The Prostate 43:31–42 (2000)

Induction of Apoptosis by Mifepristone and Tamoxifen in Human LNCaP Prostate Cancer Cells in Culture

M. Fathy El Etreby,* Yayun Liang, and Ronald W. Lewis

Section of Urology, Department of Surgery, Medical College of Georgia, Augusta, Georgia

BACKGROUND. Published data indicate that antiprogestins and antiestrogens could inhibit prostate cancer cell growth in vitro and in vivo. The main objective of the present studies was to explore the role of bcl_2 and $TGF\beta_1$ for induction of apoptosis in LNCaP prostate cancer cells growing in culture as a treatment response to the antiprogestin, mifepristone, and the antiestrogen, 4-hydroxytamoxifen.

METHODS. In vitro cell viability (cytotoxicity), DNA fragmentation, and changes in the expression of bcl_2 and $TGF\beta_1$ proteins were assessed using the sulforhodamine B protein dye-binding assay, specific ELISA, and competitive inhibition assays.

RESULTS. Both steroid antagonists induced a significant time- and dose-dependent cell growth inhibition (cytotoxicity). This inhibition of viable cells 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 4-hydroxytamoxifen-induced cytotoxicity by $TGF\beta_1$-neutralizing antibody and by the addition of mannose-6-phosphate confirmed the correlation between induction of active $TGF\beta_1$ and subsequent prostate cancer cell death. The effect of mifepristone was not significantly reduced or prevented by occupying the progesterone or glucocorticoid receptors by their corresponding high-affinity native ligands. On the contrary, the effect of a combination of mifepristone with progesterone or hydrocortisone 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 mifepristone monotherapy.

CONCLUSIONS. Our data suggest that mifepristone and tamoxifen are effective inducers of apoptosis and may represent nonandrogen-ablation, novel therapeutic approaches to overcome a potential intrinsic apoptosis resistance of androgen-independent prostate cancer cells.
Prostate 43:31–42, 2000.

KEY WORDS: **antiprogestins; antiestrogens; bcl_2; cell growth inhibition; $TGF\beta_1$**

INTRODUCTION

There are high levels of progesterone receptors in the normal prostate, in benign prostatic hyperplasia (BPH), and in prostate cancer [1–3]. Estrogen receptor mRNA and protein have been found mainly in the stromal cells of the human prostate. A periacinar arrangement of these cells was a striking feature in all prostate cancer treated with androgen ablation [4]. These results clearly indicate that stromal cells are the primary target of estrogen/antiestrogen action in the human prostate, and that androgen withdrawal upregulates the expression of estrogen receptor gene in prostate cancer tissue [4]. However, the importance of estrogens, progesterone, and their receptors in normal prostatic physiology, as well as the role of estrogens, progestins, antiestrogens, and progesterone antagonists (antiprogestins) in the treatment of abnormal prostatic growth and proliferation (BPH, prostate cancer), remains to be defined. High concentrations of glucocorticoid receptors have also been described in metastatic prostate cancer, which may be a target for a

Grant sponsor: Research Funds of the Section of Urology, Department of Surgery, Medical College of Georgia, Augusta, Georgia.

*Correspondence to: Dr. M. Fathy El Etreby, Section of Urology, BAA-8414, Department of Surgery, Medical College of Georgia, Augusta, GA 30912-4050.

Received 8 February 1999; Accepted 18 October 1999

direct therapeutic effect of glucocorticoids or glucocorticoid antagonists (antiglucocorticoids) on prostate cancer tissue [1].

Mobbs and Johnson [5] investigated the antitumor effect of mifepristone in the androgen-insensitive Dunning R3327 HI rat prostate cancer model. A significant inhibition of tumor growth was only observed when estrogen pretreatment was used to induce significant progesterone receptor (PR) concentration in the tumor tissue [5]. Similarly, the antiprogestin onapristone could achieve a significant growth inhibition of the androgen-sensitive Dunning R3327H prostate carcinoma of the rat, if given in combination with a low dose of estrogen (diethylstilbestrol-DES), which affects tumor growth only slightly but enhances PR levels. In this experiment, the tumor-inhibiting effect of onapristone plus DES was significantly superior to that of DES alone and equal to that of surgical castration [6]. Therefore, in these two prostate cancer models, the presence of PR seems to be an important prerequisite for the antitumor activity of antiprogestins. This is in agreement with our own findings in hormone-dependent breast cancer models [7–12]. In another study, Lin et al. [13] observed that mifepristone causes a growth inhibition of androgen-sensitive (LNCaP) and androgen-insensitive (PC3, DU145) human prostate cancer cell lines in vitro. Furthermore, in the same study, the in vitro growth inhibition of the PC3 cell line was reflected in an in vivo nude mouse system. Treatment with progesterone also resulted in growth inhibition of the LNCaP and PC3 cells in vitro. At an equal concentration, the degree of growth inhibition of PC3 cells by mifepristone or progesterone was partially diminished by simultaneous exposure to a glucocorticoid [13].

The molecular mechanism of action of the antiestrogen tamoxifen seems to be a complex mixture of antagonism of the mitogenic action of estrogens at the level of the estrogen receptor (ER), plus a range of other activities including induction of apoptosis, $TGF\beta_1$ modulation, and microtubule and enzyme inhibition, as well as reversing the (MDR) phenotype [11,14–20]. Inhibition or translocation of protein kinase C (PKC), inhibition of the Ca^{2+}/calmodulin-dependent cAMP phosphodiestrases, and activitation of cellular phospholipase C and D also seem to be important mechanisms of the antitumor action of tamoxifen [14–19]. All these events may contribute to effects beyond the scope of ER-dependent actions of tamoxifen and justify the interest in studying its antiproliferative (antitumor) activities in experimental and clinical prostate cancer [21–23]. In fact, Schneider et al. [21] demonstrated antitumor activity for tamoxifen and zindoxifene (2-phenylindole antiestrogen) in both the Dunning and Noble rat prostate adenocarcinoma models. Tamoxifen was also effective as a chemopreventive agent in inhibiting the development of androgen-promoted carcinomas of the seminal vesicle and of the anterior prostate of male Lobund-Wistar rats [22]. In contrast, Pienta et al. [23] found little or no activity for tamoxifen alone in suppressing the growth of the anaplastic MAT-LyLu subline of the Dunning rat prostate adenocarcinoma, although it was effective both in vitro and in vivo in the same model in combination with the microtubule inhibitor vinblastine [23].

