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Report

Androgens inhibit basal and estrogen-induced cell proliferation in the ZR-75-1 human breast cancer cell line

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Summary

This study describes the inhibitory effect of 5α-dihydrotestosterone (5α-DHT) and its precursors testosterone (T) and androst-4-ene-3,17-dione (Δ^4-DIONE) on the growth of the estrogen-sensitive human breast cancer cell line ZR-75-1. In the absence of estrogens, cell proliferation measured after a 12-day incubation period was 50–60% inhibited by maximal concentrations of 5α-DHT, T, or Δ^4-DIONE with half-maximal effects (IC_{50} values) observed at 0.10, 0.15 and 15 nM, respectively. This growth inhibition by androgens was due to an increase in generation time and a lowering of the saturation density of cell cultures. The antiestrogen LY156758 (300 nM) induced 25–30% inhibition of basal cell growth, its effect being additive to that of 5α-DHT. The mitogenic effect of 1 nM estradiol (E_2) was completely inhibited by increasing concentrations of 5α-DHT with a potency (IC_{50} = 0.10 nM) similar to that measured when the androgen was used alone. E_2 had a more rapid effect on cell proliferation than 5α-DHT, the latter requiring at least 5 to 6 days to exert significant growth inhibition. As found in the absence of estrogens, maximal inhibition of cell proliferation in the presence of E_2 was achieved by the combination of the antiestrogen and 5α-DHT. Supraphysiological concentrations of E_2 (up to 1 μM) were needed to completely reverse the growth inhibitory effect of a submaximal concentration of 5α-DHT (1 nM). The antiproliferative effect of androgens was competitively reversed by the antiandrogen hydroxyflutamide, thus indicating an androgen receptor-mediated mechanism. The present data suggest the potential benefits of an androgen-antiestrogen combination therapy in the endocrine management of breast cancer.

Introduction

Androgens such as testosterone propionate [1–3], fluoxymesterone [4, 5], and calusterone [6] have long been used in the adjuvant therapy of breast cancer with an efficacy comparable to that achieved with other types of endocrine manipulations [3, 7–9]. Since tumor regression induced by treatment with androgens is not restricted to premenopausal patients [7], it is likely that the therapeutic activity of androgens is not limited to an inhibitory effect on gonadotropin secretion.

While the presence of androgen receptors has been documented in normal [10, 11] and neoplastic [11] breast tissue, as well as in several established breast cancer cell lines [12], very little is known about their functional significance. In the widely used *in vitro* model of estrogen-responsive human breast cancer, namely the MCF-7 cell line, pharmacological concentrations (0.1–1 μM) of 5α-di-

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hydrotestosterone (5α-DHT) are mitogenic [13] and induce the secretion of a 52,000 Da glycoprotein, a protein known to be specifically regulated by estrogens [14]. This effect of 5α-DHT has been shown to be mediated by the low-affinity binding of high concentrations of androgens to the estrogen receptor [15, 16]. On the other hand, physiological (0.1–10 nM) concentrations of androgens can counteract induction of the progesterone receptor by 17β-estradiol (E_2) in MCF-7 cells through an androgen receptor-mediated mechanism [17, 18]. Moreover, in the T47-D human breast cancer cell line, 5α-DHT specifically induces the secretion of several proteins, an effect which is reversed by the antiandrogen flutamide [19, 20]. Androgens have also been found to modulate the number of specific prolactin binding sites in another human breast cancer cell line [21].

Although the above-mentioned studies indicate that breast cancer cells possess functional androgen receptors mediating various biochemical responses, no specific effect of androgens on cell proliferation has yet been reported. In the present study, we present evidence that androgens strongly inhibit proliferation of the well-characterized, estrogen-sensitive human breast cancer cell line ZR-75-1 [22]. Moreover, we have used the pure antiandrogen hydroxyflutamide (OH-FLU) [23, 24] to assess the specificity of the growth-inhibitory action of androgens. Finally, we have compared the ability of androgens and of the antiestrogen [6-hydroxy-2-(4-hydroxyphenyl)benzo[b]thien-3-yl], (4-[2-(10piperidinyl) ethoxy]-phenyl) methanone hydrochloride (LY156758) [25, 26] to influence basal cell growth and to counteract the mitogenic effect of E_2.

Materials and methods

Chemicals

All media and supplements for cell culture were purchased from Sigma, except for fetal bovine serum which was obtained from Hyclone (Logan, UT). The antiandrogen hydroxyflutamide (OH-FLU, SCH 16423) was kindly supplied by Drs J. Nagabhushan and R. Neri (Schering Corporation, Kenilworth, NJ). The antiestrogen LY156758 was generously provided by Dr. J.A. Clemens (Lilly Research Laboratories, Indianapolis, IN). 17β-[2,4,6,7-^{3}H]estradiol (specific activity, 110 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). [17α-*methyl*^{3}H]methyltrienolone (R1881; 17β-hydroxy, 17α-methyl-4,9,11-estratrien, 3-one; specific activity, 87 Ci/mmol) and unlabeled R1881 were obtained from New England Nuclear (Lachine, Quebec, Canada). Steroids were obtained from Steraloids (Pawling, NY).

Maintenance of stock cultures

The ZR-75-1 human breast cancer cell line (83rd passage) was obtained from the American Type Culture Collection (Rockville, MD). The cells were routinely cultured in phenol red-free [27, 28] RPMI 1640 medium supplemented with 10 nM E_2, 15 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU penicillin per ml, 100 μg streptomycin sulfate per ml, and 10% (v/v) fetal bovine serum (FBS), in a water-saturated atmosphere of 95% air: 5% CO_2 at 37°C. The cell cultures used for the experiments herein described were between passages 90 and 102.

