Mifepristone Induces Growth Arrest, Caspase Activation, and Apoptosis of Estrogen Receptor-Expressing, Antiestrogen-Resistant Breast Cancer Cells

Virgil T. Gaddy, John T. Barrett, Jennifer N. Delk, Andre M. Kallab, Alan G. Porter, and Patricia V. Schoenlein

1 Departments of Cellular Biology and Anatomy, 2 Radiology, and 3 Medicine, Section of Medical Oncology, Medical College of Georgia, Augusta, Georgia, and 4 Institute of Molecular and Cell Biology, Singapore, Republic of Singapore

ABSTRACT

Purpose: A major clinical problem in the treatment of breast cancer is the inherent and acquired resistance to antiestrogen therapy. In this study, we sought to determine whether antiprogestin treatment, used as a monotherapy or in combination with antiestrogen therapy, induced growth arrest and active cell death in antiestrogen-resistant breast cancer cells.

Experimental Design: MCF-7 sublines were established from independent clonal isolations performed in the absence of drug selection and tested for their response to the antiestrogens 4-hydroxytamoxifen (4-OHT) and ICI 182,780 (fulvestrant), and the antiprogestin mifepristone (MIF). The cytostatic (growth arrest) effects of the hormones were assessed with proliferation assays, cell counting, flow cytometry, and a determination of the phosphorylation status of the retinoblastoma protein. The cytotoxic (apoptotic) effects wereanalyzed by assessing increases in caspase activity and cleavage of poly(ADP-ribose) polymerase.

Results: All of the clonally derived MCF-7 sublines expressed estrogen receptor and progesterone receptor but showed a wide range of antiestrogen sensitivity, including resistance to physiological levels of 4-OHT. Importantly, all of the clones were sensitive to the antiprogestin MIF, whether used as a monotherapy or in combination with 4-OHT. MIF induced retinoblastoma activation, G1 arrest, and apoptosis preceded by caspase activation.

Conclusions: We demonstrate that: (a) estrogen receptor-progesterone receptor+, 4-OHT-resistant clonal variants can be isolated from an MCF-7 cell line in the absence of antiestrogen selection; and (b) MIF and MIF plus 4-OHT combination therapy induces growth arrest and active cell death of the antiestrogen-resistant breast cancer cells. These preclinical findings show potential for a combined hormonal regimen of an antiestrogen and an antiprogestin to combat the emergence of antiestrogen-resistant breast cancer cells and, ultimately, improve the therapeutic index of antiestrogen therapy.

INTRODUCTION

The hormone 17-β estradiol (E2) regulates the proliferation and differentiation of reproductive and other target tissues (1, 2). E2 also drives the proliferation of breast cancer cells that express estrogen receptors (ER; Refs. 2–4). The engagement of E2 with ER promotes the dissociation of the receptors from multiprotein chaperone complexes and the recruitment of coregulators (coactivators/corepressors) to the receptors. Ultimately, the activated ER binds to DNA and effects the transcription of target genes, such as cyclin D1, and other proteins that control the cell cycle (5–8). In addition, ER influences cellular activities through nongenomic (nontranscriptional) actions, such as activation of the mitogen-activated protein kinase signaling pathways (9).

For the treatment of ER-expressing breast cancer, tamoxifen has been the most commonly used antiestrogen (10). Tamoxifen therapy improves 5-year survival rates for breast cancer patients in the adjuvant setting (11) and is used as a cancer chemopreventive agent in high-risk women (12). Tamoxifen blocks the prosurvival and proliferative effects mediated by E2-stimulated ER. However, tamoxifen resistance is a major clinical limitation for the successful management of breast cancer (13–17). Inherent mechanisms of tamoxifen resistance are commonly expressed in breast cancer cells and almost all of the breast cancers that are initially responsive acquire tamoxifen resistance with prolonged treatment (13, 18, 19). To overcome tamoxifen resistance, other antiestrogens are used as second-line endocrine therapy after the failure of tamoxifen therapy (20, 21). One example is fulvestrant (ICI 182,780), a pure antiestrogen with no apparent agonist activity in any tissues, that has demonstrated some clinical benefit as a second-line endocrine treatment (22, 23). However, resistance to fulvestrant has been demonstrated in preclinical studies (24) and in patients where fulvestrant has been used to treat patients with tamoxifen-resistant breast cancer (25). Although recent studies with the aromatase inhibitors showed superior outcome (overall response and time to progression) when compared with tamoxifen in the first-line metastatic setting, and after tamoxifen failure, almost all of the patients with hormone-dependent metastatic breast...
cancer become refractory to ER-targeted therapies and subsequently die (26, 27). Thus, improved treatments for hormone-dependent breast cancer are needed.

