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Characteristics of the Biphasic Action of Androgens and of the Potent Antiproliferative Effects of the New Pure Antiestrogen EM-139 on Cell Cycle Kinetic Parameters in LNCaP Human Prostatic Cancer Cells[1]

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ABSTRACT

The most potent steroid in human prostatic carcinoma LNCaP cells, *i.e.*, dihydrotestosterone (DHT), has a biphasic stimulatory effect on cell proliferation. At the maximal stimulatory concentration of 0.1 nM DHT, analysis of cell kinetic parameters shows a decrease of the G_0-G_1 fraction with a corresponding increase of the S and G_2 + M fractions. In contrast, concentrations of 1 nM DHT or higher induce a return of cell proliferation to control levels, reflected by an increase in the G_0-G_1 fraction at the expense of the S and especially the G_2 + M fractions. Continuous labeling for 144 h with the nucleotide analogue 5'-bromodeoxyuridine shows that the percentage of cycling LNCaP cells rises more than 90% after treatment with stimulatory concentrations of DHT, whereas in control cells as well as in cells treated with high concentrations of the androgen, this value remains below 50%. Although LNCaP cells do not contain detectable estrogen receptors, the new pure steroidal antiestrogen EM-139 not only reversed the stimulation of cell proliferation and cell kinetics induced by stimulatory doses of DHT but also inhibited basal cell proliferation.

INTRODUCTION

Cancer of the prostate has become the most frequent cancer in men with a predicted annual incidence of 122,000 new cases and 32,000 deaths due to this disease in the USA alone in 1991 (1). From 1973 to 1987, the number of new prostate cancer cases rose 46% (1). Due to the rapid rise in aging of the U.S. population, it is estimated that by the year 2000, there will be a 37% increase in prostate cancer deaths (2). Despite the major medical problems caused by this disease, there are a very limited number of prostatic cancer cell lines of human origin which can be used as models for *in vivo* or *in vitro* studies. The cell line LNCaP, which was isolated from a metastatic lesion of human prostatic cancer (3, 4), is the most widely used *in vitro* model. LNCaP cells contain androgen receptors, and androgens are well known to modulate their growth (4–6).

A special characteristic of this cell line is its biphasic response to increasing concentrations of steroids (7–9), thus providing a useful model for studying cell kinetic parameters during proliferation followed by cell inhibition as a function of increasing concentrations of the same steroid. In the present study, we have characterized the effect of the natural androgen DHT[5] on cell cycle kinetic parameters, especially the distribution of cells in the G_0-G_1, S, and G_2 + M phases of the cycle, as well as the percentage of cells involved in cell cycling.

Although antiandrogens such as flutamide and its analogues are used successfully for the treatment of prostate cancer in men (10–14), the growth of LNCaP cells is paradoxically stimulated by these compounds. This particular behavior of LNCaP cells is possibly explained by a mutated androgen receptor which changes steroid and antiandrogen binding characteristics but has kept a normal affinity for natural androgens (15, 16). Since the role of estrogens on androgen action, especially in prostatic cells, has been suggested by many studies (17–21), we have studied the effect of a new pure antiestrogen, the steroidal derivative EM-139 (22–24), on the same cell kinetic parameters in LNCaP cells.

MATERIALS AND METHODS

Maintenance of Stock Cultures. The LNCaP human prostatic cancer cell line was obtained from the American Type Culture Collection at passage 19. Cells were routinely cultured in RPMI 1640 medium supplemented with 5% fetal calf serum, 2 mM L-glutamine, 10 IU penicillin/ml, and 50 μg streptomycin/ml in a water-saturated atmosphere of 95% air-5% CO_2 at 37°C. Cells were subcultured weekly by gentle digestion in a solution of 0.1% pancreatin in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer containing 3 mM EDTA (pH 7.2). Cells were collected by centrifugation, resuspended in culture medium, counted in a Coulter Counter (model ZM; Coulter Electronics, Hialeah, FL), and reseeded in 75-cm² flasks at a density of 1×10^6 cells/15 ml.