The objective of this study was to explore the role of bcl_2 expression and $TGF\beta_1$ protein for induction of apoptosis in human LNCaP prostate cancer cells growing in culture as a treatment response to mifepristone and 4-hydroxytamoxifen. Our intention was also to clarify the role of progesterone and glucocorticoid receptors in the induction of the effects of mifepristone. In this paper, in vitro results are presented to confirm that the antiproliferative activity of 4-hydroxytamoxifen and mifepristone is associated with a significant increase in DNA fragmentation (apoptosis), bcl_2 downregulation, and induction of $TGF\beta_1$ protein. Progesterone and hydrocortisone could not reverse the inhibitory effect of and seem rather to have an additive effect to mifepristone on induction of apoptosis in human LNCaP prostate cancer cells.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The parental LNCaP prostate carcinoma cell line (passage no. 43) was obtained from the American Type Culture Collection (ATCC, Rockville, MD), and maintained in RPMI-1640 medium with L-glutamine supplemented with 1% antibiotic-antimycotic mixture, 4.5 g/l D-glucose, 10 mM HEPES buffer, 1 mM sodium pyruvate, and 10% fetal bovine serum (FBS). The cell line is androgen-sensitive but possesses a mutation in the hormone-binding domain of its androgen receptor [24]. LNCaP cells retain the capacity to respond to androgen treatment with both cell proliferation or cell death and secretion of prostate-specific antigen (PSA). Due to this androgen sensitivity, they have been used frequently in the study of androgen regulation of gene expression [24]. Other LNCaP sublines (LNCaP-C4 passage no. 20 and LNCaP-C4-2 passage no. 25) were included only in our cytotoxicity studies and were obtained from Urocor Inc. (Oklahoma City, OK). These sublines were derived from a coinjection of parental LNCaP cells and normal human bone fibroblasts (*MS* bone stromal cell line) in athymic male nude mice [25,26]. The LNCaP-C4 and LNCaP-C4-2 cell lines were developed originally in

the laboratory of Dr. Leland W.K. Chung (M.D. Anderson Cancer Center, Houston, TX). The reason for the inclusion of these two cell lines in our cytotoxicity assays was to determine whether androgen-sensitive and more aggressive, androgen-insensitive variants of the LNCaP prostate cancer cell line exhibited stable differences in apoptosis sensitivity to mifepristone and 4-hydroxytamoxifen treatment. The cells were plated onto 6- or 12-well culture plates in the above-mentioned RPMI-1640 medium containing 10% FBS at a density of 1.0×10^4 cells/well. LNCaP cells were left undisturbed for 2 days in a 37°C incubator (7% CO_2) to facilitate attachment of the cells to the plates. Attached cells were fed with steroid-reduced medium (RPMI-1640 medium containing 5% heat-activated dialyzed FBS) and maintained at 37°C for an additional 1 or 2 days. One set of attached cells were harvested and counted as day 0. The remaining cells were allowed to grow in 3 ml of fresh steroid-reduced medium containing various concentrations of the different drugs. There was no difference in the effects of the tested drugs under conditions of growth in 5% vs. 10% FBS.

Drug Treatment and Control Experiments

The drugs were dissolved in 100% ethanol as stock solutions. Dilutions were performed 1/1,000 on a volume basis in the RPMI-1640 culture medium to achieve desired concentrations. The drug solution or ethanol in medium was added every 3 days. Cells from each set were harvested at a 2–3-day interval for up to 14 days. The harvested cells were counted with a hemacytometer and expressed as percent control, which is referred to as 100%. Once the optimal concentrations of the different steroid antagonists were established in the cell growth inhibition assays, 6- or 12-well plates were treated with mifepristone (10 and 15 μM) and 4-hydroxytamoxifen (5 μM). Cells were harvested at 2-day intervals for 14 days. In some experiments, the prostate cancer cells in culture were treated with progesterone or hydrocortisone, either alone or simultaneously with the mifepristone treatment. These studies were conducted to determine if these steroids, via occupation of the corresponding steroids receptors, will inhibit different parameters (endpoints) of the apoptotic process induced by mifepristone or will rather induce an additive effect to that of mifepristone. Other control experiments were performed, using a recombinant human $TGF\beta_1$ protein, a specific neutralizing antibody to human $TGF\beta_1$ (R&D Systems, Minneapolis, MN), and mannose-6-phosphate (Sigma Chemical Co., St. Louis, MO) to confirm the role of the changes in $TGF\beta_1$ protein for the in vitro cell growth inhibition (cytotoxicity) induced by 4-hydroxytamoxifen and mifepristone treatment.

Drugs and Chemicals

Mifepristone (Roussel-Uclaf/Population Council) was generously provided by Schering AG (Berlin, Germany). 4-hydroxytamoxifen was purchased from Sigma Chemical Co. The origin of specific antibodies, ELISA kits, and other important reagents and chemicals was mentioned under the corresponding description of the different techniques used in this investigation. Tissue culture media and reagents were purchased from Life Technologies (Grand Island, NY). All of the chemicals used were of A.C.S. or molecular biology grade and were obtained commercially.

Cytotoxicity/Cell Viability Assay

Viable cells were quantitated using the sulforhodamine B (SRB) assay, as described previously [27,28]. 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 viable cells or reduction of cell protein mass) of mifepristone and 4-hydroxytamoxifen on human LNCaP, LNCaP-C4, and LNCaP-C4-2 prostate cancer cells in vitro. Cell survival was based on detection of viable cells (cell protein mass) in cultures, which is the final outcome of both inhibition of cell proliferation and stimulation of cell death (apoptosis). The proliferative ability of these cells could not be assessed using this assay. 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 viable cells). The IC_{50} was calculated with dose-analysis computer software which employed the median effect equation [28]. The concentrations were recorded in our database in μM. Briefly, 1×10^4, 0.5×10^4, 0.25×10^4, and 0.125×10^4 cells were seeded in 12-well plates (LNCaP) or in 6-well plates (C4 and C4-2) for 3, 5, 7, or 10 days of treatment, respectively. Cells were allowed to attach for 3 days in the well. Cells were then incubated with the different drug treatments or with the same volume vehicle (equal concentration of ethanol). Cells were retreated every 3 days. Surviving or viable cells were fixed in situ by withdrawing the growth media and adding 100 μl each of cold Hank's balanced salt solution and 100 μl 50% cold trichloracetic acid, and incubated at 4°C for 60 min. The supernatants were removed, and wells were washed five times with ice

water. After the wells were dried, SRB solution (0.4% in 1% acetic acid) was added, followed by an 8-min 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 10 mM Tris buffer, and then transferred into 96-well plates (150 μl/well). The absorbency was read at 520 nm on a Spectra MAX 340 microplate reader (Molecular Devices, Menlo Park, CA).

Cell Death Detection ELISA

We used DNA fragmentation as a characteristic feature of apoptosis. DNA fragmentation was measured in control and treated LNCaP cells growing in culture, using a specific Cell Death Detection ELISA kit (Boehringer Mannheim, Indianapolis, IN). This kit 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, cells were washed once with PBS and counted using a hemacytometer, and 100 μl lysis buffer were added per 1×10^4 cells. After a 30-min incubation at room temperature, the cytosolic extract was recovered after centrifugation at 200*g* for 15 min and assayed for soluble DNA-histone fragments, according to manufacturer's protocol and as previously described [11]. 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 were expressed relative to vehicle control. The specific enrichment or induction of mono- and oligonucleosomes released into the cytoplasm was calculated from the absorbance values using the following formula:

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

where mU = 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 (Oncogene Research Products, Calbiochem) was added to the cell pellets in a resuspension buffer (1 ml buffer added per 5×10^6 cells). After a 30-min incubation on ice with occasional vortexing, the supernatant was recovered after centrifugation at 10,000 rpm for 5 min at 4°C and assayed for bcl_2 protein according to 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 (Oncogene Research Products, Calbiochem). The results from standard curve were normalized per mg cellular protein.

