Mifepristone Inhibits Ovarian Cancer Cell Growth *In vitro* and *In vivo*

Alicia A. Goyeneche, Rubén W. Carón, and Carlos M. Telleria

Abstract

Purpose: These studies were designed to determine whether the synthetic steroid mifepristone inhibits ovarian cancer growth *in vitro* and *in vivo* and the molecular mechanisms involved.

Experimental Design: The effect of mifepristone on ovarian cancer cell growth *in vitro* was studied in ovarian cancer cell lines of different genetic backgrounds (SK-OV-3, Caov-3, OV2008, and IGROV-1). In addition, the growth inhibition capacity of mifepristone on ovarian carcinoma xenografts was tested in nude mice.

Results: Mifepristone inhibited ovarian cancer cell proliferation in a dose- and time-dependent manner. The cytostatic effect of mifepristone was confirmed in a clonogenic survival assay and was not linked to loss of viability. Mifepristone blocked DNA synthesis, arrested the cell cycle at the G_1-S transition, up-regulated cyclin-dependent kinase (cdk) inhibitors $p21^{cip1}$ and $p27^{kip1}$, down-regulated transcription factor E2F1, decreased expression of the E2F1-regulated genes *cdk1* (*cdc2*) and *cyclin A*, and modestly decreased cdk2 and cyclin E levels. The abrupt arrest in cell growth induced by mifepristone correlated with reduced cdk2 activity, increased association of cdk2 with $p21^{cip1}$ and $p27^{kip1}$, increased nuclear localization of the cdk inhibitors, and reduced nuclear abundance of cdk2 and cyclin E. *In vivo*, mifepristone significantly delayed the growth of ovarian carcinoma xenografts in a dose-dependent manner and without apparent toxic effects for the animals.

Conclusions: These preclinical studies show that mifepristone is effective as a single agent *in vitro* and *in vivo*, inhibiting the growth of human epithelial ovarian cancer cells. Mifepristone markedly reduces cdk2 activity likely due to increased association of cdk2 with the cdk inhibitors $p21^{cip1}$ and $p27^{kip1}$ and reduced nuclear cdk2/cyclin E complex availability. Acting as a cytostatic agent, mifepristone promises to be of translational significance in ovarian cancer therapeutics.

Mifepristone, commonly known as RU486, was first synthesized in the early 1980s and described as a progesterone receptor antagonist (1). The potent antagonism of mifepristone on uterine progesterone receptors led to its clinical application for termination of pregnancy, emergency contraception, luteal phase contraception, and menstrual regulation (2). However, when targeting cells other than uterine cells, the progesterone antagonistic activity of mifepristone is less clear. For instance, in fibroblasts and T47D breast cancer cells, treatment with activators of protein kinase A abrogated the antagonistic activity of mifepristone that instead elicited partial agonistic effects (3, 4). In HeLa cells, mifepristone significantly induced progesterone-regulated reporter genes, and this agonistic effect was synergistically enhanced by elevating cyclic AMP or by overexpressing the catalytic subunit of protein kinase A (5). The molecular basis for the mixed agonist/antagonist transcriptional regulation of mifepristone in a cell type–specific manner seems to depend on the ratio of coactivators and corepressors of the steroid receptor–mediated transactivation (6).

Apart from its usage as a contraceptive agent, mifepristone has also been effective at diminishing the pain associated with pelvic endometriosis (7, 8), reducing the size of uterine fibroids (9, 10), and inhibiting the growth of meningioma cells *in vitro*, in experimental animal models, and in patients with inoperable meningiomas (11–13). Another non-contraceptive action of mifepristone is the interference with cancer cell growth. Mifepristone, when given with tamoxifen, inhibited the proliferation of MCF-7 and MDA-231 breast cancer cells in an additive manner (14, 15). In MCF-7 cells, mifepristone displayed synergistic cytotoxic activity with the protein kinase C inhibitor 7-hydroxystaurosporine (UCN-01; ref. 16) and arrested the growth of MCF-7 sublines made resistant to 4-hydroxytamoxifen (17). Preliminary clinical trials on metastatic breast cancers have shown a promising response to

Author's Affiliation: Division of Basic Biomedical Sciences, Sanford School of Medicine of The University of South Dakota, Vermillion, South Dakota
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Note: Present address for R. Caron: Instituto de Medicina y Biología Experimental de Cuyo-CONICET, Mendoza, Argentina.
Requests for reprints: Carlos Telleria, Division of Basic Biomedical Sciences, Sanford School of Medicine of The University of South Dakota, 414 East Clark Street, Vermillion, South Dakota 57069. Phone: 605-677-5161; Fax: 605-677-6381; E-mail: carlos.telleria@usd.edu.

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mifepristone in terms of growth inhibition (18, 19). Furthermore, in a recent study using p53/BRCA1–deficient mice, mifepristone prevented the formation of breast tumors (20), indicating its efficacy in not only impairing growth of established mammary tumors but also in inhibiting mammary tumorigenesis. Apart from breast cancer cells, mifepristone was reported to inhibit proliferation of normal and malignant endometrial cells (21–24), prostate cancer cells (25), and gastric adenocarcinoma cell lines (26).

The action of mifepristone on ovarian cancer has received limited attention. In 1996, Rose and Barnea (27) showed that mifepristone arrested OVCAR-3 and A2780 ovarian cancer cells at the G_1 phase of the cell cycle. Years later, it was reported outside the mainstream literature that mifepristone enhanced the toxic effect of cisplatin on COC1 ovarian cancer cells *in vitro* and in xenografted immunosuppressed mice (28, 29). In the only small clinical trial conducted with 44 patients having recurrent epithelial ovarian cancer whose tumors had become resistant to standard chemotherapy, mifepristone administration showed promising effects against some of the tumors (30). These initial studies indicate an anti–ovarian cancer activity of mifepristone; yet, the molecular targets involved in mediating such an effect have not been defined. The aim of the present study was to investigate the molecular mechanisms of cell growth arrest induced by mifepristone in ovarian cancer cells and to further define its efficacy in an *in vivo* preclinical setting. Exposure of ovarian cancer cells to concentrations of mifepristone likely to be achieved *in vivo* inhibited their growth by inducing G_1 cell cycle arrest without triggering cell death. This growth arrest was accompanied by a decline in cyclin-dependent kinase 2 (cdk2) protein level and activity, which correlated with increased association of cdk2 with the cdk inhibitors $p21^{cip1}$ and $p27^{kip1}$ and with reduced levels of transcription factor E2F1 needed for S phase progression. Finally, dose-dependent tumor growth inhibition by mifepristone monotherapy was shown *in vivo* in nude mice carrying s.c. tumors derived from human ovarian cancer cells.

Materials and Methods

Drugs. Mifepristone (RU486), D-(-)-norgestrel (levonorgestrel), medroxyprogesterone acetate (Depo-Provera), and progesterone were from Sigma Chemical Co.

Cell lines. SK-OV-3 and Caov-3 cells were obtained in 2003 from the American Tissue Culture Collection at passages 23 and 33, respectively. Cells were routinely maintained in RPMI 1640 (Mediatech) supplemented with 5% fetal bovine serum (Atlanta Biologicals), 10 mmol/L HEPES (Mediatech), 4 mmol/L L-glutamine (Mediatech), 0.45% D(+) glucose (Sigma), 1 mmol/L sodium pyruvate (Mediatech), 1× nonessential amino acids (Mediatech), 100 IU penicillin (Mediatech), 100 μg/mL streptomycin (Mediatech), and 0.01 mg/mL human insulin (Sigma). The human ovarian carcinoma cell lines OV2008 and IGROV-1 were obtained from Dr. Stephen Howell (University of California, San Diego, CA) and were maintained in RPMI 1640 (Mediatech) supplemented with 5% (OV2008) or 10% (IGROV-1) heat-inactivated fetal bovine serum (Atlanta Biologicals) and 10 mmol/L HEPES (Mediatech), 4 mmol/L L-glutamine (Mediatech), 1 mmol/L sodium pyruvate (Mediatech), 1× nonessential amino acids (Mediatech), 100 IU penicillin (Mediatech), and 100 μg/mL streptomycin (Mediatech). All cell lines were cultured at 37°C in a humidified atmosphere in the presence of 5% CO_2.