Cell growth experiments

Stock cultures in their logarithmic growth phase were harvested with 0.05% trypsin/0.02% EDTA (w/v) in Hanks' balanced salts solution and resuspended in E_2- and phenol red-free RPMI 1640 medium containing 5% (v/v) dextran-coated charcoal (DCC)-treated FBS and 500 ng of bovine insulin per ml [29], but otherwise supplemented as described above for maintenance of stock cultures. In some experiments, cells were cultured for 4 weeks in this medium (hereafter referred to as SD medium) prior to harvesting, in order to maximize estrogenic stimulation of cell growth, as explained in the 'Results' section. Cells were plated in 24-well Linbro culture plates (Flow Laboratories) at a final density of 0.5–4.0 × 10^4 cells/well.

Fourty-eight hours after plating, fresh SD medium containing the indicated concentrations of steroids or steroid antagonists was added. The final concentration of ethanol used for the addition of test substances did not exceed 0.12% (v/v), and had

no significant effect on cell growth and morphology. The incubation media were replaced every other day and cells were harvested by trypsinization after 12 days of treatment, unless otherwise indicated. Cell number was determined with a Coulter Counter (model ZM).

Specific uptake of [^{3}H]E_2 and [^{3}H]R1881 by intact ZR-75-1 cell monolayers

The relative binding affinity (RBA) of steroids for androgen and estrogen specific binding sites was assessed by measuring the effect of increasing concentrations of competitor on the uptake of [^{3}H]R1881 and [^{3}H]E^2, respectively, by ZR-75-1 cells in monolayer culture [18, 29, 30]. Briefly, cells from stock cultures were trypsinized, resuspended in SD medium at a density of 2–5 × 10^4 cells/ml, and distributed to 24-well culture plates. Cultures were grown to a final yield of about 3–5 × 10^5 cells/well in SD medium (± 1 nM E_2, in some experiments). Growth medium was then replaced with 0.5 ml of phenol red-free RPMI 1640 medium (supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 15 mM Hepes, and 0.1% (w/v) fatty acid acid-free bovine serum albumin) containing the indicated concentration of competitor plus either 2.5 nM [^{3}H]E_2 or 3.0 nM [^{3}H]R1881 (for the determination of estrogen and androgen specific binding sites, respectively). Triamcinolone acetonide (4.5 μM) was added for the measurement of [^{3}H]R1881 uptake in order to block binding to the progesterone receptor. Incubations with [^{3}H]R1881 and [^{3}H]E_2 were stopped after 1 h and uptake of radioligand measured as described [18, 30]. The apparent dissociation constant (K_D) and the number of androgen-specific binding sites per cell (Bmax) were estimated using Scatchard analysis [31] by adding increasing concentrations of [^{3}H]R1881 (0.1–4 nM) to monolayer cultures, plus or minus a 200-fold excess of unlabeled R1881 to account for non-specific uptake.

Calculations and statistical analyses

Apparent IC_{50} values were calculated using an iterative least squares regression [32], while apparent inhibition constants (Ki values) were calculated according to Cheng and Prusoff [33]. Mean generation times were calculated according to a least-squares regression program on triplicate log-transformed cell number values measured on at least 6 time intervals. Statistical significance was calculated according to the multiple-range test of Duncan-Kramer [34]. Values are presented as means ± SEM of measurements obtained from triplicate incubations. When no bar is shown, the SEM is smaller than the symbol used.

Results

As illustrated in Fig. 1, a 12-day incubation with increasing concentrations of 5α-DHT had a biphasic effect on the proliferation of ZR-75-1 cells incubated in the absence of estrogens in phenol red-free medium. Concentrations of 5α-DHT between 0.01 and 10 nM inhibited basal cell growth up to a maximum of 50% in a dose-dependent manner, a half-maximal effect (IC_{50}) being observed at about 0.10 nM 5α-DHT. When concentration of the androgen was increased from 10 to 2000 nM, the amplitude of the inhibitory effect gradually decreased, although cell number remained below control. A progressive decrease in cell growth was again observed at concentrations of 5α-DHT exceeding 2 μM.

While the addition of 1 nM E_2 induced a 2-fold increase in cell number, the effect of the estrogen was completely prevented when 5α-DHT was added up to 10 nM with an IC_{50} value again seen at 0.10 nM. Inhibition of the mitogenic effect of E_2 then remained on a plateau between 10 and 2000 nM 5α-DHT. The estrogen, however, prevented the growth inhibition induced by 5α-DHT alone up to 2 μM 5α-DHT, while higher concentrations of the androgen decreased cell number to a level similar to that observed in the absence of estrogen.

It can also be seen in Fig. 1 that the inhibitory effect of 5α-DHT was competitively neutralized by coincubating ZR-75-1 cells with the antiandrogen OH-FLU (3 μM) in control as well as in E_2-treated cells, the IC_{50} value of DHT action being increased

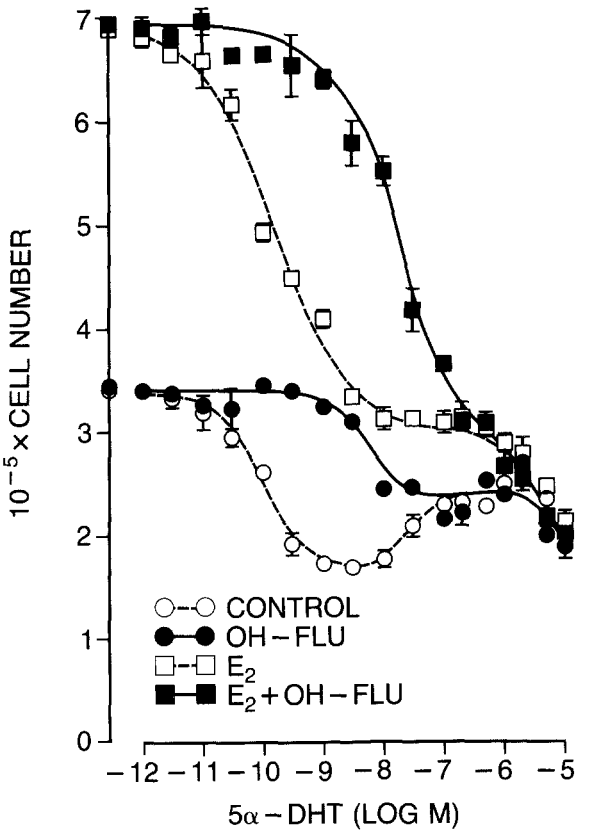

Fig. 1. Inhibitory effect of increasing concentrations of 5α-DHT on the proliferation of ZR-75-1 human breast cancer cells in culture and its prevention by the antiandrogen OH-FLU. Forty-eight hours after plating (initial cell density = 1.0×10^4 cells/well), 5α-DHT was added to cell cultures at the indicated concentrations in the presence (□, ■) or absence (○, ●) of 1 nM E_2. Cells also received 3 μM OH-FLU (●, ■) or the vehicle alone (○, □). The data are presented as means ± SEM of triplicate determinations from a representative experiment.

