicating that the decreased cell viability induced by phenoxodiol is related to induction of apoptosis. No change in caspase-3 activity was found in normal OSE cells following treatment with a similar concentration of phenoxodiol (Figure 2b).

Phenoxodiol restores the sensitivity of ovarian cancer cells to Fas-mediated apoptosis

Our next objective was to characterize the mechanism of action of phenoxodiol-induced apoptosis in ovarian

cancer cells. Chemotherapeutic agents can induce apoptosis through multiple pathways, including the extrinsic (death receptors) or intrinsic (mitochondria) pathways. We first determined whether ovarian cancer cells isolated from ascites are sensitive to Fas-mediated apoptosis, one of the main extrinsic pathways. Cells were treated with an agonistic Fas monoclonal antibody and cell viability was determined using the CellTiter 96 assay. Figure 3a shows 10 representative ovarian cancer cells treated with anti-Fas for 24 h. Fas-sensitive Jurkat T cells are included as positive controls of the assay. All ovarian cancer cell cultures ($n=22$) showed resistance to Fas-mediated apoptosis. In contrast, normal OSE cells undergo apoptosis when treated with anti-Fas (data not shown and Baldwin *et al.*, 1999). The same agonistic Fas monoclonal antibody failed to activate caspase-8 in all ovarian cancer cell cultures (Figure 3b). Therefore, primary ovarian cancer cells are resistant to Fas-mediated apoptosis possibly due to an early defect in the Fas pathway.

We investigated whether treatment with phenoxodiol would reverse the resistance of the cancer cells to Fas-mediated apoptosis. Cells were pretreated with phenoxodiol $4 \mu\text{M}$ ($1 \mu\text{g/ml}$) for 24 h followed by incubation with FasL or the agonistic anti-Fas monoclonal antibody for 12 h. In the cells treated with phenoxodiol alone, cell viability decreased by 60% compared to untreated cells, while no decrease was found in the cells treated with anti-Fas or FasL alone (Figure 3c). Phenoxodiol treatment increased the sensitivity to Fas-mediated apoptosis as demonstrated by the further decrease in cell viability followed by anti-Fas (91%) or FasL (88%). These data indicate that phenoxodiol restores the sensitivity of ovarian cancer cultures to Fas-mediated apoptosis.

Phenoxodiol induces caspase-8 activation and FLIP downregulation through the Akt-pathway

Resistance to Fas-mediated apoptosis could be because of the absence of proapoptotic elements or the presence of antiapoptotic proteins blocking the Fas pathway. The expression of the main intracellular components of the Fas pathway was evaluated using Western blot analysis. The proapoptotic proteins Fas, FasL, FADD and procaspase-8, located upstream in the Fas-pathway, are all expressed at normal levels in all primary ovarian cancer cell cultures (Figures 3b and 4a). This suggests that the cellular resistance to Fas-mediated apoptosis is probably not caused by the absence of one of the proapoptotic proteins but rather by the presence of potential apoptotic blockers.

FLIP is an important blocker of the Fas pathway competing with caspase-8 for binding into the death-inducing signalling complex (DISC). Two FLIP splice variants, FLIP-long (FLIP_L) and FLIP-short (FLIP_S), and a cleaved form of FLIP_L (FLIP_C) can be detected. These three forms of FLIP were found in the ovarian cancer cell cultures, however at different expression levels (Figure 4a). The high levels of FLIP expression

