

Effects of progesterone and anti-progestin (mifepristone) treatment on proliferation and apoptosis of the human ovarian cancer cell line, OVCAR-3

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Abstract. The study examined the effects of various progesterone and mifepristone concentrations on the proliferation and apoptosis of the human ovarian cancer cell line, OVCAR-3. OVCAR-3 cells were incubated with progesterone and mifepristone at concentrations ranging from 10^{-3} to 10^{-9} M. Proliferation and apoptosis were studied by means of inverted optical microscopy, DAPI staining, and crystal violet assay. Immunoblotting was used to study the regulation of the apoptosis-related proteins, bcl-2, caspase-3 and PARP, after incubation with various reagents. OVCAR-3 cell density was increased by progesterone concentrations of 10^{-5} M or less, and decreased by 10^{-3} M progesterone. DAPI staining showed no apoptotic bodies. Mifepristone concentrations of 10^{-3} and 10^{-4} M reduced the OVCAR-3 cell density. Immunoblotting showed PARP cleavage in the presence of mifepristone 10^{-4} M. Caspase-3 and bcl-2 expression was reduced by mifepristone 10^{-4} and 10^{-7} M. These results suggest that progesterone has a paradoxical effect on OVCAR-3 cell proliferation, stimulating it at low concentrations and inhibiting it at high concentrations, potentially through a caspase-independent non-apoptotic death pathway. Mifepristone seems to inhibit OVCAR-3 cell proliferation by down-regulating bcl-2 and up-regulating caspase-3 activity. These preliminary results suggest that progesterone and mifepristone have beneficial effects in ovarian cancer.

Introduction

Epithelial ovarian cancer is the leading cause of death by gynaecological malignancy (1,2). Hormonal regulation of

ovarian carcinogenesis is suggested by the higher incidence after menopause, owing to variations in sex steroids and gonadotropins (3-6). In addition, women using combined oral contraception have a reduced risk of epithelial ovarian cancer (7-10).

Malignant transformation of normal ovarian epithelial cells is related to genetic alterations that disrupt the regulation of apoptosis, proliferation and DNA repair (11). Apoptosis, or programmed cell death, is an active physiological process that involves endonucleases and protease cleavage of DNA and proteins (12,13). Yu *et al* (14) found that progesterone inhibited proliferation and promoted apoptosis of a human cell line derived from a malignant Brenner ovarian tumour. Bu *et al* (15) reported that progesterone induced apoptosis and up-regulated p53 expression in human ovarian cancer cell lines, without affecting bcl-2 expression. In ovarian cancer cell lines, cell proliferation and apoptosis-related markers of genetic instability have been linked to the absence of steroid hormone receptors (16,17).

Mifepristone (RU486) is a synthetic hormone antagonist that binds to progesterone receptors but not to oestrogen receptors. There is increasing evidence that mifepristone has potential as an anti-neoplastic agent (18). Although it has been reported to inhibit the growth of various malignant cell lines, the exact mechanism of cell death is unclear (19-22). An *in vitro* study suggested that mifepristone inhibited the proliferation of epithelial ovarian cancer cell lines by blocking cells in the G0/G1 phase of the cell cycle (22). Few data are available on the effects of progestins and anti-progestins on the proliferation and apoptosis of the OVCAR-3 human ovarian cancer cell line (22,23).

The aims of this *in vitro* study were to evaluate the effects of various progesterone and mifepristone concentrations on the proliferation and apoptosis of OVCAR-3 cells.

Materials and methods

Cells and culture. The human ovarian carcinoma cell line, OVCAR-3, was obtained from ATCC (American Type Culture Collection, Rockville, MD, USA). OVCAR-3 cells were established from malignant ascites of a woman with progressive adenocarcinoma of the ovary after combination chemotherapy

