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Synergistic Effect of Estramustine and [3′-keto-Bmtl]-[Val2]-Cyclosporine (PSC 833) on the Inhibition of Androgen Receptor Phosphorylation in LNCaP Cells

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ABSTRACT. Estramustine phosphate has been used frequently alone or in combination with other drugs for the treatment of hormone-refractory prostate cancer. Estramustine is one of the major active metabolites of estramustine phosphate *in vivo*. We recently demonstrated that estramustine acts as an androgen antagonist, and the combination of estramustine with [3′-keto-Bmtl]-[Val2]-cyclosporine (PSC 833) results in synergistic cytotoxicity. Unlike other regulators of microtubules, such as paclitaxel, the present study demonstrated that estramustine alone or in combination with PSC 833 did not induce bcl-2 phosphorylation in LNCaP cells. No synergism between estramustine and PSC 833 in the induction of bcl-2 phosphorylation was obtained in MCF-7 cells exposed for 16 hr to estramustine (5–15 μM) and PSC 833 (5 μM). A significant synergistic antiandrogenic effect as measured by the inhibition of dihydrotestosterone-induced reporter gene luciferase expression in both wild-type and mutated androgen receptor (AR) cDNA-transfected HeLa cells was observed when the cells were exposed to estramustine and PSC 833. Treatment of LNCaP cells with estramustine alone (5–15 μM) resulted in a decrease of AR expression and phosphorylation. This effect was enhanced markedly by PSC 833. A strong correlation between AR phosphorylation and expression of the AR target gene PSA was obtained in dihydrotestosterone-stimulated LNCaP cells. The up-regulated PSA expression is a function of the level of the phosphorylated AR ($r = 0.9814$), but not the dephosphorylated form of the receptor protein ($r = 0.4808$). Thus, our studies suggest that the synergism between estramustine and PSC 833 in LNCaP cells is a consequence of inhibition of AR expression and phosphorylation, thus leading to interruption of AR-mediated gene expression. BIOCHEM PHARMACOL **58**;7:1115–1121, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. estramustine phosphate; PSC 833; antiandrogen; androgen receptor phosphorylation; gene expression

Estramustine phosphate, an estradiol with a covalently bound nornitrogen mustard moiety, has been used, alone or in combination with other antitumor agents, for the treatment of hormone-refractory prostate cancer [1, 2] as well as breast cancer [3] and malignant glioma [4]. Estramustine is a major active metabolite of estramustine phosphate. Binding of estramustine to microtubule-associated proteins, tubulin, and proteins of the nuclear matrix currently is considered to be the most likely mechanism of its cytotoxicity in cancer cells [5–8]. In addition, estramustine has been shown to interfere with multidrug-resistant-mediated drug efflux and to enhance paclitaxel cytotoxic activity in cell lines, and it is useful clinically in the treatment of hormone-resistant prostate cancer [9–11]. This enhancement of anticancer activity was attributed either to a synergistic interaction inhibiting microtubules or to estramustine phosphate inhibition of multidrug-resistant-1-mediated efflux of the taxane [10–12]. We have demonstrated recently that estramustine also acts as an androgen antagonist in cells expressing wild-type or mutated ARs† [13].

Synergistic cytotoxicity of estramustine and [3′-keto-Bmtl]-[Val2]-cyclosporine (PSC 833), a non-immunosuppressive cyclosporin, has been observed in cancer cells [14]. The mechanisms for this synergism are not clear. In this report, we show that inhibition of AR expression and phosphorylation in LNCaP cells may be a possible mechanism to explain the synergism between estramustine and PSC 833.

MATERIALS AND METHODS

Reagents

Estramustine and PSC 833 were gifts from Pharmacia through the courtesy of Dr. B. Hartley-Asp and Novartis Ag., respectively. Wild-type and mutated AR cDNA expression plasmids, wt-pAR0 and m-pARL, respectively, and

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† *Abbreviations*: AR, androgen receptor; DHT, dihydrotestosterone; wt-pAR0, wild-type AR cDNA expression plasmids; m-pARL, mutated AR cDNA expression plasmids; FBS, fetal bovine serum; and PSA, prostate specific antigen.