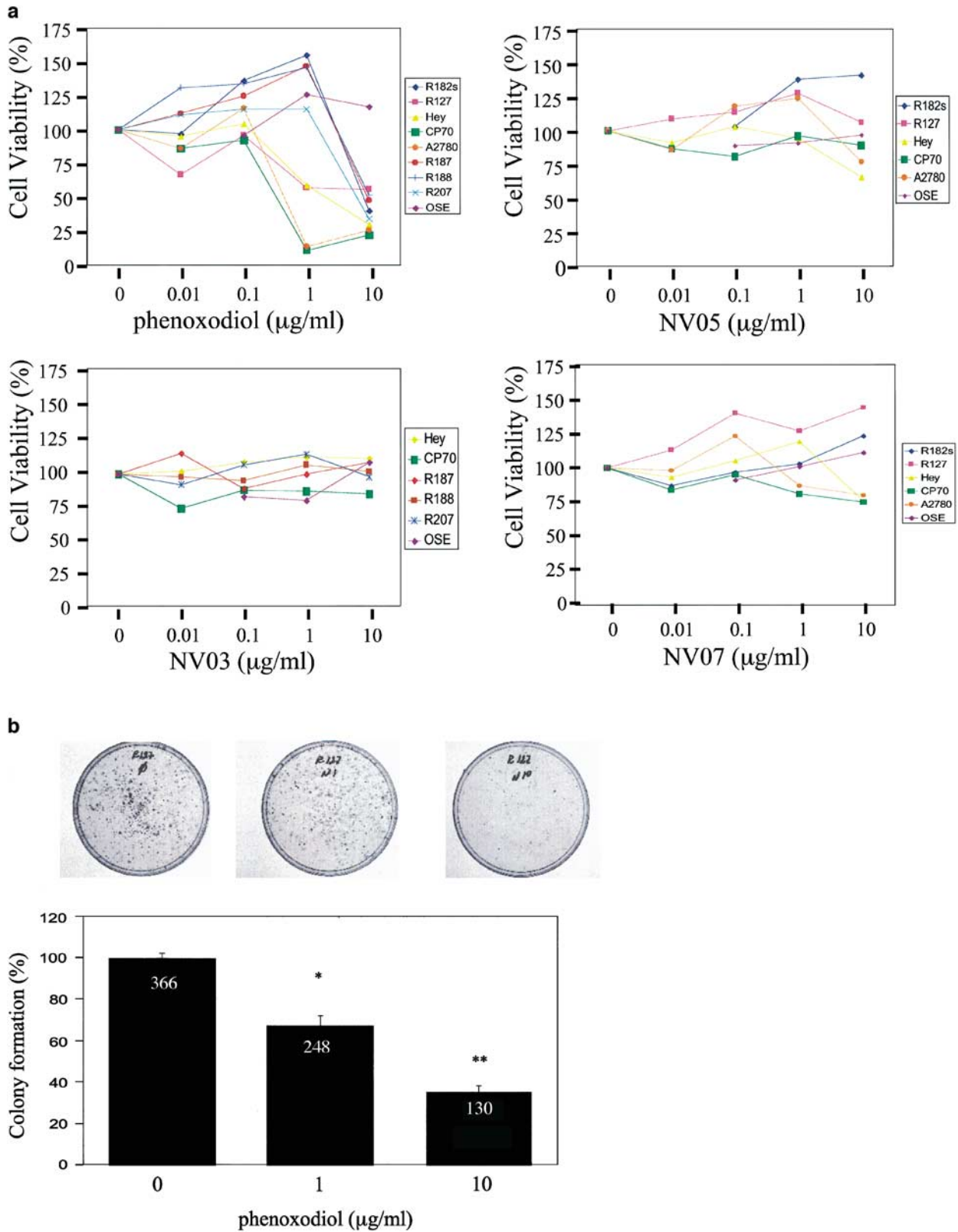


Figure 1 Phenoxodiol decreases cell viability of primary ovarian cancer cells. (a) Cell viability (in percentage, normalized to untreated cells) of primary ovarian cancer cells and normal OSE cells after treatment with phenoxodiol and three other isoforms (NV03, NV05 and NV07). (b) Colony survival (in percentage, normalized to untreated cells) of the primary ovarian cancer cells R127 after treatment with phenoxodiol 1 and 10 µg/ml (4.6 and 46 µM). Each culture dish, showing the actual colonies, corresponds to the underlying bar. Values represent the average number of colonies for one representative experiment. * $P = 0.04$, ** $P = 0.002$

