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Report

Additive effect of mifepristone and tamoxifen on apoptotic pathways in MCF-7 human breast cancer cells

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Summary

MCF-7 cells growing in culture were used to study the mechanism of the antiproliferative activity of the antiprogestin mifepristone, as compared with the antiestrogen 4-hydroxytamoxifen or the combination of both. These steroid antagonists induced a significant time- and dose-dependent cell growth inhibition (cytotoxicity). This inhibition of cell survival was associated with a significant increase in DNA fragmentation (apoptosis), downregulation of bcl_2, and induction of $TGF\beta_1$ protein. Abrogation of the mifepristone- and/or 4-hydroxytamoxifen-induced cytotoxicity by $TGF\beta_1$ neutralizing antibody confirms the correlation between induction of active $TGF\beta_1$ and subsequent cell death. The effect of a combination of mifepristone and 4-hydroxytamoxifen on cell growth inhibition, on the increase in DNA fragmentation, bcl_2 downregulation, and induction of $TGF\beta_1$ protein was additive and significantly different ($P < 0.05$) from the effect of monotherapy. A translocation of protein kinase C (PKC) activity from the soluble to the particulate and/or nuclear fraction appeared to be also additive in cells treated with a combination of both 4-hydroxytamoxifen and mifepristone. These results suggest that the mechanism of the additive antiproliferative activity of mifepristone and tamoxifen could be explained at least in part by an additive induction of apoptosis in both estrogen and progesterone receptor positive MCF 7 breast cancer cells. A bcl_2 downregulation, the PKC transduction pathway, and $TGF\beta_1$ expression seem to be involved in this additive mechanism of action. Our data further suggest that a combination of an antiprogestin with tamoxifen may be more effective than tamoxifen monotherapy in the management of human breast cancer.

Introduction

Antiprogestins are a promising new class of mammary tumor inhibitors with a unique mechanism of action which does not seem to be simply the result of progesterone withdrawal [1]. Our previously published results have shown that antiprogestins possess a potent antitumor activity in hormone-dependent *in vivo* breast cancer models [2–5]. The antiproliferative action of antiprogestins is mediated via the progesterone receptor (PR) and is related to induction of cell differentiation and apoptosis [1–5]. Moreover, it has been shown that the antiprogestin mifepristone induces PR-mediated cell death in human breast cancer cells in culture [6, 7]. Induction of apoptosis in breast cancer cells has been reported *in vitro* and *in vivo* also as a result of estrogen ablation or antiestrogen treatment [7–10]. In some of these

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studies, induction of apoptosis was associated with an early effect on 'cell death' gene expression and on induction of transforming growth factor beta$_1$ (TGFβ_1) protein [8, 9]. The addition of antiestrogens to the antiprogestin treatment showed an additive growth inhibitory effect in several hormone-dependent breast cancer models [5, 11, 12]. The initial interaction of the antiprogestin or the antiestrogen with the PR or estrogen receptor (ER), respectively, seems to be a necessary first step in initiating an as yet ill-defined cascade of events leading to apoptosis of breast cancer cells [1–7]. Details of the molecular biochemical pathway of this receptor mediated transcription of specific 'cell death genes' leading to activation of the cell suicide mechanism are still unknown. Furthermore, the precise molecular biochemical mechanism of the additive antitumor activity of antiprogestins in combination with antiestrogens needs to be clarified.

The bcl$_2$ gene is important for negative regulation of apoptosis and is highly expressed in normal human breast and in human breast cancer [13–15]. In the MCF-7 human breast cancer cells, the bcl$_2$ mRNA levels increase in a time- and concentration-dependent fashion as a result of estradiol exposure [16]. Clinical studies have shown that bcl$_2$ expression is associated with favorable prognostic factors in patients with breast cancer such as hormone receptor positivity, well differentiated tumors, intact p53, and epidermal growth factor negativity [14, 15, 17–21]. Recent results have also indicated that high bcl$_2$ expression is directly related to higher ER level in human breast cancer tissue [14, 17, 21, 22] and is associated with a better and more sustained response to tamoxifen as well as with longer time to treatment failure and better survival [23]. To our knowledge, there is no published information regarding the effect of mifepristone as compared with tamoxifen or the combination of both on bcl$_2$ expression in breast cancer.

In human breast cancer cells, (a) modulation of protein kinase C (PKC) activity by estrogens and antiestrogens [24–32], (b) elevated level of nuclear PKC in a multidrug resistant MCF 7 subline [33], and (c) an inverse relationship between PKC activity and estrogen receptor expression [28] indicate that the PKC signaling transduction pathway is involved in estrogen/antiestrogen modulated breast cancer cell growth. The antitumor activity of antiestrogens such as tamoxifen correlates with alterations of PKC activity [25, 26, 29, 30]. Furthermore, TGFβ_1 expression has been shown to be involved in the PKC mediated inhibition of breast cancer cell proliferation [34]. It is therefore reasonable to assume that tamoxifen (and mifepristone) may induce apoptosis via changes in the PKC activity and TGFβ_1 expression.

The objective of this study was to explore the role of bcl$_2$ expression, PKC, and TGFβ_1 protein for induction of apoptosis in ER and PR positive MCF-7 human breast cancer cells growing in culture as a treatment response to mifepristone, 4-hydroxytamoxifen, or the combination of both. The effect of estrogen withdrawal (ablation) was also investigated. In this paper, *in vitro* results are presented to confirm that the additive antiproliferative activity of 4-hydroxytamoxifen and mifepristone is associated with a significant increase in DNA fragmentation (apoptosis), bcl$_2$ downregulation, and induction of TGFβ_1 protein. Induction of apoptosis was also associated with alterations of PKC activity and subcellular distribution.

Materials and methods

Cell line and culture conditions

The MCF-7 human breast cancer cells (passage number: 149) were obtained from American Type Culture Collection (ATCC), Rockville, MD. The cells were cultured in Dulbecco's Modified Eagle medium (DMEM), low glucose, containing 1 mM sodium pyruvate, 10 µg/ml bovine insulin, and 10% fetal bovine serum (FBS). They were maintained in a 37 °C, 5% CO_2 incubator. Prior to experimental treatment, the cells were estrogen depleted by culturing in phenol red-free DMEM/F12 medium (containing 10 µg/ml insulin) with 10% dextran-coated charcoal (DCC) treated serum for at least 7 days. After stripping, cells were stepped down to 5% DCC treated FBS without insulin, and plated into 96-well plates or T75 flasks (PKC assays, Western analysis). On the following day, 10 nM 17β-es-

tradiol (E_2) was added to all control (except the E_2 ablation groups) and drug-treated cultures to stimulate PR induction and to maintain cell growth and proliferation in a standard fashion.

Drug treatment and control experiments

The drugs were dissolved in 100% ethanol as stock solutions. Dilutions were performed in 5% DCC treated FBS in DMEM-F_{12} medium without insulin and with 10 nM E_2. The final concentration of ethanol in all control and treated cultures was 0.05–0.5%. To determine optimal antiproliferative drug concentrations, six wells of 96-well plates were treated with a wide range (1–10,000 nM) and later a narrow range of mifepristone or 4-hydroxytamoxifen to identify the dose-response relationship. A baseline culture was harvested on day 0; remaining cultures were treated with mifepristone, 4-hydroxytamoxifen, or vehicle control, and harvested at 1–2 day intervals for 7 days to determine the time-response relationship. In some experiments, the effects of the addition of E_2 (10 nM) versus E_2 withdrawal (E_2 ablation), as well as the effects of 4-hydroxytamoxifen without and with excess E_2 (1–2 μM), were compared. Other control experiments were performed using a recombinant human $TGF\beta_1$ protein and a specific neutralizing antibody to human $TGF\beta_1$ (R&D Systems, Minneapolis, MN) to confirm the role of the changes in $TGF\beta_1$ protein for the *in vitro* growth inhibition (cytotoxicity) induced by E_2 ablation or by 4-hydroxytamoxifen and/or mifepristone treatment. Once the optimal concentrations of the different steroid antagonists were established in the cell growth inhibition assays, six wells of 96-well plates or T75 flasks were treated with mifepristone (10 μM), 4-hydroxytamoxifen (1 μM), or the combination of both (10 + 1 μM). Cells were harvested at 2 day intervals for 7 days.

Drugs and chemicals

Mifepristone (Roussel-Uclaf/Population Council) was generously provided by Schering AG, Berlin, Germany. 4-hydroxytamoxifen was purchased from ASH-Stevens, Detroit, MI. The origin of specific antibodies, ELISA kits, and other important reagents and chemicals, etc., was mentioned under the corresponding description of the different techniques used in this investigation. Tissue culture media and reagents were purchased from Sigma Chemical, St. Louis, MO, Hyclone, Logan, UT, and Life Technologies, Grand Island, NY. All of the chemicals used were of reagent grade and were obtained commercially.

