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Androgen Antagonistic Effect of Estramustine Phosphate (EMP) Metabolites on Wild-Type and Mutated Androgen Receptor

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ABSTRACT. Estramustine phosphate is used frequently, alone or in combination with other antitumor agents, for the treatment of hormone-refractory prostate cancer. Estramustine phosphate is metabolically activated *in vivo*, and its metabolites, estramustine, estromustine, estrone, and β-estradiol inhibit the assembly of microtubules [for review see: Kreis W, In: *Concepts, Mechanisms, and New Targets for Chemotherapy* (Ed. Muggia FM), pp. 163–184. Kluwer Academic Publishers, Boston, 1995]. We investigated, by displacement of [^{3}H]methyltrienolone in the presence of 2.5 mM of triamcinolone acetonide, the binding of estramustine phosphate and its metabolites, estramustine, estromustine, estrone, and β-estradiol, as well as other antiandrogen agents including α-estradiol, bicalutamide, and hydroxyflutamide, to the mutated androgen receptor (m-AR) in LNCaP cells and to the wild-type androgen receptor in wild-type AR cDNA expression plasmid (w-pAR0) cDNA-transfected HeLa cells. Analogous to the antiandrogens, bicalutamide and hydroxyflutamide, binding of estramustine phosphate metabolites to the androgen receptor was observed. The EC_{50} values (in μM) were: estramustine phosphate, >10; estramustine, 3.129 ± 0.312; estromustine; 2.612 ± 0.584; estrone, 0.800 ± 0.090; α-estradiol, 1.051 ± 0.096; β-estradiol, 0.523 ± 0.028; bicalutamide, 4.920 ± 0.361; and hydroxyflutamide, 0.254 ± 0.012. The transactivation assay demonstrated that, analogous to bicalutamide, estramustine could not induce luciferase activity in either w-pAR0 or m-pARL transfected HeLa cells. In contrast, a strong induction of the reporter activity by dihydrotestosterone was observed. Down-regulation of prostate-specific antigen (PSA) expression, an AR-target gene, by estramustine and bicalutamide was accompanied by the blockade of the mutated androgen receptor. Exposure of LNCaP cells to estramustine for 24 hr caused transcriptional inhibition of PSA in a concentration-dependent manner. The levels of PSA mRNA decreased 56 and 90% when LNCaP cells were treated with 5 and 10 μM of estramustine, respectively (IC_{50} = 10.97 ± 1.68 μM). Binding of hydroxyflutamide to m-AR in LNCaP cells resulted in a concentration-dependent stimulation of PSA expression, suggesting that hydroxyflutamide acted as an agonist of the m-AR. Our data indicate that estramustine phosphate metabolites perform as androgen antagonists of AR, an additional mechanism involved in the therapeutic effect of estramustine phosphate in patients with prostate cancer. BIOCHEM PHARMACOL **55**;9:1427–1433, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. estramustine phosphate; androgen receptor; agonist; antagonist; gene transactivation

The diagnosis of prostate cancer has increased markedly over the last decade, perhaps due to early detection. The expected new cases in the U.S.A. for 1997 were estimated to be 341,000 [1], a number substantially higher than the expected incidence of breast cancer in females or of lung cancer. The expected death rate from this disease is below the number of breast and lung cancer deaths expected for 1997; however, because the incidence of prostate cancer increases with age, the number of both newly diagnosed cases and deaths will continue to increase as the population ages.

EMP† (Emcyt®) is a compound widely used, alone or in combination with other antitumor agents, for the treatment of hormone-refractory prostate cancer [2]. The mechanism of action of EAM, the dephosphorylated metabolite, was found to be distinct from that of its constituents, β-EDO and nor-nitrogen mustard [3]. Because a large excess of EAM failed to compete with DHT binding to the specific AR of rat ventral prostate, investigators concluded

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† *Abbreviations:* EMP, estramustine phosphate; EAM, estramustine; FBS; fetal bovine serum; AR, androgen receptor; w-AR, wild-type androgen receptor; m-AR, mutated androgen receptor; pAR, androgen receptor expression plasmid; w-pAR0, wild-type AR cDNA expression plasmid; m-pARL, mutated AR cDNA expression plasmid; R1881, methyltrienolone; EOM, estromustine; EO, estrone; β-EDO, β-estradiol; RBA, relative binding affinity; DHT, dihydrotestosterone; and PSA, prostate-specific antigen.