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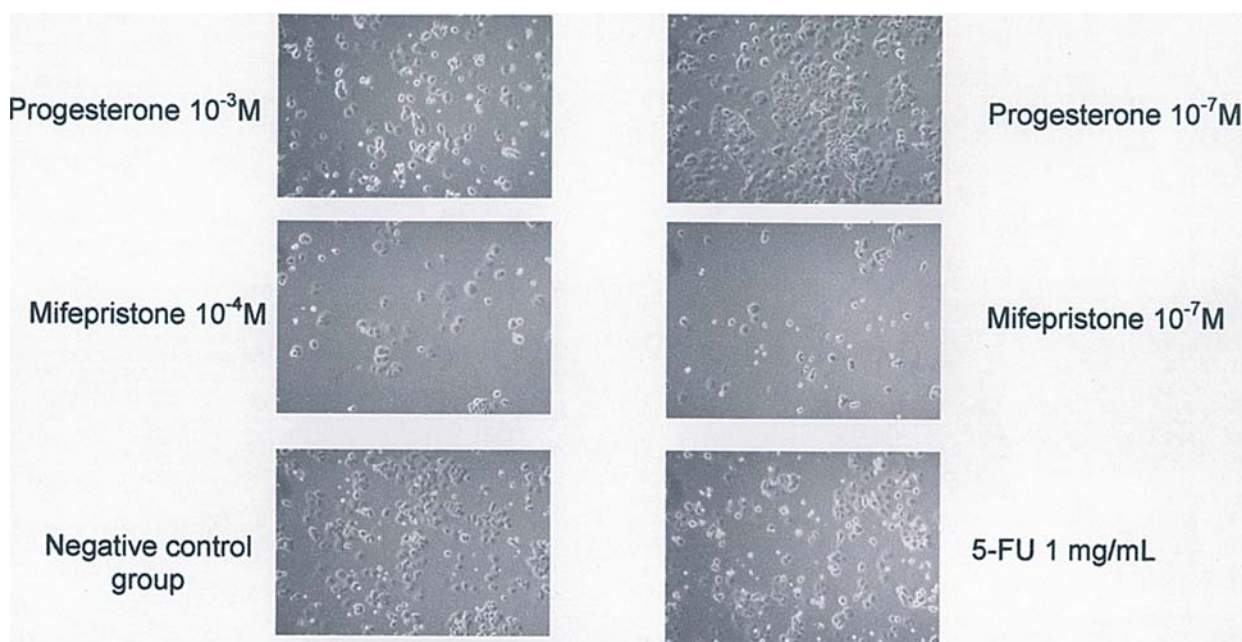


Figure 1. Inverted optical microscopy shows an increased OVCAR-3 cell density by 10^{-7} M progesterone and a reduced cell density by 10^{-3} M progesterone compared to negative control group. OVCAR-3 cell density was reduced by 10^{-7} and 10^{-4} M mifepristone, and by 5FU 1 mg/ml.

with cyclophosphamide, adriamycin and cisplatin. OVCAR-3 cells contained cytoplasmic androgen- and oestrogen-binding macromolecules having the specificity of the hormone receptors of both steroids (24). The cell line was maintained at 37°C in medium containing 90% Dulbecco's minimum essential medium (DMEM) (Life Technologies Ltd., Paisley, UK), 10% foetal bovine serum, penicillin and streptomycin (100 IU/ml) and gentamycin ($10\text{ }\mu\text{g/ml}$) (Life Technologies Ltd.), in humidified air with 5% CO_2 . All cultures were routinely tested for mycoplasma.

Inverted optical microscopy. Cells were plated in DMEM containing the test reagents at various concentrations. The reagents were omitted in negative controls and replaced by 5-FU 1 mg/ml in positive controls.

DAPI (4', 6-diamidino-2-phenylindole) staining. OVCAR-3 cell suspension ($40\text{ }\mu\text{l}$) was smeared on a glass slide, air-dried, fixed for 10 min in 70% ethanol at room temperature, and dried again. The smears were then incubated for 5 min at room temperature with 1/2000 DAPI solution (Sigma, St. Louis, MO, USA) in Dulbecco's phosphate-buffered saline (PBS) (Eurobio, Les Ulis, France) containing 0.1% Tween-20 (Sigma). They were then washed in PBS, dried and mounted in Mowiol solution (Calbiochem, San Diego, USA). Nuclear morphology was analysed under ultraviolet light with a x100 objective (Leica, Portugal).

Crystal violet assay. Cells were plated in triplicate in 96-well flat-bottomed plates at 10^5 cells/well with $200\text{ }\mu\text{l}$ of DMEM containing the test reagents at various concentrations. The supernatant was removed after 24 h of incubation and the cells were washed with $200\text{ }\mu\text{l}$ of physiological saline and then fixed with $200\text{ }\mu\text{l}$ of ethanol (70°C) for 5 min at room temperature. The ethanol was removed by aspiration and the

plates were air-dried at room temperature. The cells were then incubated with $50\text{ }\mu\text{l/well}$ crystal violet for 5 min and washed with sterile water. Crystal violet was solubilized with $100\text{ }\mu\text{l/well}$ acetic acid (33% dilution). Optical density was measured at 550 nm with an Elisa microplate reader (Labsystem).