On the basis of the abundance of progesterone receptors (PR) in ER-expressing breast cancer cells and the proliferative role of progesterone in breast cancer cells (28) and in normal breast epithelium (29), one possible strategy to treat ER$^+$ PR$^+$ breast cancer is to simultaneously block both the action of ER and PR. To block PR function, mifepristone (MIF) has been used as the prototype antiprogestin for in vivo and in vitro preclinical studies. MIF, also referred to as RU486 (Roussel Uclaf SA; Ref. 30), shows strong PR antagonist activity that blocks PR-mediated transcription (31, 32). In ER$^+$ PR$^+$ breast cancer cell models, MIF induces cytostatic and cytotoxic effects (33). Importantly, we (34, 35) and others (36) have demonstrated that tamoxifen-induced growth-inhibitory effects are increased when MIF is used in combination with tamoxifen.

In this study, we uniquely demonstrate that MIF, alone or in combination with 4-hydroxytamoxifen (4-OHT) treatment, induces growth arrest and cell death in antiestrogen-resistant MCF-7 cells that express ER and PR. The antiestrogen-resistant clones identified and characterized in this study were isolated via clonal selection of a MCF-7 population of cells without antiestrogen selection. The individual clones displayed a wide degree of variation in their responsiveness to 4-OHT. In a similar fashion, such ER-expressing antiestrogen-resistant variants are probably present in human breast cancers before antiestrogen therapy. Thus, these preclinical studies provide evidence that treatment with combination hormonal therapy (antiestrogen and antiprogestin) may more effectively circumvent the emergence of antiestrogen resistance. Furthermore, these studies demonstrate the high degree of clonal variation that can exist within MCF-7 cell populations and emphasize the caution that must be exercised when interpreting results from studies that involve clonal selections.

MATERIALS AND METHODS

Cells, Culture Conditions, and Hormone Treatments.

The MCF-7 breast cancer cell line (early passage) was purchased from American Type Culture Collection. MCF-7.0.3, MCF-7.3.6, MCF-7.3.25, and MCF-7.3.28 were established from MCF-7 cells (designated MCF-7 AP) as described previously (37). Cells were maintained in DMEM (Life Technologies, Inc.), 1% L-glutamate (Life Technologies, Inc.), 2% antibiotic/antimycotic (Life Technologies, Inc.), 10% dextran-coated, charcoal-stripped fetal bovine serum (Atlanta Biologicals, Norcross, GA), and 5% charcoal-stripped fetal bovine serum (Atlanta Biologicals, Norcross, GA) with 5% dextran-coated, charcoal-stripped fetal bovine serum (Atlanta Biologicals, Norcross, GA) and 10% fetal bovine serum (Hyclone, Logan, UT), 2% antibiotic/antimycotic (Life Technologies, Inc.), and 10% charcoal-stripped fetal bovine serum (Hyclone, Logan, UT), 2% antibiotic/antimycotic (Life Technologies, Inc.), and 10% charcoal-stripped fetal bovine serum (Hyclone, Logan, UT), 2% antibiotic/antimycotic (Life Technologies, Inc.).

Cell Proliferation Assay. Cell proliferation was quantitated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assay (38). Briefly, cells were seeded in 96-well plates and treated with hormones. At various treatment times (24–144 h), cells were incubated in 0.2% 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma) solution for 1 h at 37°C and fixed with the addition of an equal volume of 10% formaldehyde in 0.2 M Tris solution. Fixative was removed, and wells were washed with H$_2$O. The precipitate was dissolved in 6.25% 0.1 N NaOH in DMSO solution. The absorbance (570 nm) was measured using a TECAN microplate spectrophotometer and WinSelect software (TECAN United States, Research Triangle Park, NC).

Cell Counting. As a measure of cell death, cells detached from the culture vessel (referred to as floating cells) were counted. Floating cells were transferred to a conical tube, collected via centrifugation (1500 × g, 10 min, 4°C), resuspended in 1 ml of Hank’s Balanced Salt Solution, transferred to 1.5-ml Eppendorf tubes, concentrated via centrifugation (1500 × g, 10 min, 4°C), resuspended into a final volume of 50–100 μl of Hank’s Balanced Salt Solution, and counted using a hemacytometer. The remaining attached cells were trypsinized, resuspended in Hank’s Balanced Salt Solution, syringed through a 25-gauge needle twice to ensure single cell separation, and counted using a Coulter Counter at a dilution of 1:100 in Isoton II diluent (Beckman Coulter, Fullerton, CA).

Cell Cycle Analysis. Total cell populations were collected and counted using a Coulter Counter. Cells (1 × 10$^6$) were resuspended in 1 ml of ice-cold PBS, permeabilized in 10 ml of 70% ethanol, and resuspended in 1 ml of 100 μg/ml propidium iodide, 0.1% Triton X-100, and 10 μg/ml RNase A. DNA content was quantitated based on the fluorescence of propidium iodide (488 nm excitation, 585 nm emission) as measured in the MCG flow cytometry core facility using a FACSCaliber 3-color analyzer (Becton Dickinson, Franklin Lakes, NJ). The data were analyzed using ModFit LT, Version 2.0 Software (Verity Software House, Inc., Topsham, ME).