$TGF\beta_1$ ELISA

$TGF\beta_1$ protein was quantitated using the Quantikine human $TGF\beta_1$ ELISA kit (R&D Systems). Cells were processed and assayed according to the manufacturer's protocol. After treatment, conditioned medium was collected by centrifugation at 600*g* for 5 min and stored at –70°C until used for the assay. $TGF\beta_1$ protein concentrations were determined in the cell culture supernatant by plotting absorbance readings at a dual wavelength of 450/595 for unknowns on a standard curve derived from dilutions of the $TGF\beta_1$ protein standard. Each treatment was performed in triplicate. Cell numbers were determined by counting using a hemacytometer, and $TGF\beta_1$ values were normalized to cell number.

Neutralization of $TGF\beta_1$ Bioactivity

The SRB assay was used as described above to measure abrogation of cytotoxicity induced by mifepristone or 4-hydroxytamoxifen with a neutralizing anti-human $TGF\beta_1$ antibody (R&D Systems) or with mannose-6-phosphate (Sigma Chemical Co.). Previous reports indicated that mannnose-6-phosphate specifically blocks the activation of latent $TGF\beta_1$ by inhibiting its binding to insulin-like growth factor type II receptor. Inhibition of such binding prevents the proteases from cleaving an amino terminal portion of the molecule, which subsequently results in an activation of $TGF\beta_1$ [29]. Cells were treated with 200, 500, and 1,000 μg/ml of the neutralizing $TGF\beta_1$ antibody or with 100 μM mannose-6-phosphate. Similarly, cells were also treated with 2, 10, and 20 μg/ml human recombinant $TGF\beta_1$ protein (R&D Systems) to study the cytotoxic effect of exogenous $TGF\beta_1$ on the LNCaP cells growing in culture. Triple wells for each concentration were evaluated, using the SRB cytotoxicity/cell growth inhibition assay. Three independent experiments were performed.

Statistical Analysis

Differences among groups were tested using one-way analysis of variance (ANOVA) with repeated

TABLE I. Inhibitory Effect of Mifepristone on Viable LNCaP Cells In Vitro: Dose- and Time-Response Relationship, SRB Assay

	% inhibition of cell viability, mean ± SE, days posttreatment[a]		
Dose (μM)	3	7	10
2.5	7 ± 4.0	21 ± 2.2	40 ± 2.6
5.0	15 ± 4.1	39 ± 2.0	51 ± 3.7
10.0	35 ± 1.2	56 ± 1.2	69 ± 2.4
15.0	53 ± 2.6	88 ± 0.6	95 ± 1.2
20.0	70 ± 2.3	93 ± 1.3	97 ± 0.6

[a]Results of three independent experiments as compared with the vehicle control group.

TABLE II. Inhibitory Effect of 4-hydroxytamoxifen on Viable LNCaP Cells In Vitro: Dose- and Time-Response Relationship, SRB Assay

	% inhibition of cell viability, mean ± SE, days posttreatment[a]		
Dose (μM)	3	7	10
2.5	2.5 ± 0.3	11 ± 1.5	20 ± 1.2
5.0	23 ± 0.9	40 ± 2.9	56 ± 6.4
7.5	41 ± 1.3	83 ± 2.0	91 ± 3.0
10.0	66 ± 1.2	95 ± 0.9	97 ± 0.7
15.0	86 ± 0	97 ± 0.9	97 ± 0.3

[a]Results of three independent experiments as compared with the vehicle control group.

measures over time. The assumption of analysis of variance was examined, and nonparametric tests based on ranks were used if needed. Values were reported as means ± SE. 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 Statgraphics Plus Statistical Graphics Systems software.

RESULTS

Cell Growth Inhibition (Cell Viability/Cytotoxicity)

Mifepristone and 4-hydroxytamoxifen had a significant dose- and time-dependent inhibitory effect on the growth and viability of LNCaP human prostate cancer cells in culture, as determined by the SRB assay (Tables I and II). There was no significant difference between androgen-sensitive (LNCaP, LNCaP-C4) and androgen-insensitive (LNCaP-C4-2) cell lines regarding this dose- and time-response relationship (data not shown). Mifepristone appeared to be slightly less potent than 4-hydroxytamoxifen (Tables I–III). The results of the SRB assay consistently indicated that 5–10 μM 4-hydroxytamoxifen and 10–15 μM mifepristone were almost equally effective (Tables I and II). These concentrations were at about the IC_{50} values (concentration of the drug resulting in 50% inhibition of cell viability) of both drugs on day 3 posttreatment (Table III). 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 effects of these steroid antagonists on DNA fragmentation, bcl_2 expression, and $TGF\beta_1$ protein concentration.

TABLE III. Inhibitory Effect of Mifepristone and 4-hydroxytamoxifen on Viable LNCaP Cells In Vitro*

	IC_{50} in μM (mean ± SE), days posttreatment[a]		
Groups	3	7	10
MIF	13.58 ± 0.43	5.95 ± 0.20	4.04 ± 0.27
TAM	8.13 ± 0.05	4.90 ± 0.20	3.95 ± 0.22

*IC_{50} values, SRB assay. MIF, mifepristone; TAM, 4-hydroxytamoxifen.
[a]Results of three independent experiments as compared with the control group. Drugs were tested in each experiment at five dose levels to enable construction of dose-response curves and estimation of IC_{50} values (concentration of the drug resulting in 50% inhibition of cell viability).

Competitive Inhibition Assays

We investigated the possible mechanism by which the progesterone and/or glucocorticoid receptors mediate the mifepristone-induced inhibition of growth and viability of LNCaP cells. Cell-growth and cell-death modulation by mifepristone was compared with that of progesterone (P) and hydrocortisone (HC). P and HC were tested at concentrations of 1 and 10 μM, either alone or in combination with 10 μM mifepristone. Given alone to LNCaP cells in culture, P induced dose- and time-dependent inhibition of cell viability (similar to mifepristone), while HC alone had no significant effect on inhibition of cell viability (Table IV). P or HC in equimolar concentration to mifepristone could not reverse the inhibitory activity of mifepristone and had no significant additive inhibitory effect on LNCaP cell viability in the SRB assay (Table IV).