that EAM did not bind to the AR [4]. Inhibition of the assembly of microtubule proteins, preventing the formation of properly functional microtubules, was reported for EMP in 1987 [5] and for EAM in 1988 [6]. Binding of EAM to microtubules was reported shortly thereafter [7]. Further evidence for a nonclassical hormone effect is the cytotoxic effect of this agent in cell lines. EMP demonstrated substantial *in vitro* antitumor activity in the hormone-sensitive LNCaP and the hormone-resistant LNCaPr cell lines [8], as well as in hormone-resistant DU 145 and PC 3 cells [9, 10].

Antiandrogens exert their effects on target tissue by blocking androgen binding to the AR. Because the metabolites of EMP also include, besides its dephosphorylated product EAM, EOM, β-EDO, and EO [11, 12], we considered it likely that some of these estrogen relatives might also bind to the AR and thus serve as androgen antagonists. This hypothesis would explain the increased sensitivity of LNCaP cells to EMP compared with other cell lines. The present study explored this possibility and compared the binding of these molecules to the m-AR and w-AR.

MATERIALS AND METHODS

Reagents

[^{3}H]R1881 and unlabeled R1881 were purchased from New England Nuclear. EMP, EAM, EOM, α- and β-EDO, and EO were gifts from Pharmacia through the courtesy of Dr. B. Hartley-Asp. Bicalutamide and hydroxyflutamide were gifts from Zeneca Pharmaceuticals and the Schering Corp., respectively. Triamcinolone acetonide and other chemicals were purchased from the Sigma Chemical Co. w-pAR0, m-pARL and the reporter-plasmid GRE-tk-LUC were provided by Dr. Albert O. Brinkmann.

Cell Culture

The prostatic carcinoma cell lines LNCaP, PC 3, and DU-145, and the human cervical adenocarcinoma HeLa cells were purchased from the American Type Culture Collection. Cells were cultured under conditions as described previously [13].

m-AR Binding Assays in LNCaP Cells

LNCaP cells grown to 75–85% confluence were harvested, and 5×10^5 cells were reincubated for 4 hr in RPMI 1640 medium containing 10% FBS at 37° with 0.5 nM of [^{3}H]R1881 (0.25 μCi) at which concentration the binding of R1881 to m-AR was saturated. To eliminate possible interference with other steroid receptors, 2.5 mM of triamcinolone acetonide was added to the culture medium. Competition experiments of m-AR binding in LNCaP cells between [^{3}H]R1881 and EMP or its metabolites, EAM, EOM, EO, and β-EDO, or the antiandrogens bicalutamide and hydroxyflutamide were performed by adding various concentrations of those agents to the culture medium. Subsequently, cells were incubated for 4 hr at 37°. Unlabeled R1881 was used as positive control. After the incubation, the cells were collected on glass filters (Whatman LabSales) and washed three times with PBS. The labeled product was evaluated in a β-scintillation Tri-Carb 300 spectrophotometer (Packard Instruments). Each experiment was repeated three times with triplicate samples. The RBA was calculated from competitive binding curves [14] and expressed as the ratio of the concentration of unlabeled compounds versus the concentration of R1881 required to inhibit the binding of [^{3}H]R1881 by 50% (see Table 1). The RBA value of unlabeled R1881 was set at 100%.

w-AR Binding Assay in Transfected HeLa Cells

The w-AR assay was performed in HeLa cells co-transfected with wild-type AR expression plasmid (w-pAR0) and reporter-plasmid GRE-tk-LUC [15]. The transfection was carried out using standard calcium phosphate precipitation methods [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. w-pAR0 cDNA (10 μg) and GRE-tk-LUC cDNA (2 μg) in 125 mM of $CaCl_2$–HEPES buffer (0.14 M of NaCl, 0.05 M of HEPES acid, 1.5 mM of Na_2HPO_4, pH 7.05) were added to the culture and incubated for 16 hr. The precipitates were washed out with prewarmed PBS and refed with fresh RPMI 1640 medium containing 10% FBS. After incubation of the transfected cells for an additional 32 hr at 37°, the cells were harvested for AR binding assay as described above.