Cell density in the presence of the test reagents was expressed as a ratio of negative control cell density (arbitrarily 1). The mean \pm standard deviation (SD) cell density of negative controls, calculated for 12 culture wells, was 0.437 ± 0.035 .

Immunoblot analysis. After 24 h of incubation, cells were centrifuged at $1500\times g$ for 3 min. Pellets were suspended in 1 ml of physiological saline and were again centrifuged at $2000\times g$ for 2 min. Supernatants were carefully discarded and the pellets were carefully weighed. To prepare membrane/cytoplasm and nuclear extracts, cells were prepared as described by Andrews and Faller (25). Briefly, cell pellets were resuspended in 10 volumes of hypotonic buffer A [10 mM Hepes-KOH (pH 7.9), 1.5 mM MgCl_2 , 10 mM KCl, 1 mM DTT (dithiothreitol), 0.5 mM spermidine, 1 mM PMSF (phenylmethylsulfonyl fluoride), $1\text{ }\mu\text{g/ml}$ leupeptin, $1\text{ }\mu\text{g/ml}$ aprotinin (Sigma)] for 10 min on ice and then mixed and centrifuged at $500\times g$ for 1 min at 4°C . The supernatant was kept as the cytoplasmic extract. The nuclear pellet was treated with 5 volumes of hypertonic buffer B [20 mM Hepes-KOH (pH 7.9), 25% glycerol, 0.4 M NaCl, 1.5 mM MgCl_2 , 20 μM EDTA, 4 mM DTT, 1 mM PMSF, $1\text{ }\mu\text{g/ml}$ leupeptin, $1\text{ }\mu\text{g/ml}$ aprotinin] for 20 min on ice. Nuclear extracts were obtained by isolating the non-extractable chromatin residue by centrifugation at $18000\times g$ for 2 min at 4°C . The nuclear fraction was used to study poly(ADP-ribose) polymerase (PARP) cleavage, and the membrane/cytoplasmic fraction was used to analyse bcl-2 and caspase-3 expression. Membrane/cytoplasmic and nuclear extracts were submitted to SDS-PAGE

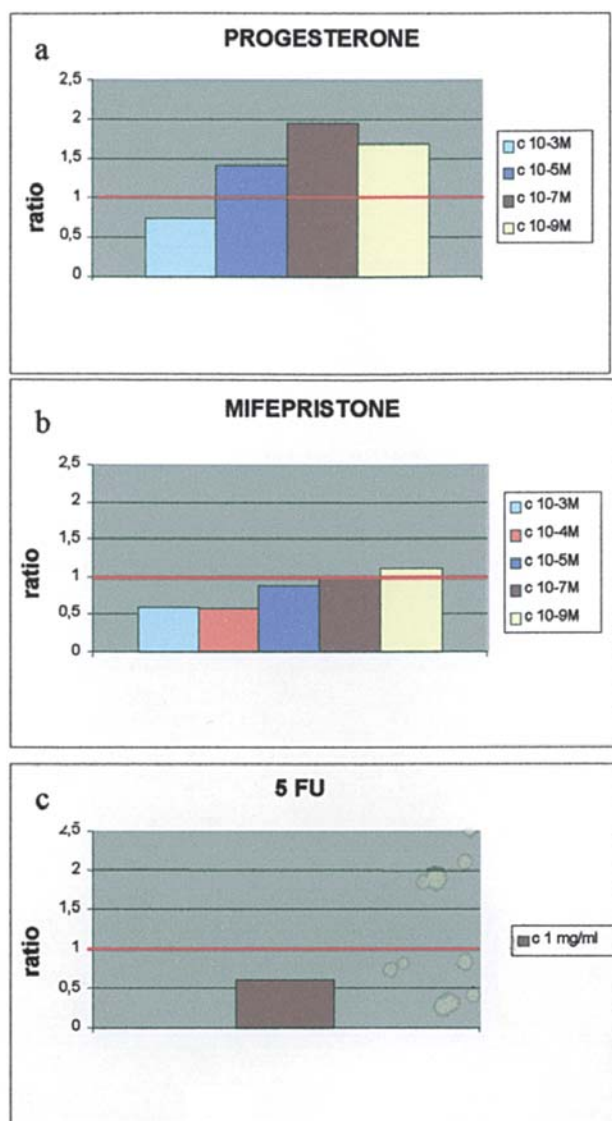


Figure 2. Crystal violet assay. Cell density was reduced by progesterone 10^{-3} M and increased by progesterone 10^{-5} , 10^{-7} and 10^{-9} M (a). Cell density was reduced by mifepristone 10^{-3} and 10^{-4} M. Cell density was unaffected by mifepristone 10^{-5} , 10^{-7} and 10^{-9} M (b). 5FU 1 mg/ml reduced cell density (c).