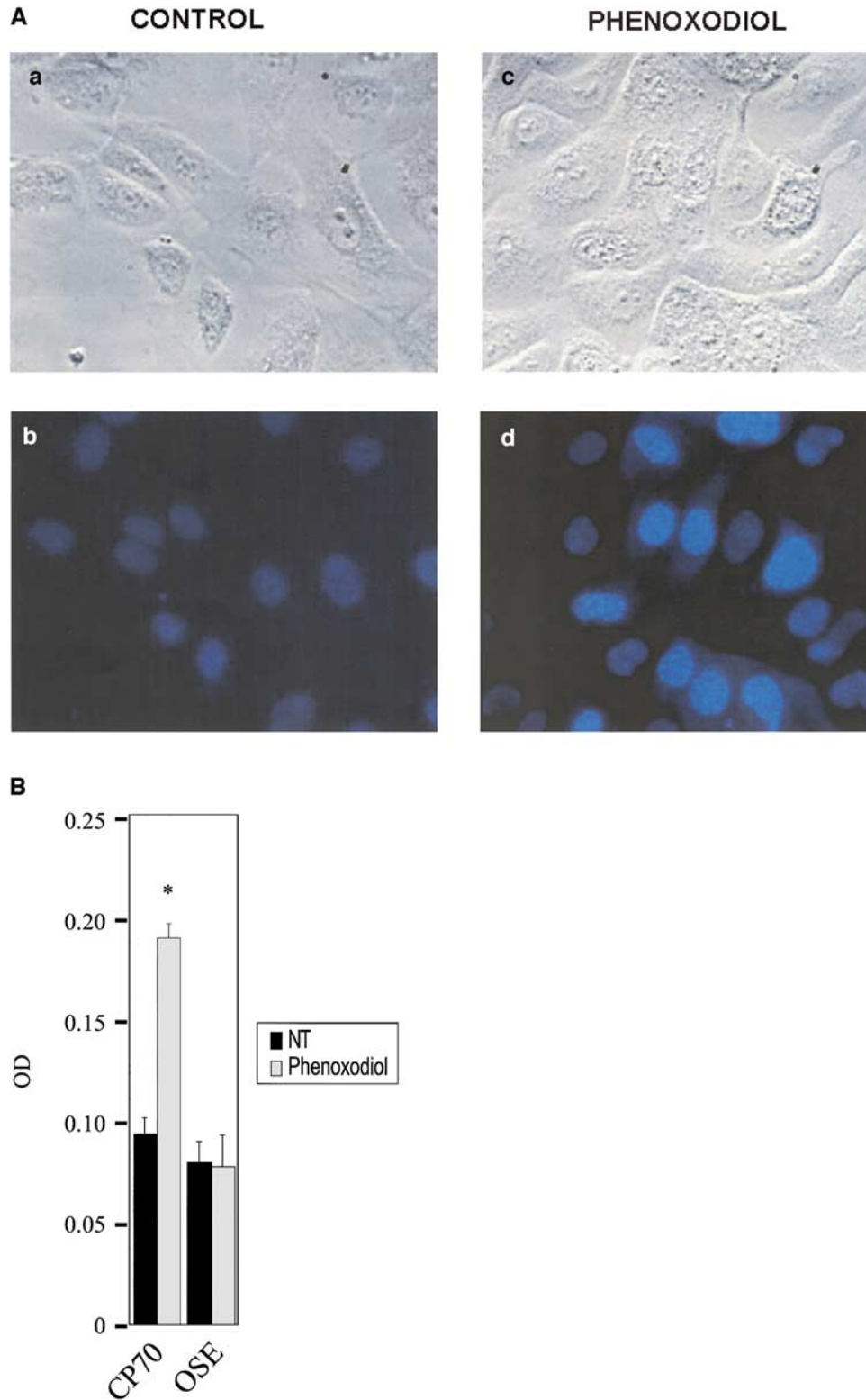


Figure 2 Phenoxodiol induces apoptosis in ovarian cancer cells. **(A)** Phase-contrast images (a, c) and Hoechst staining of apoptotic nuclei (b, d) of untreated (control) and phenoxodiol-treated primary ovarian cancer culture. **(B)** Caspase-3 activity in optical density (OD) units of untreated NT and phenoxodiol-treated CP70 ovarian cancer cells and normal OSE cells. Figures are representative experiments of the studied cell lines. * $P < 0.05$

with the absence of caspase-8 activation upon Fas stimuli suggest that FLIP could be a blocking factor of the Fas pathway conferring resistance to apoptosis. In

contrast, no detectable FLIP was observed in normal OSE cells, which are sensitive to Fas-mediated apoptosis (data not shown).

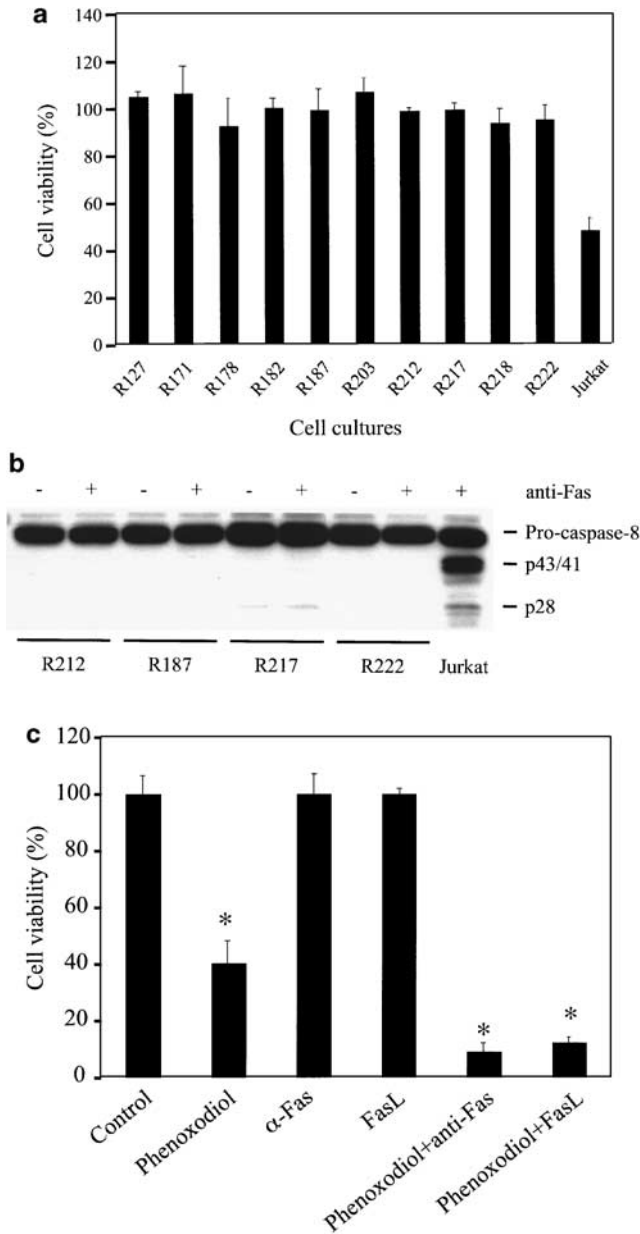


Figure 3 Phenoxodiol restores sensitivity to Fas-mediated apoptosis in ovarian cancer cells. **(a)** Cell viability (in percentage, normalized to untreated cells) of 10 representative primary ovarian cancer cell cultures after treatment with an agonistic Fas antibody. Note that ovarian cancer cells are resistant to Fas. The Fas-sensitive Jurkat cells are included as positive control. **(b)** Western blot for caspase-8 of primary ovarian cancer cells untreated (-) and treated (+) with an agonistic Fas antibody (anti-Fas). Pro-caspase-8 is present in all the ovarian cancer cells but it does not undergo activation upon Fas stimuli. Treated Jurkat cells are used as positive control showing the active forms of caspase-8. **(c)** Treatment with low doses of phenoxodiol sensitize ovarian cancer cells to Fas. Cell viability of CP70 cells untreated (control) or treated with phenoxodiol (1 μ g/ml), anti-Fas, FasL or a combination. Note the significant decrease in cell viability with anti-Fas and FasL in the groups pretreated with phenoxodiol. * $P < 0.01$

We hypothesized that phenoxodiol increases the sensitivity of ovarian cancer cells to Fas-mediated apoptosis due to caspase activation associated with the

removal of FLIP's action. Ovarian cancer cells were treated with phenoxodiol (10 μ g/ml) for 24 h and assessed for FLIP expression and activation of caspase-8. Phenoxodiol induced caspase-8 activation, characterized by cleavage of procaspase-8 into its p43/41 and p28 forms (Figure 4b) and in downregulation of the p43 form of FLIP_C in all the primary cultures as well as in the CP70 and Hey cell lines. This decrease in FLIP expression corresponded with an increase in the expression of active caspase-8 (Figure 4b).