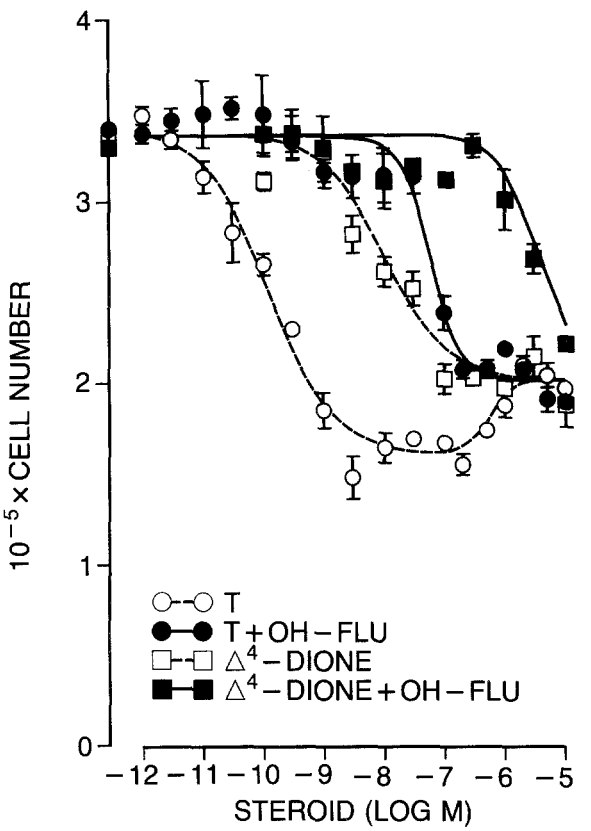

Fig. 2. Effect of increasing concentrations of T or Δ^4-DIONE on the proliferation of ZR-75-1 breast cancer cells in culture and its reversal by the antiandrogen OH-FLU. Forty-eight hours after plating (1.0×10^4 cells/well), T (○, ●), or Δ^4-DIONE (□, ■) was added at the indicated concentrations in the presence (●, ■) or absence (control, ○, □) of 3 μM OH-FLU.

from 0.10 to about 5 nM. On the other hand, the sole addition of OH-FLU had no significant effect on the growth of ZR-75-1 cells.

The effect of two other physiologically important androgens, namely testosterone (T) and its precursor androst-4-ene-3,17-dione (Δ^4-DIONE), on the proliferation of ZR-75-1 cells was next examined. Increasing concentrations of T (up to 10 nM) maximally decreased cell number by about 50% with a half-maximal effect measured at about 0.15 nM (Fig. 2). A modest but significant decrease in the inhibitory effect of T was apparent at concentrations of the androgen ranging between 0.2 and 2 μM. Inhibition of cell proliferation was also induced by Δ^4-DIONE, although the potency of this steroid was much lower ($IC_{50} \sim 15$ nM) than that observed with 5α-DHT or T. It can also be seen in Figure 2 that the maximal inhibition achieved with Δ^4-DIONE is approximately 30% lower than that achieved by 5α-DHT or T. Both T- and Δ^4-DIONE-induced inhibitory effects were efficiently counteracted by the antiandrogen OH-FLU, thus indicating that their action is also mediated by the androgen receptor. From the above-described experiments, the apparent inhibition constant (Ki) of OH-FLU action was estimated [33] at 107 ± 31 nM (mean ± SEM from 3 independent experiments), a value in close agreement with that reported for the interaction of the antiandrogen with the androgen receptor in several tissues [24].

We next studied the time course of 5α-DHT action on ZR-75-1 cell growth in the presence or absence of E_2. When cells were initially plated at a density similar to that used in experiments shown in Figs. 1 and 2, the inhibitory effect of 5α-DHT (10 nM) on the growth rate became significant following a 5- to 6-day period in both the presence and absence of 1 nM E_2 (Fig. 3A). While E_2 decreased the mean generation time from 71 ± 4 to 57 ± 2 h and increased saturation density by about 2.2-fold, a 47% lower plateau was observed in DHT-treated cells, which had a generation time of 89 ± 4 h. In the presence of E_2, 5α-DHT-treated cells had an initial growth rate similar to that of cells incubated with E_2 alone, their proliferation rate progressively decreasing after 6 days to reach cell number similar to control values after 12 days in culture. Thus, 5α-DHT decreased not only the growth rate, but also the degree of confluency reached by both control and E_2-treated ZR-75-1 cell cultures. It is also apparent that the time required by androgens to inhibit cell growth is longer than that needed by E_2 to exert its mitogenic effect.

Even more striking antiproliferative effects of 5α-DHT were observed when ZR-75-1 cells were initially plated at lower densities. As illustrated in Fig. 3B, addition of the androgen virtually stopped net cell growth after approximately one population doubling when cells were initially plated at $2.5 \times 10^3/cm^2$. Under these conditions, control and E_2-treated cells had not yet reached a plateau at the end of a 25-day incubation period. The presence of 5α-DHT abolished the net mitogenic effect of the estrogen at all time intervals examined.