Cytotoxicity/cell viability assay

Viable cells were quantitated using the sulforhodamine B (SRB) assay as described previously [35, 36]. This protein dye-binding assay is based on the measurement of whole-culture protein content to determine cell growth and cell viability (fraction of surviving cells). It was used to determine the growth inhibitory effect (inhibition of cell survival or reduction of cell protein mass) of E_2 ablation as well as of mifepristone, 4-hydroxytamoxifen or both on the MCF-7 breast cancer cells *in vitro*. Each compound was tested at five dose levels done in triplicate, to enable construction of dose-response curves and estimation of IC_{50} values (concentration of drug resulting in a ratio of test values to control values (T/C) of 50%, i.e. 50% inhibition of cell survival). The IC_{50} was calculated with dose-analysis computer software which employed the median effect equation [36]. The concentrations were recorded in our data base in μM. Briefly, cells were transferred in 100 μl aliquots (5×10^4 cells/ml) to a 96 well microplate and incubated with the different drug treatments for different periods of time. Surviving or viable cells were fixed *in situ* by withdrawing the growth media and adding 100 μl each of cold Hanks' balanced salt solution and 100 μl 50% trichloracetic acid, and incubated at 4 °C for 60 minutes. The supernatants were removed and wells were washed five times with ice-water. After the wells were dried, 50 μl of SRB solution (0.4% in 1% acetic acid) was added, followed by an 8 minute incubation at room temperature. Unbound SRB was then removed by washing five times with 1% acetic acid,

followed by air drying. Bound stain was solubilized with 150 µl of 10 mM Tris buffer. The absorbency was read at 520 nm on a Vmax microplate reader (Molecular Devices, Menlo Park, CA). Each determination was based upon six wells reading to calculate the mean ± standard error of the mean (SEM).

Cell death detection ELISA

We used DNA fragmentation as a characteristic feature of apoptosis. DNA fragmentation was measured in control and treated MCF-7 cells growing in culture using a specific Cell Death Detection ELISA kit. This ELISA kit (Boehringer Mannheim, Indianapolis, IN) is based on detection of histone coated mono- and oligonucleosomes using antihistone as the capturing antibody followed by anti-DNA conjugated antibodies for detection. After appropriate treatment, the cells were washed once with PBS, counted using a hemacytometer, and 100 µl lysis buffer was added per 1×10^4 cells. After a 30-minute incubation at room temperature, the cytosolic extract was recovered after centrifugation at 200 g for 15 minutes and assayed for soluble DNA-histone fragments according to manufacturer's protocol and as previously described [16]. Each treatment was performed in triplicate. Absorbance at 405 nm was determined on a microplate reader. The results obtained from the DNA fragmentation ELISA were then normalized for cell number, and the results are expressed relative to E_2 control. The specific enrichment or induction of mono- and oligonucleosomes released into the cytoplasm was calculated from the absorbance values using the following formula:

$$\frac{\text{mU of the treatment sample (dying/dead cells)}}{\text{mU of the corresponding } E_2 \text{ control}} = \text{Enrichment factor}$$

$$\text{mU} = \text{absorbance } [10^3]$$

Thus, an increase in DNA fragmentation and in enrichment factor indicates an increase in the number of cells undergoing apoptosis.

bcl_2 ELISA

A bcl_2 ELISA kit (Oncogene Research Products, Calbiochem, Cambridge, MA) was used for the *in vitro* quantitation of bcl_2 protein. After appropriate treatment, the cells were washed once with PBS and harvested by scraping and gentle centrifugation. Antigen Extraction Agent was added to the cell pellets in a resuspension buffer (1 ml buffer added per 5×10^6 cells). After a 30 minute incubation on ice with occasional vortexing, the supernatant was recovered after centrifugation at 10,000 RPM for 5 minutes at 4 °C and assayed for bcl_2 protein according to the manufacturer's protocol. Each treatment was performed in triplicate. The concentration was determined by interpolation from the standard curve by using Microplate Manager/PC Data Analysis Software. The results from the standard curve were normalized for mg cellular protein.

Western analysis of bcl_2 protein

The relative amounts of bcl_2 protein were quantitated using Western blot analyses. Floating cells from each flask were collected and pooled with the remaining attached cells that were released from the monolayer by trypsinization. Cells were spun and washed in 5.0 ml PBS. The cell pellet was resuspended in 300 µl of PBTDS (lysis buffer: 1 × PBS, 1% Triton × 100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1.0 mM EDTA, 0.5 µg/µl leupeptin, 1.0 µg/µl pepstatin, 1.0 µg/µl paraformethylsulfonyl fluoride, aprotinin 1.0 µg/ml). The cell suspension was incubated on ice for 10.0 minutes, and centrifuged at 10,000 × g at 4 °C to pellet cell debris. Supernatants were transferred to fresh tubes and protein content was determined using BCA Protein Assay Reagent (Pierce, Rockford, IL). Equal amounts of protein were separated by 10% SDS-PAGE and electroblotted onto nitrocellulose membrane (Amersham Life Science, Arlington Heights, IL). Bcl_2 protein was localized using an anti-bcl_2 mouse monoclonal antibody (Oncogene Research Products, Calbiochem, Cambridge, CA). The mouse monoclonal bcl_2 antibody (clone 100), raised against amino acids 41–54 of the 26 kDa bcl_2

protein, was used at a concentration of 2.5 μg/ml. Bound primary antibody was visualized using a peroxidase conjugated secondary polyclonal antimouse IgG (1:1500 dilution) (Jackson Immune Research Laboratories, Inc., West Grove, PA), detected by enhanced chemical luminescence (ECL detection kit, Amersham Life Science). Control blots, incubated in secondary antibody alone, were free of bands. The blots were scanned to measure band intensity using an imaging densitometer (Model Gs-671, BIORAD, Hercules, CA). Because protein transfer conditions routinely resulted in approximately 50% transfer, protein loading differences were determined as follows: gels were stained with Coomassie blue following protein transfer to nitrocellulose membrane, and scanned using the imaging densitometry in reflectance mode, and individual protein band intensities were quantitated using Molecular Analysis Software (BIORAD, Hercules, CA).

TGF β_1 ELISA

The TGFβ_1 protein was quantitated using the Quantikine human TGFβ_1 ELISA kit (R&D Systems, Minneapolis, MN). Cells were processed and assayed according to the manufacturer's protocol. Briefly, cells were treated with mifepristone, 4-hydroxytamoxifen, or their combination. After treatment, conditioned medium was collected by centrifugation at 600 g for 5 minutes and stored at – 70 °C until used for the assay. TGFβ_1 protein concentrations were determined in the cell culture supernatant by plotting absorbance readings at dual wavelength of 450/595 for unknowns on a standard curve derived from dilutions of the TGFβ_1 protein standard. Each treatment was performed in triplicate. Cell numbers were determined by counting using a hemacytometer, and TGFβ_1 values were normalized to cell number.

Neutralization of TGF β_1 bioactivity

The SRB assay was used as described above to measure abrogation of cytotoxicity induced by E_2 ablation or by mifepristone, 4-hydroxytamoxifen, and their combination with a neutralizing anti-human TGFβ_1 antibody (R&D Systems, Minneapolis, MN). Cells were treated with 50, 100, 200, 500, and 1000 μg/ml of the neutralizing antibody. Similarly, cells were also treated with 1.0, 10, and 20 μg/ml human recombinant TGFβ_1 protein (R&D Systems, Minneapolis, MN) to study the cytotoxic effect of exogenous TGFβ_1 on the MCF-7 cells growing in culture. Triple wells for each concentration were evaluated using the SRB cytotoxicity/cell growth inhibition assay. Three independent experiments were performed.

Western blotting and assay of PKC

Utilizing control and drug-treated (12 and 36 hours) MCF-7 cells, soluble, particulate, or nuclear fractions, prepared as previously described [37, 38], were sonicated in radioimmune precipitation (RIP) buffer containing protease and phosphatase inhibitors (50 mM Tris, pH 7.4, 150 mM NaCl, 0.5 mM sodium vanadate, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% deoxycholic acid, 20 μg/ml leupeptin, 20 μg/ml PMSF, 100 U/ml aprotinin, and 10 mg/ml p-nitrophenyl phosphate). An appropriate volume of 2 × sample buffer containing SDS and 6% mercaptoethanol was added and the sample heated at 100 °C for 5 min prior to electrophoresis. Following SDS-PAGE, proteins were Western blotted onto PVDF membranes. Membranes containing proteins of interest were blocked overnight in 10 mM Tris buffer, pH 7.2, containing 150 mM NaCl, 3% calf serum, and 1% nonfat milk and probed using antibodies to PKC α and PKC ε isoforms (Transduction Laboratories, Lexington, KY). Equivalency of loading was assessed by reactivity with an β-actin antibody (Sigma Chemical, St. Louis, MO). Blots were then washed in 10 mM Tris-Cl, pH 7.2, 150 mM NaCl (TBS) containing 0.5% Tween-20, reblocked, and incubated with antimouse IgG (goat) coupled to peroxidase (Transduction Laboratories, Lexington, KY). Following extensive washing in TBS-Tween 20 and TBS, the blot was processed for chemiluminescence detection (ECL detection kit; Amersham Life Science) and exposed to X-ray film

(for further details, see Western analysis of bcl_2 protein).