For the transactivation assay, various concentrations of DHT, EAM, bicalutamide, and hydroxyflutamide were added to the cultures after 24-hr cotransfection of w-pAR0 or m-pARL with reporter-plasmid GRE-tk-LUC, and the cells were incubated for an additional 24 hr. Then cells were harvested, and the cell extracts were used for luciferase activity assay as described above.

Northern Blotting Assay

LNCaP grown to 65–75% confluence in RPMI 1640 medium containing 10% FBS were exposed for 24 hr to various concentrations of EAM, or bicalutamide or hydroxyflutamide. The cells were harvested and washed with cold PBS, and total RNA was extracted; the northern blotting assay was performed as described previously [17].

RESULTS

The specificity of the simplified method described was established by comparing the binding of [^{3}H]R1881 in the presence of 2.5 mM of triamcinolone acetonide to the m-AR in LNCaP cells, the w-AR in transfected HeLa cells (pAR-HeLa), and the AR negative cell lines (PC-3 and DU145) as well as nontransfected HeLa cells. As shown in Fig. 1, significant binding of [^{3}H]R1881 to ARs was

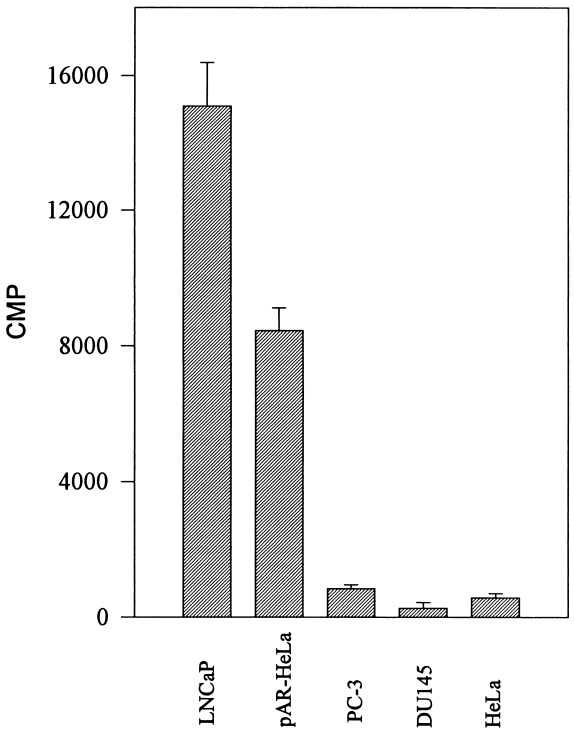

FIG. 1. Specificity of [^{3}H]R1881 binding to the m- and w-ARs. One milliliter of cells (5 × 10^5 cells/mL) of the AR-positive cell line, LNCaP, and of the -negative cell lines, PC 3 and DU-145, HeLa, as well as wild-type AR expression plasmid transfected cells, pAR-HeLa, were incubated with 0.25 μCi of [^{3}H]R1881 for 4 hr in the presence of 2.5 mM of triamcinolone acetonide at 37°. The cells were collected on glass filters and washed three times with PBS. The radioactivity was counted by β-scintillation in a Tri-Carb 300 spectrophotometer. Values are the means ± SD of three separate experiments with triplicate samples.

observed only in the AR-positive cell lines (LNCaP and pAR-HeLa) but not in the AR-negative cell lines (PC-3 and DU 145) or in nontransfected HeLa cells.

Analogous to the antiandrogen agents bicalutamide and hydroxyflutamide, the EMP metabolites, EAM, EOM, EO, and, β-EDO, as well as α-EDO, showed significant binding to the m-AR in a concentration-dependent manner (Fig. 2). The sequence of the affinity of these metabolites to m-AR in LNCaP cells, as determined by their EC_{50} values, as outlined in Table 1, was: β-EDO (0.523 ± 0.028 μM) > EO (0.800 ± 0.090 μM) > α-EDO (1.051 ± 0.096 μM) > EOM (2.612 ± 0.584 μM) > EAM (3.129 ± 0.312 μM). All of the EMP metabolites showed stronger binding than that of bicalutamide (EC_{50}: 4.920 ± 0.361 μM) but weaker binding than hydroxyflutamide (EC_{50}: 0.254 ± 0.012 μM). Poor binding of EMP to the m-AR was observed (>10 μM).