Recent studies have reported that Akt regulates FLIP expression and blocks Fas-mediated apoptosis (Rohn *et al.*, 1998). Treatment with phenoxodiol for 24 h also decreased the levels of Akt expression in the ovarian cancer cells (Figure 4b). Altogether, phenoxodiol-induced apoptosis corresponds to a reduction in Akt and FLIP expression, resulting in caspase-8 activation.

Phenoxodiol-induced apoptosis involves activation of the mitochondrial pathway

The induction of apoptosis by chemotherapeutic agents involves multiple pathways, many of them with preferential activation of the mitochondrial pathway. This pathway can be activated by a small amount of active caspase-8, resulting in caspase-9 activation. As in the same experiments previously described, we evaluated the expression and cleavage of caspase-9. Western blot analysis showed the presence of the pro-form of caspase-9 in all the ovarian cancer cells and with no cleavage of caspase-9 after anti-Fas treatment (data not shown). However, treatment with 10 μ g/ml phenoxodiol resulted in cleavage of caspase-9 into its p36 form (Figure 4b), indicating activation of the mitochondrial pathway.

Phenoxodiol treatment results in downregulation and cleavage of XIAP

XIAP is a member of intracellular antiapoptotic proteins that confers protection from death-inducing stimuli by directly blocking the activation of caspases (Deveraux *et al.*, 1998). However, XIAP can be inactivated by its own cleavage. To determine the fate of cellular XIAP during phenoxodiol-induced apoptotic execution, ovarian cancer cells were treated with phenoxodiol (10 μ g/ml) for 24 h. Western blot analysis demonstrated the full-length (53 kDa) XIAP in the ovarian cancer cells. Phenoxodiol-treated ovarian cancer cells resulted in XIAP downregulation and cleavage to its 30 kDa inactive form (Figure 4b). The decreased expression and cleavage of XIAP correlates with an increase in caspase-9 and -8 activation (Figure 4b).

The levels of the pro-form of caspase-8 and XIAP showed a significant decrease only after 48 h of phenoxodiol treatment while the cleavage forms appeared as early as 12 h (Figure 4c and data not shown).

Phenoxodiol-induced apoptosis is caspase dependent

The importance of caspase activation from phenoxodiol-induced apoptosis was demonstrated by treating