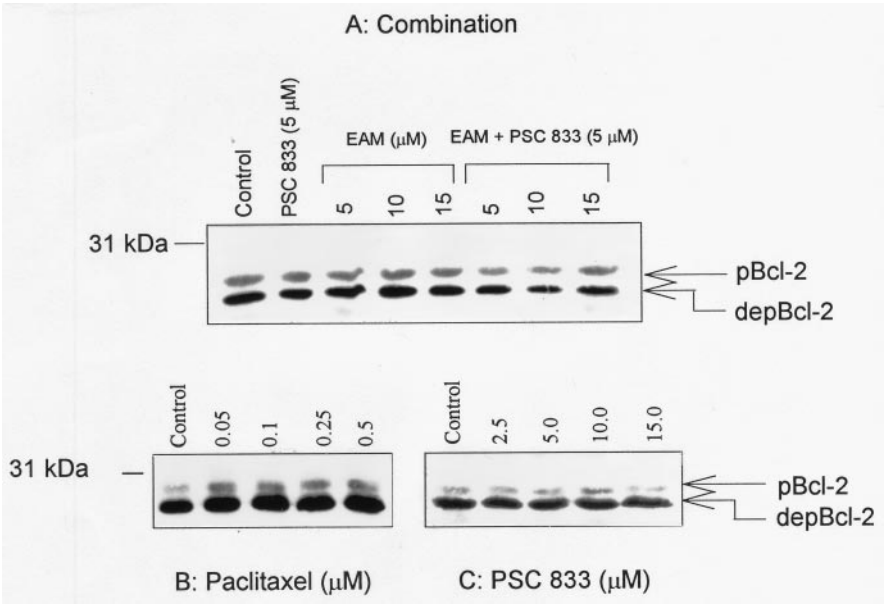

FIG. 1. Induction of bcl-2 phosphorylation by the combination of estramustine and PSC 833 (A), paclitaxel (B), or PSC 833 (C) in LNCaP cells. LNCaP cells growing exponentially were exposed to different concentrations of estramustine alone or to the indicated concentrations of estramustine in the presence of 5 μM PSC 833 for 24 hr. The cells were harvested in serum-free RPMI 1640 medium and washed once with ice-cold PBS, and total proteins were extracted. One hundred micrograms of total protein was subjected to 4%/10% stack SDS–PAGE, electrotransferred to nitrocellulose membrane, and immunoblotted with bcl-2 antibody.

the reporter-plasmid GRE-tk-LUC were provided by Dr. Albert O. Brinkmann. Luciferase assay systems were purchased from Promega. PSA antibody was purchased from the DAKO Corp. Antibodies against bcl-2, AR, and anti-rabbit IgG-horseradish peroxidase were purchased from Santa Cruz Biotechnology, Inc. A western blotting detection kit was provided by Amersham. Reagents for SDS–PAGE and a protein determination kit were obtained from Bio-Rad. RPMI 1640 and FBS were purchased from Life Technologies, Inc. Other chemicals for this study were purchased from the Sigma Chemical Co.

Cell Culture

The prostatic carcinoma cell line LNCaP, the breast cancer cell line MCF-7, and the human cervical adenocarcinoma cell line HeLa were purchased from the American Type Culture Collection. Cells were cultured under conditions described previously [15].

Gene Transactivation Assay

Transfection was carried out in HeLa cells using standard calcium phosphate precipitation methods [13, 16], and the efficiency of the transfection was monitored by the luciferase activity (Promega Assay Systems). Briefly, 3×10^5 HeLa cells in RPMI 1640 medium containing 10% FBS were sown in 10-cm dishes and incubated at 37° for 24 hr. Ten micrograms of the wild-type androgen receptor expression plasmid wt-pAR0 cDNA or the mutated AR expression plasmid m-pARL created from LNCaP cells and 2 μg of the AR-driven reporter gene GRE-tk-LUC cDNA in 125 mM $CaCl_2$–HEPES buffer (0.14 M NaCl, 0.05 M HEPES acid, 1.5 mM Na_2HPO_4, pH 7.05) were added to each culture and incubated for 16 hr. The precipitates were washed with pre-warmed PBS, and the cells were refed with fresh RPMI 1640 medium containing 10% FBS. After incubation of the transfected cells for an additional 32 hr at 37° in the presence of 10 nM DHT and different concen-