Caspase Activity Assays. Caspase activity was determined using kits (Oncogene Research Products, San Diego, CA). Cells were collected, concentrated by centrifugation (250 × g, 10 min, 4°C), and lysed in extraction buffer [50 mM HEPES (pH 7.4), 0.1 mM EDTA, 0.1% CHAPS, and 1.0 mM dithiothreitol] for 30 min with gentle agitation on ice. Cell extracts were cleared of debris by centrifugation (20,000 × g, 5 min, 4°C), and equal amounts of protein per treatment group were incubated with fluorogenic tetrapeptide substrates DEVD-AFC, IETD-AFC, or LEHD-AFC for 1 h at 37°C to measure caspase-3, -8, and -9 activity levels, respectively. Liberated AFC fluorescence was measured (400 nm excitation, 505 nm emission) using a TECAN microplate fluorometer and WinSelect software (TECAN United States).

Detection of Fragmented DNA. To detect oligonucleosomal DNA laddering, the method of Ishida et al. (39) was performed. In brief, total oligonucleosomal DNA obtained from 2 × 10$^7$ cells was loaded into one lane of a 2% agarose gel and
subjected to conventional gel electrophoresis. The gel was stained with ethidium bromide, exposed to UV, and photographed.

**Protein Extraction and Immunoblotting Analysis.**
Proteins were extracted from total cell populations in hot (100°C) lysis buffer [0.125 M Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, and 5% (v/v) β-mercaptoethanol in H2O]. DNA was sheared by pulling lysates through an 18-gauge needle. Isolated proteins were resolved under denaturing, reducing conditions in Tris-glycine SDS gels, transferred to Hybond-P polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ), blocked with TBS [0.1% Tween 20 and 5% nonfat dried milk], and probed with mouse anticaspase-3 antibody (Transduction Laboratories, Lexington, KY), anti-ERα, anti-PR A/B, antiretinoblastoma (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or anticleaved poly(ADP-ribose) polymerase antibody (Cell Signaling Technology, Inc., Beverly, MA) followed by a 1-h incubation with either anti-mouse IgG or antirabbit IgG peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Protein loading differences were determined by probing membranes with mouse anti-actin (Oncogene Research Products) or anti-β actin (Sigma) antibodies. Immunoreactive proteins were visualized with the enhanced chemiluminescence detection system (Pierce, Rockford, IL).

**Statistical Analysis.** To determine whether hormone therapy affected the cells, treated cells were compared with estradiol-treated control cells with the Student's t test using SigmaStat software (SPSS Science, Chicago, IL). Significance was assumed at $P < 0.05$. The graphed data are expressed as the mean ± SE.

**RESULTS**

**MCF-7.0.3 and MCF-7.3.28 Cells Are Resistant to 4-OHT but Effectively Growth Inhibited by MIF.** Our published studies determined that MIF and 4-OHT plus MIF combination therapy induce apoptosis in MCF-7 cells (35). However, MCF-7 cells lack caspase-3. Caspase-3, a member of the aspartate-specific caspase family of proteases, is an effector caspase that is activated by proteolytic cleavage in an enzymatic cascade in which upstream caspases (initiators) activate downstream caspases (effectors; Ref. 40). In earlier studies that addressed the mechanism of tumor necrosis factor α-induced cell death, several MCF-7 caspase-3-expressing sublines were constructed by clonal selection after transfections with caspase-3 cDNA (37). We reasoned that these sublines, designated MCF-7.3.6, MCF-7.3.25, and MCF-7.3.28, would provide good models in which to determine whether 4-OHT and/or MIF induce caspase-3 activation as part of the death process and ultimately define the role of caspase-3 in hormonally induced cell death.

In initial studies, we used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay to assess the response of MCF-7.3.28, MCF-7.3.25, and MCF-7.3.6 cells to 4-OHT, MIF, and combination treatment (4-OHT plus MIF). In addition, MCF-7.0.3 cells transfected with empty vector DNA were analyzed. Within 24 h of treatment the proliferation of MCF-7 American Type Culture Collection cells was inhibited significantly by 4-OHT, MIF, and 4-OHT plus MIF when compared with E2-treated cells (data not shown). Cell growth inhibition persisted for at least 144 h after the initial drug exposure (Fig. 1A). In contrast, 4-OHT-induced growth inhibition was severely attenuated in MCF-7.0.3, MCF-7.3.25, MCF-7.3.6, and MCF-7.3.28 cells at all of the times after treatment when compared with E2-treated cells (Fig. 1A; data not shown). However, MIF and MIF plus 4-OHT combination treatment effectively inhibited the cell growth of all of these sublines (Fig. 1A; data not shown).