As shown in Figs. 2 and 3, and Table 1, the binding of β-EDO to w-AR was slightly weaker than that to the m-AR. The RBA values of β-EDO and EO were 2.524 and 1.650 to the m-AR and 1.29 and 1.11 to w-AR, respectively ($P < 0.05$). No significant differences of the RBA values between m-AR and w-AR for EAM and EO were observed.

To explore whether EMP metabolites compete with androgens for receptor binding, a transactivation assay was carried out. HeLa cells were cotransfected with either w-pAR0 or m-pARL and an androgen responsive reporter plasmid, GRE-tk-LUC. Twenty-four hours after the transfection, various concentrations of EAM, bicalutamide and hydroxyflutamide, as well as DHT, were added to the cultures. Transactivation of luciferase activity by DHT was observed in both w- and m-pAR transfected cells in a concentration-dependent manner (Fig. 4). Hydroxyflutamide showed a slight induction of the luciferase activity in w-pAR transfected cells and a moderate induction in m-pARL transfected cells. In contrast, EAM analogues to bicalutamide could not induce enzyme activity in either w-pAR0 or m-pARL transfected cells (Fig. 4A). Moreover, at EC_{50} of the receptor binding, almost 50% of the induced luciferase activity by 10 nM DHT in both w-pAR0 and m-pARL transfected cells was blocked by EAM and bicalutamide. Under the same conditions, DHT-induced enzy-

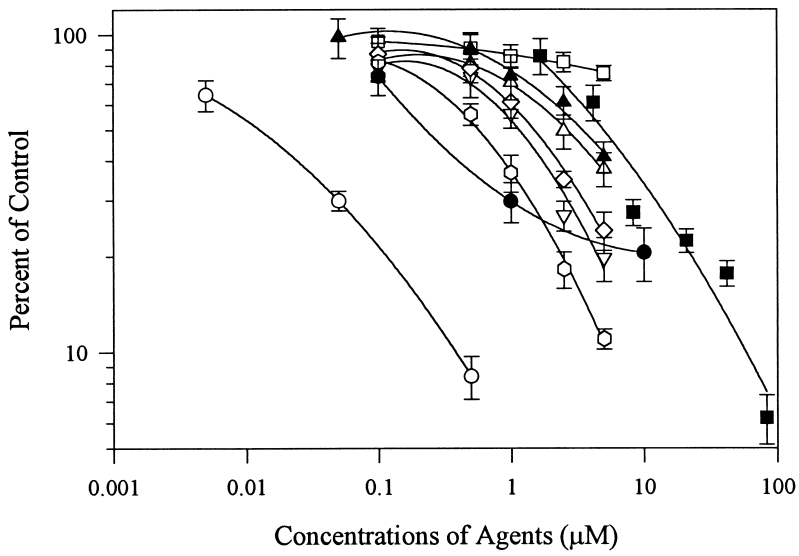

FIG. 2. Concentration-dependent binding of EMP metabolites and other antiandrogens to m-ARs of LNCaP cells. Different concentrations of EMP metabolites, bicalutamide, and hydroxyflutamide were incubated with 5 × 10^5 LNCaP cells for 4 hr in the presence of 0.25 μCi of [^{3}H]R1881 and 2.5 mM of triamcinolone acetonide at 37°. The cells were collected on glass filters and washed three times with PBS. The radioactivity was counted by β-scintillation in a Tri-Carb 300 spectrophotometer. The absolute value of the control was 15,240 cpm that was used for calculation of the percentages. All other values are means ± SD of three separate experiments with triplicate samples. Key: (○) R1881; (□) estramustine phosphate; (△) estramustine; (▽) estrone; (◇) α-estradiol; (○) β-estradiol; (●) hydroxyflutamide; (■) bicalutamide; and (▲) estromustine.