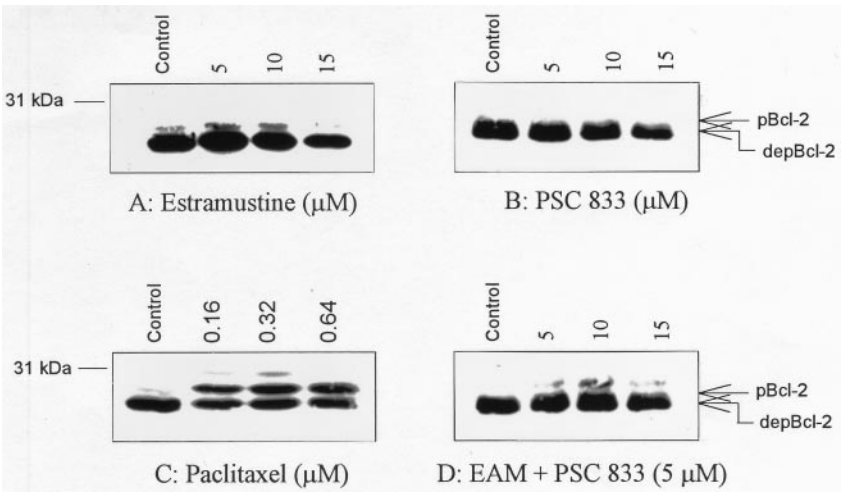

FIG. 2. Induction of bcl-2 phosphorylation by estramustine (A), PSC 833 (B), paclitaxel (C), and the combination of estramustine and PSC 833 (D) in MCF-7 cells. MCF-7 cells growing exponentially were exposed to different concentrations of the indicated agents for 24 hr. The cells were harvested in serum-free RPMI 1640 medium and washed once with ice-cold PBS, and total proteins were extracted. Fifty micrograms of total protein was subjected to 4%/10% stack SDS–PAGE, electrotransferred to nitrocellulose membrane, and immunoblotted with bcl-2 antibody.

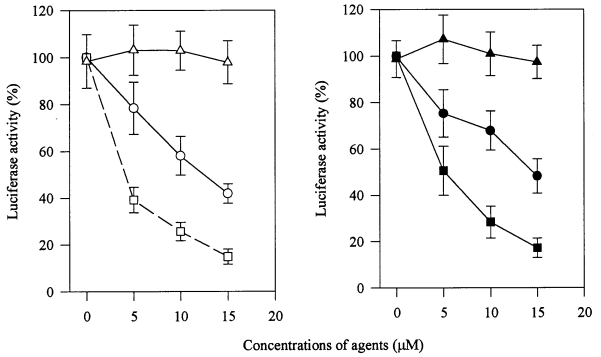

FIG. 3. Inhibition by estramustine or its combination with PSC 833 of transactivated luciferase reporter gene expression induced by DHT in wt-pAR0 (left panel) or m-pARL (right panel) transfected HeLa cells. HeLa cells were cotransfected with wt-pAR0 or m-pARL and AR-driven luciferase reporter gene for 48 hr in RPMI 1640 medium containing 10% FBS in the presence of DHT (10 nM) and/or indicated concentrations of estramustine in the absence or presence of PSC 833. The cells were harvested, and the luciferase activity was measured using the Promega assay kit. Key: PSC 833 alone (△; ▲); estramustine alone in wt-pAR0 (○) and m-pARL (●) transfected HeLa cells; and the combination of PSC 833 and estramustine (□ , ■). The values are means ± SD of three separate experiments with triplicate samples, and the absolute value of control used for the calculation of the percentage was 18,540 cpm.

trations of estramustine, PSC 833, or estramustine plus 5 μM PSC 833, respectively, the cells were harvested and washed, proteins were extracted, and luciferase activities were determined using the Promega assay kit.

Western Blotting Assay

LNCaP and MCF-7 cells grown to 65–75% confluence in RPMI 1640 medium containing 10% FBS were exposed for 24 hr to various concentrations of estramustine, PSC 833, or paclitaxel alone or to different concentrations of estramustine with 5 μM PSC 833. The cells were harvested and washed with cold PBS, and the total proteins were extracted as described previously [17]. Cellular extracts (100 μg) were separated by 4%/10% or 4%/7.5% stack SDS—PAGE, electrotransferred to nitrocellulose filters, and immunoblotted with antibodies against bcl-2, PSA, or AR. Quantitation by densitometry of ECL films was performed using an Imaging Densitometer model GS-700 (Bio-Rad Laboratories).

