The Prostate 37:51–59 (1998)

Prostate Cancer Cell Growth Inhibition by Tamoxifen Is Associated With Inhibition of Protein Kinase C and Induction of p21$^{waf1/cip1}$

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BACKGROUND. Inhibition of protein kinase C (PKC) and modulation of transforming growth factor-β (TGF-β) are both associated with tamoxifen treatment, and both appear to be important in the regulation of prostate cancer cell growth. Investigations were performed which sought to measure the efficacy, and to elucidate the mechanism of growth inhibition by tamoxifen, in hormone-refractory prostate cancer.

METHODS. Growth assays were performed on PC3, PC3-M, and DU145 prostate cancer cells. TGF-β was measured by ELISA; p21$^{waf1/cip1}$ and retinoblastoma (Rb) protein levels were measured by Western blot; PKC activity was measured by kinase assay; and effects upon cell cycle were measured by flow cytometric analysis.

RESULTS. IC_{50}s for growth inhibition ranged from 5.5–10 μM, and were not affected by estrogen. Tamoxifen-mediated growth inhibition was not associated with induction of TGF-β. However, tamoxifen treatment was associated with inhibition of PKC, which was followed by induction of p21$^{waf1/cip1}$, Rb dephosphorylation, and G1/S phase cell cycle arrest. Similar effects were observed with the known PKC inhibitor, Ro31-8220.

CONCLUSIONS. These data suggest that micromolar concentrations of tamoxifen inhibit prostate cancer cell growth by inhibition of PKC, resulting in induction of the p21$^{waf1/cip1}$ protein. *Prostate 37:51–59, 1998.* © 1998 Wiley-Liss, Inc.†

KEY WORDS: **transforming growth factor beta; retinoblastoma; signal transduction; drug therapy; cell cycle**

INTRODUCTION

Factors regulating the growth of prostate cancer cells are not well-understood. A greater understanding of prostate cancer cell growth regulatory pathways is a requisite for the development of more effective therapy for this disease, which is not curable once it has metastasized. Though a variety of growth factors and hormones have been shown to alter prostate cancer cell growth in vitro, the majority stimulate cell growth [1,2]. Agents which modulate negative regulatory factors therefore serve as valuable tools with

Abbreviations: PKC, protein kinase C; TGF-β, transforming growth factor-beta; Rb, retinoblastoma; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; DMSO, dimethylsulfoxide; MTT, dimethylthiazol-diphenytetrazolium bromide; SDS, sodium dodecyl sulfate; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; ND_{50}, neutralization dose, 50%; IC_{50}, inhibitory concentration, 50%.

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Received 16 December 1997; Accepted 31 March 1998

which to study important growth control pathways in prostate cancer.

Transforming growth factor-β (TGF-β) is a growth factor which is capable of inhibiting prostate cell growth in vitro [3–6], and has apparent prostate cell growth regulatory roles in vivo [7–10,11]. In fact, early loss of responsiveness to TGF-β-mediated growth inhibition has been associated with clinical progression of prostate cancer [10,12–14]. A second element of potential importance in regulating the growth of prostate cancer cells is the serine/threonine kinase, protein kinase C (PKC) [15,16]. PKC is a signaling enzyme of known importance in regulating the growth and/or differentiation of a variety of cell types; inhibition of its kinase activity is associated with loss of regulatory function [17].

Tamoxifen (1-[p dimethylaminoethoxyphenyl]-1,2-diphenyl-1-butene) is a drug known to have TGF-β modulatory [18,19] and PKC inhibitory effects [20,21]. Tamoxifen has been shown to increase TGF-β secretion in breast cancer cells [18,19], as well as in supporting stromal cells [22]. Tamoxifen inhibits the Ca^{2+}/phospholipid-dependent activity of PKC in vitro, with IC_{50}s as low as 6.1 μM [23]; efficacy in vivo may be greater [20].

In this study, investigations were undertaken to determine whether tamoxifen could inhibit the growth of prostate cancer cells, and if so, to determine the mechanism of growth inhibition. Tamoxifen was shown to inhibit prostate cancer cell growth in a manner independent of estrogen. Growth inhibition was associated with inhibition of Ca^{2+}/phospholipid-dependent PKC activity, which preceded the induction of the cell cycle-inhibitory protein $p21^{waf1/cip1}$ [24].

MATERIALS AND METHODS

Materials

PC3 and DU-145 prostate carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD); PC3-M prostate carcinoma cells were previously described [25], and are a metastatic variant of PC3 cells, as described by Kozlowski et al. [26]. Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and phosphate-buffered saline (PBS) were from Biofluids (Rockville, MD), RPMI-1640 from GIBCO BRL (Grand Island, NY), and microtiter plates from Becton-Dickinson (Lincoln Park, NJ).

The following were obtained from Sigma Chemical Co. (St. Louis, MO): F-12 HAM culture media, dimethylsulfoxide (DMSO), tamoxifen, 4-hydroxytamoxifen, estradiol, diltiazem, verapamil, and MTT (dimethylthiazol-diphenytetrazolium bromide).

Anti-$p21^{waf1/cip1}$ mouse monoclonal antibody (clone EA10) was purchased from Oncogene Science (Cambridge, MA).