For direct determinations of enzyme activity, cellular preparations in buffer A (20 mM Tris/Cl, pH 7.5, 2 mM EDTA, 50 mM mercaptoethanol) containing protease and phosphatase inhibitors were utilized. PKC activity was measured by determination of the incorporation of ^{32}P from (γ-^{32}P) ATP into a peptide substrate corresponding to residues 4–14 of myelin basic protein. The reaction mixture (100 µl) contained 20 mM Pipes, pH 6.8, MBP peptide (25 µM), 10 mM $MgCl_2$, 250 µM EGTA with or without 500 µM $CaCl_2$, 5 µg phosphatidyl-serine, 500 ng *sn*-1,2-diacylglycerol, and the appropriate sample (45–50 µg/tube). The reaction was initiated by addition of 1 nmol (γ-^{32}P)ATP (0.5–1.0 × 10^6 cpm), incubated for 5 min at 30 °C, and stopped by addition of 45 µl of absolute acetic acid. Aliquots of reaction mixture were spotted onto small squares of Whatman P81 paper and washed sequentially with 30% and 15% acetic acid, followed by acetone. Radioactivity was determined by liquid scintillation counting. The details of this assay have been previously described by Wrenn and Herman [38].

Statistical analysis

Differences among groups were tested using one-way analysis of variance with repeated measures over time. The assumption of analysis of variance was examined and nonparametric tests based on ranks used if needed. Values were reported as means ± standard deviation (SD) or standard errors of the mean (SEM). Statistical analysis was made by ANOVA. When ANOVA indicated significant treatment effects (F-ratio, $P < 0.05$), the Student-Newman-Keuls multirange test was employed to compare individual treatment means. For the statistical evaluation of the IC_{50} Two-Sample Analysis of variance (one-way ANOVA) was performed using Statigraphics Plus statistical graphics system software. Results of the PKC assay were evaluated using analysis of variance with a Tukey post-test.

Results

Cell growth inhibition (cell viability/cytotoxicity)

Mifepristone and 4-hydroxytamoxifen had a significant dose- and time-dependent inhibitory effect on the growth and survival of MCF-7 cells in culture, as determined by the SRB assay (Tables 1–3). 4-hy-

Table 1. Inhibitory effect of 4-hydroxytamoxifen (TAM) and mifepristone (MIF) on MCF-7 cell survival *in vitro* (dose- and time-response relationship, SRB assay)

Groups[a]		% Inhibition of cell survival (mean ± SEM)[b] Days post-treatment			
		1	3	5	7
E_2 + TAM	0.1 µM	1.4 ± 0.70	7.9 ± 3.02	11.4 ± 1.97	17.6 ± 3.31
	0.5 µM	2.9 ± 0.52	21.7 ± 2.41	36.7 ± 0.41	55.2 ± 4.47
	1.0 µM	4.5 ± 1.01	32.2 ± 0.57	53.4 ± 1.59	69.5 ± 2.64
	5.0 µM	7.0 ± 1.39	45.9 ± 2.79	73.8 ± 0.97	84.6 ± 1.23
	10.0 µM	13.2 ± 1.89	65.4 ± 4.19	90.0 ± 1.35	94.7 ± 2.08
E_2 + MIF	1.0 µM	1.9 ± 1.00	4.6 ± 0.93	6.8 ± 0.72	10.6 ± 0.59
	5.0 µM	3.2 ± 0.32	14.0 ± 0.58	21.3 ± 1.98	27.6 ± 0.93
	7.5 µM	6.1 ± 0.74	20.5 ± 2.00	30.8 ± 1.16	45.9 ± 2.39
	10.0 µM	10.1 ± 0.95	34.1 ± 2.97	53.0 ± 3.35	63.2 ± 3.27
	25.0 µM	48.5 ± 1.35	83.7 ± 1.68	96.5 ± 0.60	98.5 ± 0.23

[a] 10 nM 17β-estradiol (E_2) was added to the culture medium in all groups.

[b] Results of three independent experiments as compared with the E_2 control group.

Table 2. Inhibitory effect of 4-hydroxytamoxifen (TAM) and mifepristone (MIF) on MCF-7 cell survival *in vitro* (IC_{50} values, SRB assay)

Groups[a]	IC_{50} in μM (mean ± SEM)[b] Days post-treatment		
	3	5	7
E_2 + TAM	4.45 ± 0.50	1.01 ± 0.07	0.45 ± 0.06
E_2 + MIF	12.34 ± 0.80[c]	7.27 ± 0.07[d]	5.16 ± 0.06[d]

[a] 10 nM 17β-estradiol (E_2) was added to the culture medium in all groups.
[b] Results of three independent experiments as compared with the E_2 control group. Each compound was tested in each experiment at five dose levels (see Table 1), to enable construction of dose-response curves and estimation of IC_{50} values (concentration of the drug resulting in 50% inhibition of cell survival).
[c] Significantly different ($P < 0.002$) as compared with 4-hydroxytamoxifen.
[d] Significantly different ($P < 0.0001$) as compared with 4-hydroxytamoxifen.

droxytamoxifen was significantly more potent than mifepristone (Table 2). The results of the SRB assay consistently indicated that 1 μM 4-hydroxytamoxifen and 10 μM mifepristone were almost equieffective (Table 1). These concentrations could be considered as pharmacologically relevant dose levels clearly below the IC_{50} values (concentration of the drug resulting in 50% inhibition of cell survival) of both drugs up to day 3 (72 hours) post-treatment (Table 2). Therefore, these dose levels were selected as the optimal drug concentrations to be used for monotherapy and combination therapy, throughout all other experiments designed to study the effect of these steroid antagonists on DNA fragmentation, bcl_2 expression, $TGF\beta_1$ protein, and PKC activity and subcellular expression. A significant ($P < 0.05$) additive cell growth inhibition of the combination of 4-hydroxytamoxifen and mifepristone was evident and resulted in an average 20 or 30% more inhibition of cell survival on days 3–7 post-treatment (Table 3). This effect was also significantly different ($P < 0.05$) as compared with that induced by monotherapy as early as one day post-treatment (Table 3). The addition of excess E_2 (1 or 2 μM) to the culture medium protected the MCF-7 cells and significantly ($P < 0.05$) decreased or completely prevented the inhibitory effect of 4-hydroxytamoxifen on cell survival (Table 4). Conversely, removal of E_2 (10 nM) from the culture medium (estrogen ablation) induced a significant time-dependent inhibition of cell survival (up to 65% inhibition on day 7) as compared with E_2 control (Table 3). However, inhibition of cell survival induced by 1 μM 4-hydroxytamoxifen, 10 μM mifepristone, and their combination (in the presence of 10 nM E_2) significantly ($P < 0.05$) exceeded the inhibitory effect of estrogen ablation (Table 3). These results clearly indicate a direct inhibitory effect of these steroid antagonists beyond blocking the stimulatory effect of E_2 on cell viability.

Table 3. Inhibitory effect of 1 μM 4-hydroxytamoxifen (TAM), 10 μM mifepristone (MIF), and their combination, as compared with E_2 ablation, on MCF-7 cell survival *in vitro* (Time-response relationship-SRB assay)

Groups[a]	% Inhibition of cell survival (mean ± SEM)[b] Days post-treatment			
	1	3	5	7
E_2 ablation	3.1 ± 0.28	34.0 ± 2.50	48.3 ± 1.40	65.5 ± 2.20
E_2 + TAM	14.4 ± 1.08[c]	37.7 ± 2.49	55.5 ± 1.47[c]	76.7 ± 1.75[c]
E_2 + MIF	18.6 ± 2.40[c]	53.0 ± 1.05[c]	69.4 ± 2.96[c]	75.0 ± 4.04[c]
E_2 + TAM + MIF	21.3 ± 3.15[d]	68.9 ± 1.59[d]	88.0 ± 1.11[d]	93.5 ± 1.52[d]

[a] 10 nM 17β-estradiol (E_2) was added to the culture medium in all groups except E_2 ablation group.
[b] Results of four independent experiments as compared with E_2 control.
[c] Significantly different ($P < 0.05$) as compared with the E_2 ablation group.
[d] Significantly different ($P < 0.05$) as compared with the E_2 ablation and both 4-hydroxytamoxifen and mifepristone monotherapy groups.

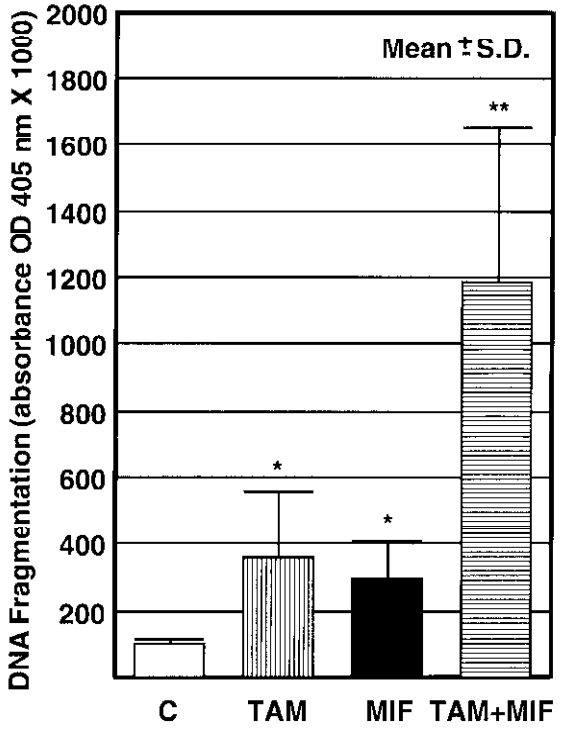

Figure 1. Effect of 1 μM 4-hydroxytamoxifen (TAM), 10 μM mifepristone, (MIF) and their combination on DNA fragmentation in the MCF-7 human breast cancer cells *in vitro*. 10 nM 17β estradiol was added to the culture medium in all groups. Mean difference between control (C) and treated groups (Mean ± SD of three independent measurements during 6 days of treatment). * $P < 0.05$ versus control; ** $P < 0.05$ versus control, 4-hydroxytamoxifen or mifepristone monotherapy. The absorbency was read at 405 nm on a microplate reader. The results were reproducible in two independent experiments (see Table 5).