electrophoresis (6% polyacrylamide for PARP, 15% for bcl-2 and caspase-3) and electrophoretically transferred onto nitrocellulose membranes [B83 Schleicher & Schuell (Dassel, Germany)]. Protein loading controls were used. To block non-specific antigenic sites, the membranes were incubated in 5% non-fat dry milk in PBS containing 0.1% Tween-20.

Blots were probed for 1 h at room temperature with the following species-specific antibody diluted 1/1000, as appropriate: anti-PARP monoclonal antibody, mouse IgG1 (clone C2-10) and anti-bcl-2 polyclonal rabbit antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and anti-pro-caspase-3 polyclonal rabbit antibody was from Pharmingen (San Diego, CA, USA).

Blots were then probed for 1 h with a second specific antibody (horseradish peroxidase-conjugated IgG) diluted 1/5000. After washing, chemiluminescence detection was performed with the ECL Western blot detection system from Amersham (Buckinghamshire, UK) according to the manufacturer's

instructions, and the membrane was exposed to X-ray film for 5-10 min. All techniques were duplicated.

Statistical analysis. Continuous variables were compared with the Kruskal-Wallis and Mann-Whitney tests. P-values <0.05 were considered significant.

Results

Microscopic analysis (Fig. 1). OVCAR-3 cells were incubated for 24 h with progesterone 10^{-3} and 10^{-7} M, mifepristone 10^{-4} and 10^{-7} M, 5FU 1 mg/ml (positive control) or medium alone (negative control).

Compared to the negative controls, the cell density was increased by 10^{-7} M progesterone and reduced by 10^{-3} M progesterone. The cell density was reduced by 10^{-7} and 10^{-4} M mifepristone, and by 5FU 1 mg/ml.

Crystal violet assay (Fig. 2). OVCAR-3 cells were incubated for 24 h with progesterone 10^{-3} , 10^{-5} , 10^{-7} and 10^{-9} M, mifepristone 10^{-3} , 10^{-4} , 10^{-5} , 10^{-7} and 10^{-9} M, and 5FU 1 mg/ml.

The cell density was reduced by progesterone 10^{-3} M ($p=0.02$; ratio = 0.74 ± 0.03) and increased by progesterone 10^{-5} M ($p=0.03$; ratio = 1.42 ± 0.22), 10^{-7} M ($p=0.02$; ratio = 1.95 ± 0.05) and 10^{-9} M ($p=0.03$; ratio = 1.69 ± 0.36).

The cell density was reduced by mifepristone 10^{-3} M ($p=0.02$; ratio = 0.60 ± 0.03) and 10^{-4} M ($p=0.02$; ratio = 0.59 ± 0.04), and was unaffected by mifepristone 10^{-5} M (ratio = 0.87 ± 0.05), 10^{-7} M (ratio = 1.00 ± 0.03) and 10^{-9} M (ratio = 1.12 ± 0.04).

5FU 1 mg/ml reduced the cell density ($p=0.02$; ratio = 0.61 ± 0.06), as did mifepristone 10^{-3} and 10^{-4} M and 5FU 1 mg/ml, to a similar extent ($p=0.96$).

DAPI staining (Fig. 3). Microscopic examination of apoptotic segmented nuclei/cells stained by DAPI showed that 5FU 1 mg/ml induced apoptosis. No apoptotic bodies were observed with progesterone 10^{-3} M or with mifepristone 10^{-3} M. Progesterone enlarged cells but did not produce genuine apoptotic bodies.

Immunoblotting (Fig. 4). OVCAR-3 cells were incubated for 24 h with progesterone 10^{-3} and 10^{-7} M, mifepristone 10^{-4} and 10^{-7} M, 5FU 1 mg/ml (positive control) or medium alone (negative control). Spontaneous apoptosis was observed in the negative control group, as confirmed by PARP cleavage.