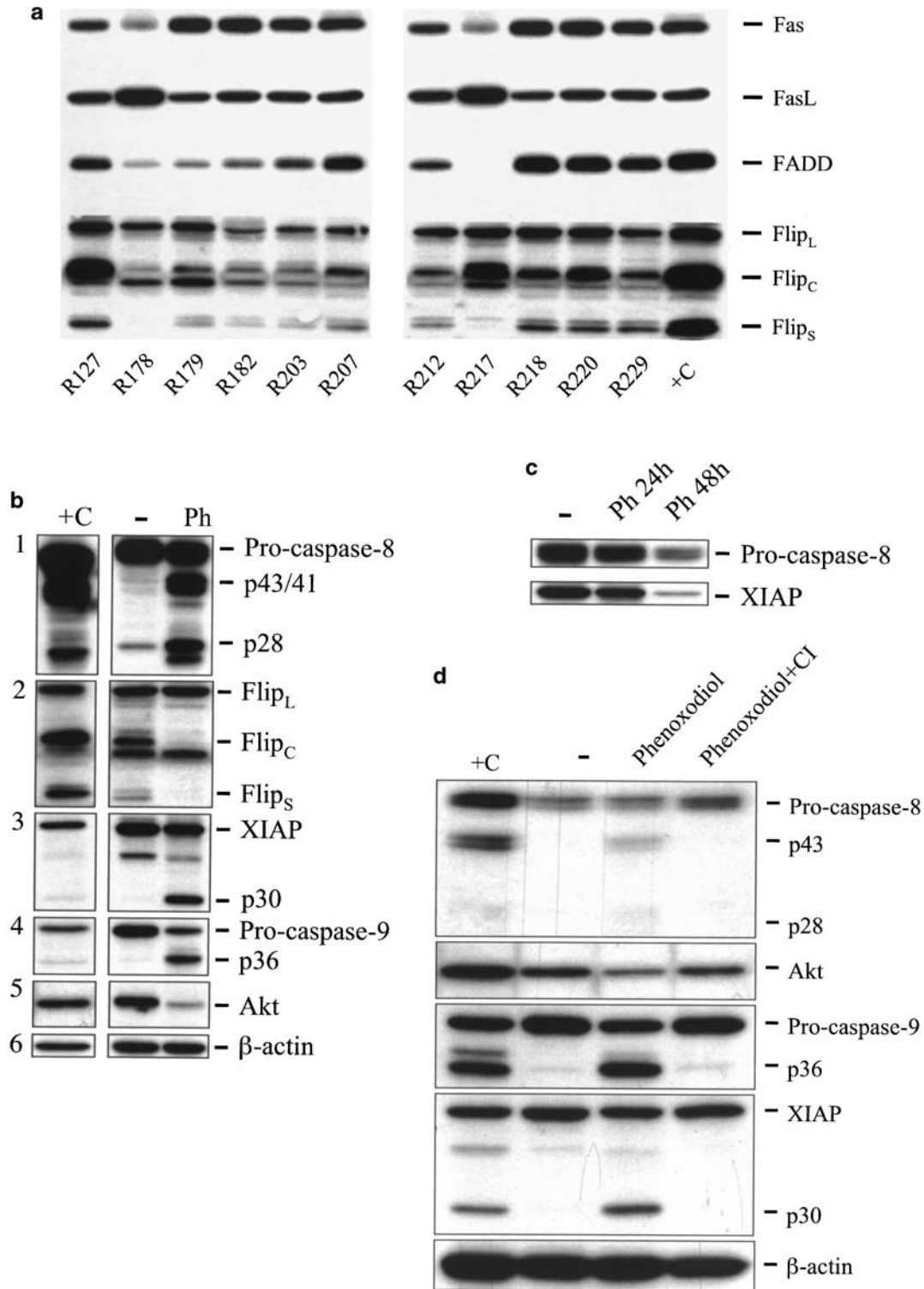


Figure 4 Characterization of phenoxodiol-induced changes in the Fas pathway in ovarian cancer cells. **(a)** Western blot analysis showing the expression of Fas, Fas-ligand, FADD and FLIP (indicated) by primary ovarian cancer cell cultures. +C represents a specific positive control for the antibody showing the pro- and active forms for each protein investigated. **(b)** Western blot analysis for caspase-8, FLIP, Akt, caspase-9, XIAP and β -actin (indicated) of ovarian cancer cells untreated (-) and 10 μ g/ml phenoxodiol-treated (Ph). Graphic is a representative experiment of the cell lines. **(c)** Time-dependent changes on the pro-forms of caspase-8 and XIAP expression following phenoxodiol treatment. Note the significant decrease in caspase-8 and XIAP expression after 48 h of phenoxodiol treatment. **(d)** Effect of the general-caspase inhibitor Z-VAD-FMK on phenoxodiol-induced apoptosis. Western blot analysis of caspase-8, Akt, caspase-9, XIAP and β -actin of cells untreated (-), treated with phenoxodiol alone or in combination with caspase inhibitor Z-VAD-FMK (phenoxodiol + CI)

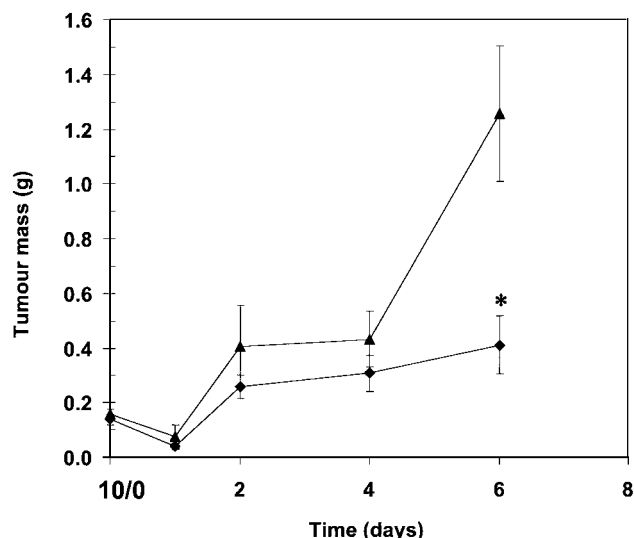


Figure 5 Xenograft studies of A2780 ovarian cancer cells injected s.c. in nude mice. The antitumor activity of daily administration of phenoxodiol (i.p.) or vehicle was determined after 6 days. Tumour mass (mean \pm s.e.m.) was calculated from the formula (width² \times length)/2. Data represent the average of 10 animals/group. * $P \leq 0.02$

ovarian cancer cells with phenoxodiol (10 μ g/ml) in the presence or absence of Z-VAD-FMK (20 μ M), a permeable general caspase inhibitor. Addition of this inhibitor blocked the apoptotic effect of phenoxodiol (data not shown). Furthermore, Z-VAD-FMK treatment blocked the phenoxodiol-induced activation of caspase-8 and -9 (Figure 4d). Similarly, the expression levels of Akt and XIAP remained equal to the nontreated control.

Tumor inhibition following phenoxodiol therapy

We examined the antitumor effect of i.p.-delivered phenoxodiol in BALB/c nude mice bearing A2780 xenografts (injected subcutaneously). When phenoxodiol was dosed i.p. at 20 mg/kg every day for 6 days, the optimal T/C was 24.7% ($P < 0.02$), thus indicating that phenoxodiol was effective at inhibiting tumor growth (Figure 5a). There was a 3.1-fold reduction in terminal tumour mass in phenoxodiol-treated groups when compared with control ($P < 0.02$). No toxic side effects were noted at this dosage, and animals continued to gain weight throughout the therapeutic window (data not shown).