The following were purchased from R & D Systems (Minneapolis, MN): anti-TGF-β1 neutralizing antibody, pan-specific TGF-β neutralizing antibody, recombinant human TGF-β1, porcine TGF-β2, recombinant human TGF-β3, and ELISA assay kits for TGF-β1 and -β2 (performed according to the manufacturer).

Sheep anti-mouse horseradish peroxidase-conjugated secondary antibody was purchased from Amersham Corporation (Arlington Heights, IL). Ro31-8220, a specific inhibitor of Ca^{2+}/phospholipid-dependent protein kinase C, whose properties have been described elsewhere [27], was kindly provided by Roche Products, Ltd. (Hertfordshire, UK). Murine monoclonal antibody (clone G3-245) to the human retinoblastoma (Rb) protein was obtained from PharMingen (San Diego, CA). cAMP levels were measured with a cyclic AMP [^{3}H] Biotrak radioimmunoassay system, according to the manufacturer (Amersham Corporation).

Cell Culture

PC3 and PC3-M cells were grown in RPMI-1640 supplemented with 10% FBS; DU-145 cells were grown in DMEM supplemented with 5% FBS. All cells were maintained at 37°C in a humidified atmosphere of 5% carbon dioxide, with biweekly media changes.

Growth Assays

Three-day growth assays were performed in Falcon TC microtiter plates, as described [28]. Briefly, 800–2,000 prostate cancer cells per 100 μl of cell culture media were plated into each well. Twenty-four hours later, the various drugs to be tested, suspended in cell culture media, were added to give a final volume of 200 μl per well. After 3 days (unless otherwise indicated), viable cells were detected by dimethylthiazol-diphenytetrazolium bromide (MTT) reduction, or else DNA synthesis was measured by thymidine uptake. MTT reduction and thymidine uptake were measured as described [28]. All microtiter assays in this study were run in replicates of 3–5, and were performed at least twice. Prior to use, tamoxifen was suspended in DMSO stock solution and stored at −70°C. Tamoxifen was thawed just prior to use. The final DMSO concentration did not exceed 0.3% in any experiment. In some experiments, cells were grown in serum-free media as described; in these experiments, cells were placed in serum-free media 24 hr after plating. Estradiol (Sigma Chemical Company), used in some experiments, was suspended in ethanol and stored as a stock solution at −70°C; final ethanol concentrations in growth assays did not exceed 0.25%.

In some experiments, cells were treated with a combination of TGF-β1 and tamoxifen. The pharmacologic effects of the combination (i.e., antagonism, additivity, or synergism) was determined by the fractional product method of Webb [29], and expressed as the ratio of calculated to observed cell viability when cells were treated with both TGF-β1 and tamoxifen. Ratios above one indicate synergism, while those below indicate antagonism. Additive effects are associated with a ratio of one. Calculated viability was determined as follows for each concentration of tamoxifen and TGF-β1 tested: (fraction of viable cells treated with tamoxifen only) × (fraction of viable cells treated with TGF-β1 only) × 100.

Flow Cytometric Analysis

Cell preparation, DNA staining, and flow cytometric analysis were performed as described [30], on a FACStar Plus (Becton-Dickinson, San Jose, CA).

Western Analysis

Cell lysis and Western blotting for $p21^{waf1/cip1}$ were performed as described previously [25]. Briefly, PC3-M cells were lysed in TNESV lysis buffer (50 mM Tris, pH 75, 1% NP40, 2 MM EDTA, 100 MM NACL) [25], and the resultant clarified lysates, normalized for protein, were run on a 15% sodium dodecyl sulfate (SDS) polyacrylamide gel. For detection of Rb, nuclear and cytoplasmic proteins were isolated and separated on a 10% SDS polyacrylamide gel as described [31]. Proteins were then transferred onto 0.45 μm nitrocellulose (Schleicher and Schuell, Keene, NH) in a semi-dry transfer apparatus (Pharmacia-Upjohn, Uppsala, Sweden). Blots were blocked with nonfat dry milk, and probed with either anti-$p21^{waf1/cip1}$ monoclonal antibody diluted 1:100, or anti-Rb antibody diluted 1:500. Proteins were then detected by anti-mouse-HRP conjugated secondary antibody diluted 1:1,000, and visualized with the ECL detection system (Amersham Corporation) according to the manufacturer's instructions.

Protein Kinase C Assay

Assay for total protein kinase C (PKC) activity was performed as described [32], utilizing the PKC-specific substrate [ser^{25}]PKC (GIBCO BRL), according to the manufacturer's instructions. Briefly, cells were first sonicated for 5 min at 4°C in cell lysis buffer consisting of 1% Triton X-100, 50 mM Tris, pH 7.5, 2 mM EGTA, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 200 μg/ml leupeptin, 400 μg/ml soybean trypsin inhibitor, and 5 μg/ml aprotinin (all from Sigma Chemical Company). After clarification at 14,000*g* for 10 min at 4°C, the supernatant was incubated with DEAE-sepharose (Pharmacia-Upjohn), and the batch was eluted with buffer A (20 mM HEPES, pH 7.5, 10 mM 2-mercaptoethanol, 0.5 mM EDTA, 0.5 mM EGTA, and 0.2 M NaCl). The kinase reaction was initiated by the addition of [^{32}P]-ATP into reaction mixtures with or without Ca^{2+}/phosphatidyl-serine; after 10 min, the reaction was stopped by placing on ice, and spotting onto glass fiber filters (Whatman, Hillsboro, OR). After washing with cold 10% trichloroacetic acid × 3, filters were dried, and incorporated [^{32}P] was counted by Cerenkov counting. All PKC assays were run in replicates of at least three; all reactions were repeated at a separate time at least once.