We then studied in detail the influence of cell density on the effects of E_2 and DHT. As shown in Fig. 4, increasing the initial inoculum allowed a higher initial growth rate and a much shorter lag period, thus resulting in a smaller relative decrease in cell number and saturation density at a fixed harvesting time following incubation with the androgen. This effect was especially marked in the case of E_2-treated cells, where the effect of 5α-DHT measured at 12 days became non-significant at initial cell densities exceeding $1.5–2 \times 10^4$ cells/cm^2. These results are consistent with the observation that the action of estrogens on cell growth is exerted faster than that of androgens, and that E_2-treated cultures were reaching confluency within 6 to 7 days at the highest cell densities studied (data not shown).

In order to better understand the opposite influences of androgens and estrogens on the proliferation of ZR-75-1 cells, we next examined the ability of increasing concentrations of E_2 to prevent growth inhibition induced by 5α-DHT and/or the antiestrogen LY156758. In preliminary experiments, we observed that following prolonged deprivation (4 weeks) of ZR-75-1 cells from estrogenic influence in SD medium, the maximal amplitude of the E_2-induced mitogenic effect was increased. Using such a protocol, which allows an optimal stimulatory effect of E_2, increasing concentrations of E_2 induced a maximal 4-fold stimulation of cell proliferation, a half-maximal increase in cell number being observed at approximately 8 pM E_2 (Fig. 5A). Addition of a submaximal concentration of 5α-DHT alone (1 nM) inhibited basal cell growth by 54%. The androgen effect was then reversed by E_2 in a biphasic manner, an intermediary plateau of the stimulatory estrogenic effect being observed between 0.3 and 10 nM E_2. This plateau corresponds to an approximately 75% inhibition by 5α-DHT of the maximal estrogenic stimulation of cell growth. At E_2 concentrations >10 nM, there was a second rise in growth of androgen-treated cells which reached values similar to those observed in the absence of 5α-DHT at 1 μM E_2. The presence of OH-FLU (1 μM), while having no effect by itself on the mitogenic effect of E_2, completely reversed the androgen-induced inhibition of cell growth.

LY156758 (200 nM) competitively blocked stimulation of cell proliferation by increasing concentrations of E_2 (Ki of LY156758 = 0.14 nM) and caused a maximal 25% decrease in basal cell number (Fig. 5B). In the absence of E_2, the simultaneous presence of both LY156758 and 5α-DHT slightly enhanced the inhibition induced by the androgen alone (54 and 63% for 5α-DHT and 5α-DHT + LY156758, respectively). Moreover, the antiestrogen completely counteracted the partial reversal by E_2 (up to about 10 nM) of the growth-inhibitory effect of 5α-DHT in a competitive manner. Again, OH-FLU (1 μM) selectively and com-

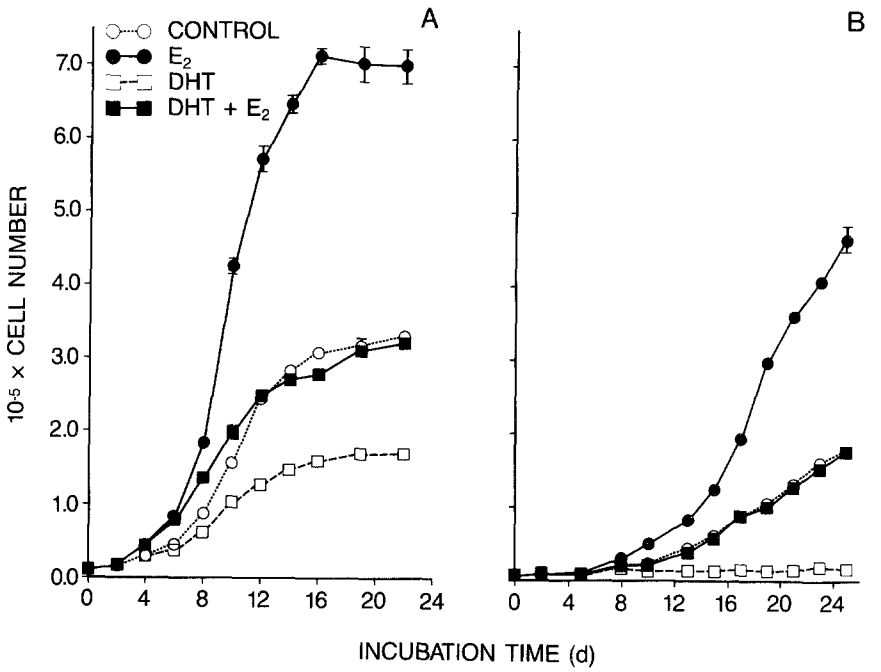

Fig. 3. Time course of the effect of 5α-DHT and/or E_2 on the proliferation of ZR-75-1 cells. A) Cells were plated at 1×10^4 cells/2.0-cm^2 well and 48h later (zero time), 1 nM E_2 (●), 10 nM 5α-DHT (□), or both steroids (■) were added and cell number determined at the indicated times. Control cells received the ethanol vehicle only. B) Same as in A, except that the initial density was 5.0×10^3 cells/2.0-cm^2 well.

pletely abolished the effect of low 5α-DHT on E_2-sensitive cell proliferation.

Additivity of the inhibitory effects of 5α-DHT and LY156758 is even more clearly illustrated in Fig. 6. In the absence of E_2, there was a dose-dependent inhibition (up to 30%) of cell proliferation by increasing concentrations of LY156758, with a half-maximal effect observed at about 0.3 nM (Fig. 6A). Again, incubation with 5α-DHT (1 nM) induced a 55% inhibition of basal cell growth, while the addition of LY156758 further decreased cell number to a maximal 70% inhibition. In good agreement with the data presented in Figs. 1 and 5, the addition of 1 nM 5α-DHT alone inhibited the mitogenic effect of E_2 by 80% (Fig. 6B). In the presence of increasing concentrations of LY156758, 5α-DHT further decreased cell number below the value reached with maximally effective concentrations of LY156758 alone to approximately 30% of basal cell growth (Fig. 6B). In every instance, OH-FLU (1 μM) completely reversed the effect of 5α-DHT without affecting the pattern of inhibition achieved with the antiestrogen.