Cell proliferation. Cell growth was evaluated in the various ovarian cancer cell lines that were subjected to dose-response or time course treatments. Medium containing each of the doses of fresh steroids was replaced every 24 h. Control groups of cells were treated with vehicle ethanol at a final concentration of less than 0.05%. The number of viable cells was evaluated by trypsinization and counting in a hemocytometer chamber using trypan blue dye exclusion (Sigma). Experiments were conducted in media without phenol red and supplemented with charcoal-extracted fetal bovine serum, or media containing unextracted serum and having phenol red. Similar results were obtained with both media preparations; therefore, after performing the growth curves, all subsequent experiments were conducted using media with unextracted serum and in the presence of phenol red. When indicated, the proliferation IC_{50} values were calculated using software designed to study drug interaction (Calcusyn, Biosoft).

Clonogenic assay. OV2008 or SK-OV-3 cells were seeded into six-well plates at 250 per well in a final volume of 2 mL of medium containing either vehicle or appropriate drug concentrations. Triplicate cultures were used for each drug concentration and time tested. At the end of the drug exposures, the drug-containing medium was replaced with fresh media. All cultures were incubated for an additional 7 days until colonies were large enough to be clearly discerned. At this point, the medium was aspirated, and the dishes were washed once with PBS, fixed with 100% methanol for 30 min, and stained with a filtered solution of 0.5% (w/v) crystal violet (Sigma) for 10 min. The wells were then washed with tap water and dried at room temperature. The colonies, defined as groups of $\geq$50 cells, were scored manually with the aid of an Olympus IMT-2 inverted microscope. Clonogenic survival was expressed as the percentage of colonies formed in mifepristone-treated cells with respect to vehicle-treated cells.

Viability assay. Cells were cultured in sterile, eight-well chamber slides at a concentration of 10,000 per well and subjected to treatment with vehicle or mifepristone. At the end of the incubation, cells were analyzed by a Live/Dead viability/cytotoxicity assay following the instructions of the manufacturer (Molecular Probes). Briefly, cells were washed with PBS and incubated for 45 min at room temperature in the presence of appropriate concentrations of cell-permeant calcein AM, a substrate for ubiquitous intracellular esterases, and ethidium homodimer (EthD-1), which enters the cells having damaged plasma membranes and stains their DNA. When permeabilization of the plasma membrane was needed, cells were exposed to 0.1% saponin (Acros Organics) for 5 min. At the end of the incubation, excess solutions were removed, the slides were mounted, and the labeled cells were observed with a Leica DM/LB microscope (Leica Microsystems) and photographed with a Leica DC 300F digital camera (Leica Microsystems). Calcein formed from calcein AM is well retained within live cells, producing an intense uniform green fluorescence. EthD-1 undergoes enhancement of fluorescence upon binding to DNA, producing a bright red fluorescence in dead cells, but it is excluded by the intact plasma membrane of live cells.

Bromodeoxyuridine labeling and immunocytochemical analysis. Cells were cultured on sterile multi-well chamber slides at a concentration of 10,000 per well and treated with vehicle or mifepristone for 24 h. Cells were exposed to 10 μmol/L bromodeoxyuridine (BrdUrd; Molecular Probes) for 12 h. At the end of the incubation, cells were fixed with 4% paraformaldehyde, and BrdUrd incorporated into the DNA was visualized by immunocytochemistry. The slides were incubated overnight at 4°C with an anti-BrdUrd monoclonal antibody conjugated to biotin (A-21303; Molecular Probes), washed in PBS, incubated with streptavidin-peroxidase (Jackson ImmunoResearch Laboratories), and stained with 3,3′-diaminobenzidine (Sigma) solution for developing peroxidase activity. After washing with tap water, the sections were counterstained with hematoxylin, dehydrated in serial alcohols to xylene, and mounted. In the negative control slides, the primary antibody was replaced with PBS. Random fields were viewed with a Leica DM/LB microscope (Leica Microsystems), and the number of

labeled and unlabeled cells was determined. At least 500 cells were counted for each sample.

Cell cycle analysis. After drug treatment, cells were trypsinized, pelleted by centrifugation at 500 × *g* for 5 min, washed with PBS, and resuspended in cell cycle buffer [3.8 mmol/L sodium citrate (Sigma), 7 units/mL RNase A (Sigma), 0.1% (v/v) Triton X-100 (Sigma), and 0.05 mg/mL propidium iodide (Sigma)] at a concentration of 1×10^6 per milliliter and stored in the dark at 4°C until analysis (24 h). Cells were analyzed using a Becton Dickinson FACScan flow cytometer and Verity Winlist software (Verity Software).

SDS-PAGE and Western blotting. Cells were washed twice with PBS, scrapped, pelleted, and lysed by the addition of radioimmunoprecipitation assay buffer containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP40 (Sigma), 0.25% sodium deoxycholate (Sigma), 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride (Sigma), 1 μg/mL pepstatin (Sigma), 1 mmol/L orthovanadate (Sigma), and 1 mmol/L sodium fluoride (Sigma). Cells were disrupted by passage through a 21-gauge needle and gently rocked on ice for 30 min. Lysates were centrifuged at 16,000 × *g* at 4°C for 15 min, and the supernatant was considered the whole-cell extract. This was assayed for protein content using the bicinchoninic acid method (Pierce). The whole-cell extracts were appropriately diluted in 6× concentrated electrophoresis sample buffer, boiled for 10 min, and stored at -80°C until electrophoresed. Equivalent amounts of protein (50 μg) per point were loaded in 10% or 12% (w/v) acrylamide gels, subjected to SDS-PAGE, and transferred to polyvinylidene difluoride membranes. The blots were blocked in 5% (w/v) nonfat milk in TBS containing 0.1% (v/v) Tween 20 (TBS-T). Blots were then probed overnight with the appropriate dilution of each of the primary antibodies. The membranes were washed 3 × 5 min in TBS-T and incubated with 1:5,000 to 1:10,000 dilution of peroxidase-conjugate secondary antibody for 1 h at room temperature. The blots were again washed and developed by chemiluminescence and exposed to radiographic film. Blots were stripped and reprobed with an antibody directed against the ubiquitous proteins glyceraldehyde-3-phosphate dehydrogenase or β-actin to control for protein loading.

Antibodies for Western blot analysis. Primary antibodies for the following proteins were used at the indicated dilutions. Cyclin E (clone HE12; 0.5 μg/mL), cyclin A (clone BF683; 1 μg/mL), E2F1 (clone KH95/E2F; 0.5 μg/mL), and $p21^{cip1}$ (clone 6B6; 2 μg/mL) were from BD PharMingen. $p27^{kip1}$ (clone 57; 1: 2,000) and Cdk1/cdc2 (clone 1; 1:2,500) were from BD Transduction Laboratories. Cyclin B1 (clone V152; 1:2,000) and poly (ADP-ribose)polymerase (#9592; 1:1,000) were from Cell Signaling Technology. Cdk2 (M2; 1:1,000) was from Santa Cruz Biotechnology. Glyceraldehyde-3-phosphate dehydrogenase (ab9485; 1:10,000) was from Abcam, Inc. β-Actin (clone AC-15; 1:20,000) was from Sigma. Peroxidase-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories.