RESULTS

Induction of Bcl-2 Phosphorylation

Previous studies had indicated that the apoptosis process suppressed by bcl-2 can be restored to normal function by bcl-2 phosphorylation [18, 19]. Bcl-2 phosphorylation can be induced by drugs that affect microtubule depolymerization or polymerization [20]. Since estramustine generally is considered to be an antimicrotubule agent [5–8], a likely

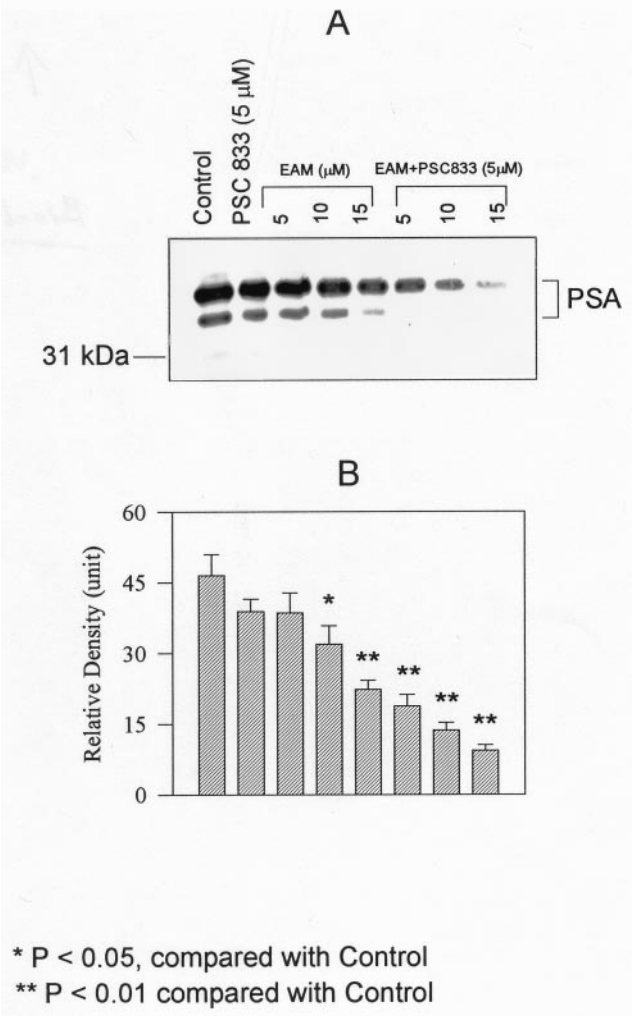

FIG. 4. Synergistic down-regulation of PSA expression by the combination of estramustine and PSC 833. LNCaP cells grown to 65–75% confluence in RPMI 1640 medium containing 10% FBS were exposed to PSC 833 alone (5 μM, lane 2), indicated concentrations of estramustine (lanes 3–5), or PSC 833 (5 μM) + indicated concentrations of estramustine (lanes 6–8) for 24 hr. Lane 1 is the control. The cells were harvested and washed with cold PBS, and total proteins were extracted. Fifty micrograms of total protein was subjected to 4%/10% stack SDS–PAGE, electrotransferred to nitrocellulose membrane, and immunoblotted with PSA specific antibody (panel A). The density of the plots of PSA proteins was determined from ECL films by an Imaging Densitometer, model GS-700 (panel B). Data are means ± SD of three separate experiments scored by the densitometer.

mechanism of action of this agent on cancer cell apoptosis is through induction of bcl-2 phosphorylation. As shown in Fig. 1A, estramustine did not induce bcl-2 phosphorylation either alone or in combination with PSC 833 (5 μM) in prostate cancer cells (LNCaP) at concentrations as high as 15 μM (the IC_{50} of estramustine for LNCaP is 11.0 ± 1.68 μM [14]), and only minor induction of bcl-2 phosphorylation by estramustine was observed in the breast cancer cell line MCF-7 (Fig. 2A). Similarly, PSC 833, a non-immunosuppressive cyclosporin [21], had no effect on bcl-2 phosphorylation in either cell line. A slight decrease of the