TABLE I. Growth Inhibition of Prostate Cancer Cells Treated With Tamoxifen for Three Days

Cell line	IC_{50}, μM (mean ± SE)
PC3	10 ± 3.0
PC3-M	8.0 ± 2.0
DU145	5.5 ± 0.7

RESULTS

Prostate Cancer Cell Growth Is Inhibited by Tamoxifen in an Estrogen-Independent Manner

Tamoxifen's growth-inhibitory effect was quantitated by treating several prostate cancer cell lines (PC3, PC3-M, and DU-145) with tamoxifen for 3 days, and measuring thymidine uptake. As shown in Table I, IC_{50} values ranged from 5.5–10 μM. In-depth investigations, aimed at elucidating the mechanism of growth inhibition by tamoxifen, were undertaken utilizing the PC3-M prostate cancer cell line. The PC3-M cell line is a more metastatic variant of the androgen-independent PC3 cell line, and as such is representative of clinical hormone-refractory metastatic prostate cancer [25,26].

The concentration of estrogen was <1 pM in these studies (>5 logs below the concentration of tamoxifen), and separate studies have shown that the PC3-M cell line lacks functional estrogen receptors [28]. However, as tamoxifen is a well-known estrogen agonist/antagonist, and as estrogens are widely used to treat prostate cancer clinically, the ability of estradiol to affect tamoxifen-mediated growth inhibition was measured. PC3-M cells were treated with tamoxifen for 3 days, in the presence or absence of 0.1 μM estradiol, and growth inhibition was measured. As shown in Figure 1, estradiol had no direct effect upon cell growth, nor did it affect tamoxifen-mediated growth inhibition of prostate cancer cells.

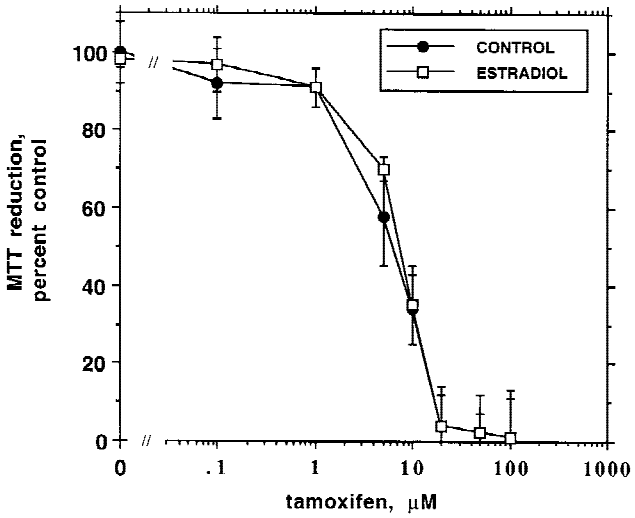


Fig. 1. Effect of estrogens upon tamoxifen-mediated prostate cancer cell growth inhibition. PC3-M cells were treated with tamoxifen for 3 days in the presence or absence of 0.1 μM estradiol, as described in Materials and Methods. MTT reduction was measured, and expressed as the mean ± SEM (n = 3) of control.

TGF-β Inhibits Growth of Prostate Cancer Cells

The effects of TGF-β upon prostate cancer cell growth were examined by exposing cells to TGF-β1, -β2, or -β3 for 24 hr and then measuring thymidine uptake (Fig. 2A). All TGF-β subspecies tested inhibited the growth of PC3-M cells, with essentially identical IC_{50} values (range, 0.22–0.28 ng/ml). The specificity of growth inhibition was shown by demonstrating that TGF-β blocking antibody reversed TGF-β-mediated growth inhibition (Fig. 2B). This was determined by treating cells as in Figure 2A, except that prior to adding a fixed dose of TGF-β (the minimal dose of each particular TGF-β subspecies associated with maximal growth inhibition), TGF-β blocking antibody was added. ND_{50}s (50% neutralization doses) were essentially identical for the three TGF-β subspecies tested (range, 1.2–2.7 μg/ml).

Tamoxifen Does Not Increase TGF-β Secretion by Prostate Cancer Cells

To determine whether tamoxifen was inducing TGF-β secretion by PC3-M cells, TGF-β levels post-tamoxifen treatment were measured by ELISA. Even after exposure to tamoxifen for up to 3 days, no increase in TGF-β1 or -β2 was detected (data not shown). This was confirmed by demonstrating that TGF-β1–3 blocking antibody did not prevent tamoxifen-mediated PC3-M growth inhibition (Fig. 3).