Chemicals. DHT, BrdUrd, Hoechst 33342, and propidium iodide were purchased from Sigma Chemical Co. (St. Louis, MO). The pure steroidal antiestrogen EM-139 was synthesized in the chemical division of our laboratory (22). Stock solutions of DHT and EM-139 were prepared in ethanol and were added to the culture medium at final ethanol concentrations below 0.1% (v/v). Such a concentration of ethanol has no detectable effect on cell growth and morphology.

Cell Proliferation and Sensitivity to Steroids. Cells harvested in their logarithmic growth phase were plated on poly-L-lysine-treated 24-well plates at a density of $7–10 \times 10^3$ cells/cm² in RPMI 1640 medium containing 2% dextran-coated charcoal-treated fetal calf serum. After 3 days in culture, the medium was changed, and the compounds to be studied were added to triplicate dishes. Cells were grown for 10–12 days with medium changes every second day.

The number of cells was evaluated either by direct counting in a Coulter Counter or by measurement of DNA content by a modification (23, 25) of the method of Fiszer-Szafarz (26).

Cell Kinetic Parameters. Cells were plated in 6-well plastic culture plates. After 72 h, medium was replaced with fresh medium of identical composition containing, in addition, the indicated concentrations of DHT and/or antiestrogen EM-139. After steroid and/or antiestrogen treatment for the indicated time periods, cells were harvested by incubation with 0.5 ml of a pancreatin solution (GIBCO) for 5–10 min at 37°C before addition of 0.5 ml of RPMI 1640 containing 5% dextran-coated charcoal-treated fetal calf serum in order to inhibit enzymatic activity. Cell number (0.1-ml aliquot) was measured with a Coulter Counter. The remaining cells were briefly centrifuged ($400 \times g$), and after removal of the supernatant, the pelleted cells were fixed in 0.5 ml

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[5] The abbreviations used are: DHT, 5α-dihydrotestosterone; EM-139, *N,n*-butyl-*N*-methyl-11-[16′α-chloro-3′,17′β-dihydroxy-estra-1′,3′,5′-(10′)triene-7′α-yl] undecanamide; BrdUrd, 5′-bromodeoxyuridine; PBS, phosphate-buffered saline; R1881, 17β-hydroxy-17α-methyl-estra-4,9,11-trien-3-one; EC_{50}, median effective concentration; IC_{50}, median inhibitory concentration.

ethanol/isotone (70/30, v/v) and stored at −20°C. In the BrdUrd experiments, 10 μM BrdUrd was added concomitantly with medium change. Cells were then harvested at the indicated time intervals and fixed as described above.

Flow Cytometry. Following incubation with the steroid and/or antiestrogen, fixed cells were centrifuged and washed with PBS (pH 7.2) before resuspension and incubation in PBS containing 1 mg RNase (40 units/ml/10^6 cells) for 30 min at 37°C. Cells were then separated by centrifugation before resuspension in 500 μl of PBS containing the DNA dye propidium iodide (50 μg/ml) and incubation for 20 min at 0°C. The proportions of cells in the G_0-G_1, S, and G_2 + M phases of the cell cycle were calculated as previously described (26). For all DNA histograms, the coefficients of variation ranged from 4 to 6.

In the BrdUrd studies, fixed cells were incubated with the DNA dye Hoechst 33342 (2 μg/ml) for 1 h at 20°C. The percentage of cells that had incorporated BrdUrd during the S phase (BrdUrd-positive cells) was determined using the above-indicated cytometer following UV excitation (351–364 nm, 50 mW) and emission over 408 nm (24, 27). Since the BrdUrd-positive cells show "quenching" with the Hoechst dye (decrease in fluorescence intensity), they were detected at the left of the DNA content histogram obtained from cells incubated in the absence of BrdUrd (24, 27).