Since high concentrations of 5α-DHT and T are known to bind to the estrogen receptor and induce nuclear retention of the resulting complexes with subsequent estrogenic responses [35–39], the binding affinity of ligands used in this study for the androgen and estrogen receptors present in ZR-75-1 cells was determined. As shown in Fig. 7, intact ZR-75-1 cells grown under steroid-deprived conditions contain 2.53×10^4 androgen specific binding sites/cell with an apparent dissociation constant (K_D) or 0.69 nM, in agreement with data already published for the ZR-75-1 cell line [12, 22]. Incubation of ZR-75-1 cells for up to 7 days in the presence of 1 nM E_2 had no significant effect on either the number (2.69×10^4 sites/cell) nor K_D (0.79 nM) of the androgen specific binding sites, while increasing cell number by 58% (data not shown) during the same period.

The RBA of various ligands for androgen and estrogen specific binding sites in ZR-75-1 cell monolayers was next studied (Fig. 8). Table 1 summarizes the apparent Ki values of the steroids used, as calculated [33] from the K_D values of R1881 (Fig.

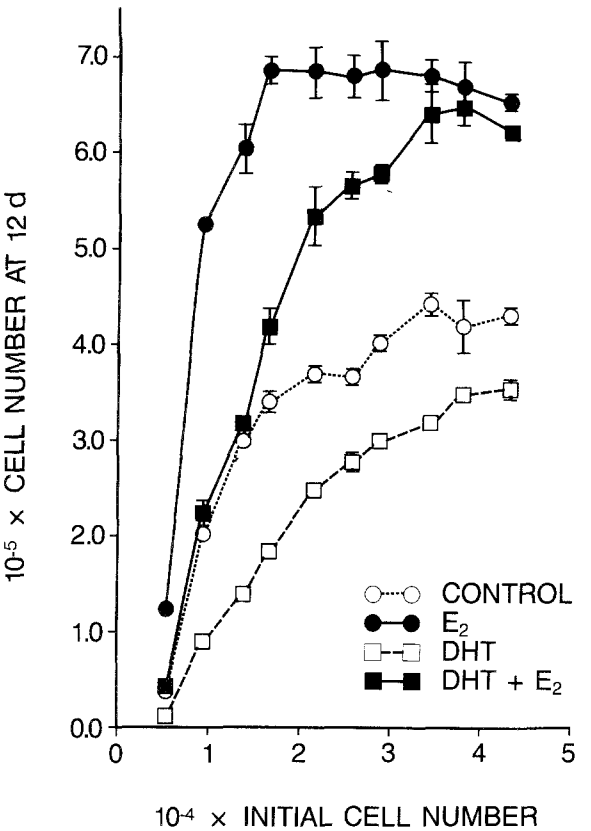

Fig. 4. Influence of seeding density on the effect of androgens and estrogens on the proliferation of ZR-75-1 cells measured after a 12-day incubation period. Cells were plated at the indicated density (per 2.0-cm^2 well) and treated for 12 days with no steroid (○), 1 nM E_2 (●), 10 nM 5α-DHT (□), or both steroids (■). Cell number was determined at the end of the incubation period.

7) and E_2 [29] of their respective receptors. The competition studies show that E_2, Δ^4-DIONE, and OH-FLU were much weaker ligands of the androgen receptor than 5α-DHT and T, which had affinities close to that of R1881 (Fig. 8A). The apparent Ki values of 5α-DHT and T were somewhat higher than the IC_{50} values measured for the same androgens on the proliferation of ZR-75-1 cells (0.10 and 0.15 nM, respectively; Figs. 1 and 2). Similar discrepancies between the measured affinity of E_2 for the estrogen receptor and the half-maximal effective concentration of the estrogen needed to induce a mitogenic effect have been noted [29, 40, 41]. While the apparent Ki value found for the antiandrogen OH-FLU agrees well with the calculated Ki for its reversal of the androgen effect on cell growth (110 nM), the affinity of Δ^4-DIONE for androgen specific binding sites was about 13-fold lower than that predicted from its potency to inhibit cell proliferation ($IC_{50} \approx 15$ nM; cf. Fig. 2). Interestingly, the calculated Ki value of E_2 for the specific uptake of [^{3}H]R1881 was about 50 nM.

Except for E_2 itself, only 5α-DHT and T were found to efficiently compete with the high affinity uptake of [^{3}H]E_2 in intact ZR-75-1 cells, although only at high concentrations (Ki = 441 and 135 nM, respectively) (Fig. 8B and Table 1). Likewise, OH-FLU had no significant affinity for estrogen specific binding sites as reported in other tissues [42].

Discussion

The present study provides the first demonstration that naturally occuring androgens exert a potent inhibitory action on the proliferation of an established, estrogen-sensitive human breast cancer cell line. Thus, in ZR-75-1 cells, concentrations of 5α-DHT similar to the plasma levels found in normal women [35, 43, 44] and breast cancer patients [45] (0.3–0.7 nM) are potent inhibitors of the mitogenic effect of E_2 and inhibit growth in the absence of estrogens. Furthermore, T, at concentrations within the physiological range of concentrations ob-

Table 1. Apparent dissociation constants (Ki) of various ligands towards the androgen (AR) and estrogen receptors (ER) present in ZR-75-1 cells. Ki values were calculated according to Cheng and Prusoff [33], using the RBA values determined from Fig. 8. The K_D values of R1881 and E_2 were estimated by Scatchard analysis under identical experimental conditions (Fig. 7.) [29].

Compound	Apparent Ki values (nM)	
	AR	ER
R1881	0.69	ND[a]
5α-DHT	0.53	491
T	1.06	135
Δ^4-DIONE	197	≥10,000
E_2	50.5	0.60
OH-FLU	145	≥10,000

[a] N.D., not determined.