Cdk2 immunoprecipitation and histone H1 kinase assay. An aliquot (600 μg of protein) from each cell lysate [NP40 lysis buffer; 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.5% NP40, 1 mmol/L DTT, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 2 μg/mL pepstatin, 1 mmol/L phenylmethylsulfonyl fluoride, 50 mmol/L sodium fluoride, 1 mm/L activated sodium orthovanadate] was incubated with 2 μg polyclonal rabbit antibody to cdk2 (M2; Santa Cruz Biotechnology) overnight at 4°C. Immunocomplexes associated with cdk2 were collected after incubating for 2 h with protein A/G Plus-agarose beads (Santa Cruz Biotechnology). The immune complexes were washed twice with lysis buffer, mixed with 2× electrophoresis sample buffer, boiled, and then resolved by SDS-PAGE and analyzed for their components by immunoblotting using the corresponding antibodies for cdk2, cyclin E, $p27^{kip1}$, or $p21^{cip1}$ as described above.

For cdk2-dependent kinase assays, cell lysates (100 μg protein) were incubated overnight at 4°C with constant rotation in 0.5 mL of NP40 lysis buffer containing 1 μg anti-cdk2 antibody (M2; Santa Cruz Biotechnology). The mixture was then incubated for 2 h at 4°C with 25 μL of protein A/G Plus-agarose beads (Santa Cruz Biotechnology). Immunocomplexes were washed thrice with lysis buffer and twice with kinase buffer [50 mmol/L HEPES (pH 7.2), 10 mmol/L $MgCl_2$, 1 mmol/L DTT, 1 mmol/L sodium fluoride, and 10 mmol/L β-glycerophosphate]. Subsequently, the beads were resuspended in 30 μL of kinase buffer containing 2 μg histone H1 (Upstate Cell Signaling Solutions), 5 μmol/L ATP (Upstate Cell Signaling Solutions), and 5 μCi [γ^{32}P]ATP (MP Biomedicals). The reaction mixtures were incubated at 30°C for 30 min; the reactions were terminated with 30 μL of 2× electrophoresis sample buffer, boiled, and separated on 12% SDS/polyacrylamide gels. Gels were stained with Coomassie blue (Sigma) to visualize the histone H1 bands, dried, and autoradiographed.

Subcellular fractionation. Upon treatment with vehicle or mifepristone, cells were washed in PBS, scrapped, pelleted, and resuspended in low salt lysis buffer [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 2 μg/mL aprotinin, 2 μg/mL leupeptin, and 2 μg/mL pepstatin], incubated on ice for 15 min, and homogenized with a hand pestle. The lysates where then centrifuged for 10 min at 800 × *g* at 4°C, and the supernatant was collected (cytosolic fraction). The pellet was resuspended in NP40 lysis buffer (described above) and rocked for 1 h at 4°C. The suspension was centrifuged at 16,000 × *g* for 20 min to remove debris, and the supernatant was considered the nuclear fraction. The protein concentrations of both factions were determined as described above; 50 μg of each of the protein fractions were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes, and immunoblotted with antibodies against $p27^{kip1}$, $p21^{cip1}$, cdk2, cyclin E, poly (ADP-ribose)polymerase (for nuclear fraction purity), and actin (for cytoplasmic fraction purity and loading control).

Nude mouse studies of SK-OV-3 tumor xenografts. *In vivo* studies were done following approval by the University of South Dakota Institutional Animal Care and Use Committee (study protocol 50-01-05-08B). Immunodeficient (athymic nude-*Foxn1*^{nu}) female mice (Harlan) at 6 to 8 weeks of age (~23 g in weight) were inoculated s.c. with 1×10^6 log-phase SK-OV-3 cells per site at two sites (right and left flanks). When tumors had developed, and the average tumor volume was ~50 mm^3, animals were randomized into three groups of 10 animals each and were implanted s.c. with constant release pellets (Innovative Research of America) containing mifepristone (0.5 or 1 mg/d) or placebo, using a 10-gauge precision trochar. Tumor size and animal weight were measured every 5 days beginning on the day of pellet implantation (day 0). After 40 days, the animals were sacrificed. Tumors were measured, excised, fixed in 4% paraformaldehyde, and embedded in paraffin for histologic studies. Tumor volume (in mm^3) was calculated according to the following formula: $[(W)^2 \times L] / 2$, where W and L are the width (perpendicular smaller diameter) and length (perpendicular larger diameter) of the tumor, respectively. The W and L of the tumors were measured using an electronic digital caliper with a resolution of 0.01 mm. Because the range of volumes of experimental tumors was normally large (typically a 5-fold range between the largest and the smallest tumor used in an experiment), we compared relative tumor volumes for individual tumors rather than absolute values. For an individual tumor, the relative tumor volume (V_t/V_0) was calculated from its volume at a particular time (V_t) divided by its volume at the start of treatment (V_0) on day 0. The mean relative tumor volume for the group was plotted against time for each group to compare effects. The tumor mass doubling times were estimated by calculating the tumor volume variations over 40 days using the following formula (31): doubling time = $40 \times \log 2 / \log(V_{40}/V_0)$, where V_{40} is the volume of the tumor before sacrifice, and V_0 is the volume of the tumor at the beginning of treatment (day 0). Body weight was expressed in grams and was used to monitor relative systemic toxicity of mifepristone.

Statistics. All data are reported as means ± SE, and statistical significance was defined at $P < 0.05$. To compare the efficacy of steroid compounds on cell growth inhibition, clonogenic survival, and cell

cycle distribution, one-way ANOVA followed by the Dunnett's multiple comparison test or two-way ANOVA followed by the Bonferroni's multiple comparison test were used as appropriate. To determine significant differences in relative tumor volume (V_t/V_0) between groups, repeated-measures two-way (time × treatment) ANOVA followed by the Bonferroni method as post-test was used. The variance of tumor volume increased as the mean volume increased with time in accordance with exponential growth. The variation of tumor volume was much larger on any given day in the control group than in the treated groups, suggesting greater differences in tumor growth among the animals in the control group. A transformation of the data [Y = Log (Y)] was done to reduce this variance difference and to normalize the data before analysis.

Results

Mifepristone inhibits ovarian cancer cell growth displaying progesterone-like effect. The effect of mifepristone was first studied together with a panel of steroids composed of progesterone and synthetic progestins, on the growth of SK-OV-3 ovarian cancer cells. The cells were subjected for 4 days to treatment with different doses of progesterone, the synthetic progestins levonorgestrel and medroxyprogesterone acetate, and the progesterone receptor modulator mifepristone. In all cases, the number of attached cells was counted for each experimental group before the initiation of the treatment. Medium containing each of the doses of fresh steroids was replaced every 24 h for 4 days. At the end of the experiment, the number of viable cells was evaluated by trypsinization and counting in a hemacytometer chamber using trypan blue dye exclusion. Micromolar concentrations of progesterone, levonorgestrel, medroxyprogesterone acetate, and mifepristone significantly inhibited the growth of SK-OV-3 cells when compared with the growth achieved by cells treated only with vehicle (Fig. 1). The results also show that mifepristone was a more potent suppressor of SK-OV-3 cell growth than the progestins tested. Overall, the results depicted in Fig. 1 clearly show the efficacy of progestins and mifepristone as growth inhibitors in SK-OV-3 cells and show that in terms of growth suppression, mifepristone has progesterone-like effects.