Cellular DNA Fragmentation

The treatment with mifepristone (10 and 15 μM) increased the DNA fragmentation of LNCaP cells

TABLE IV. Inhibitory Effect of Mifepristone, Progesterone, Hydrocortisone, and Their Combinations on Viable LNCaP Cells In Vitro: SRB Assay*

Groups and dose (μM)	% inhibition of cell viability, mean ± SE, days posttreatment	
	3	7
MIF (10)	25 ± 2.0	50 ± 1.2
MIF (15)	49 ± 3.4	87 ± 2.5
P (1)	22 ± 0.9	56 ± 5.3
P (10)	37 ± 0.6	73 ± 1.2
HC (1)	–2 ± 1.2	–5 ± 3.5
HC (10)	–0.4 ± 0.3	–6 ± 2.3
MIF + P (10 + 1)	25 ± 4.2	53 ± 7.2
MIF + P (10 + 10)	40 ± 2.4	76 ± 2.2
MIF + HC (10 + 1)	22 ± 0.9	51 ± 2.0
MIF + HC (10 + 10)	27 ± 1.0	49 ± 4.3

*MIF, mifepristone; P, progesterone; HC, hydrocortisone.
[a]Results of three independent experiments as compared with the vehicle control group.

growing in culture in a dose-dependent manner. An almost 5-fold increase of DNA fragmentation was observed as early as 1 day posttreatment with 15 μM mifepristone. The increase in DNA fragmentation as a result of 15 μM mifepristone was significantly ($P < 0.05$) different from the control group at all time points (days 1–7 posttreatment). However, 10 μM mifepristone induced only on day 3 posttreatment a significant ($P < 0.05$) increase in DNA fragmentation as compared with the control group (Table V). In the groups treated with 5 μM 4-hydroxytamoxifen, the average increase in DNA fragmentation was about 3–4-fold that of the control group ($P < 0.05$ vs. control on days 1, 3, and 7 posttreatment). The addition of 1 μM P to 10 μM mifepristone induced a slight but not significant ($P > 0.05$) additive increase in DNA fragmentation on day 1 posttreatment (Table V). DNA fragmentation as a result of mifepristone (10 μM) treatment could be slightly but not significantly ($P > 0.05$) inhibited on days 1 and day 3 posttreatment by the addition of 1 μM HC to the culture medium (Table V). Both P and HC exhibited a significant ($P < 0.05$) additive effect to mifepristone on the induction of DNA fragmentation on day 7 posttreatment. This additive effect was more prominent for P compared with that of HC ($P < 0.05$) (Table V).

bcl_2 Protein Expression

The bcl_2 concentration in control LNCaP cells treated with the vehicle alone decreased on day 3 to about 50% of its value on day 1 posttreatment (Table VI). A significant ($P < 0.05$) downregulation of bcl_2 (over 70% inhibition) was observed as early as 1 day posttreatment with 4-hydroxytamoxifen and mifepristone. On day 3 posttreatment, the downregulation of bcl_2 induced by the steroid antagonists was less prominent (30–50% inhibition) as compared with that seen on day 1 posttreatment (Table VI). The decrease in bcl_2 concentration at day 3 posttreatment was amplified to about 65% inhibition ($P < 0.05$ vs. control) by simultaneous administration of mifepristone and P or HC. This additive effect of a combination therapy was significantly different ($P < 0.05$) from that of mifepristone monotherapy (Table VI).

$TGF\beta_1$ Protein Concentration

The $TGF\beta_1$ protein concentration was measured in supernatants from LNCaP cells treated in culture with the vehicle alone (control) or with the different drugs dissolved in the same vehicle. $TGF\beta_1$ protein levels increased to approximately 2.5-fold those of control levels on day 7 posttreatment with 5 μM 4-hydroxytamoxifen. This increase was significantly different ($P < 0.05$) from the control group (Table VII). The concentration of $TGF\beta_1$ protein increased in a dose-dependent fashion as a result of the treatment with mifepristone. The maximum protein levels (about 5–6-fold induction) were observed as an effect of mifepristone (15 μM) on days 3 and 7 posttreatment ($P < 0.05$). Also in this group, a slight but still statistically significant increase ($P < 0.05$) in $TGF\beta_1$ protein was observed as early as day 1 posttreatment (Table VII). Both P and HC exhibited a significant ($P < 0.05$) additive effect to mifepristone (10 μM) on the induction of $TGF\beta_1$ protein on days 1 and 7 posttreatment. The additive effect of P was more prominent and significantly different ($P < 0.05$) from that of HC on day 7 posttreatment (Table VII).

In Vitro Inhibition of Cytotoxicity by Mannose-6-Phosphate

The SRB assay was used to measure abrogation of cytotoxicity induced by 4-hydroxytamoxifen and mifepristone with the addition of mannose-6-phosphate. At a concentration of 100 μM mannose-6-phosphate, there was a significant ($P < 0.05$) abrogation of the cytotoxic effect of 4-hydroxytamoxifen and mifepristone (Table VIII).

In Vitro Inhibition of Cytotoxicity by Anti-Human $TGF\beta_1$-Neutralizing Antibody

The SRB assay was used to measure abrogation of cytotoxicity induced by 4-hydroxytamoxifen and

TABLE V. Effect of Mifepristone as Compared With 4-hydroxytamoxifen on DNA Fragmentation in LNCaP Cells In Vitro†

Group and dose (μM)	Absorbance (OD 405 nm × 1,000)[a] (mean ± SE),[b] days posttreatment			Enrichment factor[c] (mean ± SE)[d] days posttreatment		
	1	3	7	1	3	7
Control	45 ± 5.2	42 ± 2.8	104 ± 3.5			
TAM (5)	142 ± 16.6*	154 ± 8.6*	289 ± 3.5*	3.14 ± 0.25	3.64 ± 0.06	2.78 ± 0.06
MIF (10)	73 ± 8.3	94 ± 6.4*	194 ± 4.0	1.61 ± 0.07	2.23 ± 0.01	1.86 ± 0.04
MIF (15)	213 ± 27.4*	135 ± 6.4*	359 ± 7.8*	4.70 ± 0.20	3.21 ± 0.08	3.45 ± 0.07
MIF + P (10 + 1)	117 ± 21.9*	80 ± 4.4*	380 ± 14**	2.54 ± 0.19	1.88 ± 0.04	3.65 ± 0.06
MIF + HC (10 + 1)	47.5 ± 8.5	65 ± 4.3*	302 ± 11.8***	1.04 ± 0.02	1.53 ± 0.01	2.89 ± 0.05

†MIF, mifepristone; TAM, 4-hydroxytamoxifen; P, progesterone; HC, hydrocortisone.
[a]Absorbance was read at OD 405 nm on a microplate reader. Absorbance × 1,000 = mU.
[b]Results of three independent experiments using the Cell Death Detection ELISA Kit (Boehringer Mannheim, Indianapolis, IN).
[c]Enrichment factor = mU of treated sample/mU of control sample.
*$P < 0.05$ vs. control.
**$P < 0.05$ vs. control and MIF (10 μM).
***$P < 0.05$ vs. control, MIF (10 μM) and MIF + P (10 + 1 μM).