Noting the reduced sensitivity of MCF-7.0.3, MCF-7.3.28, MCF-7.3.6, and MCF-7.3.25 to 4-OHT therapy, we analyzed ERα expression in these sublines to determine whether 4-OHT resistance correlated to loss of ERα expression. Western analyses demonstrated that MCF-7 American Type Culture Collection, MCF-7.0.3, and MCF-7.3.28 cells express ER and that 4-OHT treatment results in the typical increase in ERα levels (Fig. 1B), most probably due to 4-OHT-induced inhibition of

![Graph](https://clinicalcancerresearch.aacrjournals.org/content/5/11/5217/F1.large.jpg)
ER degradation by the ubiquitin pathway (41, 42). Similar results were obtained with MCF-7.3.25 and MCF-7.3.6 cells (data not shown).

Analyses by flow cytometry confirmed the 4-OHT-resistant phenotype of the MCF-7.0.3 and MCF-7.3.28 cell lines and additionally established that MIF, alone and in combination with 4-OHT, induces growth arrest in these cells (Fig. 2A). At 48 h, MIF and combination treatment (4-OHT plus MIF) resulted in a significant increase in G0/G1 phase arrest in MCF-7.0.3 (48% and 64%, respectively) and MCF-7.3.28 (16% and 31%, respectively) cells relative to E2-treated (control) cells. In addition, MIF and 4-OHT plus MIF treatments induced corresponding significant decreases in S phase in MCF-7.0.3 (50% and 66%, respectively) and MCF-7.3.28 (21% and 47%, respectively; Fig. 2A). Treatment with 4-OHT did not induce significant changes in either G0/G1 or S phases of these clones. In contrast, the 4-OHT-sensitive MCF-7 American Type Culture Collection cells showed a significant increase in the percentage of cells in G0/G1 phase (23%) and a corresponding decrease in the percentage of cells in S phase (42%) when treated with 4-OHT relative to E2-treated (control) cells. Similar results were obtained when cells were analyzed 24 and 72 h after hormonal treatment (data not shown).

We next analyzed the phosphorylation status of retinoblastoma, a key regulator of the G1-to-S-phase transition (43) and a major growth inhibitory effector in 4-OHT-treated MCF-7 cells (44–46). Consistent with the results of the flow cytometry, 4-OHT treatment did not induce retinoblastoma activation (hypophosphorylation) in MCF-7.0.3 or MCF-7.3.28 cells (Fig. 2B, lanes 6 and 10, respectively) as seen in MCF-7 American Type Culture Collection cells (Fig. 2B, lane 2). In contrast, treatment of MCF-7.0.3 and MCF-7.3.28 cells with MIF induced a marked retinoblastoma activation within 48 h that was not seen in MCF-7 American Type Culture Collection cells (Fig. 2B, compare lanes 7 and 11 to lane 3). Similar results were obtained when MCF-7.3.28 and MCF-7.0.3 cells were analyzed 72 h after hormonal therapies and with MCF-7.3.6 and MCF-7.3.25 cells (data not shown). These combined experiments demonstrate that MCF-7.0.3, MCF-7.3.28, MCF-7.3.25, and MCF-7.3.6 are ERα-expressing, 4-OHT-resistant MCF-7 sublines that can be effectively growth-inhibited by MIF treatment.

MCF-7.0.3 and MCF-7.3.28 Show an Attenuated Response to the Pure Antiestrogen ICI 182,780. Previous studies have demonstrated that 4-OHT-resistant MCF-7 variants (47) and patients with advanced, tamoxifen-resistant breast cancers (48) can be sensitive to other antiestrogens. Therefore, we determined whether 4-OHT-resistant MCF-7.0.3 and MCF-7.3.28 cells were sensitive to the pure ER antagonist ICI 182,780. In addition, we analyzed the antiestrogen sensitivity profile of the MCF-7 subline, MCF-7 AP, from which these clones were isolated to determine whether it also expressed antiestrogen resistance (37). After 96 h of ICI 182,780 treat-
ment, the total cell number was not significantly decreased in MCF-7.0.3 cells as compared with the E2-treated cells (Fig. 3). MCF-7.3.28 cells also showed an attenuated response to ICI 182,780 (Fig. 3). In agreement with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays, 4-OHT did not significantly alter the total number of MCF-7.0.3 or MCF-7.3.28 cells relative to that of E2-treated cells. Cells were, however, sensitive to MIF and MIF plus 4-OHT treatment (Fig. 3). In comparison, MCF-7 American Type Culture Collection and MCF-7 AP cells treated for 96 h with 4-OHT, ICI 182,780, MIF, and 4-OHT plus MIF showed significant decreases in cell number when compared with E2-treated cells (Fig. 3). These results suggest that the mechanism of 4-OHT resistance in these sublines also confers partial resistance to other antiestrogens. Furthermore, the fact that these cells had never been exposed to 4-OHT suggested that MCF-7.0.3 and MCF-7.3.28 cells were pre-existing, antioestrogen-resistant clonal variants in the MCF-7 AP population or, alternatively, that these cells acquired antioestrogen resistance during the transfection and selection of these sublines (37).