TABLE 1. Binding of EMP metabolites and other antiandrogen agents to m- and w-ARs as expressed by the EC_{50} and relative binding affinity

	LNCaP		pAR-HeLa*	
Agents†	EC_{50} (μM)	RBA‡ (%)	EC_{50} (μM)	RBA‡ (%)
R1881	13.2 ± 0.23 nM	100	8.90 ± 0.19 (nM)	100
EMP	>10 μM	<0.13		
EAM	3.129 ± 0.312	0.506	1.210 ± 0.19	0.72
EOM	2.612 ± 0.584	1.168		
EO	0.800 ± 0.090	1.650	1.220 ± 0.16	1.11
α-EDO	1.051 ± 0.096	1.251		
β-EDO	0.523 ± 0.028	2.524	0.690 ± 0.078	1.29
Bicalutamide	4.920 ± 0.361	0.268		
HDF	0.254 ± 0.012	5.190		

The EC_{50} values are means ± SD of three separate experiments with triplicate samples.

*HeLa cells were transfected with wild-type androgen receptor cDNA expression plasmid (w-pAR0).

†R1881, methyltrienolone; EMP, estramustine phosphate; EAM, estramustine; EOM, estromustine; EO, estrone; EDO, estradiol; and HDF, hydroxyflutamide.

‡RBA: relative binding affinity. The RBA value of R1881 was set at 100. The absolute values of controls in mutated LNCaP cells and w-pAR0 transfected HeLa cells were 15,240 and 9,680 cpm, respectively, that were used in the calculation of RBA.

matic activity blocked by hydroxyflutamide was only 28% in w-pAR0 and 14% in m-pARL (Fig. 4C). These data indicate that EAM, a major metabolite of EMP, acts as an androgen antagonist.

To verify the antagonistic effect of EAM, the effects of EAM, bicalutamide, or hydroxyflutamide on the expression of *PSA*, a native AR-targeted gene [18, 19], were evaluated further by northern blotting. As shown in Fig. 5, analogous to bicalutamide, exposure of LNCaP cells to EAM for 24 hr caused a dramatic decrease of PSA transcription in a concentration-dependent manner. The levels of PSA mRNA decreased 56 and 90% when LNCaP cells were treated with 5 and 10 μM of EAM (IC_{50} = 10.97 ± 1.68 μM in LNCaP cells), confirming that EMP metabolites serve as androgen antagonists to m-AR. In contrast, binding of hydroxyflutamide to m-AR in LNCaP cells resulted in a moderate concentration-dependent stimulation of PSA expression, consistent with the results shown above.

DISCUSSION

The effects of steroids on target cells are mediated by their respective receptors. After binding of the hormone, these ligand-dependent transcription factors activate their target genes by tightly and selectively binding to areas of hormone responsive elements in the target genes [20–22].

AR is a member of the superfamily of ligand-dependent transcription factors that comprise receptors for retinoids, thyroid hormones, and steroids [20, 22]. The binding of AR to the hormone responsive element of its targeted genes is