TABLE VI. Effect of Mifepristone, as Compared With 4-hydroxytamoxifen, on bcl_2 Expression in LNCaP Cells In Vitro†

Groups and dose (μM)	bcl_2 units/mg protein, mean ± SEM, days posttreatment[a]		% inhibition, days posttreatment	
	1	3	1	3
Control	16.46 ± 1.80	8.78 ± 0.67		
TAM (5)	4.45 ± 0.40*	6.20 ± 0.28*	73	29
MIF (10)	4.40 ± 0.32*	5.12 ± 0.46*	73	42
MIF (15)	4.32 ± 0.23*	4.32 ± 0.40*	74	51
MIF + P (10 + 1)	4.20 ± 0.11*	3.10 ± 0.34**	74	65
MIF + HC (10 + 1)	4.56 ± 0.30*	3.23 ± 0.34**	72	63

†MIF, mifepristone; TAM, 4-hydroxytamoxifen; P, progesterone; HC, hydrocortisone.
[a]Results of two independent experiments using the bcl_2 ELISA kit (Oncogene Research Products, Cambridge, MA).
*$P < 0.05$ vs. control.
**$P < 0.05$ vs. control and MIF (10 μM).
% inhibition vs. control.

mifepristone with the addition of an anti-human $TGF\beta_1$-neutralizing antibody. At antibody concentrations up to 1 mg/ml there was a trend toward dose-dependent abrogation of cytotoxicity induced by the treatment with 4-hydroxytamoxifen and mifepristone. This resulted in an increase in the viability of the LNCaP cells in culture (decrease in the inhibition of cell viability) (Table IX). An antibody concentration of 1.0 mg/ml almost completely inhibited the cytotoxic effect of 5 μM 4-hydroxytamoxifen and 10 μM mifepristone (Table IX). LNCaP cells were stimulated to grow slightly ($P > 0.05$) above control when neutralizing $TGF\beta_1$ antibody was added alone at concentrations of 0.2, 0.5, and 1 mg/ml (Table X). On the contrary, the addition of as low as 2.0–20 μg/ml recombinant human $TGF\beta_1$ protein to the culture medium induced dose-dependent inhibition of cell viability (up to 42%), as measured in the SRB assay (Table XI).

DISCUSSION

DNA Fragmentation and Modulation of Bcl_2 Expression

This is the first report to demonstrate an effect of mifepristone and 4-hydroxytamoxifen on the increase in DNA fragmentation (induction of apoptosis) in human prostate cancer cells. A role of bcl_2 downregula-

TABLE VII. Effect of Mifepristone as Compared With 4-hydroxytamoxifen on TGFβ$_1$ Protein Concentration in LNCaP Cells In Vitro†

Group and dose (μM)	TGFβ$_1$ protein[a] (pg/10^4 cells), mean ± SE,[b] days posttreatment			Ratio (T/C), mean ± SE,[b] days posttreatment		
	1	3	7	1	3	7
Control	21.1 ± 0.3	49.1 ± 2.1	9.8 ± 0.4			
TAM (5)	24.7 ± 1.1	64.8 ± 2.4	24.2 ± 1.1*	1.17 ± 0.04	1.32 ± 06	2.47 ± 0.09
MIF (10)	23.3 ± 1.1	90.0 ± 1.8*	19.9 ± 0.8*	1.10 ± 0.04	1.83 ± 0.04	2.03 ± 0.09
MIF (15)	26.0 ± 0.4*	264 ± 6.9*	57.9 ± 1.26*	1.23 ± 0.01	5.37 ± 0.13	5.92 ± 0.09
MIF + P (10 + 1)	30.3 ± 2.2**	118 ± 11.5*	31.7 ± 1.44**	1.44 ± 0.09	2.40 ± 0.15	3.26 ± 0.27
MIF + HC (10 + 1)	29.9 ± 0.9**	100.9 ± 6.5*	26.2 ± 0.60***	1.42 ± 0.03	2.05 ± 0.05	2.68 ± 0.06

†T/C, treatment/control; MIF, mifepristone; TAM, 4-hydroxytamoxifen; P, progesterone; HC, hydrocortisone.
[a]TGFβ$_1$ protein was quantitated in cell culture supernatant using the Quantikine Human TGFβ$_1$ ELISA kit (R&D Systems, Minneapolis, MN).
[b]Results of three independent experiments.
*$P < 0.05$ vs. control.
**$P < 0.05$ vs. control and MIF (10 μM).
***$P < 0.05$ vs. control, MIF (10 μM) and MIF + P (10 + 1 μM).

TABLE VIII. Abrogation of Inhibitory Effect of Mifepristone and 4-hydroxytamoxifen on Viable LNCaP Cells In Vitro by Addition of Mannose-6-phosphate: SRB Assay, Day 3 Posttreatment*

Groups and dose (μM)	% inhibition of cell viability, mean ± SE, mannose-6-phosphate (μM)[a]	
	0	100
TAM (5)	24.3 ± 1.45	5.9 ± 2.35[b]
MIF (10)	36.0 ± 6.11	18.8 ± 7.22[b]
MIF (15)	61.0 ± 6.56	49.3 ± 8.51[b]

*MIF, mifepristone; TAM, 4-hydroxytamoxifen.
[a]Results of three independent experiments as compared with the vehicle control group.
[b]Significant difference ($P < 0.05$) as compared with the corresponding group without the addition of mannose-6-phosphate.

tion in the induction of apoptosis by mifepristone and 4-hydroxytamoxifen in prostate cancer cells has not previously been published. Our results suggest that the antitumor activity of mifepristone (antiprogestins) and antiestrogens such as tamoxifen could be the result of their interaction with the cell suicide mechanism, which involves an early downregulation (as early as 24 hr posttreatment) of bcl$_2$ in human prostate cancer cells. In this study, there was a nice correlation between induction of apoptosis (extent of DNA fragmentation) and bcl$_2$ downregulation as a result of treatment with 4-hydroxytamoxifen and mifepristone. The increase in DNA fragmentation in our ELISA assay was associated with the appearance of DNA laddering on agarose gel electrophoresis (data not shown). 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. Moreover, the increase in DNA fragmentation and the downregulation of bcl$_2$ confirm that both 4-hydroxytamoxifen and mifepristone are specific inducers of apoptosis in prostate cancer cells.