4-OHT Sensitivity Varies Among Individual Cells within the MCF-7 Population. To determine whether clonal variants existed in the MCF-7 AP cell population, we used conventional cloning methodologies and established 13 independent clones from the MCF-7 AP cell line, designated clones 1–9 and 11–14. These clones were tested for sensitivity to 4-OHT, MIF, and 4-OHT plus MIF combination therapy (Fig. 4). The individual clonal variants expressed a large degree of variation in 4-OHT sensitivity, ranging from a 59.8% reduction in growth (clone 11) to no reduction in growth (clone 2) when compared with E2-treated cells (Fig. 4A). All of the clones expressed ERα that was downregulated by E2. In addition, each of the clonal variants expressed E2-inducible PR that was inhibited in the presence of 4-OHT. E2 stimulation of PR in MCF-7.0.3 cells was observed with prolonged exposure of the film (data not shown). Regardless of the sensitivity profiles of the clones to 4-OHT, MIF as a monotherapy or as a combination therapy with 4-OHT inhibited their growth (Fig. 4C), even when the dose of MIF was reduced to 2.0 μM (Fig. 4D). These data identify clonal diversity within the MCF-7 AP cell line that includes 4-OHT resistance (clone 2). In addition, these data demonstrate that 4-OHT resistance is not due to the loss of ERα expression or aberrant ER function and indicate that the mechanism(s) that impart 4-OHT resistance to ER- and PR-expressing breast cancer cells do not confer MIF resistance.

![Fig. 4](image-url) The isolation of 4-hydroxytamoxifen (4-OHT)-resistant, mifepristone (MIF)-sensitive, and estrogen receptor (ER)-expressing clonal variants from an MCF-7 subline. The MCF-7 AP cell line was subjected to clonal selection in the absence of 4-OHT selection. Thirteen individual clones were isolated and expanded for additional analyses, which included determining their sensitivity to 1 μM 4-OHT (A), their expression levels of ERα and progesterone receptor (PR; A and B forms; B), their sensitivity to 10 μM MIF (C) and to 2 μM MIF (D). Cell counting and immunoblotting was performed as described in “Materials and Methods.” For cell quantitation experiments, three independent experiments were performed. Statistical significance (P < 0.05) was determined using Student’s t test and is indicated by * The data are graphed as mean; bars, ±SE. 4-OHTS = 4-OHT-sensitive; 4-OHTR = 4-OHT-resistant; ATCC, American Type Culture Collection; E2, 17β estradiol.
MIF Induces Cell Death with Poly(ADP-Ribose) Polymerase Cleavage and Caspase-3 Activation as Measurable Outcomes in Antiestrogen-Resistant MCF-7 Cells. Because inherent clonal variation explains the antiestrogen-resistant phenotype of MCF-7.0.3 and MCF-7.3.28 sublines, we believe that these sublines provide valuable ERα-expressing, antiestrogen-resistant model systems in which to study the underlying mechanism of MIF antitumor activity. Therefore, we performed studies to determine whether MIF induced active cell death, in addition to growth arrest, in the antiestrogen-resistant MCF-7.0.3 and MCF-7.3.28 cells. We used cell detachment from the monolayer, poly(ADP-ribose) polymerase cleavage (49, 50), caspase activation, and DNA laddering as outcome measures of cell death. 4-OHT and MIF induced cell lifting and poly(ADP-ribose) polymerase cleavage in MCF-7 American Type Culture Collection (Fig. 5, A and B) and MCF-7 AP cells (Fig. 5A; data not shown). Treatment of MCF-7.0.3 and MCF-7.3.28 cells with MIF also induced cell lifting and poly(ADP-ribose) polymerase cleavage (Fig. 5, A and B). In contrast, 4-OHT treatment of MCF-7.0.3 or MCF-7.3.28 cells did not induce cell lifting or poly(ADP-ribose) polymerase cleavage. Thus, MIF induced active cell death in antiestrogen-resistant breast cancer cells. Furthermore, MIF treatment induced a significantly higher number of floating cells in caspase-3-expressing MCF-7.3.28 cells when compared with caspase-3-deficient MCF-7.0.3 cells (Fig. 5A). Although inherent clonal variation could potentially explain this difference, clones isolated from MCF-7 AP did not demonstrate the same degree of variation in response to MIF treatment as they did in response to 4-OHT treatment (Fig. 5, A and B). So, the increased cell lifting could be explained by MIF-induced caspase-3 activation with an enhanced death response. An enhanced death response with secondary proteolysis of the 89 kDa fragment could also account for the reduced levels of cleaved poly(ADP-ribose) polymerase observed in MCF-7.3.28 cells after 72 h of MIF or MIF plus 4-OHT treatment (Fig. 5B).