Specific Uptake of [^{3}H]R1881 by LNCaP Cells. The relative binding affinity of steroids for androgen-specific binding sites was assessed by measuring the effect of increasing concentrations of the indicated unlabeled steroid on the uptake of [^{3}H]R1881 by LNCaP cells in monolayer culture (25, 28). The apparent dissociation constant (K_d) and the number of androgen-specific binding sites per cell (B_{max}) were estimated by Scatchard analysis of the effect of increasing concentrations of [^{3}H]R1881 added to triplicate dishes, in the presence or absence of a 200-fold excess of unlabeled R1881 to account for nonspecific uptake, in the presence of 4.5 μM triamcinolone acetonide (24, 28).

Calculations. Radioimmunoassay data were calculated using a program based on model II of Rodbard and Lewald (29). Dose-response curves and EC_{50} values were calculated using a weighted iterative nonlinear least squares regression (30). The experiments were performed at least twice in triplicate or quadruplicate. Results are presented as means ± SEM of one of these experiments. Statistical significance was determined according to the multiple range test of Duncan-Kramer (31).

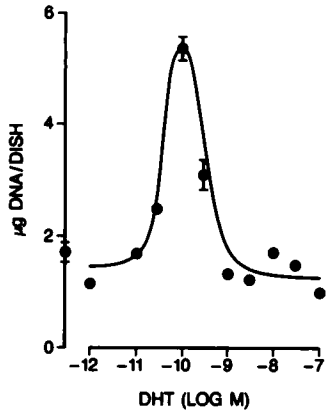

Fig. 1. Effect of increasing concentrations of DHT on the proliferation of LNCaP human prostatic carcinoma cells. Seventy-two h after plating (15×10^3 cells/well), the indicated concentrations of DHT or vehicle were added, and media were changed every second day. After 11 days, cells were fixed with 150 μl of methanol, and DNA content was measured by a modification of the method of Fiszer-Szafarz (23, 24). Results are presented as means ± SEM (*bars*) of triplicate or quadriplicate measurements.

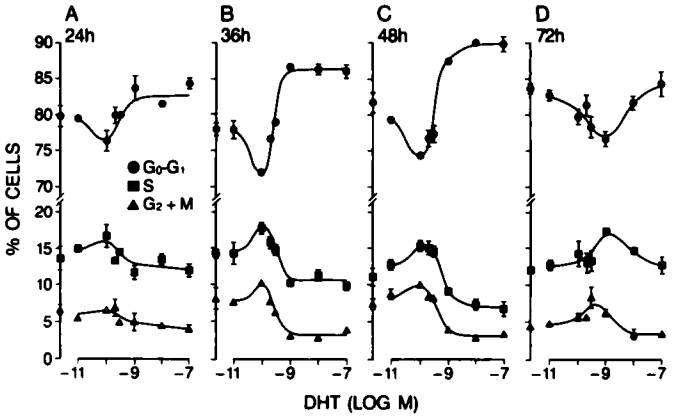

Fig. 2. Effect of increasing concentrations of DHT on the distribution of LNCaP cells in the G_0-G_1 (●), S (■), or G_2 + M (▲) phases of the cell cycle. After 24, 36, 48, or 72 h, cells were harvested and fixed before staining with the DNA dye propidium iodide and analyzed with an Epics 753 flow cytometer. The distribution of cells in the G_0-G_1, S, and G_2 + M fractions was calculated using the PARA 1 software. *Bars*, SEM.

RESULTS

Stimulation of Cell Proliferation by DHT. Since we have previously observed that the maximal stimulatory effects of steroids on the proliferation of LNCaP human prostate cancer cells in culture are observed between 10 and 14 days (9), we studied the effect of increasing concentrations of DHT on the growth of this cell line after 11 days of treatment. As can be seen in Fig. 1, DHT causes a maximal 3.1-fold stimulation of LNCaP cell proliferation at approximately 0.1 nM concentration, whereas a half-maximal effect (EC_{50} value) is measured at 0.03 nM concentration. At concentrations above 0.1 nM, however, there is a progressive decrease in the stimulatory effect of DHT, with a return to basal levels measured between 1 nM and 100 nM concentration.