Cellular DNA fragmentation

In a first experiment, the treatment with 4-hydroxytamoxifen and/or mifepristone increased the DNA fragmentation of the MCF-7 cells growing in culture for 6 days. A 3–4-fold increase of DNA fragmentation was observed as a result of monotherapy with 4-hydroxytamoxifen or mifepristone. This increase was significantly ($P < 0.05$) different from the control group. In the group treated with the combination of 4-hydroxytamoxifen and mifepristone, the average increase in DNA fragmentation was 12-fold that of the control group ($P < 0.05$ versus control). This additive or synergistic effect of the combination therapy on the extent of the increase in DNA fragmentation was also significant ($P < 0.05$) when compared with the effect of 4-hydroxytamoxifen or mifepristone monotherapy. A summary of the results of this first experiment is illustrated in Figure 1.

In a second independent experiment, the time-dependent effect of mifepristone and/or 4-hydroxytamoxifen on DNA fragmentation, was compared with the effect of E_2 treatment, estrogen ablation, and 4-hydroxytamoxifen in combination with excess E_2. A significant ($P < 0.05$) increase in DNA fragmentation was observed as early as 12 hours post-treatment with 4-hydroxytamoxifen, mifepristone or the combination of both, as well as following E_2 ablation (steroid depletion without the addition of 10 nM E_2 to the culture medium) (Table 5). A time-dependent further increase in DNA fragmentation was observed at 36 and 72 hours post-treatment. E_2 treatment of control cultures kept the

Table 4. Effect of excess 17β-estradiol (e E_2) on the inhibitory effect of 1 μM 4-hydroxytamoxifen (TAM) on MCF-7 cell survival *in vitro* (SRB assay)

Groups[a]	% Inhibition of cell survival (mean ± SEM)[b] Days post-treatment			
	1	3	5	7
E_2 + TAM	4.5 ± 1.01	32.2 ± 0.57	53.4 ± 1.59	69.5 ± 2.64
E_2 + TAM + eE_2 1 μM	0.0 ± 0.00[c]	2.7 ± 2.65[c]	8.7 ± 1.65[c]	8.0 ± 3.00[c]
E_2 + TAM + eE_2 2 μM	0.0 ± 0.00[c]	1.5 ± 1.50[c]	1.6 ± 0.95[c]	1.8 ± 0.70[c]

[a] 10 nM E_2 was added to the culture medium in all groups.
[b] Results of three independent experiments as compared with the E_2 control group.
[c] Significantly different ($P < 0.05$) as compared with 4-hydroxytamoxifen without e E_2

DNA fragmentation at low levels up to 72 hours post treatment. An additive effect of a combination of 4-hydroxytamoxifen and mifepristone on the increase in DNA fragmentation was significantly different ($P < 0.05$) from the effect of 4-hydroxytamoxifen or mifepristone monotherapy and from the effect of E_2 ablation (Table 5). At 36 and 72 hours post-treatment, the DNA fragmentation induced by 4-hydroxytamoxifen significantly ($P < 0.05$) exceeded that induced by E_2 ablation (Table 5). These results clearly indicate a direct effect of these steroid antagonists on induction of DNA fragmentation beyond blocking the E_2 stimulatory effect on cell survival. DNA fragmentation as a result of 4-hydroxytamoxifen treatment appeared to be an ER-mediated effect which could be significantly ($P < 0.05$) inhibited at 36 and 72 hours post-treatment by the addition of excess E_2 (2 μM) to the culture medium (Table 5).

Bcl_2 protein expression (ELISA)

In a first experiment, the treatment with 4-hydroxytamoxifen or mifepristone induced approximately 60% inhibition of bcl_2 concentration in the MCF-7 cells growing in culture for 7 days. This effect was significant ($P < 0.05$) in both treatment groups as compared with the bcl_2 concentration in the control group. The decrease in bcl_2 concentration was amplified to about 85% inhibition ($P < 0.05$ versus control) by simultaneous administration of 4-hydroxytamoxifen and mifepristone. This additive effect of a combination therapy was significantly different ($P < 0.05$) from that of 4-hydroxytamoxifen or mifepristone monotherapy (Figure 2).

In a second independent experiment; we continued our investigations to study the time-dependent effect of mifepristone and/or 4-hydroxytamoxifen on bcl_2 protein expression, as compared with that of E_2 treatment, estrogen ablation, and of 4-hydroxytamoxifen in combination with excess E_2. A significant ($P < 0.05$) downregulation of bcl_2 (49–70% inhibition) was observed as early as 12 hours post-treatment with 4-hydroxytamoxifen, mifepristone, or the combination of both, as well as following E_2 ablation (steroid depletion without the addition of 10 nM E_2 to the culture medium) (Table 6). E_2 treat-

Table 5. Effect of 1 μM 4-hydroxytamoxifen (TAM), 10 μM mifepristone (MIF), and their combination, as compared with E_2 ablation, on DNA fragmentation in the MCF-7 human breast cancer cell *in vitro*

Groups[a]	Absorbance (OD 405 nm × 1000)[b] (Mean ± SEM)[c] Hours post-treatment			Enrichment Factor[d] (Mean ± SEM)[c] Hours post-treatment		
	12 hours	36 hours	72 hours	12 hours	36 hours	72 hours
E_2 control	166 ± 11.6	127 ± 10.1	149 ± 5.5			
E_2 ablation	232 ± 9.4[e]	221 ± 15.9[e]	437 ± 6.4[e]	1.4 ± 0.1	1.8 ± 0.2	3.0 ± 0.1
E_2 + TAM	225 ± 8.1[e]	309 ± 21.9[f]	570 ± 14.7[f]	1.4 + 0.1	2.5 ± 0.1	3.9 ± 0.2
E_2 + MIF	242 ± 21.9[e]	230 ± 44.3	327 ± 6.7[e]	1.5 ± 0.1	1.8 ± 0.3	2.2 ± 0.1
E_2 + TAM + MIF	342 ± 15.4[g]	460 ± 67.7[g]	989 ± 84[g]	2.1 ± 0.2	3.6 ± 0.4	6.6 ± 0.4
E_2 + TAM + e E_2	186 ± 29.6	167 ± 7.5[h]	185 ± 26[h]	1.2 ± 0.1	1.3 ± 0.1	1.2 ± 0.2

[a] 10 nM 17β-estradiol (E_2) was added to the culture medium in all groups except E_2 ablation group.

[b] Absorbance was read at 405 nm on a microplate reader. Absorbance × 1000 = mU.

[c] Results of three independent experiments using the Cell Death Detection ELISA Kit – Boehringer Mannheim, Indianapolis, IN.

[d] Enrichment Factor = $\frac{\text{mU of treated sample}}{\text{mU of } E_2 \text{ control}}$

[e] $p < 0.05$ versus E_2 control.

[f] $p < 0.05$ versus E_2 control and E_2 ablation.

[g] $p < 0.05$ versus E_2 control, E_2 ablation, mifepristone, and 4-hydroxytamoxifen monotherapy.

[h] $p < 0.05$ versus 4-hydroxytamoxifen monotherapy.

e E_2 – excess E_2 (2 μM).