PARP cleavage was also observed with mifepristone 10^{-4} M and with 5FU. All mifepristone concentrations reduced caspase-3 and bcl-2 expression.

Progesterone did not affect caspase-3 or bcl-2 expression. The 115-kD PARP band was reduced by progesterone 10^{-3} M, without PARP cleavage.

Discussion

This *in vitro* study confirms that proliferation and apoptosis of the OVCAR-3 cell line are differently affected by various progesterone and mifepristone concentrations.

Progesterone has been used in the treatment of ovarian cancer, but its mechanism of action is not fully understood.

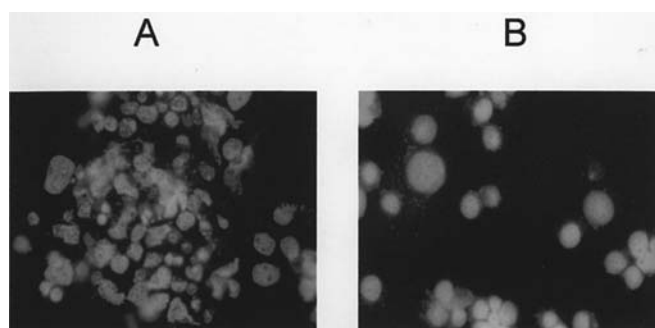


Figure 3. Fluorescence microscopic examination (DAPI staining after 24 h of incubation) shows apoptotic bodies induced by 5FU 1 mg/ml (A). Progesterone 10^{-3} M enlarged cells but did not produce genuine apoptotic bodies (B).

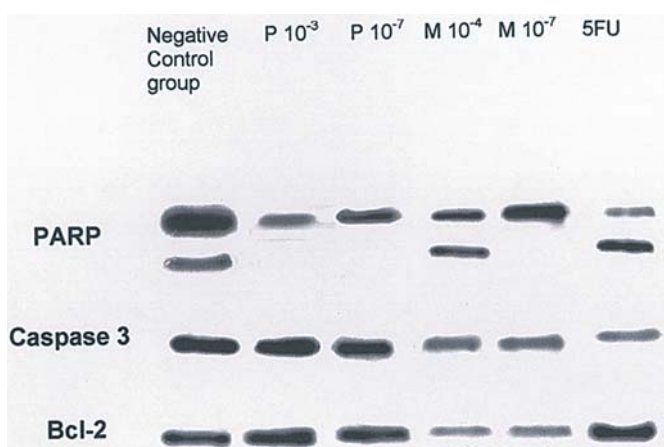


Figure 4. Immunoblot analysis shows a spontaneous apoptosis in the negative control group, as confirmed by PARP cleavage. PARP cleavage was also observed with mifepristone 10^{-4} M and with 5FU. Mifepristone at 10^{-4} and 10^{-7} reduced caspase-3 and bcl-2 expression. The 115-kD PARP band was reduced by progesterone 10^{-3} M, without PARP cleavage.

In this setting, as in the current study, progesterone is generally used at a concentration 10-100 times higher than that observed during the luteal phase (26). We found that OVCAR-3 cell density was increased by progesterone concentrations of 10^{-5} M or less, while it was reduced by a concentration of 10^{-3} M. Our results partly disagree with those of Feng *et al* (27), who reported that progesterone concentrations of 10^{-5} M or more inhibited the growth of ovarian cancer cells *in vitro*. This apparent discrepancy may be due to the use of different ovarian cancer cell lines (3AO and AO) with different steroid receptor affinity for progesterone. We found that progesterone stimulated cell proliferation at a low concentration but inhibited it at high concentrations. In contrast, Bu *et al* (15) reported that the inhibitory effect of progesterone on ovarian cancer cell growth *in vitro* was concentration-dependent. However, these latter authors only tested two concentrations of progesterone. Our results are in keeping with those of Syed and Ho (28) showing that low progesterone concentrations promoted the growth of normal and malignant ovarian epithelial surface cells while high concentrations had an inhibitory effect. Although 10^{-3} M progesterone reduced OVCAR-3 cell density

in our study, it is important to note that this inhibitory effect was weaker than that of 5FU.