Effect of DHT on Cell Kinetic Parameters. Fig. 2 illustrates the effect of increasing concentrations of DHT on the three cell cycle fractions, *i.e.*, G_0-G_1, S, and G_2 + M phases. Whereas no significant effect is observed at 24 h (Fig. 2*A*), it can be seen that 36 and 48 h after the addition of DHT, the G_0-G_1 fraction significantly decreased at 0.1 nM DHT, when compared with control cells [36 h: control, 78.0 ± 0.8% *versus* DHT, 72.0 ± 0.5% ($P < 0.01$); 48 h: control, 81.7 ± 1.4% *versus* DHT, 74.3 ± 0.5% ($P < 0.01$)] (Fig. 2, *B* and *C*). On the other hand, at 1 nM and higher concentrations, DHT increased the G_0-G_1 fraction [36 h: control *versus* 1 nM DHT, 86.7 ± 0.5% ($P < 0.01$); 48 h: control *versus* 1 nM DHT, 87.3 ± 0.3% ($P < 0.01$)] with an opposite effect on the S and G_2 + M fractions (Fig. 2, *B* and *C*). In addition, as shown in Fig. 2, *B* and *C*, after 36 or 48 h incubation with 0.1 nM DHT, the decrease in the G_0-G_1 fraction is reflected by an increase in the other two subphases of the cell cycle [48 h: S phase, control, 11.0 ± 1.2% *versus* 0.1 nM DHT, 15.3 ± 0.7% ($P < 0.01$); G_2 + M fraction, control, 7.3 ± 0.7% *versus* 0.1 nM DHT, 10.0 ± 0.5% ($P < 0.01$)].

On the other hand, the stimulatory effect of DHT observed after 36 or 48 h at concentrations of 1 nM DHT or above on the G_0-G_1 fraction is compensated by a significant decrease in the S phase [36 h: control, 14.2 ± 0.7% *versus* 1 nM DHT, 10.3 ± 0.5% ($P < 0.01$); 48 h: control, 11.0 ± 1.2% *versus* 1 nM DHT, 9.0 ± 0.5% ($P < 0.05$)], and by a dramatic decrease in the G_2 + M fraction [36 h: control, 8.0 ± 1.1% *versus* 1 nM DHT, 3.0 ± 0.1% ($P < 0.01$); 48 h: control, 7.3 ± 0.7% *versus* 1 nM DHT, 3.7 ± 0.3% ($P < 0.01$)]. The effects observed on cell cycle kinetic parameters at 1 nM DHT are similar to those induced by 10 or 100 nM DHT, except at 72 h, at which a significant decrease of the G_0-G_1 fraction is seen at 1 nM, whereas at 10 and 100 nM DHT, the G_0-G_1 fraction becomes

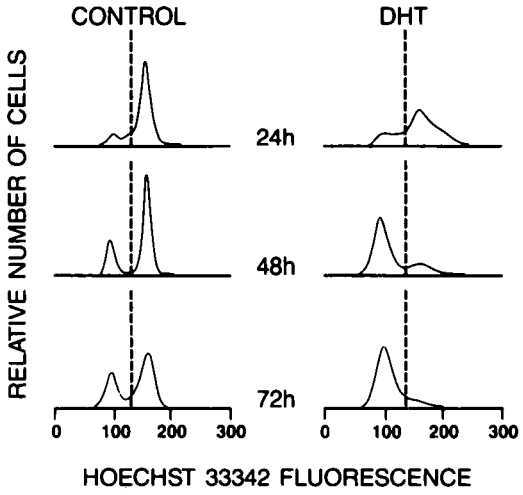

Fig. 3. Effect of incubation with 0.1 nM DHT for 72 h on the time course of BrdUrd incorporation. Cells were pretreated for 72 h in the presence or absence of DHT before medium was removed and replaced with new medium containing the same steroid and 10 μM BrdUrd. At the indicated time intervals, cells were harvested in a total volume of 1 ml medium, and 0.1 ml medium was used for cell counting with a Coulter Counter. The remaining cells were fixed and stained with the DNA dye Hoechst 33342. The percentage of BrdUrd-positive cells was calculated as follows: since the cells that have gone through the S phase and have incorporated BrdUrd contain less fluorescence after DNA staining with Hoechst 33342, the BrdUrd-positive cells are those located at *left* of the *dashed lines*.