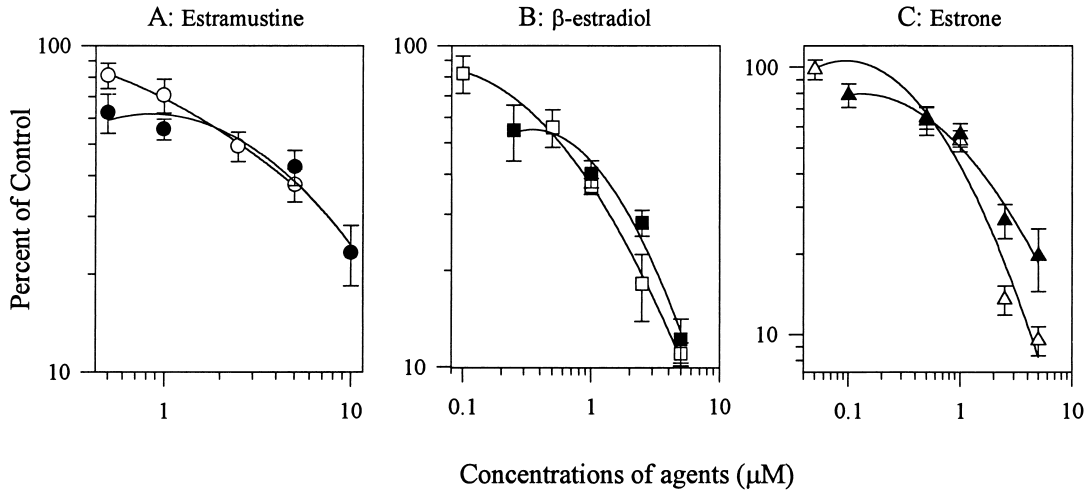

FIG. 3. Binding of EMP metabolites to m-ARs (open symbols) and w-ARs (closed symbols). HeLa cells were transfected with w-AR cDNA expression plasmid (w-pAR0). After 48-hr transfection, 5×10^5 cells were incubated with different concentrations of EMP metabolites for 4 hr in the presence of 0.25 μCi of [^{3}H]R1881 and 2.5 mM of triamcinolone acetonide at 37°. The cells were collected on glass filters and washed three times with PBS. The radioactivity was counted by β-scintillation in a Tri-Carb 300 spectrophotometer. Values are the means ± SD of three separate experiments with triplicate samples. The absolute values of controls in m-ARs and w-ARs were 9680 and 16,020 cpm, respectively, which were used for calculation of the percentages.