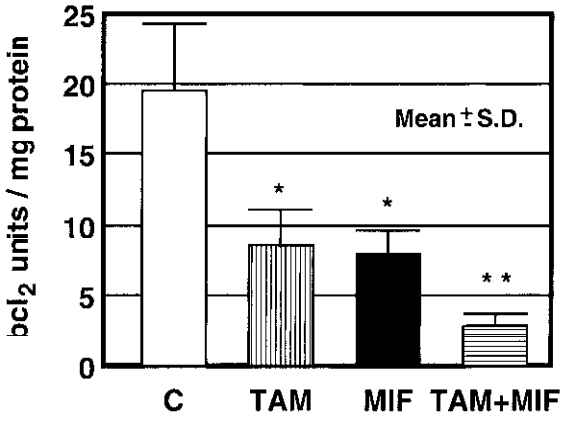

Figure 2. Effect of 1 μM 4-hydroxytamoxifen (TAM), 10 μM mifepristone (MIF), and their combination on bcl_2 expression in the MCF-7 human breast cancer cells *in vitro*. 10 nM 17β-estradiol was added to the culture medium in all groups. Mean difference between control (C) and treated groups (Mean ± SD of five independent measurements during 7 days of treatment). * $P < 0.05$ versus control; ** $P < 0.05$ versus control, 4-hydroxytamoxifen, or mifepristone monotherapy. The results were reproducible in three independent experiment (see Table 6).

ment induced upregulation of bcl_2 in a time-dependent fashion (12 versus 72 hours). Conversely, at both 12 and 72 hours post-treatment, bcl_2 concentration could be significantly ($P < 0.05$) inhibited by E_2 ablation (55% inhibition) or by 4-hydroxytamoxifen (55 and 72% inhibition) and mifepristone (49 and 65% inhibition) treatment. An additive effect of a combination of 4-hydroxytamoxifen and mifepristone on downregulation of bcl_2 (70 and 84% inhibition) was also significantly different ($P < 0.05$) from the effect of 4-hydroxytamoxifen and/or mifepristone monotherapy (Table 6). At 72 hours post-treatment, the downregulation of bcl_2 induced by the steroid antagonists significantly ($P < 0.05$) exceeded that induced by E_2 ablation (Table 6). These results clearly indicate a direct effect of these steroid antagonists on the downregulation of bcl_2 beyond blocking the E_2-induced upregulation of bcl_2 protein expression. The downregulation of bcl_2 as a result of 4-hydroxytamoxifen treatment appeared to be an ER-mediated effect which could be significantly ($P < 0.05$) inhibited at 72 hours post-treatment by the addition of excess E_2 (2 μM) to the culture medium (Table 6).

Western analysis for bcl_2 protein

To confirm the results of the bcl_2 ELISA assays described above, two independent experiments were conducted in which immunoblotting with a bcl_2 specific antibody was performed to quantitate the levels of bcl_2 protein in MCF-7 cells following treat-

Table 6. Effect of 1 μM 4-hydroxytamoxifen (TAM), 10 μM mifepristone (MIF), and their combination, as compared with E_2 ablation, on bcl_2 expression in the MCF-7 human breast cancer cells *in vitro*

Groups[a]	12 hours post-treatment		72 hours post-treatment	
	bcl_2 units/mg protein (mean ± SEM)[b]	% Inhibition	bcl_2 units/mg protein (mean ± SEM)[b]	% inhibition
E_2 control	26.54 ± 1.31		43.00 ± 0.70	
E_2 ablation	12.04 ± 1.07[c]	55	19.55 ± 1.55[c]	55
E_2 + TAM	11.88 ± 0.53[c]	55	11.84 ± 1.55[d]	72
E_2 + MIF	13.41 ± 1.35[c]	49	15.18 ± 1.63[d]	65
E_2 + TAM + MIF	7.91 ± 1.07[e]	70	6.87 ± 0.35[f]	84
E_2 + TAM + e E_2	16.44 ± 1.34[d]	38	34.06 ± 2.32[f]	21

[a] 10 nM 17β-estradiol (E_2) was added to the culture medium in all groups except E_2 ablation group.
[b] Results of three independent experiments using the bcl_2 ELISA Kit-Oncogene Research Products, Cambridge, MA.
[c] $P < 0.05$ versus E_2 control.
[d] $P < 0.05$ versus E_2 control and E_2 ablation.
[e] $P < 0.05$ versus E_2 control and mifepristone monotherapy.
[f] $P < 0.05$ versus E_2 control, E_2 ablation, mifepristone and 4-hydroxytamoxifen monotherapy.
e E_2 = excess E_2 (2 μM).
% Inhibition versus E_2 control.

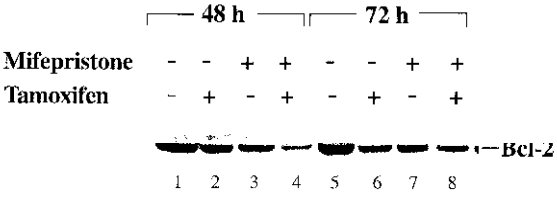

Figure 3. Effect of 1 μM 4-hydroxytamoxifen, 10 μM mifepristone, and their combination on bcl_2 in the MCF-7 human breast cancer cells *in vitro*. 10 nM 17β-estradiol was added to the culture medium in all groups. Western analysis of bcl_2 protein levels 48 and 72 hours post-treatment. These results are typical of two independent experiments.

ment with 4-hydroxytamoxifen, mifepristone, or a combination of both. A representative immunoblot is shown in Figure 3. At 48 and 72 hours post-treatment, MCF-7 cells treated with 4-hydroxytamoxifen, mifepristone, or a combination of both showed a decrease of approximately 31% and 63% (lane 2 and 6), 52% and 58% (lane 3 and 7), or 80% and 78% (lane 4 and 8) respectively, as compared to MCF-7 cells treated with E_2 alone (lane 1 and 5). Thus, combined treatment with 4-hydroxytamoxifen and mifepristone had an additive effect on decreasing the steady-state levels of bcl_2 protein. Furthermore, an approximately 50% increase in bcl_2 protein was observed in MCF-7 cells treated with E_2 alone for 72 hours as compared to those treated for only 48 hours (compare lanes 1 and 5). This time-dependent upregulation in bcl_2 expression following E_2 exposure is consistent with the results of our bcl_2 ELISA assay (see Table 6) and correlates nicely with the above mentioned time-dependent down-regulation of bcl_2 as a result of 4-hydroxytamoxifen treatment at 72 versus 48 hours post-treatment (63% versus 31% reduction). Using Western analysis, we were unable to consistently detect a decrease in bcl_2 protein levels in all drug treatment groups at 12 hours post-treatment. However, a slight decrease of bcl_2 protein level (approximately 30% as determined by densitometry) was observed in the 4-hydroxytamoxifen and mifepristone combination group as compared with the E_2 control group at 24 hours post-treatment (data not shown). Therefore, our ELISA assay was clearly more sensitive than Western Analysis in detecting decreased bcl_2 levels at early treatment times.

TGF β_1 protein concentration

The $TGF\beta_1$ protein concentration was measured in supernatants from MCF-7 cells treated in culture with E_2 alone (control) or in combination with 4-hydroxytamoxifen, mifepristone, or the combination of both. In the control group, E_2 treatment induced a decrease in $TGF\beta_1$ protein concentration in a time-dependent fashion (72 versus 36 hours) (Table 7). However, $TGF\beta_1$ protein levels increased to

Table 7. Effect of 1 μM 4-hydroxytamoxifen (TAM), 10 μM mifepristone (MIF), and their combination on $TGF\beta_1$ protein concentration in the MCF-7 human breast cancer cells *in vitro*

Groups[a]	36 hours post-treatment		72 hours post-treatment	
	$TGF\beta_1$ protein ($pg/10^4$ cells) (mean ± SEM)[b]	Ratio (T/C)	$TGF\beta_1$ protein ($pg/10^4$ cells) (mean ± SEM)[b]	Ratio (T/C)
E_2 control	36.9 ± 2.3		25.9 ± 1.45	
E_2 + TAM	51.3 ± 0.95[c]	1.39	39.1 ± 2.20[c]	1.51
E_2 + MIF	56.8 ± 0.05[c]	1.54	48.2 ± 2.20[c]	1.86
E_2 + TAM + MIF	104.7 ± 4.65[d]	2.84	78.0 ± 4.00[d]	3.01

[a] 10 nM 17β-estradiol (E_2) was added to the culture medium in all groups.

[b] $TGF\beta_1$ protein was quantitated in cell culture supernatant using the Quantikine human $TGF\beta_1$ ELISA kit (R&D Systems, Minneapolis, MN). Results of three independent experiments.

[c] $P < 0.05$ versus E_2 control.

[d] $P < 0.05$ versus E_2 control, 4-hydroxytamoxifen and mifepristone monotherapy.

T/C = Treatment/Control.

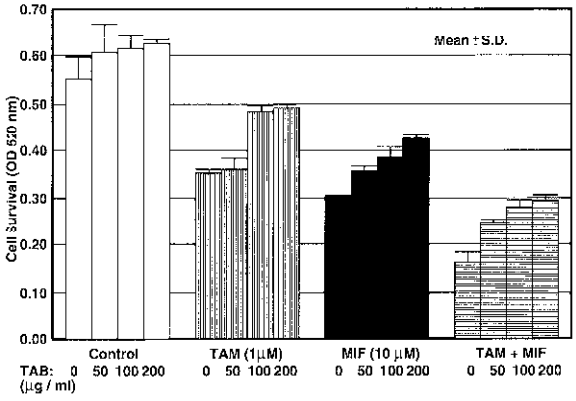

Figure 4. Effect of the addition of neutralizing antibody to human TGFβ$_1$ (TAB) on the *in vitro* inhibition of cell survival (SRB assay-72 hours post-treatment) induced by 1 μM 4-hydroxytamoxifen, 10 μM mifepristone, or the combination of both as compared with E_2 control. 10 nM E_2 was added to the culture medium in all groups.

approximately 1.5 and 2-fold that of the control levels at 36 and 72 hours post-treatment with 4-hydroxytamoxifen or mifepristone. This increase was significantly ($P < 0.05$) different from the control group (Table 7). The maximum protein levels (about 3-fold induction) were observed as an effect of the combination of 4-hydroxytamoxifen and mifepristone treatment. This additive effect of the combination therapy was significantly different ($P < 0.05$) from that of 4-hydroxytamoxifen or mifepristone monotherapy (Table 7). Only in this combination group, a slight increase (1.6-fold that of the control) in TGFβ$_1$ protein was observed as early as 24 hours post-treatment. No increase in TGFβ$_1$ protein concentration was observed in the monotherapy groups before 36 hours post-treatment (data not shown).