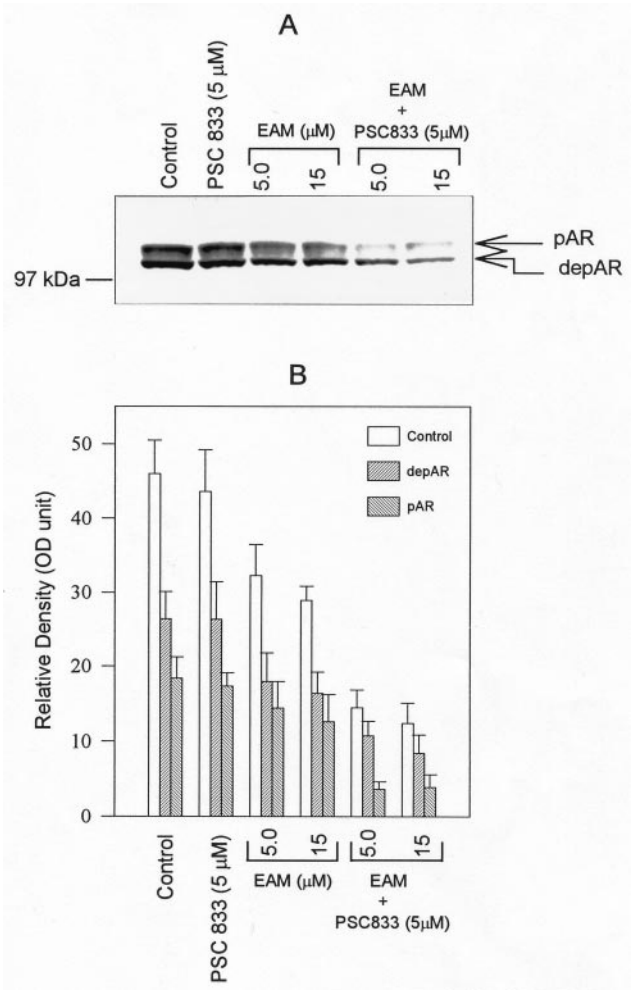

FIG. 5. Effect of estramustine and its combination with PSC 833 on the expression and phosphorylation of ARs in LNCaP cells. LNCaP cells grown to 65–75% confluence in RPMI 1640 medium containing 10% FBS were exposed to PSC 833 alone (5 μM, lane 2), various concentrations of estramustine (lanes 3 and 4), or PSC 833 (5 μM) + indicated concentrations of estramustine (lanes 5 and 6) for 24 hr. The cells were harvested and washed with cold PBS, and total proteins were extracted. One hundred micrograms of total protein was subjected to 4%/7.5% stack SDS–PAGE, electrotransferred to nitrocellulose membrane, and immunoblotted with AR specific antibody (panel A). The density of the plots of AR proteins (total AR, phosphorylated AR, and dephosphorylated AR) was determined from ECL films by an Imaging Densitometer, model GS-700 (panel B). Data are means ± SD of three separate experiments scored by the densitometer.

level of bcl-2 protein, but not phosphorylation, occurred when MCF-7 cells were treated with 15 μM estramustine or with 15 μM PSC 833 in MCF-7 cells (Fig. 2, A and B). However, no synergism was obtained when the cells were exposed to 15 μM estramustine in the presence of 5 μM PSC 833 (Fig. 2D). In contrast, as shown in Figs. 1B and 2C, paclitaxel significantly ($P < 0.01$) induced bcl-2 phosphorylation in both LNCaP and MCF-7 cells, consistent with previous reports [20]. These findings thus indicate that the increased anti-cancer activity of estramustine in the presence of PSC 833 was not due to the induction of bcl-2 phosphorylation leading to apoptosis.