In a recent study, the apoptotic responses of LNCaP and its androgen-independent derivative LNCaP C4-2 cells were investigated after treatment with the differentiation-promoting agent phenylbutyrate [30]. The results of this study showed that the downregulation of bcl$_2$ and upregulation of bax are key elements mediating phenylbutyrate-induced prostate cancer cell death. With respect to human prostate cancer, data have accumulated that upregulation of bcl$_2$ is associated with cancer progression and the acquisition of an androgen-independent phenotype [31,32], although the relative significance of bcl$_2$ in the process has more recently been disputed [33]. Other published data indicate that androgens promote bcl$_2$ expression [34], and that the effects of androgen withdrawal are blocked by enforced overexpression of bcl$_2$ [35]. This is consistent with some reports of bcl$_2$ overexpression in androgen-insensitive tumors as detected in immunohistochemical analyses of patient specimens [31–33,36]. The possibility that disruption of the cell death pathway may contribute to multidrug resistance (androgen-independent progression) and tumor metastasis was suggested in a recent study using nonmeta-

TABLE IX. Effect of Anti-Human TGFβ$_1$-Neutralizing Antibody on the Inhibitory Effect of 4-hydroxytamoxifen and Mifepristone on Viable LNCaP Cells In Vitro: SRB Assay, Day 3 Posttreatment*

Groups	% inhibition of cell viability, mean ± SE[a]	% abrogation of cytotoxicity, mean ± SE[a]
TAM 5 μM + TAB (mg/ml)		
0.0	26 ± 1.9	
0.2	19 ± 2.0	25 ± 7.7[b]
0.5	11 ± 1.5	57 ± 8.6[b]
1.0	5 ± 1.3	81 ± 5.9[b]
MIF 10 μM + TAB		
0.0	33 ± 1.2	
0.2	24 ± 5.3	27 ± 13.6[b]
0.5	11 ± 5.9	68 ± 16.8[b]
1.0	0.9 ± 1.2	97 ± 10.4[b]
MIF 15 μM + TAB		
0.0	60 ± 2.6	
0.2	54 ± 5.2	9 ± 7.7
0.5	39 ± 6.6	35 ± 9.3[b]
1.0	26 ± 2.1	56 ± 5.5[b]

*TAB, TGFβ$_1$-neutralizing antibody; TAM, 4-hydroxytamoxifen; MIF, mifepristone.
[a]Results from three independent experiments as compared to the control group.
[b]Significantly different ($P < 0.05$) as compared with the corresponding control group (without the addition of TAB).

TABLE X. Effect of Anti-Human TGFβ$_1$-Neutralizing Antibody on Viable LNCaP Cells In Vitro: SRB Assay, Day 3 Posttreatment*

TAB (mg/ml)	% cell viability (mean ± SE)[a]
0.2	100 ± 00.0
0.2	146 ± 20.8
0.5	128 ± 11.9
1.0	115 ± 11.3

*TAB, TGFβ$_1$-neutralizing antibody.
[a]Results from three independent experiments as compared to the control group.

TABLE XI. Effect of a Recombinant Human TGFβ$_1$ Protein on Viable LNCaP Cells In Vitro: SRB Assay, Day 3 Posttreatment

TGFβ$_1$ protein[a]	% inhibition of cell viability (mean ± SE)[b]
2.0 μg/ml	26 ± 7.9[c]
10.0 μg/ml	31 ± 7.2[c]
20.0 μg/ml	42 ± 6.5[c]

[a]TGFβ$_1$ protein was added to the culture medium in all groups.
[b]Results of three independent experiments as compared with the control group, using the recombinant human TGFβ$_1$ protein (R&D Systems, Minneapolis, MN).
[c]Significantly different ($P = 0.05$) as compared with the control group.

static and metastatic variants of the LNCaP human prostate cancer cell line [37]. In this study, of the five members of the bcl$_2$ gene family analyzed, upregulation of bcl$_2$ was the most dramatic alteration of expression observed in the metastatic cells, indicating that it was directly selected for by the metastatic cells in this model. Downregulation of bax and bak was also observed, which may contribute to the apoptosis-resistant phenotype of these cells [37]. The expression of the other members of the bcl$_2$ gene family (e.g., bcl-x$_L$, bad) was indistinguishable among these cell lines, indicating the selectivity of the process [37]. These data support the hypothesis that apoptosis resistance contributes to androgen-independent prostate cancer progression and metastasis, and that elevated expression of bcl$_2$ and downregulation of bax are involved in the acquisition of such an intrinsic survival advantage. If prostate cancer progression and metastasis select for apoptosis resistance and alterations in expression of the bcl$_2$ family, it is conceivable that apoptosis resistance is an important cause of drug resistance. Therefore, the emergence of androgen-independent clones of cells that do not respond to androgen ablation may involve the acquisition of intrinsic apoptosis resistance [37]. Antiprogestins and tamoxifen as effective inducers of apoptosis could rep-

resent nonandrogen ablation, leading to novel therapeutic approaches to overcome such intrinsic apoptosis resistance of androgen-independent cells.

Role of TGFβ$_1$ Protein

This is the first report to demonstrate that the in vitro antiproliferative activity of mifepristone and 4-hydroxytamoxifen is associated with an 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 most of the drug treatment groups did not start to be significant before day 3 posttreatment. This appeared to coincide with the inhibition of cell viability in the cell growth inhibition (cytotoxicity) assay. On the contrary, the bcl$_2$ downregulation and the increased DNA fragmentation appeared to start to be significant as early as 24 hr posttreatment. The hypothesis that tamoxifen and mifepristone operate through induction of TGFβ$_1$ is supported by their effect of increasing TGFβ$_1$ protein concentration, as measured in supernatants (ELISA), and by the ability of TGFβ$_1$-neutralizing antibodies and mannose-6-phosphate 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 and mifepristone 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 prostate cancer cell death. However, LNCaP cells treated with anti-TGFβ$_1$ antibody alone showed a tendency of a slight increased growth as compared with control cells (see Table X). These findings imply that the antibody also blocked TGFβ$_1$ that may have been present from constitutive expression by LNCaP cells or from TGFβ$_1$ in FBS. Our findings also indicate that the TGFβ$_1$ protein induced by the antihormone treatments in our studies was in an active and not in a latent form. Our results are in agreement with published data showing that although all intermediate steps of apoptosis in prostate cells have not been fully identified, an increase in TGFβ production is known to be a key element [29,38–42]. TGFβ$_1$ shows upregulation in gene expression parallel to the apoptosis induced by androgen ablation in both normal prostate (e.g., rat ventral prostate) and prostate cancer tissues (e.g., PC82 androgen-dependent human prostate cancer xenografts transplanted into nude mice) [39]. Furthermore, the fenretinide-induced cytotoxicity in PC3 cells was abrogated by the addition of anti-TGFβ$_1$ antibody [41]. Hsing et al. [42] reported direct evidence that TGFβs induce apoptosis of prostatic epithelial cells in vitro, using two unique nontumorigenic and tumorigenic dorsal-lateral rat prostatic cell lines. TGFβ$_1$ induces apoptosis of both cell lines within 24 hr, as shown by a decrease in cell viability, in situ DNA nick-end labeling, and internucleosomal DNA fragmentation [42]. Using human prostate cancer cell lines, it has been suggested that LNCaP cells do not express TGFβ$_1$, as both Northern blot analysis of LNCaP RNA and immunoprecipitation of LNCaP-conditioned media did not detect TGFβ$_1$ [40]. In a recent study, RT-PCR was used to increase the sensitivity of detection. The results of this study showed that LNCaP cells express TGFβ$_1$ but not TGFβ$_2$ and TGFβ$_3$ mRNA. However, the possibility exists that these cells may also express TGFβ$_2$ and TGFβ$_3$, but at a level undetectable by the RT-PCR primers used in this study [29]. The above-mentioned results, together with findings suggesting that TGFβ$_1$ suppresses the immune system [43], stimulates angiogenesis [44], and enhances the invasive potential of tumors [45], indicate that TGFβ$_1$ might play an important but still undefined role in the pathogenesis and management of prostatic diseases. The molecular mechanism of TGFβ$_1$ and its possible role in mifepristone- and tamoxifen-induced apoptosis in prostate cancer cells remain to be established.