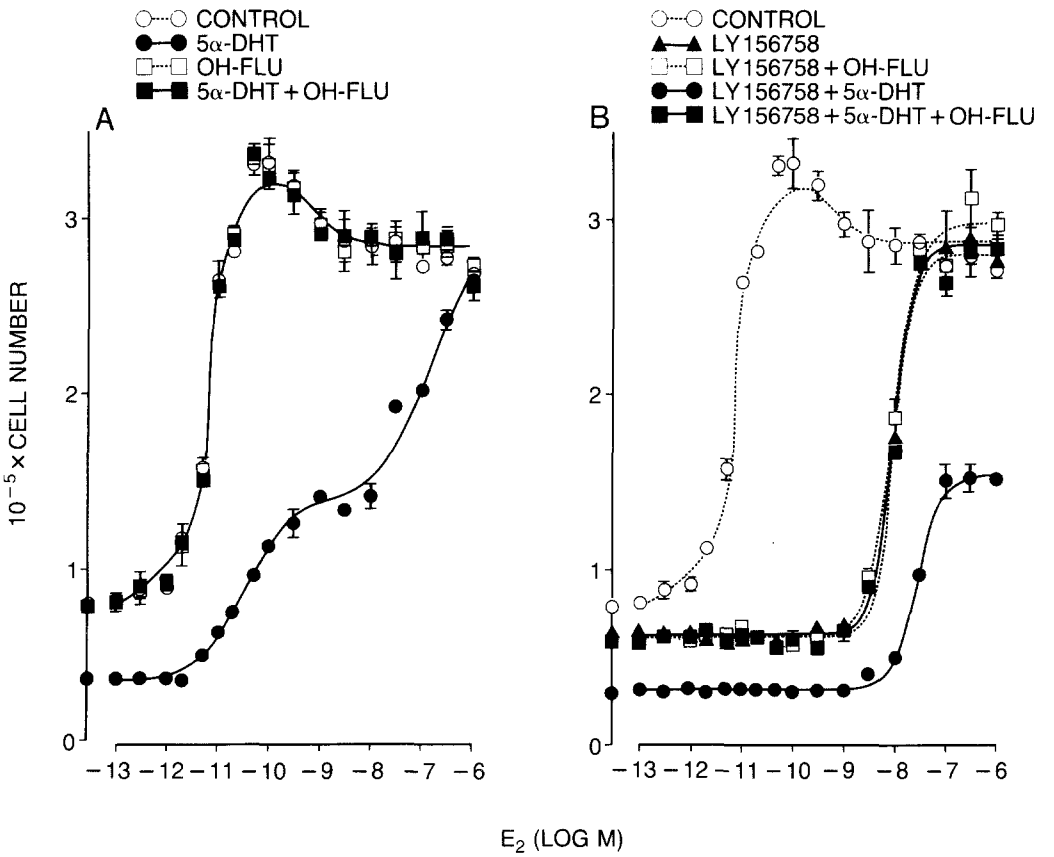

Fig. 5. Effect of increasing concentrations of E_2 on the proliferation of ZR-75-1 cells, and its inhibition by 5α-DHT and/or the antiestrogen LY156758. Prior to the experiment, cells were cultured for 4 weeks in SD medium. Cells were then plated at 8.0×10^3 cells/well in fresh medium of identical composition. A) 48 hours after plating, E_2 was added at the indicated concentrations alone (control, ○), or concomitantly with 1 nM 5α-DHT (●), 1 μM OH-FLU (□), or both compounds (■). B) Same as in A, except that 200 nM LY156758 was also added alone (△) or in the presence of 1 nM 5α-DHT (●), 1 μM OH-FLU (□), or both compounds (■).

served in adult women (1–3 nM) [35, 43–45], is also a potent inhibitor of cell growth. Δ^4-DIONE also led to significant growth inhibition in ZR-75-1 cells, although the active concentrations ($IC_{50} \sim$ 15 nM) are in the upper range of the plasma concentrations (1–10 nM) found in women [35, 43–45].

Several lines of evidence show that the potent growth-inhibitory effect of androgens observed in ZR-75-1 cells is mediated through their specific interaction with the androgen receptor. Firstly, the potency of 5α-DHT and T to induce antiproliferative effects ($IC_{50} \sim$ 0.10 and 0.15 nM, respectively) is in agreement with their relative binding affinity for androgen specific binding sites in intact ZR-75-1 cells as well as in other human breast cancer cells [12, 46]. It also compares well with the potency of 5α-DHT to specifically stimulate the secretion of the Zn-α_2-glycoprotein [19] and the GCDFP-15 glycoprotein [19, 20] in T47-D human breast cancer cells. The ability of Δ^4-DIONE to induce an antiproliferative effect ($IC_{50} \sim$ 15 nM) is more likely to result from its metabolic transofrmation into T and 5α-DHT [47–49] than from its direct interaction with the androgen receptor ($K_D \sim$ 200 nM). Secondly, the antiandrogen OH-FLU competitively reversed the effect of 5α-DHT, and Δ^4-DIONE with an apparent dissociation constant (Ki ~ 110 nM) consistent with its known affinity for the androgen receptor [24]. This compound is devoid of any significant affinity for steroid receptors other than the androgen receptor [42].