Synergistic Androgen Antagonistic Effect

We have reported previously that estramustine acts as an androgen antagonist in hormone-dependent LNCaP cells [13] with an IC_{50} of 11.0 ± 1.68 μM [14]. To explore whether the synergism between estramustine and PSC 833 in prostate cancer results from their antiandrogenic effect, the inhibition of AR-driven gene expression by estramustine alone or in combination with PSC 833 was studied. As shown in Fig. 3, the stimulation of luciferase activity by DHT (10 nM) in HeLa cells transfected with m-pARL (AR-mutated LNCaP cell cDNA) or wt-pAR0 (wild-type AR cDNA) was inhibited by estramustine alone in a concentration-dependent manner. This inhibition was consistent with our previous observation [13]. The action of estramustine was enhanced significantly by the presence of 5 μM PSC 833 ($P < 0.01$). Exposure of wt-pAR0 cDNA-transfected HeLa cells to 5 μM estramustine alone resulted in a 20.8% decrease of the luciferase activity, whereas a 60% decrease of the enzyme activity was observed in the presence of PSC 833 ($P < 0.01$, Fig. 3, left panel). PSC 833 alone at this concentration had no effect. Concentration-dependent synergistic inhibition by estramustine plus PSC 833 of the induction of luciferase activity induced by 10 nM DHT was observed both in m-pARL (Fig. 3, right panel) and in wt-pAR0-transfected HeLa cells (Fig. 3, left panel).

To confirm a synergistic antiandrogenic effect, regulation of the expression of PSA, a native AR target gene [22], by the combination of estramustine and PSC 833 was evaluated. Concentration-dependent inhibition of PSA expression was observed when LNCaP cells were exposed to various concentrations of estramustine for 24 hr (Fig. 4, lanes 3–5). This inhibition of PSA expression was enhanced significantly by the presence of 5 μM PSC 833 (lanes 6–8, $P < 0.01$), whereas no significant effect was obtained by PSC 833 alone (lane 2). The IC_{50} of estramustine for inhibition of PSA expression decreased from approximately 20 μM to 5 μM in the presence of 5 μM PSC 833 ($P < 0.01$).

Synergistic Effect on AR Expression and Phosphorylation

We have demonstrated previously that finasteride, a 5α-reductase inhibitor, significantly inhibits expression of the AR, and that down-regulation of PSA expression by finasteride occurs through inhibition of complex formation between the AR and the steroid receptor-binding consensus sequence in the promoter region of the PSA gene in LNCaP cells [17]. In addition, the function of the AR as a transcription factor also is believed to be regulated by phosphorylation [23, 24]. To explore a possible mechanism of the synergistic down-regulation of PSA expression by the combination of estramustine and PSC 833, levels of total AR protein and its phosphorylated and dephosphorylated forms were measured after exposure of LNCaP cells to estramustine and PSC 833. As shown in Fig. 5, treatment of

TABLE 1. Effect of estramustine (EAM) alone or in combination with PSC 833 (PSC) on AR expression and phosphorylation in LNCaP cells.

Treatment	Total AR T/C (%)	Dephosphorylated AR T/C (%)	Phosphorylated AR T/C (%)
Control	100	100	100
PSC (5 μM)	106.3 ± 0.55	111.7 ± 18.7	96.9 ± 9.8
EAM (5 μM)	69.9 ± 9.1*	87.5 ± 7.0	44.1 ± 7.9†
EAM (10 μM)	93.4 ± 8.4	117.6 ± 11.3	58.5 ± 8.7*
EAM (15 μM)	58.3 ± 4.1*	84.2 ± 10.7*	21.0 ± 3.6†
EAM + PSC (5 μM + 5 μM)	31.8 ± 5.2†	48.7 ± 7.0†	7.7 ± 5.1†
EAM + PSC (10 μM + 5 μM)	22.6 ± 3.6†	33.0 ± 5.4†	7.7 ± 6.1†
EAM + PSC (15 μM + 5 μM)	30.5 ± 2.6†	45.1 ± 8.7†	8.7 ± 8.7†

Total -, dephosphorylated-, and phosphorylated-, and phosphorylated-AR were determined from ECL films of western blot by an Imaging Densitometer, model GS-700. Data are the means ± SD of three separate experiments. The absolute value of control for the calculatoin of the percentage is 54.5.

* $P < 0.05$, significantly different from control.

† $P < 0.01$, significantly different from control.

LNCaP cells with estramustine alone resulted in a moderate decrease of total AR as well as the phosphorylated protein. A greater decrease of AR phosphorylation was obtained when LNCaP cells were exposed to the same concentrations of estramustine in the presence of 5 μM PSC 833; PSC 833 when used alone caused no effect. The levels of total AR decreased 30% ($P < 0.05$, compared with the control) in the absence of PSC 833 and 68.2% ($P < 0.01$, compared with the control) in the presence of PSC 833. Exposure of LNCaP cells to 5 μM estramustine plus 5 μM PSC 833 resulted in a 92.3% decrease in the level of phosphorylated AR, whereas the level of phosphorylated AR protein decreased by 55.9% in LNCaP cells exposed to 5 μM estramustine alone (Table 1). These results parallel the down-regulation of PSA expression under the same experimental conditions as shown in Fig. 4. Therefore, inhibition of AR phosphorylation correlated with down-regulated PSA expression induced by the combination of estramustine and PSC 833.