The anti-tumoural action of progesterone involves apoptosis (14), modulation of inducible nitric oxide synthase (29), altered plasma membrane fluid dynamics (30) and alternative expression of transforming growth factors (31). In our study, DAPI staining revealed large cells but no apoptotic bodies in the presence of progesterone. This suggests that progesterone at a concentration of 10^{-3} M reduces OVCAR-3 cell density potentially via a cell death pathway other than the classical apoptosis pathway described by Kerr *et al* (32). However, further analysis using electron microscopy or pan-caspase inhibitor z-VAD-fmk would be required to confirm this hypothesis. Immunoblotting showed no effect of progesterone on caspase-3 expression, suggesting a caspase-independent death pathway.

Mifepristone reduced OVCAR-3 cell density when used at concentrations of 10^{-3} and 10^{-4} M, to an extent similar to that observed with 5FU 1 mg/ml. Lower mifepristone concentrations had no such effect. These results are in keeping with those of studies showing an anti-proliferative effect of mifepristone on various cancer cell lines (22,33-35). Rose and Barnea (22) reported mifepristone concentration-dependent growth inhibition of several human ovarian epithelial cancer cell lines expressing progesterone receptors. However, we found that mifepristone concentrations lower than 10^{-4} M had no inhibitory action on OVCAR-3 cell proliferation. This apparent discrepancy could be explained by our relatively short incubation period (24 h) with mifepristone. Indeed, mifepristone effects are both concentration- and time-dependent (22,36). Mifepristone down-regulates progesterone-receptor expression in cancer cell lines and blocks cells in G0/G1 phase, thereby reducing the number of cells in S phase (22,34,35,37). Our immunoblotting results show that mifepristone plays a crucial role in OVCAR-3 apoptosis. Indeed, PARP cleavage and reduced bcl-2 and caspase-3 expression were observed in the presence of mifepristone, at levels similar to those observed with 5FU. Our results are in keeping with those of Wang *et al* (35), showing that mifepristone induced concentration-dependent apoptosis of the ovarian cancer cell line, SKOV3. Moreover, Li *et al* (36) found that the anti-tumoural effect of mifepristone on the ovarian cancer cell line, 3AO, was related to bcl-2 protein down-regulation. This effect has also been found in other cancer cell lines (33,38). Qin and Wang (39) suggested that mifepristone acts by enhancing the sensitivity of ovarian cancer cells to chemotherapy, possibly by regulating bcl-2 and bax protein expression. Some studies have suggested a regulatory effect of mifepristone on apoptosis-related proteins, with up-regulation of p53 in ovarian cancer cells (15,36) and p21^{WAF/cip1} in breast cancer cells (40), and down-regulation of bcl-x_L in gastric adenocarcinoma cells (34).

Our immunoblotting experiments showed that mifepristone reduced caspase-3 expression. This is in keeping with the results of Pollett *et al* (41), who studied caspase-3 regulation by mifepristone in myeloma cells. They found that caspase-3 levels were below the Western blot detection level after 24 h of incubation, and confirmed the up-regulation and activation of caspase-3 following mifepristone induction, by means of active-caspase-3-specific Western blotting. In our

study, the functional activity of caspase-3 may be confirmed by the detection of PARP cleavage products. Up-regulation of caspase-3 activity by mifepristone was also reported by Li *et al* (34), in a human gastric adenocarcinoma cell line.

From the clinical point of view, our results support the concept that mifepristone could play a role in the adjuvant treatment of ovarian cancer. Previous *in vitro* and *in vivo* studies have shown that mifepristone can inhibit the growth of chemoresistant human ovarian carcinoma and enhance its sensitivity to cis-platinum (35,39,42). Moreover, a clinical trial has shown that mifepristone is well tolerated and effective in patients with ovarian cancer resistant to cisplatin and paclitaxel (43). Some of the limitations of the current study are as follows: first, the use of only one cell line of ovarian cancer established from malignant ascites of a woman previously treated by chemotherapy; second, only data obtained after 24 h of incubation were available and should be confirmed using longer incubation periods; and finally, quantitation of apoptosis by trypan blue staining or TUNEL analysis could also be relevant.

In conclusion, our results support the potential effects of progesterone and anti-progestin treatment on the proliferation and apoptosis of the OVCAR-3 cell line. Progesterone at a concentration of 10^{-3} M inhibited OVCAR-3 cell proliferation potentially via a caspase-independent death pathway different to that of apoptosis. Moreover, mifepristone seems to inhibit ovarian cancer cell lines by down-regulating bcl-2 and up-regulating caspase-3 activity. Our preliminary results suggest that progesterone, and especially mifepristone, could be useful in the treatment of ovarian cancer.

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