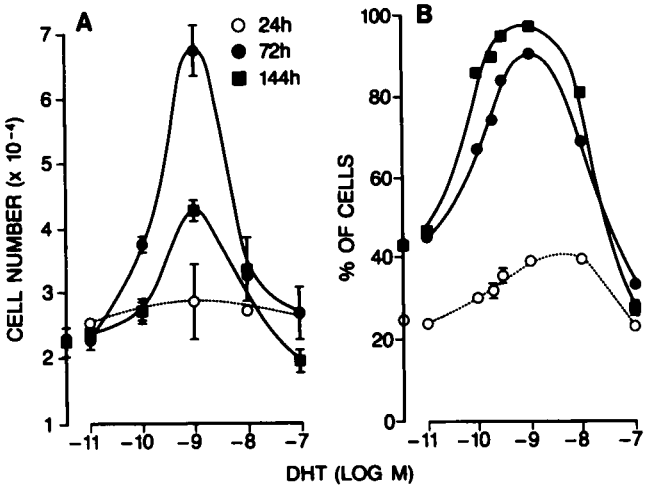

Fig. 4. Effect of increasing concentrations of DHT on the number of LNCaP cells (*A*) and on the proportion of cycling LNCaP cells (BrdUrd-positive cells) (*B*) after 24 (○), 72 (●), or 144 (■) h of exposure to BrdUrd. Cells were treated as described in legend to Fig. 3. *Bars*, SEM.

similar to the control value [control, 83.7 ± 0.7% *versus* 100 nM DHT, 84.3 ± 1.7% ($P > 0.05$)]. On the other hand, it can be seen in Fig. 2*D* that following a maximal increase of the S phase at 1 nM DHT, a progressive decrease is observed at 10 and 100 nM DHT on the proportion of cells in the S and G_2 + M fractions compared to the maximal values obtained at 1 nM concentration.

Effect of DHT on the Proportion of Cycling Cells. In order to investigate the action of DHT on the proportion of LNCaP cells which advanced through the S phase, the cells were continuously exposed to BrdUrd for the indicated time periods following a prior 72-h incubation with 1.0 nM DHT. As illustrated in Fig. 3, after a 24-h exposure to BrdUrd, 19% of control cells and 23% of cells incubated with 1 nM DHT showed a decrease in fluorescence after incorporation of the DNA dye Hoechst 33342 as reflected by fluorescence values situated at the *left* of the *dashed line*. It can be seen in Fig. 3 that after a 72-h exposure to an optimal stimulatory concentration of DHT on cell growth, 87% of cells were BrdUrd positive, whereas only 39% of control cells had gone through the S phase during the same time period.

As illustrated in Fig. 4*A*, no stimulatory effect of DHT on cell proliferation could be observed after 24 h of simultaneous exposure to BrdUrd and the androgen, whereas the cell number was increased by 3.0- and 1.9-fold after 72 and 144 h of exposure to BrdUrd and DHT, respectively. It can be seen in Fig. 4*A* that the stimulatory effect of DHT on cell number was inhibited after 144 h of incubation in the presence of BrdUrd. The biphasic action of DHT on cell growth (Fig. 4*A*) was also observed on the percentage of cells passing through the S phase (Fig. 4*B*). It can, in fact, be seen in Fig. 4*B* that the percentage of BrdUrd-positive cells was increased after incubation for 24, 72, and 144 h with 1 nM DHT by 1.6-, 2.1-, and 2.3-fold, respectively. Following the peak of stimulation of BrdUrd incorporation at 1 nM DHT, incubation with higher concentrations of the androgen led to a progressive decrease of the value of this parameter, which returned toward basal levels.