In vitro *inhibition of cytotoxicity by anti-human TGFβ$_1$ neutralizing antibody*

In two independent experiments, the SRB assay was used to measure abrogation of cytotoxicity induced by E_2 ablation, 4-hydroxytamoxifen, mifepristone and their combination with the addition of an anti-human TGFβ$_1$ neutralizing antibody. In the first experiment and at antibody concentrations up to 200 μg/ml there was a trend toward dose dependent abrogation of cytotoxicity induced by the treatment with 4-hydroxytamoxifen, mifepristone, and their combination. This resulted in an increase in the viability of the MCF-7 cells in culture (decrease in the inhibition of cell survival) (Figure 4). We continued our investigations in a second experiment using higher concentrations of the anti-human TGFβ$_1$ neutralizing antibody. In this experiment an antibody concentration of 1.0 mg/ml completely inhibited the cytotoxic effect of E_2 ablation and of 4-hydroxytamoxifen, mifepristone, and the combination of both (Table 8). Indeed, MCF-7 cells were stimulated to grow slightly above control when neu-

Table 8. Effect of anti-human TGFβ$_1$ neutralizing antibody (TAB) on the inhibitory effect of 1 μM 4-hydroxytamoxifen (TAM), 10 μM mifepristone (MIF), and their combination, as compared with estrogen ablation, on MCF-7 cell survival *in vitro* (SRB assay, 72 hours post-treatment)

Groups[a]	% Inhibition of cell survival (mean ± SEM)[b]	% Abrogation of cytotoxicity (mean ± SEM)[b]
E_2 ablation + TAB (mg/ml)		
0.0	36.6 ± 1.29	
0.2	18.7 ± 6.31[c]	48.8 ± 17.24[c]
0.5	1.8 ± 4.34[c]	94.5 ± 11.76[c]
1.0	− 7.8 ± 4.64[c]	121.7 ± 12.80[c]
E_2 + TAM + TAB		
0.0	35.6 ± 3.93	
0.2	18.1 ± 4.71[c]	49.2 ± 13.20[c]
0.5	7.4 ± 3.87[c]	79.2 ± 10.82[c]
1.0	− 6.4 ± 4.43[c]	118.0 ± 16.18[c]
E_2 + MIF + TAB		
0.0	31.5 ± 6.13	
0.2	11.6 ± 8.09[c]	63.0 ± 14.81[c]
0.5	1.4 ± 3.78[c]	95.5 ± 6.91[c]
1.0	− 6.4 ± 3.51[c]	119.0 ± 11.06[c]
E_2 + TAM + MIF + TAB		
0.0	58.3 ± 1.58	
0.2	29.7 ± 3.84[c]	49.1 ± 6.57[c]
0.5	9.6 ± 1.34[c]	83.5 ± 2.31[c]
1.0	3.0 ± 1.95[c]	105.0 ± 3.45[c]

[a] 10 nM 17β-estradiol (E_2) was added to the culture medium in all groups, except E_2 ablation group.
[b] Results from three independent experiments as compared to the E_2 control group.
[c] Significantly different ($P < 0.05$) as compared with the corresponding control group (without addition of TAB).

tralizing TGFβ$_1$ antibody was added either alone (Figure 4), or at high concentrations (1 mg/ml) in combination with the steroid antagonists or E_2 ablation (Table 8). Conversely, the addition of as low as 1.0–20 μg/ml recombinant human TGFβ$_1$ protein to the culture medium induced dose-dependent inhibition of cell survival (up to 68%) as measured in the SRB assay (Table 9).

PKC activity and subcellular distribution

4-hydroxytamoxifen, mifepristone, and their combination induced a marked decrease in the PKC activity in the soluble fraction starting at 24 hours post-treatment (Figure 5A). A significant increase of this enzyme activity in the particulate and/or nuclear fraction in 4-hydroxytamoxifen- and/or mifepristone-treated cells was evident as soon as 12 hours following drug exposure (data not shown). Further, the decrease in the soluble fraction (24 hours) and the increase in the nuclear fraction (36 hours) appears additive in cells treated with a combination of both 4-hydroxytamoxifen and mifepristone. This was significantly ($P < 0.001$) different from the effect of monotherapy (Figure 5A and B). Therefore, at least part of the movement of PKC from the soluble pool appears to involve a shift in PKC activity (an increase in enzyme localization) to the nuclear fraction (Figure 5B). Western analysis complementing the enzyme assay data indicates

Table 9. Effect of a recombinant human TGFβ$_1$ protein on MCF-7 cell survival *in vitro* (SRB assay – day 7 post-treatment)

Groups[a]	% Inhibition of cell survival (mean ± SEM)[b]
E_2 + TGFβ$_1$	
1.0 μg/ml	55.9 ± 2.61[c]
10.0 μg/ml	57.7 ± 1.28[c]
20.0 μg/ml	68.4 ± 0.52[c]

[a] 10 nM 17β-estradiol (E_2) was added to the culture medium in all groups.

[b] Results of three independent experiments as compared with the E_2 control group using the recombinant human TGFβ$_1$ protein (R&D Systems, Minneapolis, MN).

[c] Significantly different ($P < 0.05$) as compared with the E_2 control group.

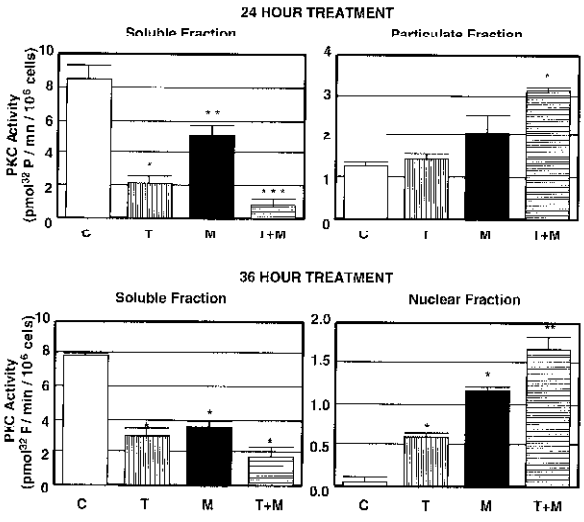

Figure 5A–B. Effect of 1 μM 4-hydroxytamoxifen (T), 10 μM mifepristone (M) and their combination on PKC activity and subcellular expression (soluble, particulate and/or nuclear fraction) in the MCF-7 human breast cancer cells *in vitro* (Mean ± SEM). 10 nM 17β-estradiol was added to the culture medium in all groups. A significant additive effect of the combination was observed on the decrease in the soluble fraction at 24 hours post-treatment (A, * $P < 0.001$ versus control; ** $P < 0.05$ versus control; *** $P < 0.001$ versus control, 4-hydroxytamoxifen or mifepristone monotherapy), and on the increase in the nuclear fraction at 36 hours post-treatment (B; * $P < 0.001$ versus control, ** $P < 0.001$ versus control, 4-hydroxytamoxifen or mifepristone monotherapy). These results are representative of three repetitions in two independent experiments with several timepoints.

that the α isoform of PKC is involved in the treatment-related shift of PKC activity from the soluble to the particulate fraction. Drug treatment with 4-hydroxytamoxifen and/or mifepristone increases the PKCα protein in the particulate fraction. An additive effect of the combination therapy on the decrease of PKCα in the soluble fraction was clearly evident (Figure 6). The effect of 4-hydroxytamoxifen appeared to be an ER-mediated effect which could be significantly inhibited by the addition of excess E_2 (2 μM) to the culture medium. However, at both 12 and 36 hours post-treatment, no clear effect of E_2 ablation was observed on the translocation of PKCα isoform from the soluble to the particulate fraction. Preliminary results also indicate increased nuclear content of PKCε in MCF-7 cells exposed to both 4-hydroxytamoxifen and mifepristone (data not shown).