Mifepristone inhibits growth of ovarian cancer cells of different genetic backgrounds. To define whether mifepristone inhibits growth of ovarian cancer cells of different genetic backgrounds, we subjected various ovarian cancer cell lines (SK-OV-3, Caov-3, IGROV-1, and OV2008) to a fixed, 20 μmol/L concentration of mifepristone for 3 days, with the compound being replaced every 24 h. In all cell lines, exposure to 20 μmol/L mifepristone for 24 h was sufficient to arrest cell growth. The arrest persisted during the 3 days of study (Fig. 2A-D). Furthermore, a dose-response experiment was done with SK-OV-3 and OV2008 cells, which were exposed to various concentrations of mifepristone for 3 days. Results shown in Fig. 2E indicate that mifepristone inhibits ovarian cancer cell growth in a dose-dependent manner with a 50% inhibition concentration achieved with ~6 to 7 μmol/L (proliferation IC_{50}: 6.25 μmol/L for SK-OV-3 and 6.91 μmol/L for OV2008).

Mifepristone decreases cloning efficiency of ovarian cancer cells inducing cytostasis. To further confirm the cytostatic effect of mifepristone, we did a clonogenic survival assay using SK-OV-3 and OV2008 cells. Cells were plated at a low density and treated with vehicle or various doses of mifepristone for different times. Treatment was then removed, and cells were cultured in mifepristone-free media for 7 days until colonies having >50 cells became visible in vehicle-treated controls. Increasing the concentration of mifepristone or extending the exposure time of the cells to mifepristone reduced the number of colonies, confirming the growth-inhibitory effect of the steroid (Fig. 3A and B). To determine whether this growth-inhibitory effect was reversible, a characteristic of cytostatic agents (32), cells were treated with various concentrations of mifepristone for 3 days, trypsinized, and re-plated at low density together with cells that had been pretreated with vehicle. Results in Fig. 3C show that the number of colonies did not significantly vary between controls and mifepristone-pretreated groups, suggesting that the inhibition of growth by mifepristone is elicited only when the drug is present, and it does not involve cell toxicity. Data shown in Fig. 1–3 suggest that the action of mifepristone as a cell growth inhibitor in ovarian cancer cells is limited to cytostasis at doses up to 20 μmol/L. To confirm the lack of cytotoxicity upon exposure to 20 μmol/L mifepristone, cells that were cultured in the presence of this dose of mifepristone for 3 days were exposed to the combination of fluorochromes calcein AM and EthD-1. Calcein AM is a cell-permeant compound that is converted by cytoplasmic esterases into a green fluorescent product. EthD-1 binds DNA with red fluorescence but only when the plasma membrane of the cells is compromised (33). The viability of the cells after mifepristone exposure was clearly indicated by the catalysis of calcein AM into a green fluorescence product. This was further confirmed by the lack of red fluorescence in the nuclei, indicative of a lack of binding of EthD-1 to DNA, confirming the integrity of the plasma membrane of mifepristone-treated cells (Fig. 3D). As a control, mifepristone-treated cells were

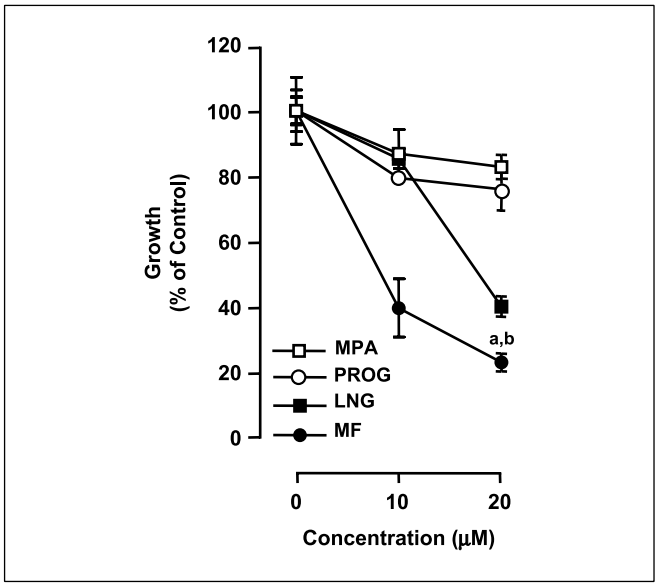

Fig. 1. Inhibition of SK-OV-3 cell growth by progestins and mifepristone. SK-OV-3 cells were cultured in the presence of the indicated concentrations of progesterone (*PROG*), levonorgestrel (*LNG*), medroxyprogesterone acetate (*MPA*), or mifepristone (*MF*). The number of viable cells was recorded at the beginning of the experiment and after 4 d of treatment. The difference between number of cells in vehicle-treated controls at 0 h and after 4 d of culture was considered to be 100%. The growth of the treated groups is expressed as percentage of control. Points, average of triplicate counts; bars, SE. a, $P < 0.05$ versus levonorgestrel; b, $P < 0.001$ versus progesterone and medroxyprogesterone acetate.

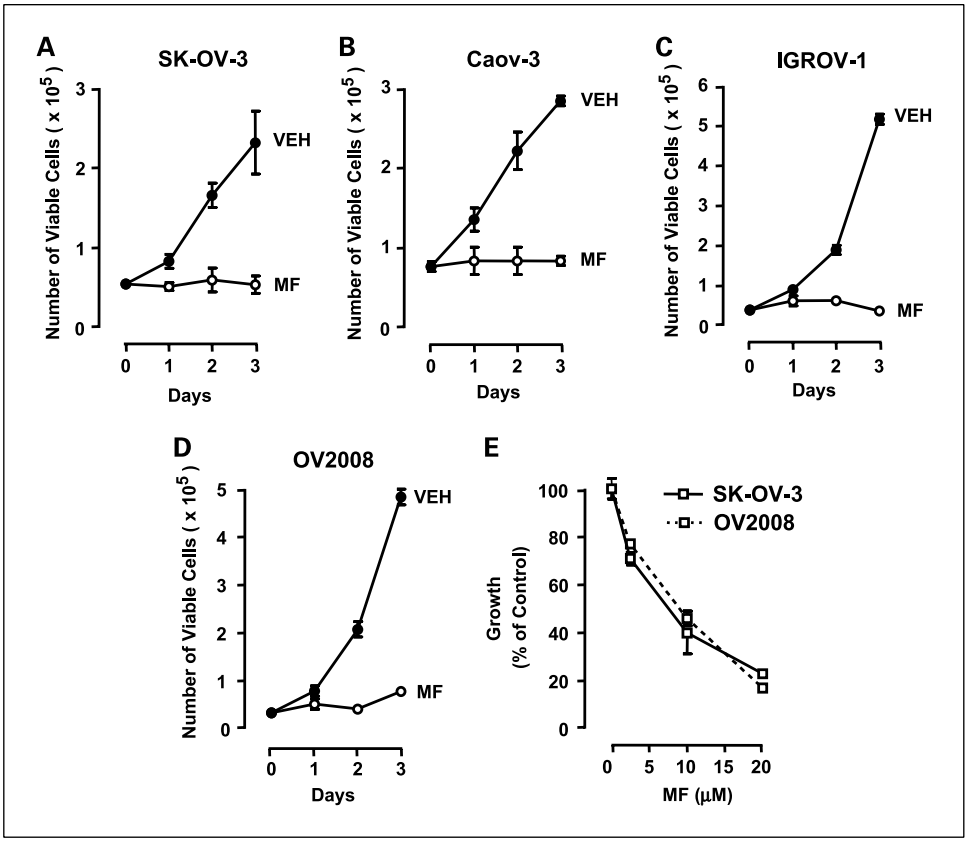

Fig. 2. Inhibition of cell growth by mifepristone in various ovarian cancer cell lines. SK-OV-3 (*A*), Caov-3 (*B*), IGROV-1 (*C*), or OV2008 (*D*) cells were cultured in the presence of 20 μmol/L mifepristone for the indicated times, and the number of viable cells was recorded. *E,* a dose-response experiment is depicted for SK-OV-3 and OV2008 cells. Because SK-OV-3 and OV2008 have a different pattern of growth, the results of this experiment, to be combined within one diagram, are displayed as percentage of vehicle-treated control (*VEH*), whose growth was considered to be 100%.

exposed to calcein AM and EthD-1 in the presence of saponin to permeabilize the plasma membrane (34). Figure 3E shows that the presence of saponin was sufficient to allow EthD-1 to enter the cells and to bind DNA as indicated by the red nuclear fluorescence. In conclusion, our results show that at doses up to 20 μmol/L, mifepristone is cytostatic but not cytotoxic to ovarian cancer cells.