Potent Antiproliferative Effects of the Pure Antiestrogen EM-139. Table 1 shows that among the androgens DHT and testosterone, their metabolites 5α-androstane-3β,17β-diol and 5α-androstane-3α,17β-diol, and the estrogens estrone (E_1) and 17β-estradiol (E_2), which are all known to stimulate LNCaP cell growth (9, 25), DHT is the most potent competitor for the uptake of [^{3}H]R1881 (3 nM), with an IC_{50} value of 0.83 nM, whereas the compounds with lowest activity, namely estrone and 5α-androstane-3α,17β-diol, have IC_{50} values of 74.4 and 53.0 nM, respectively. Testosterone, estradiol, and 5α-androstane-3β,17β-diol have IC_{50} values of 2.06, 3.45, and 1.90 nM, respectively. It can be seen in the same table that the potency of these compounds as competitors of [^{3}H]R1881 uptake is closely correlated with their mitogenic potency. Whereas the antiandrogen OH-flutamide inhibited [^{3}H]R1881 uptake at an IC_{50} value of 11.9 nM and stimulated cell proliferation at an EC_{50} value of 20 nM, the three antiestrogens tested had no significant effect on the parameters measured. In fact, no displacement of [^{3}H]R1881 uptake and no stimulation of cell proliferation were observed with the nonsteroidal antiestrogens OH-tamoxifen and LY-156758 as well as with the new pure steroidal antiestrogen EM-139 (Table 1).

It can be seen in Fig. 5 that after 10 days of incubation with 0.3 nM DHT, the stimulatory effect of the androgen is reversed

Table 1 *Comparison of the potency of various compounds to compete for [^{3}H] R1881 uptake in LNCaP human prostatic cancer cells and their mitogenic effect*

Compound	Competition of [^{3}H]R1881 uptake[a] [IC_{50} (nM)]	Mitogenic effect[b] [EC_{50} (nM)]
Dihydrotestosterone	0.83	0.21
Testosterone	2.06	1.00
Estrone	74.4	16.1
Estradiol	3.45	1.60
5α-Androstane-3α,17β-diol	53.0	100
5α-Androstane-3β,17β-diol	1.90	2.0
OH-flutamide	11.9	20.0
OH-tamoxifen	NS[c]	NS
LY-156758	NS	NS
EM-139	NS	NS

[a] Concentration of the tested compound required to displace [^{3}H]R1881 binding by 50%.

[b] The mitogenic effect corresponds to the concentration of the tested compound causing a half-maximal stimulation (EC_{50}) of cell proliferation after a 12-day incubation period. See legend to Fig. 1.

[c] NS, no significant effect.

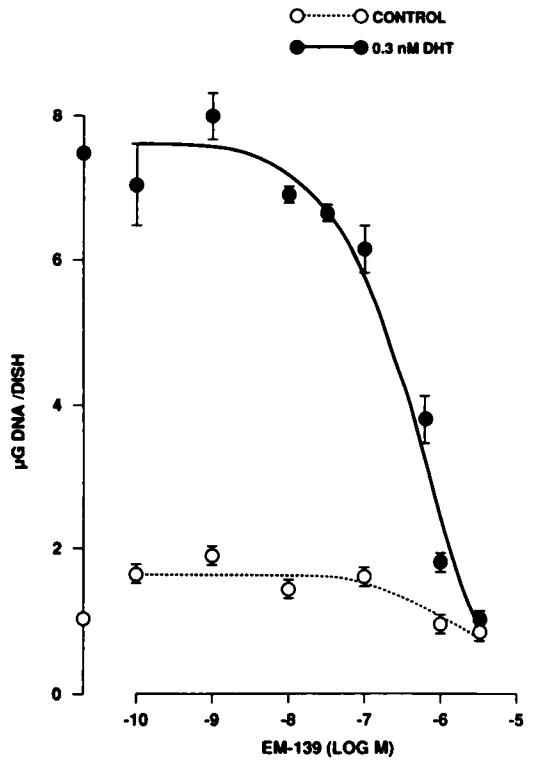

Fig. 5. Effect of increasing concentrations of the antiestrogen EM-139 on the proliferation of LNCaP cells in the absence or presence of 0.3 nM DHT. Cells were treated as described in legend to Fig. 1 and collected after 10 days of hormonal treatment. *Bars*, SEM.