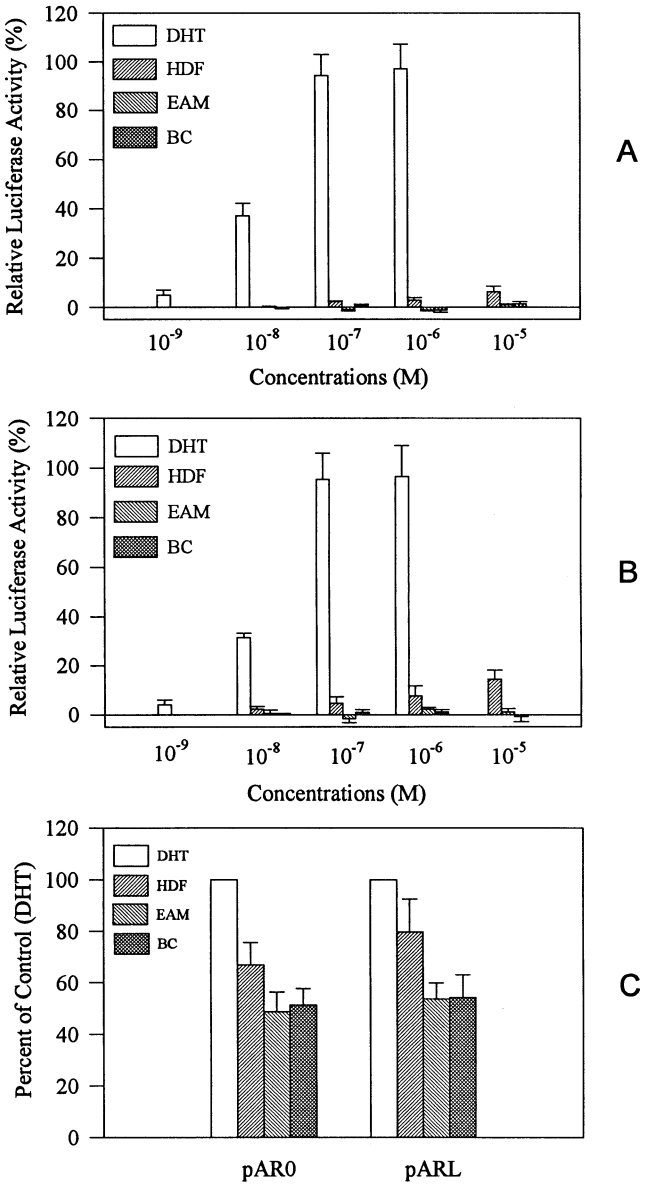

FIG. 4. Transactivation of androgen agonists or antagonists on the w-AR (A) or m-AR (B) mediated luciferase reporter gene expression. HeLa cells were cotransfected with either w-pAR0 or m-pARL and luciferase reporter gene, and the luciferase activity was measured as described in Materials and Methods. (C) Inhibition of hydroxyflutamide (HDF), EAM, and bicalutamide (BC) on the luciferase activity induced by 10 nM of DHT in both w- and m-AR transfected HeLa cells. The enzyme activity induced by DHT was set at 100. The absolute counts for luciferase activity of controls in w- and m-AR transactivation assays were 542 and 486, respectively, which were used for calculation of the percentages. The values are means ± SD of three separate experiments with triplicate samples.

an essential step of transcriptional activation. We have shown that down-regulation of PSA by finasteride, a 5α-reductase inhibitor, is accompanied by inhibition of complex formation between AR and the steroid receptor binding consensus (SRBC) in the promoter of the *PSA* gene [19] in mutated AR (codon 868) of LNCaP cells [23, 24]. DHT has been shown to stimulate significantly the expression of PSA in LNCaP cells [25, 26]. However, androgen antagonists usually compete with agonists for receptor binding without permitting the receptor to stimulate transcriptional activity [20, 21].

We previously demonstrated that PSA plays a role in the growth stimulation of the androgen responsive prostate cancer cell line LNCaP [13]. In this study, we have shown that EMP metabolites, such as EAM, EOM, EO, and β-EDO significantly bind to both m-AR and w-AR, but that EAM could not induce w-pAR- or m-pAR-mediated luciferase reporter gene transactivation. In addition, EAM inhibited expression of the native AR-target gene *PSA* in AR-mutated LNCaP cells. These data indicate that EMP metabolites act, at least partially, as androgen antagonists, a new mechanism of action for this agent. This conclusion is also supported by previous observations where LNCaP cells were more sensitive to both EAM and EOM than were the hormone-independent cell lines, PC-3 and DU145 [compared the IC_{50} value of EOM in LNCaP (9.73 μM), with those in DU-145 (65.62 μM), and PC-3 (74.20 μM)] [8, 10].

The metabolism of steroid hormones in LNCaP cells was investigated by Schuurmans *et al.* [27]. Of the five steroids investigated (progesterone, β-EDO, triamcinolone, R1881, and DHT), only DHT was metabolized. Even if the metabolism of EAM does occur to some extent in the human prostatic tumor cell line 1013 L [28], it is unlikely that the EMP metabolites investigated in our study are further metabolized by LNCaP cells, because the major site of metabolism for steroids is the liver [11, 12].

The binding of hydroxyflutamide to m-AR in LNCaP cells was found to be approximately 16 times higher than that of bicalutamide. This finding is in contrast to the binding of these two compounds to w-AR in w-pAR transfected COS-N cells, where a similar RBA value of these two drugs was observed [29]. This may have clinical relevance. For estradiol, similar relative AR binding to m-AR was reported by Veldscholte *et al.* [24] and Taplin *et al.* [30].

Recent studies suggest that the mutations of the AR may change the characteristics of some AR blockers, such as hydroxyflutamide, anandron, cyproterone acetate, estradiol, and progesterone, from antagonists to agonists. These agents can compete with androgens to bind to m-AR, but have also been reported to stimulate cell growth and excretion of PSA [11, 21, 24, 29–31]. Our data demonstrated that EMP metabolites, such as EAM and EOM (data not shown) as well as bicalutamide significantly inhibited *PSA* gene expression in m-AR LNCaP cells. These metabolites could not induce reporter gene activity in either w-pAR0 or m-pARL transfected HeLa cells (Figs. 4 and 5). These data indicate that those agents still exhibit their antagonistic effects on both m-AR and w-AR, which distinguish them from hydroxyflutamide and β-EDO. Similar results for transcription activation in w- and m-pAR transfected cells were reported by Berrevoets *et al.* [29], Veldscholte *et al.* [24], and Yeh *et al.* [31].