Mifepristone inhibits DNA synthesis and induces G_1 phase arrest in ovarian cancer cells. To examine the effect of mifepristone on the cell cycle in SK-OV-3 and OV2008 cells, the number of cells cycling was assessed by analyzing DNA synthesis with BrdUrd labeling. Cells were exposed to vehicle or mifepristone for 24 h. Twelve hours before the end of the experiment, the cells were pulsed with 10 μmol/L BrdUrd. Incorporation of BrdUrd into newly synthesized DNA was evaluated by immunocytochemistry. Figure 4A shows that the percentage of BrdUrd-labeled nuclei abruptly decreased in mifepristone-treated cells compared with vehicle-treated controls. The decrease was ~4-fold in SK-OV-3 cells and ~ 10-fold in OV2008 cells. To further evaluate the effect of mifepristone on cell cycle traverse, SK-OV-3 and OV2008 cells were cultured in the presence of vehicle or 20 μmol/L mifepristone for 1, 2, or 3 days. At the end of the experiment, the cells were stained with propidium iodide, and the distribution of cells within the cell cycle was analyzed by flow cytometry. Results in Fig. 4B and C indicate that exposure of cells to mifepristone for 1 day significantly reduced the percentage of cells in S phase while inducing a reciprocal increase in the percentage of cells in G_0-G_1. Percentage of cells in G_2-M was significantly decreased in OV2008, yet was unchanged in SK-OV-3. Exposure of the cells to mifepristone longer than 1 day induced a further decrease in the S-phase fraction while inducing additional accumulation of cells in G_0-G_1. No cells had DNA content within the sub-G_0-G_1 region of the histogram, indicating the absence of cells with apoptotic characteristics in both control and treated groups.

Mifepristone changes the expression of several cell cycle regulatory proteins. To study the molecular mechanisms involved in the G_1 cell cycle arrest induced by mifepristone in ovarian cancer cells, we explored the G_1-S transition, which is ultimately controlled by the cyclin E in association with cdk2. This complex phosphorylates the retinoblastoma tumor suppressor, which leads to the release of the E2F transcription factor. E2F controls progression through the S phase (35). Mifepristone provoked slight decreases in the expression of cdk2 and cyclin E after 2 days of treatment (Fig. 5A). In addition, expression of $p21^{cip1}$ and $p27^{kip1}$ was abruptly up-regulated 1 day after exposure to mifepristone (Fig. 5A). Mifepristone also induced down-regulation of the E2F1 transcription factor (Fig. 5B). This was associated with the down-regulation of genes whose expression is dependent on the progression of S phase of the cell cycle, such as *cyclin A*, *cyclin B1*, and *cdk1* (also known as *cdc2*; Fig. 5B). Reduction in expression of the S phase cyclin (cyclin A) and the M phase cyclin (cyclin B1) is most likely the cause of a reduced number of cycling cells in mifepristone-treated cells. Together, DNA synthesis studies, cytometry data, and cell cycle regulatory protein expression suggest that mifepristone arrests ovarian cancer cells at the late G_1 phase of the cell cycle.

Mifepristone increases association of $p21^{cip1}$ and $p27^{kip1}$ with cdk2, inhibits cdk2 activity, promotes nuclear localization of

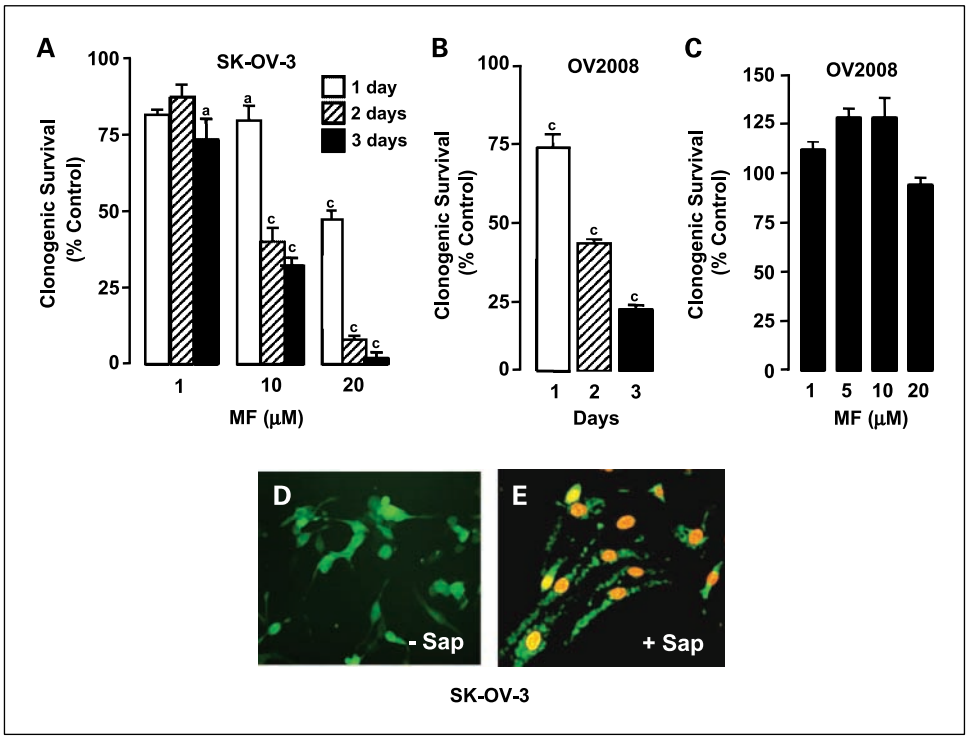

Fig. 3. Effect of mifepristone on ovarian cancer cell clonogenic survival. *A,* 250 SK-OV-3 cells were seeded in six-well plates and exposed to 0, 1, 10, or 20 μmol/L mifepristone for 1, 2, or 3 d. Following treatment, the cells were cultured in regular growth medium for 7 d. At the end of the experiment, the cells were fixed with 100% methanol and stained with 0.5% crystal violet. Colonies containing ≥50 cells were counted. Number of colonies counted for the different experimental groups is expressed as percentage of the control, which was considered to be 100%. a, $P < 0.01$; c, $P < 0.001$, compared with control. *B,* an experiment similar to that in (*A*) was done with OV2008 cells. The cells were exposed to 20 μmol/L mifepristone for 1, 2, or 3 d. c, $P < 0.001$ versus control. *C,* OV2008 cells were incubated with the indicated concentration of mifepristone for 3 d, after which the cells were trypsinized and plated in a clonogenic survival assay, and their growth compared with cells that had not been treated with mifepristone and whose growth was considered to be 100%. *D* and *E,* results of a viability/cytotoxicity assay of SK-OV-3 cells upon exposure to mifepristone. Cells were treated with 20 μmol/L mifepristone for 72 h and exposed to calcein AM plus EthD-1 in the absence (*-Sap*; *D*) or presence (*+Sap*; *E*) of 0.1% saponin. Magnification, ×200.