at an EC_{50} value of 300 nM by the antiestrogen EM-139, whereas the same compound causes a significant decrease of basal cell proliferation at 1 µM and 3 µM concentrations. As further illustrated in Fig. 6, the antiestrogen EM-139 (1 µM) has a potent inhibitory effect on LNCaP cell proliferation. In fact, after 4 days of incubation with 1 µM EM-139, basal LNCaP cell proliferation was already 35% inhibited ($P < 0.01$) compared to control cells. Moreover, cell growth was completely inhibited at later time intervals up to 12 days, whereas 0.3 nM DHT stimulated cell proliferation by 7-fold at 7 days, and simultaneous incubation with 1 µM EM-139 completely reversed the stimulatory effect of the androgen not only to basal levels but to the level achieved in cells treated with EM-139 alone (Fig. 6). Such an inhibitory effect was not obtained with tamoxifen or LY-156758 (data not shown).

Following the observation of such a marked inhibitory effect of EM-139 on LNCaP cell growth, we next investigated the effect of the antiestrogen on the percentage of cycling cells (percentage of BrdUrd-positive cells). Following 24, 48, 72, and 96 h of incubation in the presence of BrdUrd, 1 nM DHT induced increases of 9.7, 26.3, 10.9, and 13.8% ($P < 0.01$), respectively, in the proportion of BrdUrd-positive cells, whereas exposure to 1 µM EM-139 for the same time intervals reduced BrdUrd-positive cells by 48.6, 35.3, 43.2, and 46.7%, respectively ($P < 0.01$) (Fig. 7). Intermediate values were found when both DHT and EM-139 were present simultaneously. As illustrated in Table 2, cells treated for 48 h with 1 nM DHT and for the last 24 h in the presence of 10 µM BrdUrd showed a decrease in the G_0-G_1 fraction from 86.0 ± 0.8% to 80.3 ± 1.9% ($P < 0.01$), especially to the benefit of the S phase [14.0 ± 1.9% *versus* 10.0 ± 0.8% ($P < 0.01$)]. In contrast, 1 µM EM-139 increased G_0-G_1 at the expense of the S phase when compared to control cells [G_0-G_1: control, 86.0 ± 0.8% *versus* 1 µM EM-139, 92.7 ± 0.3% ($P < 0.01$); S phase: control, 10.0 ± 0.8% *versus* 1 µM EM-139, 5.7 ± 0.5% ($P < 0.01$)]. It can also be seen that EM-139 reversed the stimulatory effect of DHT on all of the parameters measured to control levels (Table 2).

DISCUSSION

The present study describes the effect of the most potent natural androgen, namely dihydrotestosterone, on LNCaP cell

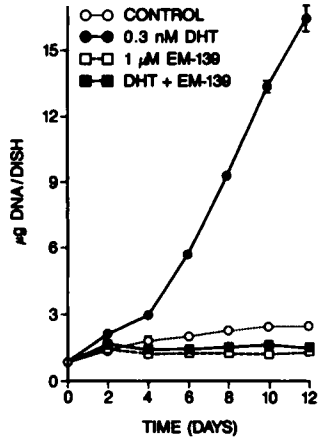

Fig. 6. Effect of the antiestrogen EM-139 on LNCaP cell proliferation. Seventy-two h after plating, cells were treated with DHT or EM-139. Media were changed every second day. At the indicated time intervals, cells were harvested and fixed, and DNA content was measured as described in legend to Fig. 1. *Bars*, SEM.