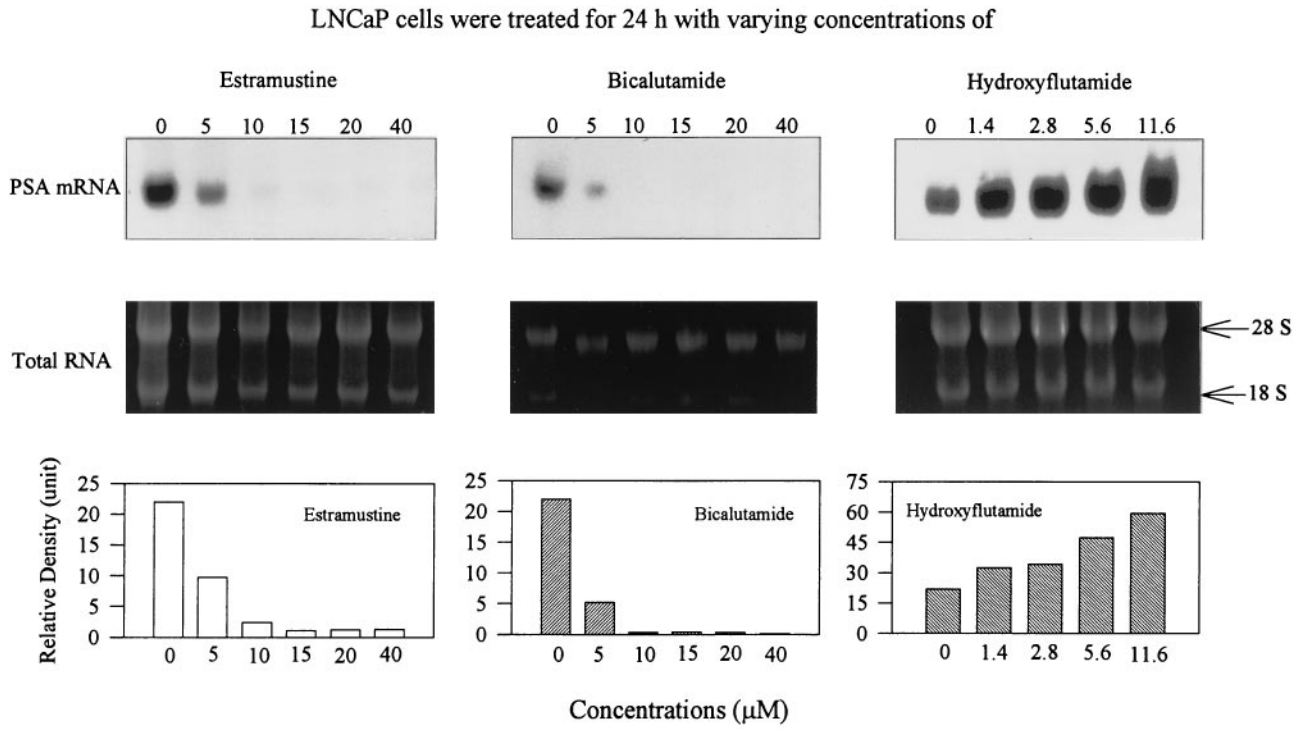

FIG. 5. Levels of PSA mRNA in AR mutated LNCaP cells as a function of concentrations of estramustine, bicalutamide, and hydroxyflutamide. LNCaP cells grown at 65–75% confluence in RPMI 1640 medium containing 10% FBS were exposed to the indicated concentrations of agents for 24 hr, respectively, the cells were harvested and washed once with cold PBS, and total RNA was extracted. The PSA mRNA was then detected by northern blotting using 5′-end labeled PSA-specific oligonucleotide as the probe. Top panels: PSA mRNA measured by northern blotting. Middle panels: the total RNA. Bottom panels: density of the plots of PSA mRNA determined from x-ray films by an Imaging Densitometer, model GS-700. Data are the means of two separate experiments scored by the densitometer and normalized to 28S RNA.

The discrepancy in bioactivities of bicalutamide and hydroxyflutamide observed in this study indicates the need for caution in therapy with antiandrogens for prostate cancer patients. It is possible that ARs mutated at various codons might further complicate the choice of treatment and might further evoke paradoxical responses to the treatment. Screening procedures using new molecular targets might allow a more rational approach to the treatment of this disease with antiandrogens.

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