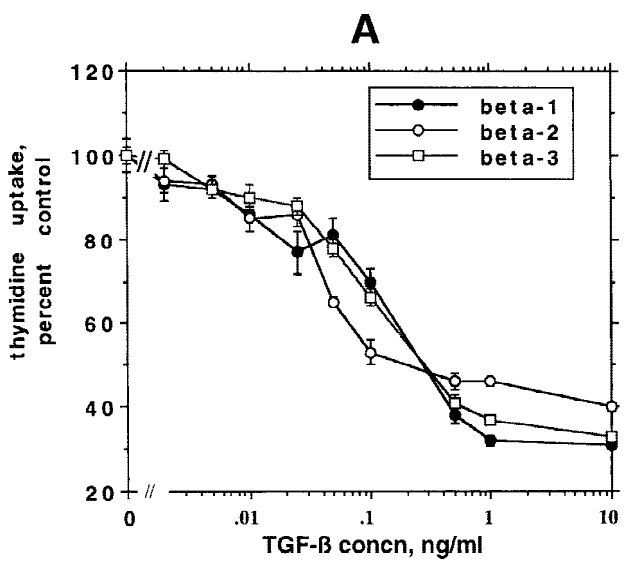
The effect of TGF-β upon thymidine uptake in PC3-M cells which had been pretreated with tamoxifen was then determined (Fig. 4A,B). Tamoxifen and TGF-β had additive effects upon thymidine uptake in PC3-M cells (Fig. 4B).

Tamoxifen Induces $p21^{waf1/cip1}$ Protein Expression

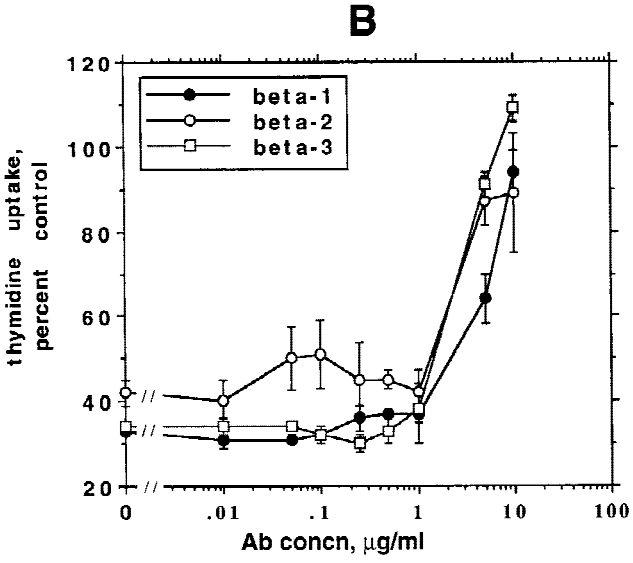
Additive effects between TGF-β and tamoxifen raised the possibility of a direct effect by tamoxifen upon signaling pathways related to TGF-β action. While little is known of the TGF-β signaling pathway, cell cycle arrest at the G1/S phase interface, and induction of the cell cycle-regulatory protein, p21, have been demonstrated after treatment with TGF-β [33]. In the current study, cell cycle-modulatory effects were demonstrated by treating PC3-M cells with tamoxifen, and measuring DNA content by flow cytometric analysis. A 33 ± 5% (mean ± SD; n = 2) increase in the number of cells in the G1 phase of the cell cycle was observed 12 hr after exposure of PC3-M cells to 10 μM tamoxifen. Further increases in cell cycle blockade were not observed with longer treatment times for tamoxifen concentrations ⩽10 μM (data not shown).

To evaluate effects upon $p21^{waf1/cip1}$ protein level, PC3-M cells were treated with 10 μM tamoxifen for various time periods, and $p21^{waf1/cip1}$ protein was measured by Western blot (Fig. 5A). Untreated PC3-M cells exhibited a low level of $p21^{waf1/cip1}$ protein expression. Twelve hours after treatment with tamoxifen, $p21^{waf1/cip1}$ protein levels increased, remaining elevated for at least 24 hr. Interestingly, treatment of PC3-M cells with the PKC inhibitor, Ro31-8220, also led to $p21^{waf1/cip1}$ induction; treatment with both tamoxifen and Ro31-8220 increased $p21^{waf1/cip1}$ protein to levels greater than those observed after treatment with either inhibitor alone. In Figure 5B, PC3-M cells were treated with various concentrations of tamoxifen for 24 hr. Induction of $p21^{waf1/cip1}$ protein was shown to occur only with concentrations of tamoxifen >0.5 μM. The ability of TGF-β to induce $p21^{waf1/cip1}$ protein in PC3-M cells was demonstrated by exposing cells to 0.5 ng/ml TGF-β for various time periods, and measuring $p21^{waf1/cip1}$ protein by Western blot. As shown in Figure 5C, $p21^{waf1/cip1}$ protein increased 3 hr after treatment with TGF-β, remaining elevated for at least 24 hr.