Discussion

The proper maintenance of the delicate balance between cell proliferation and apoptosis is essential for tissue remodelling and homeostasis in the normal ovaries. Apoptosis or programmed cell death plays an important role in OSE repair (Tilly, 2001), follicular atresia (Hughes and Gorospe, 1991; Tilly, 1996) and regression of the corpus luteum (Roughton *et al.*, 1999). Altera-

tions in the factors controlling this balance may lead to neoplastic transformation and cancer (Hanahan and Weinberg, 2000; Green and Evan, 2002). The main characteristic of a cancer cell is its resistance to undergo apoptosis (Lowe and Lin, 2000; Mor *et al.*, 2002); therefore, reversing this resistance to apoptosis may represent the best approach for the treatment of cancer.

Although chemotherapeutic agents are widely used for the treatment of ovarian cancer, chemoresistance remains a major problem. Suppression of drug-induced apoptosis, despite the drug-induced cellular damage, is considered to be a mechanism of chemoresistance.

Targeting the factors regulating apoptosis represents a new approach for the treatment of cancer cells (Reed, 2002). Phenoxodiol induces cell death in all studied ovarian cancer cells, including those cells resistant to conventional agents such as paclitaxel and carboplatin (Flick *et al.*, in preparation).

We have characterized the cytotoxic effect of phenoxodiol on ovarian cancer cells, and demonstrated that (1) this is a highly efficient drug inducing apoptosis in all studied ovarian cancer cells and (2) this apoptotic effect is due to the regulation of the Akt-FLIP-XIAP pathway. Furthermore, we demonstrated that the successful inactivation of FLIP and XIAP by phenoxodiol restores the sensitivity of ovarian cancer cells to Fas-mediated apoptosis.

The decrease in cell viability after phenoxodiol treatment was due to the induction of apoptosis as demonstrated by chromatin condensation and caspase activation. Mechanisms of apoptosis have been classified into two main groups: an intrinsic or mitochondrial apoptotic pathway and an extrinsic or receptor-mediated pathway. The extrinsic pathway is exemplified by a receptor-initiated proapoptotic signal, such as that initiated by Fas (Green and Evan, 2002).

The Fas pathway is one of the main pathways mediating tissue remodelling in reproductive organs, including the mammary gland and the ovaries (Mori *et al.*, 1997; Quirk *et al.*, 1997; Song *et al.*, 2000; Sapi *et al.*, 2002). We have shown that the expression of Fas and FasL, in normal tissues, responds to the cyclic hormonal changes and that the apoptotic pathway is activated as a result of hormone withdrawal or decrease in estrogen and progesterone (Song *et al.*, 2000, 2002; Sapi *et al.*, 2002). In this way, the Fas/FasL system controls cell number in the reproductive tissues by eliminating cells through apoptosis. Disruption of this pathway can lead to excessive tissue proliferation and cancer. Contrary to normal ovarian cells, ovarian cancer cells are resistant to Fas-mediated apoptosis (Baldwin *et al.*, 1999) and do not respond to hormone withdrawal (Song *et al.*, 2001, 2002).

The initiation of caspase activation was believed to be the 'point of no return', after which the cell was irreversibly committed to cell death. New evidence indicates that activation of caspases can be controlled at different levels (Salvesen and Duckett, 2002). We evaluated the effect of phenoxodiol on the activators and inhibitors of apoptosis as well as the effector caspases. One of the early inhibitors, FLIP, contains

caspase-8-like death effector domains (DEDs) and competes with procaspase-8 for recruitment into the DISC. Multiple splice variants of FLIP have been reported, but so far only two forms of FLIP, FLIP_L and FLIP_S, can be detected at the protein level (Scaffidi *et al.*, 1999). FLIP_L and FLIP_S inhibit different steps of caspase-8 activation at the DISC (Krueger *et al.*, 2001).

Interestingly, high expression levels of FLIP were observed in the ovarian cancer cells, which may explain the absence of caspase-8 activation upon Fas stimulation (Kirchhoff *et al.*, 2000; Krueger *et al.*, 2001). Phenoxodiol-induced apoptosis was related to a decrease in the expression of the p43 form of FLIP. However, this effect was not at the RNA level (no changes in FLIP mRNA levels following phenoxodiol treatment were identified), suggesting the involvement of ubiquitination and proteasome-dependent degradation of FLIP, as shown by a recent study where PPAR γ modulators induce ubiquitination of FLIP, without concomitant reduction in FLIP RNA (Kim *et al.*, 2002). These results indicate the existence of another intracellular pathway that may regulate FLIP expression.

Considerable crosstalk exists between the extrinsic and intrinsic pathways. Caspase-8 can activate Bid, which then facilitates cytochrome *c* release from the mitochondria followed by caspase-9 and -3 activation (Peter *et al.*, 1999). The apoptotic effect of phenoxodiol seems to involve the extrinsic (caspase-8) and the intrinsic pathway (caspase-9). Caspase-9 and its cofactor Apaf1 have been found to be essential downstream components of p53 in *myc*-induced apoptosis (Soengas *et al.*, 1999). Inactivation of Apaf1 was shown to be associated with chemoresistance in metastatic melanoma (Soengas *et al.*, 2001). Therefore, blocking caspase-9 function in ovarian cancer cells may constitute a mechanism of drug resistance that phenoxodiol is able to overcome, but how?

An important factor controlling caspase-9 function is a family of proteins known as inhibitors of apoptosis (IAPs). The IAP proteins are endogenous inhibitors of the terminal or effector caspases and provide a mechanism for limiting or halting the cascade (Salvesen and Duckett, 2002). When we checked for the expression of XIAP in our panel of ovarian cancer cells, we found that all of them express high levels of this inhibitor. These findings correlate with previous studies showing that IAPs are highly expressed in many tumors and contribute to the resistance of cancers to apoptosis (Deveraux *et al.*, 1998; Salvesen and Duckett, 2002).