To support this observation, the correlation between AR phosphorylation and expression of the AR-target gene PSA was studied. Exposure of synchronized LNCaP cells at G_1 to various concentrations of DHT (1.0 to 1000.0 nM) for 24 hr resulted in significant stimulation of AR phosphorylation. The levels of AR phosphorylation correlated with the androgen concentrations present in the medium (Fig. 6A). Even though the level of total ARs increased significantly, the increase in the amount of phosphorylated ARs was even more pronounced (increased 5 times in the presence of 100 nM DHT) than that of the dephosphorylated form (increased 1.5 times with the same concentration of DHT).

PSA, mRNA, and protein increased in a concentration-dependent manner when synchronized LNCaP cells were exposed to the same concentrations of DHT (Fig. 6B) This increased PSA expression paralleled the level of phosphorylated but not dephosphorylated AR. The correlation between PSA expression and AR phosphorylation is illustrated in Fig. 6C. The level of PSA protein is a function of the level of phosphorylated AR protein ($r_p = 0.9814$, $P < 0.01$), but not dephosphorylated AR protein ($r_{dep} = 0.4808$). Inhibition of AR phosphorylation and PSA expression by phorbol-12-myristate-13-acetate also demonstrated this correlation [25].

DISCUSSION

Estramustine phosphate is a stable conjugate of estradiol and nornitrogen mustard with antimitotic properties. Originally, this drug was believed to be an alkylating agent. Recognition of binding of the drug to microtubule-associated proteins, tubulin, and protein of the nuclear matrix has led to clinical reevaluation of estramustine phosphate [1, 2, 5–8]. Estramustine is a major active form of estramustine phosphate *in vivo*. We have demonstrated that in addition to anti-microtubule activity, estramustine also exhibits antiandrogenic activity in hormone-dependent LNCaP cells [13]. Combinations of estramustine with PSC 833, the non-immunosuppressive cyclosporin analogue, previously demonstrated synergistic antitumor activity in cancer cell lines [14]. In this study, we explored the possible mechanism of the observed synergism between estramustine and PSC 833.

Paclitaxel, a classical antimicrotubule agent, promotes apoptosis by inactivation of bcl-2 as a consequence of protein phosphorylation in several cancer cell lines [19]. In contrast to paclitaxel, our studies demonstrated that the antimicrotubule agent estramustine could not induce bcl-2 phosphorylation in LNCaP cells, even at concentrations as high as 15 μM or in the presence of 5 μM PSC 833. A slight induction of bcl-2 protein phosphorylation by estramustine in MCF-7 cells was observed. PSC 833 alone did not stimulate bcl-2 phosphorylation in either the LNCaP or the MCF-7 cancer cell line.

Hormone-related or hormone-independent chemotherapy directed against the ARs remains a major approach for the treatment of prostate cancer. Antiandrogens exert their