p21^{cip1} and p27^{kip1}, and reduces nuclear abundance of cdk2 and cyclin E. Because we observed a decrease in cdk2 abundance together with a large increase in the abundance of cdk inhibitors, we asked whether the activity of cdk2 was also affected by mifepristone. We immunoprecipitated cdk2 from control cells or from cells treated with mifepristone and did an *in vitro* kinase assay using histone H1 as substrate. The results in Fig. 5C show that within 1 day of exposure to mifepristone, the activity of cdk2 was inhibited in the absence of any change in the abundance of the enzyme that was immunoprecipitated. In addition, there is a remarkable increase in the amounts of p21^{cip1} and p27^{kip1} that coimmunoprecipitates with cdk2, suggesting that the inhibition is most likely due to an increase in the binding of the inhibitors p21^{cip1} and p27^{kip1}. To evaluate whether mifepristone affects the nucleocytoplasmic trafficking of the cdk inhibitors and cdk2, vehicle- and mifepristone-treated cells were subjected to subcellular fractionation and Western blot analysis. Results in Fig. 5D reveal that the 24-h incubation with mifepristone induced an increase in nuclear localization of p21^{cip1} and p27^{kip1}, which correlated with decreased cdk2 and cyclin E nuclear levels. These results suggest that mifepristone-mediated inhibition of cdk2 activity also involves decreased cdk2 and cyclin E nuclear abundance, together with increased nuclear abundance of the cdk inhibitors p21^{cip1} and p27^{kip1}.

Mifepristone inhibits growth of ovarian cancer xenografts in nude mice. To study whether mifepristone is capable of inhibiting ovarian cancer tumor growth *in vivo*, we generated s.c. xenografts of SK-OV-3 cells in immunosuppressed mice. Female mice of ~7 weeks of age were inoculated s.c. with

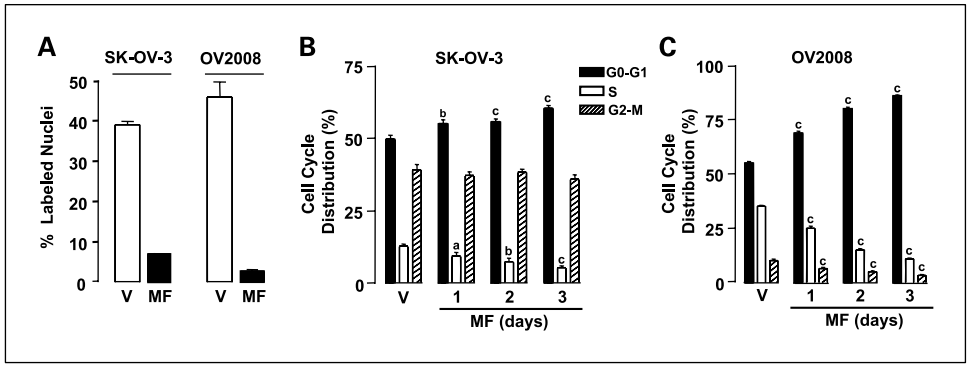

Fig. 4. Effect of mifepristone on BrdUrd incorporation into DNA and on cell cycle kinetics in ovarian cancer cells. *A,* SK-OV-3 and OV2008 cells were cultured on multi-well slides and treated with either vehicle (*V*) or 20 μmol/L mifepristone for 24 h. Cells were exposed to 10 μmol/L BrdUrd for 12 h. At the end of the experiment, the cells were fixed with 4% paraformaldehyde, and BrdUrd incorporated into the DNA was visualized by immunocytochemistry using an anti-BrdUrd monoclonal antibody. BrdUrd-positive nuclei were quantified using a microscope with a ×100 magnification, and at least 500 cells were counted for each sample. The experiment was done in triplicate. Results are expressed as percentage of BrdUrd-positive cells. *B,* logarithmically growing SK-OV-3 and OV2008 ovarian cancer cells were exposed to vehicle or 20 μmol/L mifepristone for 1, 2, or 3 d. The percentage of cells in the G_0-G_1, S, and G_2-M phases of the cell cycle was determined by cytometric analysis of propidium iodide – stained cells. Columns, means of two separate experiments done in triplicate; bars, SE. a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$ relative to the control group.

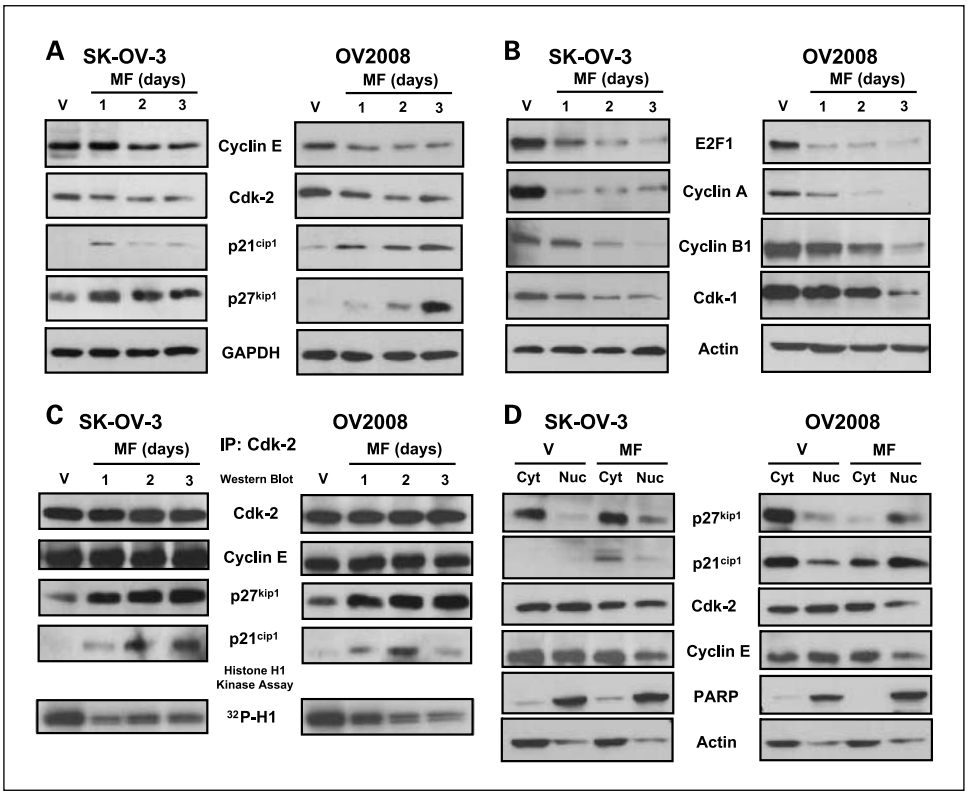

Fig. 5. Effect of mifepristone on the expression of cell cycle regulatory proteins, cdk2 activity, association of cdk2 with $p21^{cip1}$ and $p27^{kip1}$, and subcellular localization of $p27^{kip1}$, $p21^{cip1}$, cdk2, and cyclin E. SK-OV-3 or OV2008 cells were exposed to vehicle or 20 μmol/L mifepristone for the indicated times or for 24 h when not indicated. Whole-protein extracts were obtained and separated by electrophoresis, and immunoblots were probed with the indicated antibodies. The housekeeping genes *GAPDH* (*A*) and *β-actin* (*B*) were used as loading controls. *C,* whole-protein extracts were immunoprecipitated with anti-cdk2 antibody. The immunoprecipitates were Western blotted for cdk2, cyclin E, or the cdk inhibitors $p21^{cip1}$ and $p27^{kip1}$. The immunoprecipitates were also assayed for their capacity to phosphorylate histone H1 *in vitro* in the presence of [^{32}P]ATP. Products of the kinase reaction were run on polyacrylamide gels, and the phosphorylated histone H1 (^{32}P-H1) was analyzed by autoradiography. In a third experiment (*D*), cells were treated with vehicle or 20 μmol/L mifepristone for 24 h. The cells were then fractionated into nuclear and cytosolic fractions, and proteins from each respective fraction were subjected to immunoblot analysis with antibodies to $p27^{kip1}$, $p21^{cip1}$, cdk2, or cyclin E. Poly (ADP-ribose) polymerase (*PARP*) and actin were used to assess the purity of the subcellular fractions. Actin was further utilized to control for protein loading. The results were repeated twice with similar outcome.