Treatment of the ovarian cancer cells with phenoxodiol induces the inactivation of XIAP characterized by the appearance of an ~30-kDa protein which corresponds to the cleavage of XIAP (Figure 4b) (Deveraux *et al.*, 1999). Deveraux *et al.* (1999) have demonstrated that the cleavage of XIAP may be one of the mechanisms by which cell death programs circumvent the antiapoptotic barrier posed by XIAP. This may explain our findings of caspase-9 activation after phenoxodiol treatment. Furthermore, it was shown that incubation of XIAP with purified recombinant caspase-

8 results in XIAP cleavage (Deveraux *et al.*, 1999). The first effect that we found with phenoxodiol treatment is caspase-8 activation followed by caspase-9. We, therefore, propose that the decrease in FLIP expression allows caspase-8 activation which results in the cleavage/inactivation of XIAP, and consequently removes the blocking of caspase-9 and -3, allowing apoptosis. This is confirmed by our finding that incubation of the cancer cells with a general caspase inhibitor prior to phenoxodiol treatment inhibits XIAP cleavage and apoptosis.

The next question is the mechanism of action by which phenoxodiol removes FLIP and allows caspase-8 to activate the apoptotic cascade. The balance between proliferation and cell death is clearly exemplified by the antiapoptotic effect of survival signals such as the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. The PI3K/Akt pathway is crucial for protection against apoptosis by various mechanisms, including the activation of NF- κ B, phosphorylation of Bcl-2 antagonist of cell death (BAD), inhibition of caspase-9 and suppression of FasL. Akt translocates to the nucleus where it may contribute to the regulation of the transcription of genes mediating cell survival. A possible mechanism by which Akt functions as a promoter of survival is through the induction of FLIP expression and blocking the extrinsic apoptotic pathway (Panka *et al.*, 2001; Suhara *et al.*, 2001). Also, in this study, high levels of FLIP were found in all the ovarian cancer cells. When we evaluated the effect of phenoxodiol on Akt, we found a clear inhibition of its expression, followed by a decrease in FLIP expression and reversal of the sensitivity to Fas-mediated apoptosis.

The *in vitro* studies were confirmed by the results *in vivo*. Phenoxodiol treatment was active against A2780 ovarian cancer xenografts, reducing by ~80% tumor mass in relation to the control. These data show that the antitumor activity exerted by phenoxodiol is effective, *in vivo*, in treating cancer cells.

In summary, the studies described above highlight the fact that disruption of apoptosis can promote tumor initiation, progression and treatment resistance and, therefore, emphasize the importance of understanding the underlying alterations that influence apoptosis during tumorigenesis. It is a fact that anticancer agents induce apoptosis in normal tissues as well as in tumors, making the development of new treatments for ovarian cancer that can specifically restore apoptosis in cancer cells without affecting the normal tissues a crucial objective. Phenoxodiol has proven to accomplish these objectives both *in vitro* and *in vivo* by mediating the removal of blockers of apoptosis and renewing their sensitivity to cell death. Our next task is to prove whether it is also effective in patients with ovarian cancer.

Materials and methods

Isolation of primary ovarian cancer cells and cell culture

Ascites collected from patients were centrifuged for 20 min at 300 \times g to pellet cells. The cells were washed with Cellgro (Mediatech, Herndon, VA, USA), and subjected to gradient

centrifugation using Lymphocyte Separation Medium (ICN Biomedicals Inc.) to separate cancer cells and white blood cells (mononuclear cells) from red blood cells. The mononuclear cell-containing layer was collected and incubated with an anti-CD45 mAb conjugated to magnetic beads (Dynabeads 450, Dynal, Oslo, Norway) at 4°C with rotation for 30 min. Following this incubation, the cells were placed into a magnetic field and the unbound epithelial cells were collected, washed and placed into culture media. The purity of the epithelial ovarian cancer cells was 100% as determined by immunostaining for cytokeratin (Sigma). Cancer cells were cultured in 50% 199 medium and 50% 105 medium (Sigma, St Louis, MT), supplemented with 15% FBS (Gemini Bioproducts), 4 ng/ml EGF (Sigma), 1000 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES, 100 nM nonessential amino acids and 1 mM sodium pyruvate (Gibco, Carlsbad, CA, USA). Malignancy of the isolated ovarian cells was confirmed with Pap staining by the Department of Pathology (Yale University). Experiments were carried out within five passages. Jurkat cells were grown in RPMI (Gibco), supplemented with 10% FBS (Gemini Bioproducts), 1000 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES, 100 nM nonessential amino acids and 1 mM sodium pyruvate (Gibco).

Stimulation of Fas-mediated apoptosis and phenoxodiol treatment

The sensitivity to Fas-mediated apoptosis was evaluated as previously described (Mor *et al.*, 2001). In short, cells were treated in the presence or absence of 500 ng/ml Fas-antibody (PharMingen, San Diego, CA, USA) in OptiMem for 24 h or with 0.1, 1 or 10 µg/ml phenoxodiol, NV03, NV05 or NV07 in OptiMem for 24 h. For treatment with phenoxodiol and caspase inhibitors, 10 µg/ml phenoxodiol was used alone, or in combination with 20 µM general caspase-inhibitor Z-VAD-FMK (PharMingen) and incubated for 24 h.