The physiologic significance of $p21^{waf1/cip1}$ induction was determined by examining the effects of tamoxifen upon retinoblastoma (Rb) protein phosphorylation. p21, via its inhibition of cyclin-dependent kinase, is an important regulator of Rb phosphorylation [24], and dephosphorylation of Rb is associated with cell cycle arrest at the G1/S phase interface [34]. After PC3-M cells were treated with tamoxifen for up



TGF-ß IC50 values, ng/ml (mean ± SD; n=2)	
ß-1	0.28 ± 0.04
ß-2	0.22 ± 0.09
ß-3	0.28 ± 0.03



TGF-ß ND50 values, µg/ml (mean ± SD; n=2)	
ß-1	2.7 ± 0.72
ß-2	1.2 ± 0.18
ß-3	1.3 ± 0.07

Fig. 2. TGF-β subspecies 1–3 inhibit the growth of prostate cancer cells. **A:** Twenty-four hours after plating, PC3-M cells were switched to serum-free media and exposed to TGF-β1, -β2, or -β3 for an additional 24 hr. **B:** TGF-β blocking antibody was added prior to the addition of the minimal concentration of TGF-β1, -β2, or -β3 associated with maximum growth inhibition (i.e., 0.5 ng/ml). At the end of the incubation period, thymidine incorporation was measured, and expressed as mean ± SEM (n = 6) of control. Fifty percent growth inhibition (IC_{50}) values represent the concentration of TGF-β subspecies which will decrease thymidine incorporation by 50%. The 50% neutralization dose (ND_{50}) for blocking antibodies is the concentration of antibody which will reverse growth inhibition caused by the minimal concentration of TGF-β associated with maximal growth inhibition.

to 24 hr, cells were fractionated into nuclear and cytoplasmic preparations, and Rb protein was measured by Western blot. As can be seen in Figure 6, cytosolic Rb protein decreased 12 hr after treatment with tamoxifen, continuing to decline for at least 24 hr after treatment. In the nucleus, the phosphorylated form of Rb (i.e., the slower-migrating band) disappeared between 12–24 hr after treatment with tamoxifen.

Tamoxifen Inhibits Protein Kinase C Activity Prior to Induction of p21

As $p21^{waf1/cip1}$ protein levels are increased after treatment with either tamoxifen or Ro31-8220, and both tamoxifen and Ro31-8220 are known inhibitors of PKC, PKC inhibition might be involved in tamoxifen-mediated $p21^{waf1/cip1}$ induction. The effect of tamoxifen upon PKC activity in PC3-M cells was therefore determined by treating cells with 10 µM tamoxifen for various time periods, and then measuring Ca^{2+}/phospholipid-dependent and -independent PKC activity (Fig. 7). The Ca^{2+}/phospholipid-dependent activity of PKC in tamoxifen-treated cells decreased to 34 ± 4.5% (mean ± SEM, n =3) of control 5 hr after

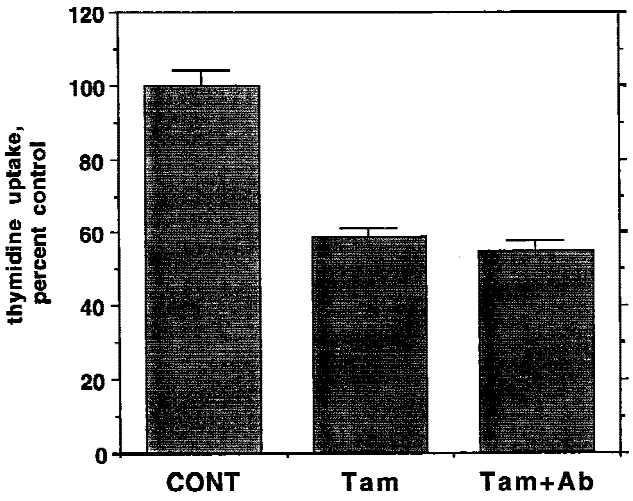


Fig. 3. The effect of TGF-β-blocking antibody upon tamoxifen-mediated growth inhibition of prostate cancer cells. Twenty-four hours after plating, PC3-M cells were placed into serum-free media. Cells were then grown for 3 days under the following conditions: no tamoxifen (CONT), 10 µM tamoxifen (Tam), or 10 µM tamoxifen plus 10 µg/ml pan-TGF-β-blocking antibody (Tam + Ab). Thymidine incorporation was then measured and expressed as mean ± SEM (n = 6) percent of control.