Cell Growth Inhibition/Interactions With Progesterone and Glucocorticoid Receptors

Under our culture conditions, mifepristone and 4-hydroxytamoxifen had a significant dose- and time-dependent inhibitory effect on the growth and viability of LNCaP, LNCaP-C4, and LNCaP-C4-2 prostate cancer cells. Our results have shown that the antiproliferative activity of mifepristone was not significantly reduced or prevented by occupying the progesterone (PR) or the glucocorticoid (GR) receptors by their corresponding high-affinity native ligands. On the contrary, progesterone exhibited growth inhibition of the LNCaP prostate cancer cells comparable to that of mifepristone, and the effect of a combination of mifepristone with P or HC was rather additive regarding induction of the apoptotic pathway. Therefore, we have no direct evidence of whether the antiproliferative effects (cytotoxicity) of mifepristone in the human LNCaP prostate cancer cells are mediated via interactions with GR or PR [5,6,13]. However, the mutation in the ligand-binding domain of the androgen receptor of LNCaP cells could affect steroid-binding characteristics and the response to these different steroids [24]. Our results in the cell growth inhibition assay also clearly indicate that both androgen-sensitive and androgen-independent variants of the LNCaP prostate

cancer cell line did not exhibit any significant differences in apoptosis sensitivity to mifepristone and 4-hydroxytamoxifen treatment.

CONCLUSIONS

Based on our results, it seems reasonable to assume that mifepristone and tamoxifen interact with prostate cancer cells to initiate a cell death command via apoptotic pathways involving downregulation of bcl_2 and induction of $TGF\beta_1$ protein. Our data further suggest that mifepristone and tamoxifen, similar to antiandrogens/androgen ablation, are effective inducers of apoptosis. Therefore, these steroid antagonists could represent nonandrogen ablation novel therapeutic approaches to overcome a potential apoptosis resistance of androgen-independent prostate cancer cells. Further studies are still needed to confirm this hypothesis.

REFERENCES

1. Ekman P, Snochowski M, Dahlberg E, Gustafsson JA. Steroid receptors in metastatic carcinoma of the human prostate. Eur J Cancer 1979;15:257–262.
2. Robel P, Eychenne B, Blondeau JP, Baulieu EE, Hechter O. Sex steroid receptors in normal and hyperplastic prostate. Prostate 1985;6:255–267.
3. Brolin J, Skoog L, Ekman P. Immunohistochemistry and biochemistry in detection of androgen, progesterone and estrogen receptors in benign and malignant human prostatic tissue. Prostate 1992;20:281–295.
4. Ehara H, Koji T, Deguchi T, Yoshii A, Nakano M, Nakane PK, Kawada Y. Expression of estrogen receptor in diseased human prostate assessed by nonradioactive in situ hybridization and immunohistochemistry. Prostate 1995;27:304–313.
5. Mobbs BG, Johnson IE. Suppression of the growth of the androgen-insensitive R3327 HI rat prostatic carcinoma by combined estrogen and antiprogestin treatment. J Steroid Biochem Mol Biol 1991;39:713–722.
6. Parczky K, Schneider MR. The future of antihormone therapy: innovations based on an established principle-review. J Cancer Res Clin Oncol 1996;122:283–396.
7. Schnieder MR, Michna H, Nishino Y, El Etreby MF. Antitumor activity of the progesterone antagonists ZK 98.299 and RU 38.486 in the hormone-dependent MXT-mammary tumor model of the mouse and the DMBA- and the MNU-induced mammary tumor models of the rat. Eur J Cancer Clin Oncol 1989;25:691–701.
8. Michna H, Schneider MR, Nishino Y, El Etreby MF. Antitumor activity of the antiprogestins ZK 98.299 and RU 38.486 in hormone dependent rat and mouse mammary tumors; mechanistic studies. Breast Cancer Res Treat 1989;14:275–288.
9. Michna H, Schneider MR, Nishino Y, El Etreby MF. The antitumor mechanism of progesterone antagonists is a receptor mediated antiproliferative effect by induction of terminal cell death. J Steroid Biochem Mol Biol 1989;34:447–453.
10. Schneider MR, Michna H, Nishino Y, El Etreby MF. Antitumor activity and mechanism of action of different antiprogestins in experimental breast cancer models. J Steroid Biochem Mol Biol 1990;37:783–787.
11. El Etreby MF, Liang Y, Wrenn RW, Schoenlein PV. Additive effect of mifepristone and tamoxifen on apoptotic pathways in MCF-7 human breast cancer cells. Breast Cancer Res Treat 1998; 51:149–168.
12. El Etreby MF, Liang Y. Effect of antiprogestins and tamoxifen on growth inhibition of MCF-7 human breast cancer cells in nude mice. Breast Cancer Res Treat 1998;49:109–117.
13. Lin M-F, Kawachi MH, Stallcup MR, Grunberg SM, Lin F-F. Growth inhibition of androgen-insensitive human prostate carcinoma cells by a 19-norsteroid derivative agent, mifepristone. Prostate 1995;26:194–204.
14. Colletta AA, Benson JR, Baum M. Alternative mechanisms of action of antiestrogens. Breast Cancer Res Treat 1994;31:5–9.
15. Rowlands MG, Budworth J, Jarman M, Hardcastle IR, McCague R, Gescher A. Comparison between inhibition of protein kinase C and antagonism of calmodulin by tamoxifen analogues. Biochem Pharmacol 1995;50:723–726.
16. Astruc ME, Chabret C, Bali P, Gagne D, Pons M. Prolonged treatment of breast cancer cells with antiestrogens increases the activating protein-1-mediated response: involvement of the estrogen receptor. Endocrinology 1995;136:824–832.
17. Gundimeda U, Chen ZH, Gopalakrishna R. Tamoxifen modulates protein kinase C via oxidative stress in estrogen receptor-negative breast cancer cells. J Biol Chem 1996;271:13504–13514.
18. Kiss Z, Anderson WH. Inhibition of phorbol ester-stimulated phospholipase D activity by chronic tamoxifen treatment in breast cancer cells. FEBS Lett 1997;400:145–148.
19. Cabot MC, Zhang Z, Cao H, Lavie Y, Giuliano AE, Han TY, Jones RC. Tamoxifen activates cellular phospholipase C and D and elicits protein kinase C translocation. Int J Cancer 1997;70: 567–574.
20. Theyer G, Schirmbock M, Thalhammer T, Sherwood ER, Baumgartner G, Hamilton G. Role of MDR-1-encoded multiple drug resistance phenotype in prostate cancer cell lines. J Urol 1993;150:1544–1547.
21. Schneider MR, Schiller CD, Humm A, von Angerer E. Effect of zindoxifene on experimental prostatic tumors of the rat. J Cancer Res 1991;117:33–36.
22. Lucia MS, Anzano MA, Slayter MV, Anver MR, Green DM, Shrader MW, Logsdon DL, Driver CL, Brown CC, Peer CW, Roberts AB, Sporn MB. Chemopreventive activity of tamoxifen, N-(4-hydroxyphenyl) retinamide, and the vitamin D analogue RO 24-5531 for androgen-promoted carcinomas of the rat seminal vesicle and prostate. Cancer Res 1995;55:5621–5627.
23. Pienta KJ, Replogle T, Lehr JE. Inhibition of prostate cancer growth by vinblastine and tamoxifen. Prostate 1995;26:270–274.
24. Veldscholte J, Ris-Stalpers C, Kuiper GG, Jenster G, Berrevoets C, Claassen E, Rooij HC, Trapman J, Brinkman AO, Mulder E. A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to antiandrogens. Biochem Biophys Res Commun 1990;173:534–540.
25. Wu HC, Hsieh JT, Gleave ME, Brown NM, Pathak S, Chung LWK. Derivation of androgen-independent human LNCaP prostatic cancer cell sublines: role of bone stromal cells. Int J Cancer 1994;57:406–412.
26. Thalmann GN, Anezinis PE, Chang S-M, Zhau HE, Kim EE, Hopwood VL, Pathak S, von Eschenbach AC, Chung LWK. Androgen-independent cancer progression and bone metastasis in the LNCaP model of human prostate cancer. Cancer Res 1994; 54:2577–2581.
27. Rubenstein LV, Shoemaker RH, Paul KD, Simon RM, Tosini S, Skehan P, Scudiero DA, Monks A, Boyd MR. Comparison of in vitro anticancer-drug-screening data generated with a tetrazo-