Cell viability assay

Cells (10 000) were plated per well in a 96-well plate. At 70% confluence, the medium was replaced with OptiMem (Gibco) for 24 h followed by treatment. Subsequently, 20 µl CellTiter (Promega, Madison, WI, USA) was added to each well, and after incubation for 1–3 h the optical density was measured at 490 nm. Cell viability, in percentage, was normalized to untreated cells. Each experiment was repeated at least three times.

Staining of apoptotic nuclei and caspase-3 activity assay

Cells (100 000) were seeded in chamber slides (B.D. Falcon, Franklin Lakes, NJ, USA) and incubated in the presence or absence of phenoxodiol (10 µg/ml) for 24 h. Subsequently, Hoechst staining was performed according to the manufacturer's instructions (Vybrant Apoptosis Assay Kit #5, Molecular Probes, Eugene, OR, USA). Caspase-3 activity was determined in duplicate on 50 µg of protein lysate by using the CasPACE™ Assay System according to the manufacturer's protocol (Promega).

Preparation of cell lysates and measurement of protein concentration

Protein was prepared as previously described (Aschkenazi *et al.*, 2002). Cells (1 000 000) were plated in 6- or 10-cm dishes. Once the cells reached 70% confluence, the medium was changed to OptiMem for 24 h. Subsequently, cells were treated with 500 ng/ml anti-Fas or 10 µg/ml phenoxodiol (NV06) or

vehicle and incubated for 24 h. Cells were scraped, pelleted at 300 × g for 10 min, resuspended in lysis buffer (1% NP40 and 0.1% SDS in PBS) and incubated for 20 min on ice. The cell lysate was centrifuged at 14 000 r.p.m. using a C3i centrifuge (AC1.14 rotor, SOCIETE JOUAN, Winchester, VI, USA), for 10 min. The supernatant was collected and stored until further use at –40°C. A cocktail of protease inhibitors was added to the supernatant prior storage. Protein concentration was determined by BSA Calibration Assay (Pierce, Rockford, IL, USA) as previously described (Gutierrez *et al.*, 1999).

Colony formation

Primary ovarian cancer cells were seeded in 35 mm Petri dishes and treated with phenoxodiol for 4 h. Following treatment, the cells were maintained in regular media. Colonies were stained with crystal violet after 10 days. Only colonies of more than 30 cells were counted. The average of three independent experiments was calculated.

Western blot analysis

Protein (20 µg) were denatured in sample buffer (2.5% SDS, 10% glycerol, 5% β-mercapto-ethanol, 0.15 M Tris (pH = 6.8) and 0.01% bromophenol blue) and subjected to 10 or 12% SDS-PAGE. Proteins were transferred to PVDF membranes (Immobilon, Millipore, Bedford, MA, USA) at 100 V for 105 min. Subsequently, the membranes were blocked for 1 h in 5% milk in PBS with 0.5% Tween-20 (PBS-T) and incubated overnight at 4°C in primary antibody in PBS-T with 1% milk. The following antibodies and concentrations were used: 1:10 000 rabbit anti-actin (Sigma), 1:1000 rabbit anti-Flip (Upstate Biotechnology, Charlottesville, VI, USA), 1:10 000 mouse anti-Fas-Ligand (Transduction Laboratories, San Jose, CA, USA), 1:1000 mouse anti-Fas (B10, Santa Cruz, Santa Cruz, CA, USA), 1:1000 mouse anti-caspase-8 (Ab-3, Oncogene San Diego, CA, USA), 1:5000 mouse anti-caspase-9 (R&D Systems, Minneapolis, MN, USA), 1:2000 rabbit anti-Akt (Cell Signaling, Beverly, MA, USA), 1:1000 mouse anti-Xiap and 1:5000 mouse anti-FADD (PharMingen). After three washes with PBS-T, membranes were incubated for 1 h at room temperature in 1:10 000 horse anti-mouse or goat anti-rabbit, both HRP labelled (Vector, Burlingame, CA, USA). Finally, proteins were visualized using enhanced chemiluminescence (Pierce).

Xenografts

Subconfluent (80%) flasks of A2780 ovarian cancer cells were trypsinized, washed in Hanks balanced salt solution (Sigma) and resuspended in the same buffer at a density of 2 × 10⁷ cells/ml. Athymic nu/nu BALB/c mice were s.c. inoculated with 2 × 10⁶ A2780 cells bilaterally midway along the dorsal surface. For the treatment group (*n* = 10), therapy commenced 8 days postinoculation. Phenoxodiol was formulated as a suspension in 1% carboxymethyl cellulose (CMC) and delivered i.p., every day for 6 days (qd × 6). Treatment commenced on day 8 postinoculation and tumor measurements commenced on day 12. Control group (*n* = 10) received 1% CMC vehicle. Tumors were measured every third day in two dimensions, length (*a*) and width (*b*), using calipers. Tumor weight (*W*) was calculated by the formula $W = ab^2/2$, where *a* is the longer of the two measurements (O'Dwyer *et al.*, 1994). Tumor proliferation curves were analysed with respect to maximal tumor inhibition (treated/control, T/C) and growth delay (difference in time (days) for test tumor to double the initial

tumor volume minus the same period required for the control group to reach these parameters).

Statistics

Statistical analysis was performed using Student's *t*-test or ANOVA analysis. Analysis of data and graphic utilized GraphPad PRISM software (version 2) (San Diego, CA, USA).

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