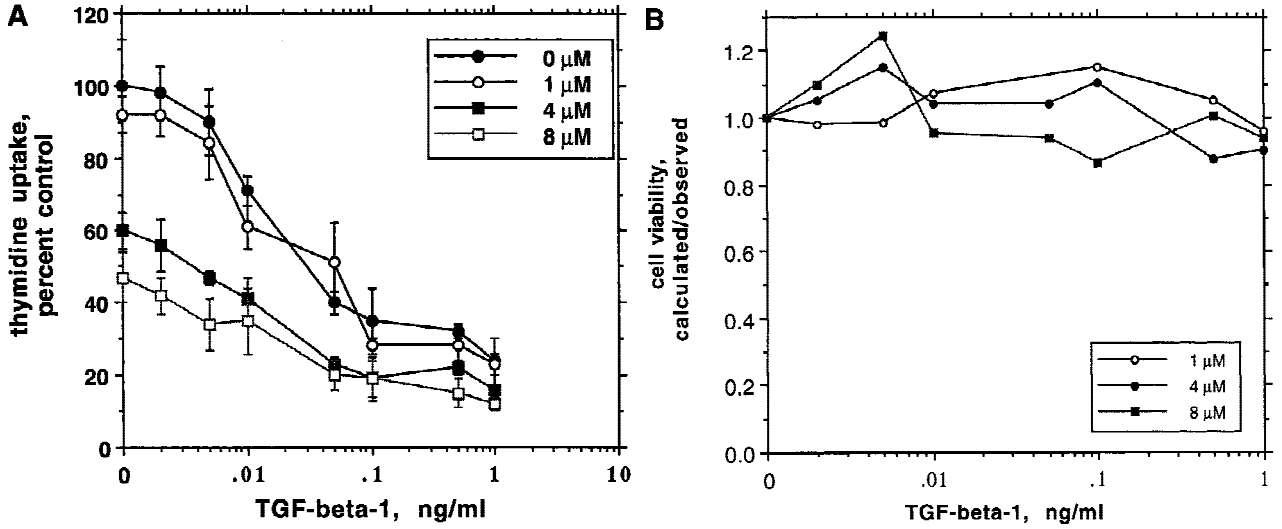


Fig. 4. Effect of tamoxifen upon TGF-β-mediated growth inhibition of prostate cancer cells. Twenty-four hours after plating, PC3-M cells were treated with either 1, 4, or 8 μM tamoxifen (or not) for 3 days. During the last 24 hr of incubation, cells were switched to serum-free media (containing the same concentration of tamoxifen) and treated with TGF-β1. **A:** Thymidine uptake was expressed as mean ± SEM (n = 6) of control. **B:** The ratio of calculated to observed cell viability is depicted.

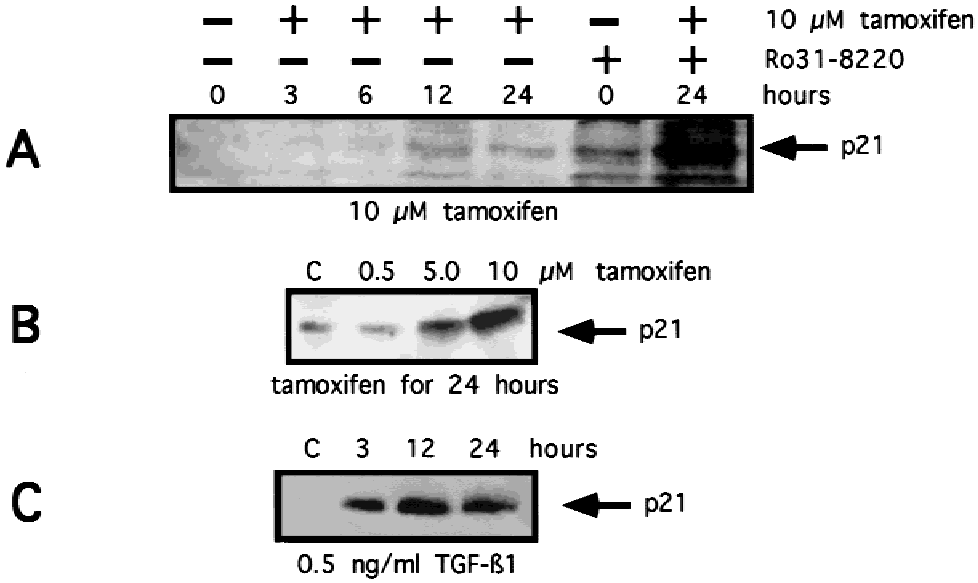


Fig. 5. Effect of tamoxifen upon $p21^{waf1/cip1}$ protein expression in PC3-M prostate cancer cells. Twenty-four hours after plating, PC3-M cells were treated as indicated, and $p21^{waf1/cip1}$ protein expression measured by Western blot, as described in Materials and Methods. **A:** Cells were treated with 10 μM tamoxifen for the indicated time periods; some cells were also treated with 2.5 μM of the PKC inhibitor, Ro31-8220, for 24 hr. **B:** Cells were treated with various concentrations of tamoxifen for 24 hr. **C:** Twenty-four hours after plating, cells were switched to serum-free media and exposed to 0.5 ng/ml of TGF-β1 for 3, 12, or 24 hr (or not, for control).

treatment, remaining depressed for at least 12 hr. No significant effect upon Ca^{2+}/phospholipid-independent activity was observed. If PKC inhibition via tamoxifen was important in regulating PC3-M cell growth, then other inhibitors of PKC should also inhibit cell growth. This was tested by first treating PC3-M cells with various concentrations of tamoxifen and Ro31-8220, and then measuring cell growth 3 days later. As shown in Figure 8, both tamoxifen and Ro31-8220 inhibited PC3-M cell growth. Interestingly, at lower concentrations of Ro31-8220 (i.e., 1 and 2.5 μM), tamoxifen increased Ro31-8220-mediated growth inhibition. However, at the 10-μM level of Ro31-8220, tamoxifen had no additional effect.