1×10^6 SK-OV-3 cells per site at two sites (right and left flanks). When tumors developed, and the average tumor volume was ~50 mm^3, animals were randomized into three groups that were implanted s.c. with pellets customized to release either placebo or mifepristone at rates of either 0.5 or 1 mg/d. The volume of the tumors was measured every 5 days. For an individual tumor, the relative tumor volumes (V_t/V_0; n = 12-19 per group) was calculated from its volume at a particular time (V_t) divided by its volume at the start of treatment (V_0) on day 0. The results show that the growth rate of tumors was significantly inhibited by mifepristone at either dose of mifepristone beginning 20 days after the beginning of the treatment (Fig. 6A). The dose-dependent growth inhibition was also confirmed by the fact that the rate of tumor growth achieved after 40 days of treatment with 1 mg/d mifepristone was significantly lower than that of animals treated with 0.5 mg/d of the drug. The calculated doubling times over the 40 days of the study were 9.5 ± 0.6 days for the placebo-treated tumors (n = 12), 14.1 ± 1.6 days for the 0.5 mg/d mifepristone-treated tumors (n = 16), and 17.2 ± 1.6 days for the 1 mg/d mifepristone-treated tumors (n = 12). This further highlight the capacity of mifepristone to impair the growth of tumors generated upon s.c. inoculation of SK-OV-3 ovarian cancer cells into immunosuppressed mice. The animals seemed to tolerate the chronic exposure to mifepristone well as evidenced by stable animal weights that were indistinguishable between the arms of the study (Fig. 6B).

Discussion

Major clinical problems in the treatment of ovarian cancer include the resistance to current therapy, the elevated toxicity of standard drugs, and the repopulation of cells that escape chemotherapy. Therefore, the development of novel strategies for ovarian cancer treatment is of significant importance. In this report, we show that the contraceptive steroid mifepristone is a potent cytostatic agent to human ovarian cancer cells of different genetic backgrounds, inducing cell cycle arrest and blocking the G_1-S phase transition. Mifepristone-induced G_1 arrest involves inhibition of cdk2 activity likely due to a combination of decreased nuclear cdk2 abundance and increased recruitment of cdk inhibitors $p21^{cip1}$ and $p27^{kip1}$ to the nucleus. The decline in cdk2 activity is associated with decreased abundance of the transcription factor E2F1 that drives S phase progression. Moreover, we provide evidence that mifepristone, at doses that are likely to be achieved clinically, has an antiproliferative action not associated with cell death.

Blockage of ovarian cancer cells at the G_1-S transition was shown by the interference of mifepristone with the incorporation

of BrdUrd into newly synthesized DNA molecules. The remarkable inhibition of DNA synthesis induced by mifepristone was consistent with the abrupt interruption in cell growth observed within 24 h of exposure to the drug, the reduction in the number of cells in S phase, and the accumulation of cells in G_1 phase. Blockage of the cell cycle in G_1 after exposure to mifepristone has also been reported in human gastric adenocarcinoma cells (26), T-47D breast cancer cells (36), and in OVCAR-3 and A2780 ovarian cancer cells (27). In T-47D breast cancer cells, the inhibition of cell cycle progression by mifepristone was accompanied by induction of the cdk inhibitor $p21^{cip1}$ (36). Similar to mifepristone, the synthetic progestin Org2058 also induced T-47D cell cycle arrest mediated through the cooperation of the cell cycle inhibitors $p27^{kip1}$ and $p18^{ink4}$ (37). The antiproliferative effect of mifepristone in ovarian SK-OV-3 and OV2008 cells was associated with an increase in the levels of $p21^{cip1}$ and $p27^{kip1}$ and with their relocalization to the nucleus; these proteins are likely responsible for the inhibition of cdk2 activity because both coimmunoprecipitated with cdk2. Further inhibition of cdk2 activity may be a result of the decrease of cdk2 protein levels in the nucleus upon sustained exposure to mifepristone. This is because nuclear localization of cdk2 is required for its full activation, leading to the progression of the cell cycle (38, 39). Hence, in ovarian cancer cells, cdk2, upon which the cells depend to pass through the G_1-S interface (40), had a dual regulation by mifepristone, which reduces its activity and its nuclear abundance. Because cdk2 is frequently up-regulated in ovarian tumors (41), the potent inhibitory effect on cdk2 displayed by mifepristone in ovarian cancer cells may be of central importance in the development of new therapeutic approaches using the steroid. Moreover, the increased abundance of $p21^{cip1}$ and $p27^{kip1}$ and their localization to the nucleus in response to mifepristone is of significance because it is generally accepted that nuclear but not cytoplasmic cdk inhibitors are the catalytic inhibitors of cdk2 (42) and also because cytoplasmic localization of $p27^{kip1}$ in ovarian cancer patients is associated with poor prognosis (43). Thus, mifepristone may be particularly useful in rescuing the tight inhibitory control of cdk inhibitors on the activity of cdk2, which is frequently lost in ovarian cancer. In addition, nuclear $p21^{cip1}$ and $p27^{kip1}$, apart from blocking cdk2 activity in response to mifepristone, might be involved as transcriptional cofactors leading to the inhibition of transcription of cell cycle–promoting genes (42).

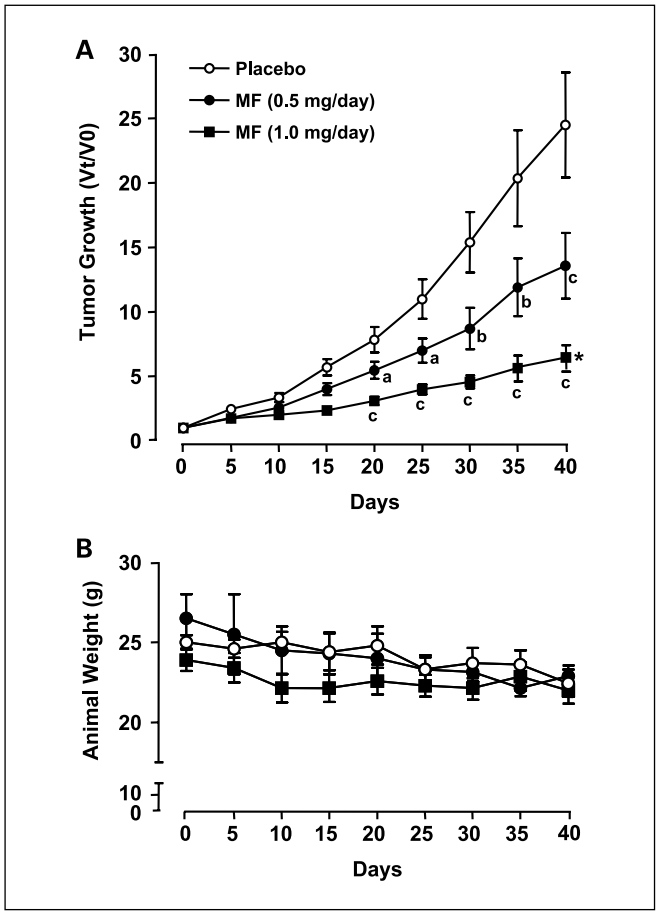

Fig. 6. Effect of mifepristone on tumor growth *in vivo. A,* growth curve for SK-OV-3 tumors in nude mice that were treated with placebo (○), 0.5 mg/d mifepristone (●), or 1 mg/d mifepristone (■). Tumor sizes are expressed as relative volume at a particular time (V_t) divided by its volume at the beginning of the treatment (V_0) on day 0. a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$ compared with its time-matched placebos. *, $P < 0.05$ compared with mifepristone-treated at 0.5 mg/d. *B,* body weights were stable during treatment and similar for the placebo-treated (○), mifepristone-treated at 0.5 mg/d (●), or mifepristone-treated at 1 mg/d (■) animals.