To determine whether MIF activated caspase-3 in MCF-7.3.28 cells, caspase-3 activity assays were performed. Before assaying caspase-3 activity in response to hormonal therapy, we repeated earlier studies (37) and used tumor necrosis factor α treatment to confirm that procaspase-3 was expressed in MCF-7.3.28 cells and activated appropriately in response to an apoptotic stimulus. The increase in caspase-3 activity levels correlated directly to a reduction in the levels of procaspase-3 (Fig. 6, A and B). The kinetics of caspase-3 activation in response to 4-OHT, MIF, and combination therapy 4-OHT plus MIF was then examined. In MCF-7.3.28 cells, MIF induced a significant increase (128.1 ± 2.2%) in caspase-3 activity as early as 24 h after treatment. Caspase-3 activity continued to increase (552.7 ± 64.1%) up to 96 h after treatment (Fig. 6C). Also, in response to combination treatment, caspase-3 activity was significantly increased after 72 h and 96 h of treatment (380.56 ± 13.58% and 524.1 ± 35.6%, respectively; Fig. 6C). In stark contrast, at 24 h and 48 h after treatment, 4-OHT appeared to suppress the activity of caspase-3 to 47.6 ± 2.3% and 65.8 ± 3.2%, respectively, of the E2-treated control cells (Fig. 6C). Statistically significant but very small increases in caspase-3 activity were measured in response to 4-OHT treatment at later time points (Fig. 6C). Furthermore, in MCF-7.3.28, MCF-7.3.6, and MCF-7.3.25 cells (Fig. 6D), the proenzyme levels of caspase-3 were easily detected and appeared to increase in response to 4-OHT treatment (Fig. 6D, lane 2) and decrease in response to MIF treatment (Fig. 6D, lane 3) when compared with levels in E2-treated cells (Fig. 6D, lane 1). However, the disappearance of the caspase-3 proenzyme did not correlate precisely with caspase-3 activation (i.e., treatment with combination therapy; Fig. 6D, lane 4), possibly because treatment with 4-OHT increases the stability of procaspase-3 as it does for ERα (41, 42; Fig. 1B). During the course of these experiments, we never detected procaspase-3 in MCF-7.0.3 (Fig. 6, B and D), MCF-7 American Type Culture Collection, MCF-7 AP cells, or the MCF-7 AP clonal variants (data not shown). Consistent with the required role of caspase-3 for internucleosomal DNA cleavage during apoptosis (37), hormonal treatment of MCF-7 or MCF-7.0.3 cells did not result in DNA laddering, whereas DNA laddering was detected in MCF-7.3.28 cells treated with MIF and combination treatment (4-OHT plus MIF; data not shown).

Fig. 5 Mifepristone (MIF) induces cell detachment and apoptosis of antiestrogen-resistant, caspase-3-expressing MCF-7 cells. A, the number of MCF-7, MCF-7 AP, MCF-7.0.3, and MCF-7.3.28 floating cells was determined 96 h after 4-hydroxytamoxifen (4-OHT), ICI 182,780, MIF, and combination therapy (4-OHT plus MIF). The procedure for floating cell quantitation and Western analysis is described in “Materials and Methods.” Three independent experiments were performed, and statistical significance (*, *P < 0.05) was determined using Student’s t test. The data are graphed as mean; bars, ±SE. B, the relative levels of poly(ADP-ribose) polymerase (PARP) proteolysis after 4-OHT, MIF, and 4-OHT plus MIF were determined using immunoblot analysis; ATCC, American Type Culture Collection; E2, 17-β estradiol.
Fig. 6 Mifepristone (MIF) induces caspase-3 activation and cell death in 4-hydroxytamoxifen (4-OHT)-resistant MCF-7.3.28 cells. In control experiments, tumor necrosis factor (TNF)α induces caspase-3 activity in MCF-7.3.28 cells after 18 h of treatment (A), and caspase-3 induction correlates to decreases in procaspase-3 in a time dependent manner (B). MIF, alone and in combination with 4-OHT, induces significant caspase-3 activity, whereas 4-OHT monotherapy only marginally increases caspase-3 activity (C). Procaspase-3 levels are reduced in response to MIF treatment of MCF-7.3.6, MCF-7.3.25, and MCF-7.3.28 cells in the absence of 4-OHT treatment (D). Caspase-3 activity and immunoblotting were performed as described in “Materials and Methods.” Three independent experiments were performed, and statistical significance (*, P < 0.05) was determined using Student’s t test. The data are graphed as mean; bars, ±SE; E2, 17β-estradiol.