It should be mentioned that growth inhibition by

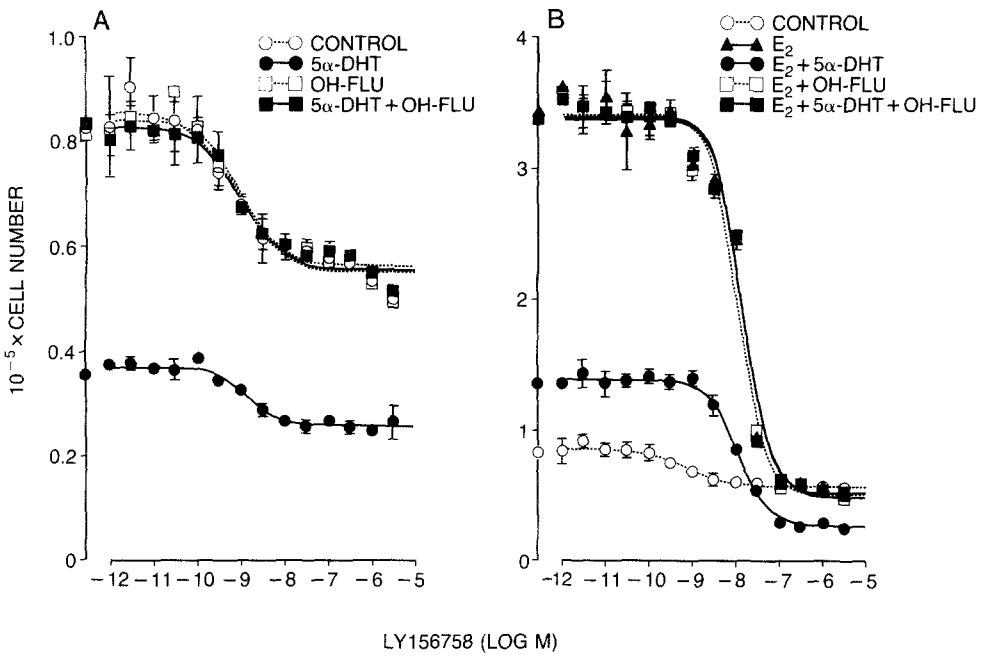

Fig. 6. Effect of increasing concentrations of the antiestrogen LY156758 on the proliferation of ZR-75-1 cells, and the influence of coincubation with 5α-DHT and/or E_2. Prior to the experiment, cells were cultured for 4 weeks in SD medium. Cells were then plated at 8.0×10^3 cells/well in fresh medium of identical composition. A) 48 h after plating, LY156758 was added at the indicated concentrations, alone (control, ○) or in the presence of 1 nM 5α-DHT (●), 1 μM OH-FLU (□), or both compounds (■). B) Same as in A, except that 1 nM E_2 was also present (△) or in association with 1 nM 5α-DHT (●), 1 μM OH-FLU (□), or both compounds (■).

5α-DHT lagged behind the mitogenic effect of E_2, either in the presence or absence of the estrogen (Fig. 3), suggesting different pathways for the action of estrogens and androgens on cell proliferation. Moreover, the growth-inhibitory effect of 5α-DHT is clearly additive to that induced by maximally effective concentrations of the antiestrogen LY156758, thus indicating an action mediated by a mechanism different from interaction with the estrogen receptor. Thus, the present evidence leaves little doubt that the antiproliferative effect of androgens does not result from competition for binding to the estrogen receptor, but rather is mediated by an androgen receptor-mediated mechanism which is additive to blockade of the estrogen receptor by LY156758.

The degree of growth inhibition exerted by 5α-DHT, especially in the presence of E_2, was critically dependent on cell density at the time of steroid addition, mainly because of the relatively long lag needed for androgen action on cell growth. On the other hand, estrogens and androgens can antagonize each other at earlier time intervals on a number of parameters or regulated gene products, as shown in the rat uterus [50, 51] and mammary tumors [50], in the Syrian hamster kidney [52], and in human hepatoma cells [53]. In the MCF-7 [17, 18] and the ZR-75-1 breast cancer cells (R.P. and F.L., manuscript in preparation), low androgen concentrations inhibit the E_2-dependent induction of progesterone receptor via an androgen-receptor-mediated mechanism. We were unable to detect any estrogen receptor-mediated 'down-regulation' of androgen specific binding sites in ZR-75-1 cells, such as that recently reported for MCF-7 cells [54]. Reasons for this discrepancy are not clear, but might reflect divergent control of cellular functions by androgens and estrogens in different human breast cancer cell lines. For instance, while T47-D and ZR-75-1 mammary tumor cells express the GCDFP-15 mRNA and glycoprotein under androgen stimulation, MCF-7 cells do not [19, 20], possibly indicating an apocrine tissue character for the former cell lines [19].

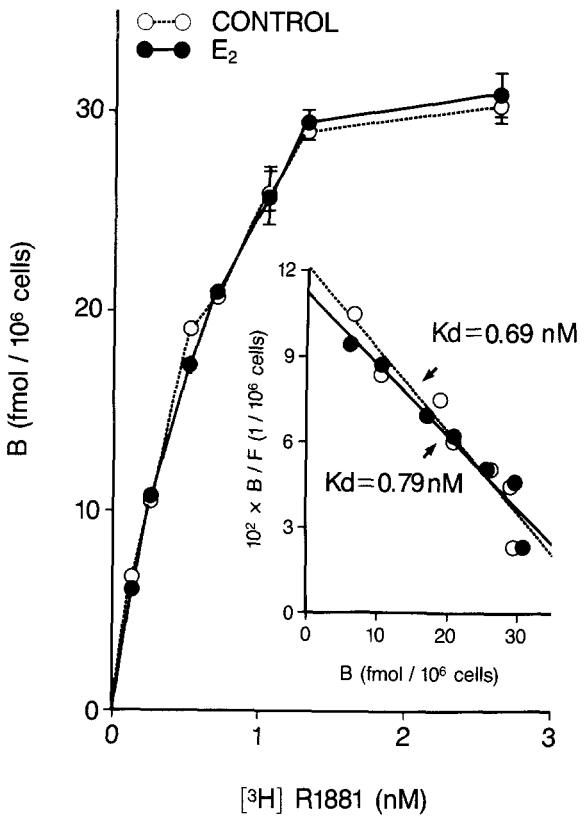

Fig. 7. Specific uptake of increasing concentrations of [^{3}H]R1881 by intact ZR-75-1 cell monolayers. Cells were grown in 24-well culture plates either in the absence (○) or in the presence (●) of 1 nM E_2, for a 7-day incubation period. The specific uptake of [^{3}H]R1881 was then determined as described in 'Materials and Methods'. Inset: Scatchard analysis of the same data.