E2F1 is a key transcription factor required for the transactivation of target genes involved in the G_1-S transition and for correct progression through the cell cycle (44). E2F1 expression was greatly inhibited by mifepristone. This was in agreement with the lack of cell cycle progression observed in ovarian cancer cells after exposure to the drug. E2F1 down-regulation may have two components. On one hand, the abrupt decline in cdk2 activity may reduce the degree of phosphorylation of the retinoblastoma protein, making E2F1 less available for gene regulation. Furthermore, a down-regulation of E2F1 by mifepristone is also evident. Because commitment of the cell to S phase progression requires cdk2 activity and E2F1 availability, the down-regulation of cdk2 (activity and abundance), together with the down-regulation of E2F1, explains the cell cycle arrest of ovarian cancer cells in G_1. As expected, mifepristone led to the down-regulation of genes needed to progress through the S phase, such as *cyclin A*, *cyclin B1*, and *cdk1* (40).

The cytostatic effect of mifepristone displayed similar potency among the ovarian cancer cell lines studied. This effect occurred irrespective of the p53 background of the cell lines, as SK-OV-3 is p53 null (45), Caov-3 cells express mutant p53 (46), whereas IGROV-1 and OV2008 cells express wild-type p53 (47, 48). This observation is of particular importance because mutations of the p53 tumor suppressor occur at extremely high frequencies in ovarian cancer (49). Thus, mifepristone would be equally effective in tumors with either wild-type p53 or mutant p53 expression. Furthermore, the similar potency of growth inhibition achieved by mifepristone in SK-OV-3 (IC_{50}, 6.25 μmol/L), which are intrinsically resistant to cisplatin (50), and OV2008 (IC_{50}, 6.91 μmol/L) that are sensitive to cisplatin therapy (51), suggest that mifepristone could be useful in abrogating cell growth in ovarian cancer irrespective of the resistance to cisplatin. This is of particular significance when considering that acquisition of resistance to cisplatin occurs with high frequency in patients with recurrent ovarian cancer disease.

At the doses used in this study, the effect of mifepristone was limited to cytostasis showed by the reversibility of the

growth inhibition observed when the drug was removed from the culture media, in association with the lack of measurable cell death. It has been reported previously that mifepristone, at concentrations higher than 20 μmol/L, can trigger cell death in OVCAR-3 cells (27). In the cell lines used in our study, concentrations of mifepristone 30 μmol/L or higher display cytotoxic activity (results not shown), suggesting that 20 μmol/L seems to be the threshold for cell cycle inhibition in these ovarian cancer cell lines. Several pharmacokinetic studies in humans report that when administered orally in doses ranging from 10 to 800 mg, mifepristone reaches concentrations in circulation ranging from 2.5 to 20 μmol/L, but not higher (52–55). Although most studies using mifepristone in humans were conducted using short-term treatment schedules, a recent study in patients with non-resectable meningioma who received long-term (months to years) therapy showed that the drug can be safely administered for prolonged periods of time with manageable side effects (56). In our study, the pellets of mifepristone used were designed to release either 0.5 or 1 mg/d. Although the actual steady-state concentrations of mifepristone attained in circulation in these mice were not measured, the fact that tumor growth rate was reduced in a dose-dependent manner and without apparent discomfort to the animals confirms the effectiveness of the compound *in vivo*. The dose of mifepristone used in our study is within the range used in another investigation in which growth inhibition of prostate cancer xenografts was observed (25); as in our study, no significant changes in the overall weight of the animals were observed upon chronic exposure to mifepristone.

Mifepristone behaved similar to synthetic progestins in inducing cell growth arrest, although with greater potency. It is possible that the cytostatic effect of mifepristone is mediated by an agonistic action on progesterone receptors. Support for this idea is that in breast cancer cells, mifepristone interfered with cell proliferation, displaying progesterone-like effects (57). In MDA-MB-231 breast cancer cells transfected with the progesterone receptor, mifepristone, akin to progesterone, inhibited cell growth by arresting the cells in the G_0-G_1 phase of the cell cycle (58). In another study, the antiproliferative effect of mifepristone was additive to tamoxifen in MCF-7 cells expressing both estrogen and progesterone receptors (14). However, such antiproliferative action of mifepristone was also observed in estrogen receptor– and progesterone receptor–negative MDA-231 cells (15), suggesting that the presence of progesterone receptors may not be required for mifepristone to elicit its growth-inhibitory effect. Furthermore, the reported level of expression of progesterone receptors in ovarian cancer cell lines is not without controversy. For example, Caov-3 cells were reported to express progesterone receptor mRNA in one study (59), but not in another report (60). Similarly, studies in SK-OV-3 cells showing some and no expression of progesterone receptor mRNA have been published (60–62). We have detected progesterone receptor immunoreactive proteins in OV2008 cells but failed to show any reactive proteins in SK-OV-3 cells (data not shown), leading to more doubt about the involvement of progesterone receptors in mifepristone-induced growth inhibition.

Another possibility is that mifepristone may drive its anticancer action through glucocorticoid receptors. This is because it can bind to glucocorticoid receptors with an affinity similar to that for progesterone receptors (63), and ovarian cancer cells have been reported to express glucocorticoid receptors (64). In this regard, we have detected abundant levels of glucocorticoid receptor immunoreactive proteins in both SK-OV-3 and OV2008 cell lines (results not shown). However, when SK-OV-3 and OV2008 cells were cultured in the presence of the glucocorticoid agonist dexamethasone at concentrations equimolar to cytostatic mifepristone, no modifications in cell growth were observed (data not shown), suggesting that even if mifepristone binds glucocorticoid receptors in the ovarian cancer cells it may not trigger receptor transactivation.

A final possibility is that mifepristone may have an effect that does not involve specific hormone receptors. In this regard, mifepristone was shown to have a potent antioxidant effect manifested at micromolar concentrations and attributed to the presence of the dimethylamino phenyl side chain of the molecule (65). Furthermore, the growth-inhibitory effect of mifepristone in endometrial cells and macrophages has been attributed, at least in part, to the antioxidant property of the compound (23, 66). A putative antioxidant effect of mifepristone on ovarian cancer cells could be interesting in the context of G_1 arrest associated with $p21^{cip1}$ up-regulation because $p21^{cip1}$ can be induced in response to some antioxidants in a p53-independent fashion (67, 68). We have shown that mifepristone-induced cell growth arrest is associated with $p21^{cip1}$ increase in both wild-type p53 expressing OV2008 cells and in p53 null SK-OV-3, opening the possibility that mifepristone acts as an antioxidant to drive G_1 arrest through a p53-independent $p21^{cip1}$ up-regulation.

In summary, we have shown that mifepristone is a potent blocker of ovarian cancer growth *in vitro* and *in vivo*. The feasibility of using mifepristone to enhance the efficacy of conventional chemotherapy for ovarian cancer is encouraging and requires further investigation.

Acknowledgments

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