MIF Induces Caspase-8 and -9 Activation in Antiestrogen-Resistant Breast Cancer Cells. To additionally define the mechanism of MIF-induced cell death, we characterized caspase-8 and caspase-9 activity profiles during a course of hormone treatment (Fig. 7). Traditionally, caspase-8 activation is used as an indicator of activation of the extrinsic pathway of apoptosis that typically involves activation of a death receptor, whereas caspase-9 activation indicates involvement of the intrinsic mitochondrial pathway of apoptosis (51). However, recent studies have demonstrated a significant degree of cross-talk between these two death pathways, in part mediated by caspase-3 activation. Once activated, caspase-3 can amplify apoptosis by activating caspase-8 or -9 as part of a positive feedback loop (52–54).

After 48 h, MCF-7.0.3 cells showed an increase in caspase-8 activity in response to MIF (164.4 ± 4.1%) and 4-OHT plus MIF treatments (175.3 ± 4.6%) as compared with control cells (Fig. 7A). Similarly, in MCF-7.3.28 cells, 48 h of treatment with MIF (230.9 ± 13.3%) and 4-OHT plus MIF (122.6 ± 5.1%) induced significant increases in caspase-8 activity in comparison with control cells (Fig. 7B). Similar changes were observed in caspase-9 activity. In MCF-7.0.3 cells, 48 h of MIF (138.1 ± 3.5%) or 4-OHT plus MIF treatment (148.4 ± 2.9%) induced significant increases in caspase-9 activity relative to that in control cells (Fig. 7C). In MCF-7.3.28 cells, relative activity compared with control cells was also increased for caspase-9 to 252.5 ± 11.3% and 126.0 ± 6.2% for MIF and 4-OHT plus MIF treatments, respectively, after 48 h (Fig. 7D). In contrast, 4-OHT significantly decreased caspase-8 and -9 activity in MCF-7.3.28 cells by 48 h of treatment (Fig. 7, B and D). 4-OHT increased caspase-8 activity at 48 h after treatment in MCF-7.0.3 cells, but this increase is not remarkable (Fig. 7, A and C). These studies show that 4-OHT inhibits and MIF stimulates the activation of caspase-3, -8, and -9 as part of an active cell death program in ER−PR−, antiestrogen-resistant breast cancer cells.

DISCUSSION

Antiestrogen-resistant breast cancer is still a major obstacle in the clinical setting even after decades of research devoted to the development of selective estrogen receptor modulators, ER down-regulators, and aromatase inhibitors. Although understanding the underlying mechanisms of antiestrogen resistance are crucial to the development of specific therapeutic targets, it is also important to identify adjuvant therapies that can inhibit the growth of antiestrogen-resistant tumor cells. Our preclinical in vitro and in vivo studies demonstrate that antiestrogen therapy in combination with antiprogestin therapy induces growth arrest and cell death of antiestrogen-sensitive breast cancer cells more effectively than either monotherapy (34, 35). In the current study, we uniquely demonstrate that MIF induces growth arrest, caspase activation, and cell death in ER-expressing, antiestrogen-resistant breast cancer cells. Importantly, when MIF was administered to antiestrogen-resistant tumor cells, the presence of 4-OHT did not significantly abrogate MIF-induced antitumor activity. To our knowledge, this is the first preclinical study to demonstrate that antiprogestin therapy, in the presence or absence of 4-OHT, is effective against antiestrogen-resistant breast cancer cells.

The antiestrogen-resistant sublines used in this study were derived in two independent experiments. Initially, we charac-
characterized MCF-7 sublines that were selected after transfecting a population of MCF-7 cells with either the pcDNA3 vector (designated MCF-7.0.3) or the pcDNA3 vector containing the caspase-3 cDNA (designated MCF-7.3.25, MCF-7.3.28, and MCF-7.3.6; Ref. 37). All four of these sublines expressed resistance to 4-OHT. In addition, these sublines showed an attenuated response to the pure antiestrogen ICI 182,780 as compared with the MCF-7 American Type Culture Collection cell line. These 4-OHT-resistant sublines expressed ER and PR. Therefore, antiestrogen resistance was not due to loss of the ER receptor. The possibility existed that either the selection protocol with geneticin (selective drug for the pcDNA3 expression vector) or the DNA transfection itself resulted in the clonal selection of antiestrogen-resistant clonal variants. However, MCF-7 cells transfected with the pcDNA vector were obtained from the laboratory of Derek LeRoith (National Cancer Institute, NIH, Bethesda, MD) and were antiestrogen sensitive under our treatment conditions (data not shown). Thus, it did not appear that transfection per se selected for these 4-OHT-resistant variants. We then postulated that 4-OHT-resistant variants existed in the parental MCF-7 population of cells and could be identified by clonal selection. To test this possibility, clonal selections were performed, in the absence of antiestrogen selective pressure, on the parent MCF-7 cell line used in the laboratory of A. G. P. (designated MCF-7 AP) from which the MCF-7.0.3, MCF-7.3.25, MCF-7.3.28, MCF-7.3.6, and MCF-7.0.3 sublines were derived. Fourteen clones of various sizes were selected and expanded. One clone died with propagation. The remaining 13 clonally derived sublines expressed ER and PR. Therefore, antiestrogen resistance was not due to loss of the ER receptor.