lium assay versus a protein assay against a diverse panel of human tumor cell lines. J Natl Cancer Inst 1990;82:1113–1118.
28. Hannan CJ, Liang Y, Allison JD, Searle JR. In vitro cytotoxicity against human cancer cell lines during pulsed magnetic field exposure. Anticancer Res 1994;14:1517–1520.
29. Kim IY, Kim JH, Zelner DJ, Ahn HJ, Sensibar JA, Lee C. Transforming growth factor-β_1 is a mediator of androgen-regulated growth arrest in an androgen-responsive prostatic cancer cell line, LNCaP. Endocrinology 1996;137:991–999.
30. Ng YK, Bales W, Box E, Pickens C, Veltri RW. Bcl_2 and bax but not p53 or Fas are involved in prostate cancer cell apoptosis induced by phenylbutyrate. In: Proceedings of the 89th Annual Meeting of the American Association for Cancer Research in New Orleans, LA, March 28–April 1, 1998, abstract #3882.
31. McDonnell TJ, Troncoso P, Brisbay SM, Logothetis C, Chung LWK, Hsieh JT, Tu SM, Campbell ML. Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. Cancer Res 1992;52:6940–6944.
32. Colombel M, Symmans F, Gil S, O'Toole KM, Chopin D, Benson M, Olsson CA, Korsmeyer S, Buttyan R. Detection of the apoptosis-suppressing oncoprotein bcl-2 in hormone-refractory human prostate cancers. Am J Pathol 1993;143:390–400.
33. Krajewska M, Krajewski S, Epstein JI, Shabaik A, Sauvageot J, Song K, Kitada S, Reed JC. Immunohistochemical analysis of bcl-2, bax, bcl-X and mcl-l expression in prostate cancers. Am J Pathol 1996;148:1567–1576.
34. Berchem GJ, Bosseler M, Sugars LY, Voeller HJ, Zeitlin S, Gelmann EP. Androgens induce resistance to bcl-2 mediated apoptosis in LNCaP prostate cancer cells. Cancer Res 1995;55:735–738.
35. Raffo AJ, Perlman H, Chen MW, Day ML, Streitman JS, Buttyan R. Overexpression of bcl-2 protects prostate cancer cells from apoptosis in vitro and confers resistance to androgen depletion in vivo. Cancer Res 1995;55:4438–4445.
36. Bubendorf L, Sauter G, Moch H, Jordon P, Blochlinger A, Gasser TC, Mihtsch MJ. Prognostic significance of bcl-2 in clinically localized prostate cancer. Am J Pathol 1996;48:1557–1565.
37. McConkey DJ, Greene G, Pettaway CA. Apoptosis resistance increases with metastatic potential in cells of the human LNCaP prostate carcinoma line. Cancer Res 1996;56:5594–5599.
38. Furuya Y, Isaacs JT. Proliferation-dependent vs. independent programmed cell death of prostatic cancer cells involves distinct gene regulation. Prostate 1994;25:301–309.
39. Kyprianou N, Isaacs JT. Expression of transforming growth factor-β in the rat ventral prostate during castration induced programmed cell death. Mol Endocrinol 1989;3:1515–1522.
40. Wilding G, Zugmeier G, Knabbe C, Flanders K, Gelmann E. Different effects of transforming growth factor β on human prostate cancer cells in vitro. Mol Cell Endocrinol 1989;62:79–87.
41. Roberson KM, Penland SN, Padilla GM, Selvan RS, Kim C-S, Fine RL, Robertson CN. Fenretinide: induction of apoptosis and endogenous transforming growth factor β in PC3 prostate cancer cells. Cell Growth Differ 1997;8:101–111.
42. Hsing AY, Kadomatsu K, Bonham MJ, Danielpour D. Regulation of apoptosis induced by transforming growth factor-β_1 in nontumorigenic and tumorigenic rat prostate epithelial cell lines. Cancer Res 1996;56:5146–5149.
43. Torre-Amione G, Beauchamp RD, Koeppen H, Park BN, Schreiber H, Moses HL, Rowley DA. A highly immunogenic tumor transfected with a murine transforming growth factor type β_1 cDNA escapes immune surveillance. Proc Natl Acad Sci USA 1990;87:1486–1490.
44. Roberts AB, Spron MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, Keine UI, Liotta LA, Flanaga V, Kehrl JH, Fauci AS. Transforming growth factor type β: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Acad Sci USA 1986;83:4167–4171.
45. Welch DR, Farba A, Nakajima M. Transforming growth factor β stimulates mammary adenocarcinoma cell invasion and metastatic potential. Proc Natl Acad Sci USA 1990;87:7678–7682.