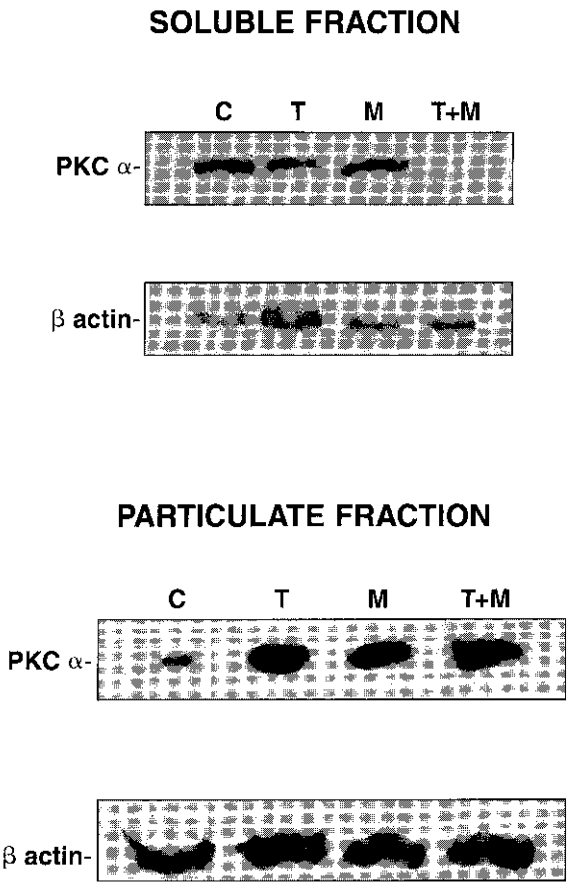

Figure 6. Effect of 1 μM 4 hydroxytamoxifen (T), 10 μM mifepristone (M), and their combination on subcellular distribution of PKCα in the MCF-7 human breast cancer cells *in vitro*. 10 nM 17β-estradiol was added to the culture medium in all groups. Cells were treated for 36 hours. Western blots were probed using anti-PKCα antibody. An antibody to β-actin was used as control for loading equivalency.

Discussion

DNA fragmentation and modulation of bcl_2 expression

This is the first report to demonstrate an additive effect of mifepristone in combination with 4-hydroxytamoxifen on the increase in DNA fragmentation (induction of apoptosis) and on bcl_2 downregulation in human breast cancer cells. A role of bcl_2 downregulation in the induction of apoptosis by mifepristone alone and in combination with 4-hydroxytamoxifen has not been published before. These results suggest that the additive antitumor activity of mifepristone (antiprogestins) and antiestrogens such as tamoxifen [5, 11, 12], could be the result of their interaction with the cell suicide mechanism, which involves an early downregulation (as early as 12 hours post-treatment) of bcl_2 in both ER and PR positive breast cancer cells. In this study, there is a nice correlation between induction of apoptosis (extent of DNA fragmentation) and bcl_2 downregulation in response to E_2 ablation and as a result of treatment with 4-hydroxytamoxifen and/or mifepristone. The changes in the bcl_2 protein concentrations in our Western analysis confirm the bcl_2 measurements in our ELISA. However, the time- and dose-dependent effects of these steroid antagonists (either alone or in combination) on the extent of DNA fragmentation and on the bcl_2 protein and mRNA expression need further clarification in future studies.

The increase in DNA fragmentation (ELISA) and the downregulation of bcl_2 confirm that both 4-hydroxytamoxifen and mifepristone are specific inducers of apoptosis. Using the pulsed field gel electrophoresis technique, Schoenlein et al. [39; manuscript in preparation] could show, in agreement with our result in this study, a significant breakdown of chromosomal DNA into high molecular weight DNA fragments of > 300 kb along with detectable levels of 50 kb fragments in the MCF-7 cells growing in culture as a result of the treatment with 4-hydroxytamoxifen, mifepristone, or the combination of both. However, apoptosis was not associated with the pattern of DNA fragmentation, detectable in agarose gels as multiples of oligonucleosomal DNA, that is characteristic of the DNA of cells undergoing apoptosis [39]. The lack of oligonucleosomal DNA laddering has also been reported in previous studies using MCF-7 cells treated *in vitro* or *in vivo* with antiestrogens [9, 10]. Therefore, the absence of DNA laddering is not an accurate indication of lack of apoptosis in the MCF-7 human breast cancer cells.

In this study, the time-dependent E_2-induced upregulation of bcl_2 protein expression as well as the decrease in bcl_2 protein in response to E_2 ablation and to treatment with 4-hydroxytamoxifen are in agreement with recent published data showing that the bcl_2 mRNA levels in the MCF-7 human breast

cancer cells increased in a time- and concentration-dependent fashion as a result of E_2 exposure [16]. These authors found further that addition of 4-hydroxytamoxifen inhibited the stimulatory effects of estrogens on bcl_2 mRNA levels, thus supporting the possibility that increased bcl_2 mRNA levels involve the interaction of E_2 with the ER [16]. Also, our data have confirmed that downregulation of bcl_2 as a result of 4-hydroxytamoxifen treatment was ER mediated and could be inhibited by excess E_2 (2 μM). We have further shown that these steroid antagonists had a direct effect on the bcl_2 downregulation beyond blocking the E_2-induced upregulation of bcl_2 protein expression. Therefore, our results complement and confirm the studies of Wang and Phang [16]. Both studies define an important role for bcl_2 expression in the estrogen/antiestrogen modulation of hormone-dependent breast cancer cell death (apoptosis) and cell survival.

Also, in clinical studies, a relationship has been shown between estrogen receptor expression and bcl_2 protein level [14, 17, 21, 22]. Despite its anti-apoptotic effects which favor tumor survival, expression of bcl_2 has also been shown to be associated with favorable prognostic factors [14, 15, 17–21], and with disease-free and overall survival in breast cancer patients [23]. The favorable prognosis could be explained by recent results showing that transfection of breast cancer cells with bcl_2 prolongs the cell cycle and inhibits growth [40]. It has also been shown that bcl_2 promotes increased survival of breast cancer cells exposed to different chemotherapeutic drugs and that drug resistance develops through bcl_2 protein induction [41]. Therefore, the results of our study seem to be clinically relevant.

Involvement of PKC transduction pathway

PKC is comprised of a family of at least 12 isozymes. These serine/threonine protein kinases have different structures, functions, and tissue expression patterns. Over-expression of certain isoforms can lead to enhanced rates of cell growth whereas other isoforms may inhibit growth and induce apoptosis [42]. PKC isoforms also play a pivotal role in mediating signal transduction through the activator protein-1 (AP-1) pathway. Recently, tamoxifen was shown to stimulate the AP-1 pathway, which may represent a mechanism of tamoxifen-resistant breast cancer [28, 42]. Our results in this study have shown a potential linkage of mifepristone- and 4-hydroxytamoxifen-induced cell growth inhibition to alteration of PKC expression (a decrease in the soluble fraction and an increase in the particulate and/or nuclear fraction), as well as an evidence for the involvement of PKC in the antiproliferative effect (via induction of apoptosis) of these steroid antagonists. The shift in PKC activity (an increase in enzyme localization) from the soluble pool to the nuclear and/or particulate fraction appears also additive in cells treated with a combination of both 4-hydroxytamoxifen and mifepristone. To our knowledge, similar findings have not been published before. However, our results are consistent with recent published data demonstrating a strong correlation of the presence and absence of membrane PKCα with induction of apoptosis and resistance to apoptosis, respectively [43]. These authors have used phorbol ester-induced apoptosis in the androgen-sensitive LNCaP human prostate cancer cells as their *in vitro* model [43]. Our results are also in agreement with other investigations indicating that the PKC signaling transduction pathway is involved in the antitumor activity of antiestrogens such as tamoxifen [24–32]. In a recent study, PKCα overexpression in breast cancer cell systems has also been found to correlate with hormone-independence as characterized by response to E_2 and antiestrogens as well as by ER and PR status. These results suggest that PKC α may mediate the AP-1 response in tamoxifen-resistant breast cancer [42].

The observed elevation in nuclear PKC in our experiments as a result of the treatment of human breast cancer cells in culture with 4-hydroxytamoxifen, mifepristone, or the combination of both raises the intriguing possibility that these steroid antagonists may induce apoptosis via alterations in genomic transcription (phosphorylation of one or more protein transcription regulators of apoptosis) mediated by one or more PKC isoforms. These isoforms may be linked to the nuclear matrix during the apoptotic process [44]. Tamoxifen and mifepristone may also differ in a component of the PKC sig-

naling pathway or in a separate cell death regulating mechanism that involves different PKC isoforms, such as the α and ε isoforms [43]. However, confirmation of PKC involvement will require examination of the effects of selective inhibition and overexpression of PKC isoforms on tamoxifen and/or mifepristone-induced apoptosis. These experiments are planned for future studies.

The molecular mechanism of action of tamoxifen seems to be a complex mixture of antagonism of the mitogenic action of estradiol at the level of the ER, plus a range of other activities from enzyme inhibition to growth factor modulation. Inhibition of the PKC activity and of the Ca^{2+}/calmodulin dependent cAMP phosphodiesterases seems to be an important mechanism of the antitumor action of tamoxifen [26–28]. An involvement of PKC in the cytotoxic action of different antiestrogens other than tamoxifen has also been suggested [25]. Recent results suggest that tamoxifen induces a generation of transmembrane signals and an oxidative stress to elicit a membrane association of PKC, followed by an irreversible activation and subsequent downregulation of this enzyme which, in part, may lead to cell growth inhibition [29]. In a further investigation to study the mechanisms of ER-independent interactions of tamoxifen and its effect on the initial steps of cell signal transduction, it was found that tamoxifen activates cellular phospholipase C and D and elicits PKC translocation [31]. These authors concluded that addition of tamoxifen to cultures initiates a selective membrane association of PKC ε and that tamoxifen exerts considerable extra-nuclear influence at the transmembrane signaling level. These events may contribute to effects beyond the scope of ER-dependent actions of tamoxifen [31]. It is also known that acute exposure of the MCF-7 cells to a stimulatory dose of E_2 revealed significant increase in PKC activity over that of untreated control cells while antiestrogens inhibited the E_2 stimulated increase in PKC activity in a dose-responsive fashion [24].