To investigate the possibility that tamoxifen may be acting through other mechanisms related to its calcium channel [35], calmodulin-stimulated cAMP phosphodiesterase [36], or antiestrogen binding site (AEBS) activities [36], a series of other experiments was conducted. Neither diltiazem nor verapamil (known calcium channel-blocking agents), at concentrations one log greater than that associated with calcium channel-blocking activity, altered cell growth when exposed to PC3-M cells for 3 days (Fig. 8). No

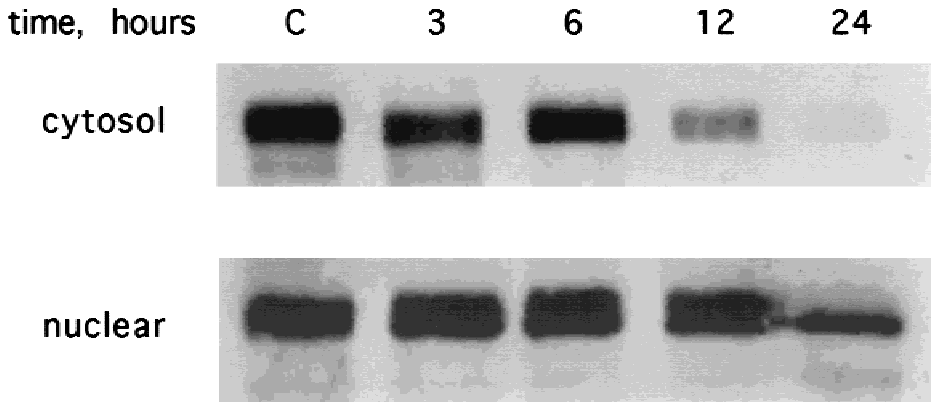


Fig. 6. Effect of tamoxifen treatment upon retinoblastoma (Rb) protein levels and phosphorylation in prostate cancer cells. PC3-M cells were first treated with 10 &M tamoxifen (or not, for control, **lane C**); at the indicated time periods, nuclear and cytoplasmic preparations were made, and equal amounts of protein from each preparation were then loaded onto each lane of a polyacrylamide gel. Rb was then detected by Western blot, as described in Materials and Methods.

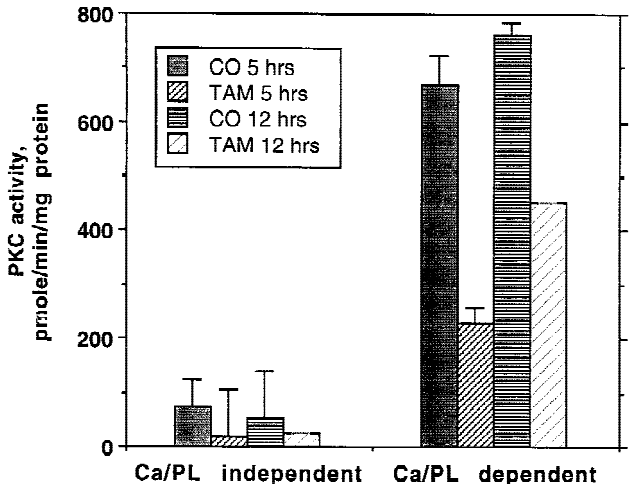


Fig. 7. Effect of tamoxifen upon PKC activity. Twenty-four hours after plating, PC3-M cells were treated with 10 μM tamoxifen for either 5 or 12 hr. Ca^{2+}/phospholipid (Ca/PL)-dependent and -independent PKC activity was then measured in untreated control (CO) and tamoxifen-treated (Tam) cells, as described in Materials and Methods. Results are the mean ± SEM of a single experiment run in triplicate; similar results were obtained in a separate experiment, also run in triplicate.

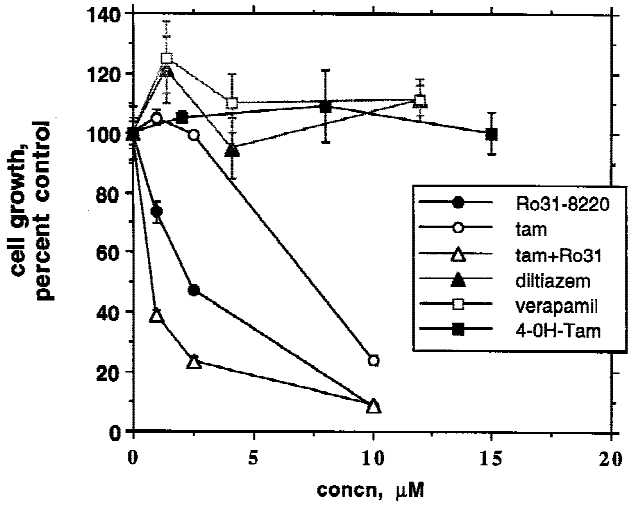


Fig. 8. Modulation of PC3-M cell growth by PKC inhibition, calcium channel blockade, or treatment with 4-hydroxytamoxifen (4-OH-Tam). PC3-M cells were plated for 3 days in the presence of the following, at the indicated concentrations: tamoxifen or Ro31-8220, or the combination of both, 4-OH-Tam, diltiazem, or verapamil. After 3 days, thymidine uptake was measured. Results are the mean ± SEM of at least two separate experiments, each run in replicates of 3–6.

alterations in cAMP levels were detected by radioligand binding assay when PC3-M cells were exposed to 10 μM tamoxifen for up to 9 hr (data not shown). Other studies utilized the tamoxifen metabolite, 4-hydroxytamoxifen (4-OH-Tam); 4-OH-Tam retains AEBS activity, but is a less potent inhibitor of PKC activity in vivo [20,21]. If PKC inhibition were the predominant mechanism of growth inhibition, then 4-OH-Tam should be less active than tamoxifen. This hypothesis was supported by demonstrating that the thymidine uptake of PC3-M cells exposed to 15 μM 4-OH-Tam for 3 days was not different from that observed in control cells (Fig. 8).