According to the antagonistic relationship between estrogen and androgen receptor-mediated events, only when the antiestrogen LY156758 was added together with E_2 did the antiproliferative effect of 5α-DHT reach the degree observed in estrogen-deprived, androgen-treated ZR-75-1 cells (Figs. 5, 6). On the other hand, high concentrations of E_2 can interact with the androgen receptor (Fig. 8) [35], a fact which may account for the progressive reversal of androgen-induced inhibition of cell proliferation observed at concentrations of E_2 exceeding 10 nM (Fig. 5A).

Antiestrogens such as LY156758 inhibit both E_2-induced and basal cell proliferation through an estrogen receptor-mediated mechanism. Although the physiological basis for growth inhibition of estrogen-deprived cells by antiestrogens is still debated [55, 56], it cannot be solely attributed to the estrogenic effect of phenol red [27, 28], which was absent from the media used in the present experiments. Furthermore, the possible prolonged retention of estrogens following steroid withdrawal [57] was unlikely to contribute significantly to the effect of LY156758 on basal cell growth, since a 4-week period of estrogen deprivation did not attenuate this phenomenon. Whatever the underlying mechanism for antiestrogen-mediated inhibition of growth in the apparent absence of estrogens, the present data clearly show that complete suppression of estrogen action by an antiestrogen is a prerequisite in order to observe the full inhibitory effect of androgens on breast cancer cell growth.

Interpretation of the role of androgens in mammary cancer has been complicated by the known dual affinity of 5α-DHT and T for both the androgen ($K_D \sim$ 0.01–1 nM) and the estrogen ($K_D \sim$ 100–1000 nM) receptors in various tissues (Fig. 8) [15, 16, 35, 36]. Moreover, binding of androgens to the estrogen receptor results in the induction of several characteristic estrogenic responses in the rat uterus [36, 37], in 7,12-dimethylbenz(a)anthracene-induced rat mammary tumors [38, 39], and in human breast cancer cells [13–16]. In ZR-75-1 cells, high (100–2000 nM) concentrations of 5α-DHT and to a lesser degree T, are less inhibitory than those in the 0.1–10 nM range (Figs. 1, 2), this biphasic effect probably resulting from increasingly significant interaction of DHT and T with the estrogen receptor at higher concentrations. Similar observations have been made *in vivo* concerning carcinogen-induced rat mammary tumors [58, 59] and in the immature mouse uterus [38], where intermediary doses of various androgens were the most effective in counteracting estrogen-induced tissue weight increases.

In conclusion, we have presented unequivocal evidence that physiologically relevant concentrations of 5α-DHT, T, and Δ^4-DIONE can potently counteract the mitogenic effect of E_2 as well as markedly accentuate growth inhibition induced by an antiestrogen in an established human breast cancer cell line. These effects are mediated via

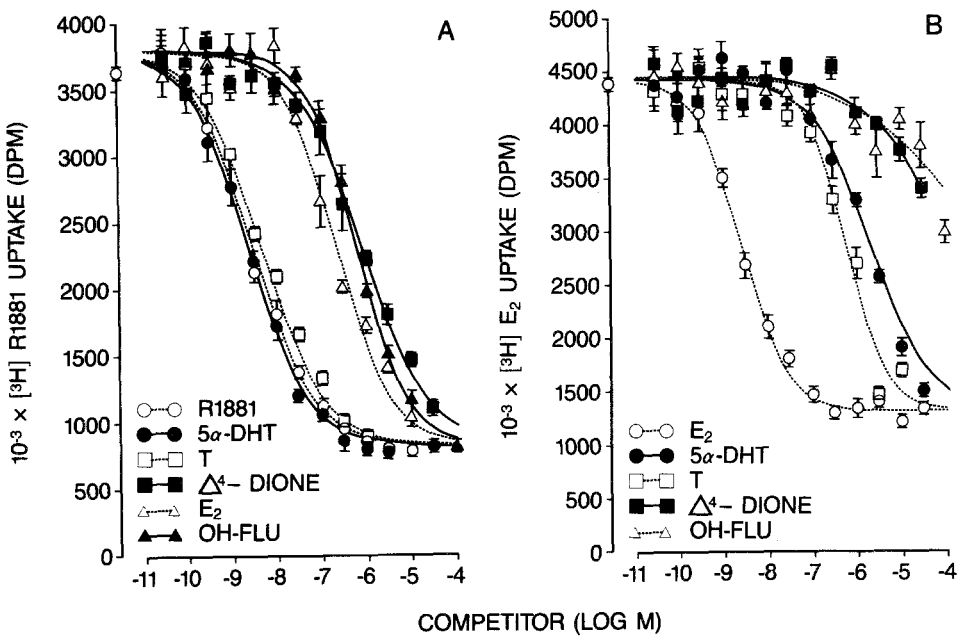

Fig. 8. Competition for the uptake of [^{3}H]R1881 (A) or [^{3}H]E_2 (B) by increasing concentrations of various unlabeled ligands. Cells were grown in SD medium and the uptake of radiolabeled ligand determined, as described in 'Materials and Methods', in the presence of the indicated concentrations of competitor.

specific interaction of these steroids with the androgen receptor. Whether similar growth-inhibitory action of androgens can apply to human breast tumor cells in the clinical situation remains to be established. However, the overwhelming clinical evidence for tumor regression observed in 20 to 50% of pre- and post-menopausal breast cancer patients treated with various androgens [7] favors the view that naturally occuring androgens might constitute an as yet overlooked, direct hormonal control of mammary cancer cell growth. It is thus reasonable to suggest that an imbalance between androgenic and estrogenic influences could modify the overall growth rate of breast tumors in much the same way as that suggested for progestins in estrogen target tissues [60]. Interestingly, the indication that an increased response rate might be obtained by combining androgens and an antiestrogen therapy in breast cancer patients [5] is in agreement with our observation that the mechanisms of inhibition by both types of agents are different, and their effects, at least in part, additive.

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