Role of TGF β_1 protein

The *in vitro* additive antiproliferative activity of mifepristone in combination with 4-hydroxytamoxifen was associated with an additive interaction of both steroid antagonists (antiprogestin and antiestrogen) on increased secretion of endogenous TGFβ_1 protein. The induction of TGFβ_1 protein appeared to be a relatively late event in the apoptotic cascade induced by these steroid antagonists. Its increased expression in the drug treatment groups did not start to be significant before 36 hours post treatment. This appeared to coincide with the inhibition of cell survival in the cell growth inhibition (cytotoxicity) assay (SRB). In contrast, bcl_2 downregulation, alteration of PKC activity, and increased DNA fragmentation appeared to start to be significant as early as 12 hours post treatment. The hypothesis that tamoxifen and mifepristone operate through induction of TGFβ_1 is supported by their ability to increase TGFβ_1 protein concentration measured in supernatants (ELISA) and by the ability of TGFβ_1 neutralizing antibodies to block the antiproliferative (cytotoxic) activity of these steroid antagonists *in vitro*. This novel observation that anti-TGFβ_1 antibody was able to block the cytotoxicity induced by 4-hydroxytamoxifen, mifepristone, and their combination, is the first observation to show evidence of a possible cause-and-effect relationship between induction of TGFβ_1 by these steroid antagonists and subsequent cell death. To our knowledge, our results show also for the first time that the neutralizing antibody to TGFβ_1 blocks the inhibition of cell survival induced as a result of E_2 ablation. These results indicate that estrogen-induced cell growth and survival is mediated via inhibition of endogenous TGFβ_1 secretion. Therefore, in both our control and treated cells, the endogenous expression of TGFβ_1 protein was maintained at a relatively low level due to the expected inhibition induced by the addition of E_2 (10 nM). However, MCF-7 cells treated with anti-TGFβ_1 antibody alone or with high antibody concentrations (1 mg/ml) in combination with the steroid antagonists or E_2 ablation showed a tendency of a slight increased growth as compared with control cells (see Table 8 and Figure 4). These findings imply that the antibody also blocked TGFβ_1 that may have been present from constitutive expression by MCF-7 cells or from TGFβ_1 in FBS. Our findings also indicate that the TGFβ_1 protein in-

duced by the antihormone treatments in our studies is in an active and not in a latent form.

Our *in vitro* data contradict the results of *in vivo* experiments published before suggesting that 1) $TGF\beta_1$ augments tumor growth of MCF-7 cells in nude mice [45], and 2) $TGF\beta_1$ does not seem to play a role in the antiestrogen inhibition of human breast cancer cells [46]. On the contrary, other publications have consistently shown that the human breast cancer cells respond to this growth factor with a reduced rate of cell growth and proliferation [47–49]. Furthermore, evidence for the role of increased $TGF\beta_1$ expression in the induction of apoptosis in hormone-dependent breast cancer models following estrogen ablation [8, 48], antiestrogen [9, 10, 47], or antiprogestin [1, 49] treatment has been previously suggested. Similarly, our own data confirm that MCF-7 breast cancer cells are inhibited by the addition of exogenous recombinant human $TGF\beta_1$ protein to the culture medium (see Table 9).

It has been shown also in previous studies that longer (> 12 hours) incubation of ER positive MCF-7 or ER negative MDA-231 human breast cancer cells with high dose (10 μM) 4-hydroxytamoxifen induced $TGF\beta_1$ protein (about 3-fold) and mRNA (about 2-fold) in both cell lines [47]. The 4-hydroxytamoxifen-induced internucleosomal DNA cleavage was inhibited by the addition of a specific anti-$TGF\beta_1$ antibody. These effects could not be reversed by the addition of estrogens and seem to be independent of ER status (non-ER mediated mechanisms), and may be the result of a direct regulatory effect of a high dose of 4-hydroxytamoxifen on $TGF\beta_1$ transcription [47]. In our study, we did not use higher concentrations of tamoxifen (> 1 μM), as it is known that high non-pharmacological concentrations (> 4 μM tamoxifen) are clinically irrelevant and induce a non-specific cytotoxic effect, which is not reversed by estrogens and may involve antiestrogen-specific sites [7].

Cell growth inhibition/interactions with ER and PR

Although the human breast cancer cells of our MCF-7 subline express both ER and PR [50], 10 nM E_2 was added to the steroid-depleted cells in our control and drug-treated cultures to stimulate PR induction and to maintain cell growth and proliferation in a standard fashion. Under our culture conditions and in the presence of 10 nM E_2, mifepristone and 4-hydroxytamoxifen had a significant dose- and time-dependent inhibitory effect on the growth and survival of MCF-7 cells. A significant additive cell growth inhibition of the combination of 4-hydroxytamoxifen and mifepristone correlated with a significant additive effect on DNA fragmentation, bcl_2 downregulation, induction of $TGF\beta_1$ protein, and changes of PKC activity and subcellular expression. In this study, the addition of excess E_2 (1 or 2 μM) to the culture medium protected the MCF-7 cells and significantly decreased or completely prevented the effect of 4-hydroxytamoxifen on cell growth inhibition, DNA fragmentation, bcl_2 downregulation, and translocation of PKC α from the soluble to the particulate fraction. These results clearly indicate ER-mediated effects. Conversely, E_2 ablation induced a significant time-dependent inhibition of cell survival associated with an increase in DNA fragmentation and bcl_2 downregulation. Moreover, a specific anti-human $TGF\beta_1$ neutralizing antibody induced complete abrogation of the cytotoxic effect of E_2 ablation. These findings clearly indicate that the effect of E_2 ablation on inhibition of cell survival is mediated via $TGF\beta_1$ protein expression. However, the steroid antagonists seem to have a direct effect beyond blocking the effect of estrogens. Furthermore, our previous results have shown that the antiproliferative activity of 4-hydroxytamoxifen and mifepristone was significantly reduced or prevented by occupying the respective receptors by their corresponding high affinity native ligands [50]. These results are in agreement with other published data which have shown that treatment of MCF-7 breast cancer cells growing in culture with nanomolar concentrations of 4-hydroxytamoxifen (and mifepristone) produced both cytotoxic and cytostatic effects [7]. In these particular studies, the cytotoxic effects of 4-hydroxytamoxifen and mifepristone have been shown also to be mediated via the ER and PR, respectively. These effects were not observed in ER- and PR-negative breast cancer cell lines, and were prevented or rescued by the occupation of their respective

receptors by estradiol or progestin [7]. Therefore, our conclusion is justified that the antiproliferative effects (cytotoxicity) of 4-hydroxytamoxifen and mifepristone in the MCF-7 human breast cancer cells are mediated via their interactions with the ER and PR, respectively. However, it has been shown in other investigations that either removal of E_2 from the culture medium or the addition of 4-hydroxytamoxifen was only cytostatic (i.e. cells accumulate in the G_0–G_1 phase of the cell cycle) and not cytotoxic to the MCF-7 cells [51]. The MCF-7 cell variant used, culture conditions (presence of phenol red), as well as dose of 4-hydroxytamoxifen and experimental design may explain these variable results. Future studies are planned to determine the relative importance of growth arrest (inhibition of cell proliferation) versus induction of apoptosis for the antiproliferative activity of mifepristone, 4-hydroxytamoxifen, or both as compared with estrogen ablation.

In all experiments, mifepristone counteracted the E_2-induced cell proliferation (antiestrogenic effect). However, the antiproliferative activity of mifepristone (inhibition of cell survival) as well as its effect on bcl_2 downregulation (72 hours post-treatment) exceeded the effect of E_2 ablation (see Tables 3 and 6). These results clearly indicate a direct antiproliferative effect of mifepristone beyond blocking the E_2-induced cell proliferation and bcl_2 upregulation. In this study, we did not find any difference in the effect of mifepristone in the presence or absence of E_2. However, previous studies have shown that mifepristone, in the absence of E_2, was able to stimulate proliferation and similar to E_2, regulate ER target gene expression (PR, PS_2, TGFβ) in human breast cancer cells (MCF-7, T47D) in culture [52–54]. In these previous studies, 4-hydroxytamoxifen and the pure antiestrogen ICI 164,384 blocked the mifepristone-induced cell proliferation. This estrogenic activity of mifepristone was also not due to impurities or aromatization to estrogenic metabolites [52]. Our results in this study cannot confirm such a weak estrogenic activity of mifepristone and do not suggest that mifepristone induces an ER-mediated cell proliferation.

Conclusions

Based on our results and other published data, it seems reasonable to assume that antiprogestins and antiestrogens interact with their respective receptors in both ER and PR positive breast cancer cells to initiate an additive cell death command via converging apoptotic pathways involving downregulation of bcl_2, changes in the PKC enzyme activities and subcellular expression, and induction of $TGF\beta_1$ protein. Further studies are still needed to confirm this hypothesis.

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