DISCUSSION

As tamoxifen is a known estrogen agonist/antagonist [37], it was important to examine the potential role of estrogen in tamoxifen-mediated prostate cancer cell growth inhibition. These studies demonstrate that tamoxifen-mediated growth inhibition of prostate cancer cells was not dependent upon estrogen receptor-related effects. The IC_{50}s for growth inhibition by tamoxifen were one log higher than concentrations associated with estrogen receptor antagonism. Tamoxifen inhibited the growth of PC3-M cells, a cell line previously shown to lack estrogen receptors [28]. And finally, high concentrations of estrogen did not affect prostate cancer cell growth, and neither did they affect tamoxifen-mediated growth inhibition of prostate cancer cells.

TGF-β has known growth-inhibitory effects upon a variety of epithelial cell types [11]. TGF-β1 has been shown to directly inhibit the growth of prostate cell

lines [3], while TGF-β2 likely inhibits their growth [4]. This study confirms these findings and demonstrates that TGF-β2 and -β3 directly inhibit prostate cancer cell growth. It further demonstrates that TGF-β1, -β2, and -β3 have essentially equivalent growth inhibitory efficacy in vitro.

In contrast to its effect upon breast cancer cells [18] and upon stromal cells [22], tamoxifen did not increase TGF-β secretion by prostate cancer cells. While PC3-M cells do secrete TGF-β, and treatment with TGF-β-blocking antibody increases thymidine uptake (unpublished observations), treatment of PC3-M cells with TGF-β blocking antibody did not reverse tamoxifen-mediated growth inhibition. Tamoxifen and TGF-β did, however, have additive effects with respect to growth inhibition.

Additive effects between tamoxifen and TGF-β raised the possibility that tamoxifen was directly affecting pathways associated with TGF-β action. This was confirmed by demonstrating that tamoxifen induced $p21^{waf1/cip1}$ protein, dephosphorylation of retinoblastoma protein (Rb), and G1/S phase cell cycle arrest, i.e., effects associated with TGF-β [33,38]. The functional significance of $p21^{waf1/cip1}$ induction was demonstrated by showing that it was associated with Rb dephosphorylation.

Inhibition of PKC was identified as a possible cause of $p21^{waf1/cip1}$ induction in prostate cancer cells. Treatment of cells with either Ro31-8220, an inhibitor of Ca^{2+}/phospholipid-dependent PKC activity [27], or tamoxifen (also an inhibitor of Ca^{2+}/phospholipid-dependent PKC activity [20,21]) directly induced $p21^{waf1/cip1}$ protein. Tamoxifen was shown to inhibit in vivo Ca^{2+}/phospholipid-dependent PKC activity in PC3-M cells prior to induction of $p21^{waf1/cip1}$ protein. Finally, both tamoxifen and Ro31-8220 inhibited PC3-M cell growth. Interestingly, tamoxifen treatment further reduced cell growth at Ro31-8220 concentrations below 10 μM, consistent with at least additive effects by submaximal concentrations of each agent. At high Ro31-8220 concentrations (i.e., 10 μM), however, tamoxifen treatment had no additional effect. Such a finding is consistent with the notion that tamoxifen-mediated growth inhibition is largely due to PKC inhibition.

Tamoxifen has been shown to alter calcium channel function [35], as well as calmodulin-dependent phosphodiesterase activity [36]. However, the failure of calcium channel-blocking agents to alter cell growth at concentrations associated with calcium channel-blocking activity, and the lack of detectable change in cAMP levels after tamoxifen treatment, further support the role of PKC inhibition. While it was possible that tamoxifen may have been acting in part through AEBS, IC_{50}s well above 1 μM would suggest that binding to high-affinity AEBS was an unlikely mechanism [21]. This is also supported by the inability of 4-OH-Tam (an agent with known AEBS activity) to inhibit cell growth [21].

PKC plays an important role in cellular signal transduction [17]. However, the physiology of PKC is complicated by the fact that there are at least 10 different isoenzymes, and the particular role of each appears to vary from system to system. While more studies will need to be conducted in order to determine which PKC isoenzymes are involved in the current system, specific PKC isoenzymes likely play an important role. As tamoxifen did not alter Ca^{2+}/phospholipid-independent PKC activity, the Ca^{2+}-independent PKC isoenzymes delta, epsilon, and zeta likely were not involved. It is interesting to note, however, that different PKC isoenzymes appear to be associated with prostate cancer cells [15], as compared to those that are associated with breast cancer cells [39]. It is possible that differences in PKC isoenzyme distribution may underlie the apparent difference in TGF-β secretory response observed between prostate and breast cancer cells after treatment with tamoxifen.

Taken together, these findings suggest that inhibition of PKC and induction of $p21^{waf1/cip1}$ protein are potential mechanisms by which tamoxifen exerts growth-inhibitory effects upon prostate cancer cells. These effects are observed with micromolar concentrations of tamoxifen, and such concentrations can be attained in serum with high-dose tamoxifen therapy [40]. It is possible that similar effects in vivo are responsible for the clinical activity being observed with high-dose tamoxifen therapy in patients with hormone-refractory prostate cancer [41].

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