The antiestrogen-resistant phenotype of the MCF-7.0.3 and MCF-7.3.28 sublines corresponded to the inability of 4-OHT to induce G1 arrest, retinoblastoma activation, caspase activation, and cell death. As in our earlier studies (34, 35), we used 1 μM 4-OHT and 10 μM MIF to treat MCF-7 cells, with the exception of studies detailed in Fig. 4D that were performed with 2 μM MIF treatments every 48 h. A circulating level of ~1.3 μM tamoxifen has been demonstrated in the serum of patients on tamoxifen therapy (55, 56); therefore, we consider this dose physiologically relevant. A 1 μM 4-OHT concentration results in approximately 20–30% growth inhibition of MCF-7 cells treated for 72–96 h. In a similar fashion, 72–96 h of 10 μM MIF treatment inhibits MCF-7 cell growth by approximately 20–30%. Because 10 μM MIF and 1 μM 4-OHT are ineffective in blocking the growth of ER- and PR-negative MDA-231 cells.
(57), we believe that a significant amount of the MIF and 4-OHT activity are mediated by binding to PR and ER, respectively.

The fact that 4-OHT-resistant cells were growth arrested and underwent apoptosis in response to MIF treatment suggests that MIF-induced antitumor activity occurs via a unique set of signaling events than growth arrest and cell death induced by 4-OHT. If the molecular events that lead to cell death differ when ER is blocked as compared with when PR is blocked, then a combination treatment regimen of antiestrogen plus antiprogestin treatment may have additive therapeutic potential. Other preclinical studies have demonstrated antitumor efficacy for MIF in the treatment of prostate cancer cells (58, 59). In these studies, MIF up-regulated death receptor expression in prostate tumor cells and sensitized cells to additional therapeutic approaches (60). Thus, MIF may have value as a chemosensitizing agent, chemotherapeutic agent, or an adjuvant hormonal agent.

The presence of inherently antiestrogen-resistant clonal variants in an MCF-7 cell line underscores the caution that must be used in interpreting data based on the isolation and expansion of clones stably transfected with various cDNAs. The heterogeneity of the MCF-7 population with regard to 4-OHT resistance is consistent with earlier studies by Osborne et al. (61) detailing distinct differences between the MCF-7 American Type Culture Collection cell line and cell lines passaged in various independent laboratories, including a slower growth rate for the MCF-7 American Type Culture Collection cells. In a similar fashion, we consistently observed that MCF-7 American Type Culture Collection cells grow slower than the MCF-7 AP cell line in E2-supplemented medium, although we did select at least one clone (clone 12) that showed a similar growth rate to that of MCF-7 cells. E2-stimulated growth of clone 12 was similar to that of MCF-7 American Type Culture Collection in that it was reduced significantly when compared with that of the 4-OHT-resistant clones like MCF-7.0.3, MCF-7.3.28, and clone 2 (Fig. 4C). We have not yet determined the underlying mechanism(s) of 4-OHT resistance in clone 2 selected in this study or in the sublines MCF-7.3.28, MCF-7.3.25, and MCF-7.3.6. We believe that the mechanism of resistance, once identified, will also account for the overall reduction in the number of detached cells that we noted consistently in these studies for the MCF-7 AP, MCF-7.0.3, and MCF-7.3.28 cell populations that were treated with E2, 4-OHT, and/or MIF (Fig. 5A).

We are currently analyzing several potential mechanisms of the observed 4-OHT resistance, including mechanisms that have been described in other in vitro models including altered binding of coactivator and corepressor molecules to the antiestrogen/ER complex (24, 62, 63), constitutive activation of the mitogen-activated protein/extracellular signal-regulated kinase kinase/mitogen-activated protein kinase signaling pathway (64–66), and altered ER protein due to altered mRNA splicing (67).

In human patients, many of the molecular changes that occur when breast tumors transition from a tamoxifen-sensitive to a tamoxifen-resistant phenotype are still not well defined. Although it is not known what percentage of breast cancers that acquire tamoxifen resistance with treatment actually express ER and PR, it is clear that in most cases acquired resistance is not due to a loss or mutation of the ER (68). The positive ER status of cancer cells surviving tamoxifen therapy is supported by a recent clinical study that demonstrates that postmenopausal women placed on letrozole, as compared with placebo, after 5 years of tamoxifen treatment for early stage breast cancer show a marked reduction (43%) in cancer recurrence (69). The frequency of inherent tamoxifen resistance expressed by ER-expressing tumors is also quite high (approximately 25–30%). Because the majority of ER− antiestrogen-resistant tumors likely express PR, antiprogestin treatment in combination with antiestrogen treatment may offer some clinical benefit to patients, particularly in light of the studies described herein. In summary, the data presented in this study support the consideration of MIF to be used in conjunction with antiestrogen strategies for the treatment of breast cancer.

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