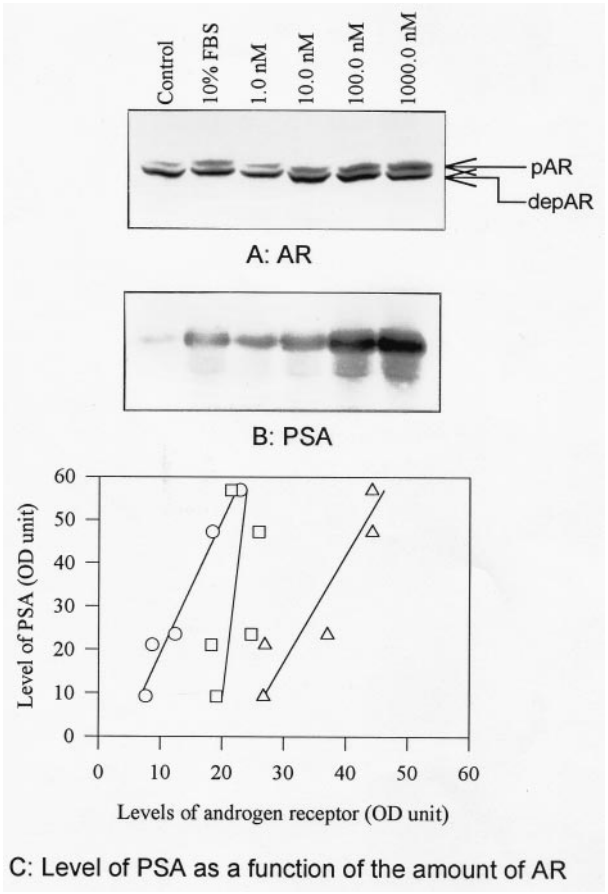

FIG. 6. Expression of PSA as a function of phosphorylated, but not dephosphorylated forms of the AR. LNCaP cells grown exponentially were synchronized at G_1 phase by incubation of the cells in serum-free RPMI 1640 medium for 48 hr. Then the cells were exposed to indicated concentrations of DHT for 24 hr in serum-free RPMI 1640 medium. The cells were harvested and washed with cold PBS, and total proteins were extracted. One hundred micrograms of total protein was subjected to 4%/7.5% (panel A) or 4%/10% (panel B) stack SDS–PAGE, electrotransferred to nitrocellulose membrane, and immunoblotted with antibodies specifically against AR (panel A) or PSA (panel B). The density of the plots of AR proteins (total AR, phosphorylated AR, and dephosphorylated AR) and PSA were determined from ECL films by an Imaging Densitometer, model GS-700 (panel C). Data are means of two separate experiments scored by the densitometer. The linear regressions (panel C) were made by the levels of PSA against the levels of phosphorylated (○, $r = 0.9814$), dephosphorylated (□, $r = 0.4808$), or total AR proteins (Δ, $r = 0.8673$).

effects on prostate cancer cells through blocking androgen binding to the AR, leading to interruption of AR-mediated signal transduction. In this study, we demonstrated that the antiandrogenic effect of estramustine in LNCaP cells occurred by inhibition of AR phosphorylation. This effect was enhanced significantly in the presence of PSC 833.

The functional significance of AR phosphorylation remains to be established definitively. However, AR phosphorylation/dephosphorylation is believed to play a crucial role in the regulation of AR target gene expression. Deletion of AR putative phosphorylation sites causes loss of receptor function [26]. Dephosphorylation of AR by forskolin, a protein kinase A stimulator, results in down-regulated expression of two endogenous PSAs and the β1-subunit of Na/K-ATPase [27]. In the present study, we found that the amount of PSA protein expressed was a function of the level of phosphorylated but not dephosphorylated AR. Inhibition of AR phosphorylation caused by differentiation inducers, such as all-*trans*-retinoic acid, or true androgen antagonists, such as bicalutamide, results in significant down-regulation of PSA expression [25]. *In toto*, these data suggest that synergism observed in LNCaP cells as a consequence of exposure to estramustine and PSC 833 may be attributed to inhibition of AR phosphorylation, leading to interruption of receptor-mediated gene transcription. Other mechanisms also may be involved.

At the present time, regulation of AR phosphorylation by exogenous drugs is not well understood. Previous reports suggested a role of receptor phosphorylation in receptor cycling between nucleus and cytoplasm [26–29], protein–protein interaction, such as binding of heat shock protein p90 to the glucocorticoid receptor [30, 31], or interaction between AR and other transcription factors, such as AP-1/c-Jun [32], and protein–DNA interaction as well as glucocorticoid receptor regulation of tight junction permeability. We have demonstrated previously that down-regulation of prostate-specific antigen expression by finasteride is achieved through inhibition of complex formation between the AR and the steroid receptor-binding consensus sequence in the promoter region of the gene for PSA in LNCaP cells [14]. Whether or not the inhibition of AR phosphorylation by the combination of estramustine and PSC 833 affects AR-mediated protein–protein interaction, protein transport, and protein–DNA interaction needs to be defined by future studies.

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NOTE ADDED AT PROOF

Preliminary experiments revealed minimal inhibition by PSC 833 of the phosphorylation of P 170-glycoprotein in MCF7 cells resistant to doxorubicin.

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