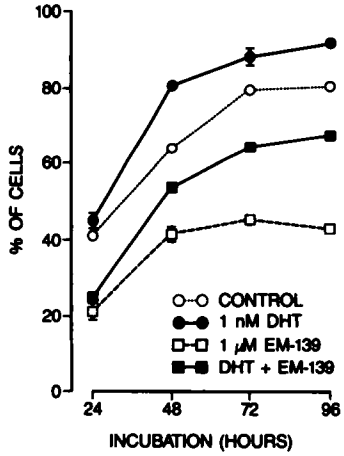

Fig. 7. Time course of the effect of DHT, the antiestrogen EM-139, or the combination of both compounds on BrdUrd incorporation in LNCaP cells. Seventy-two h after plating, media were removed and replaced by media containing either vehicle, 0.1 nM DHT, 1 µM EM-139, or 0.1 nM DHT and 1 µM EM-139. After 72 h, each medium was replaced by new medium containing the same steroid(s) and 10 µM BrdUrd. After the indicated time intervals, cells were harvested and stained as described in legend to Fig. 3. *Bars*, SEM.

Table 2 *Cell kinetic parameters of LNCaP cells incubated for 48 h in the presence of DHT and/or EM-139*[a]

Treatment	$G_0 - G_1$	S	$G_2 + M$
Control	86.0 ± 0.8	10.0 ± 0.8	4.0 ± 0.5
1 nM DHT	80.3 ± 1.9**	14.0 ± 1.9**	5.3 ± 0.7
1 µM EM-139	92.7 ± 0.3**	5.7 ± 0.5**	3.3 ± 0.7
EM-139 + DHT	87.0 ± 0.5	9.3 ± 0.5	3.7 ± 1.0

[a] Cell kinetic measurements were performed as described in legend to Fig. 2 except that 10 µM BrdUrd was present during the last 24 h of incubation. **, $P < 0.01$, experimental *versus* control.

cycle kinetic parameters. At optimal concentrations of DHT, cell proliferation is accompanied by an increase of the S and G_2 + M fractions and by an increase in the percentage of cycling cells. In contrast, concentrations of DHT above the optimal value cause a progressive decrease in cell proliferation to the control levels by reversing the distribution of cells in the various phases of the cycle. This effect of high concentrations of the androgen is associated with a marked increase in the G_0-G_1 fraction at the expense of the S phase and G_2 + M fractions. In fact, both S and G_2 + M fractions decrease dramatically below the control level at 48 h. The present data also demonstrate that a new pure steroidal antiestrogen, EM-139, which does not bind to the androgen receptor, not only reversed the stimulatory effect of DHT on cell proliferation and cell kinetics but also inhibited basal levels of the same parameters.

The stimulatory effects of steroids on the growth of the human prostatic carcinoma LNCaP cell line have been reported by our group (9, 25), as well as by others (7, 8). As previously demonstrated, the proliferation of LNCaP cells is stimulated by estrogens, progestins, and androgens as well as by the main androgen metabolites, namely androsterone, 5α-androstane-3α,17β-diol, and 5α-androstane-3β,17β-diol, in a biphasic manner (9, 25). This model is thus particularly interesting for studies of cell cycle parameters, since among the few human prostatic carcinoma cell lines available, LNCaP is the only one that possesses a high level of sensitivity to hormones. In the present study, we show that after a 12-day incubation in the presence of increasing concentrations of DHT, 0.1 nM concentration induces a maximal increase in cell proliferation followed by a progressive decrease, with a return to control levels at doses of 1 nM concentration or higher. This implies that the effect of DHT on cell proliferation is restricted to a range of two orders of magnitude, thus suggesting the involvement of a proliferative process and an antiproliferative process modulated by steroids (25).

Cytometric methods have been widely used for studying the effect of hormones on DNA content and cell cycle distribution and more particularly for investigating the effect of steroids in breast cancer cell lines (24, 32–34). As proposed by Dean (35), a cell population analyzed by flow cytometry should be divided into three classes, and the analysis is based on the following assumptions: (*a*) all of the cells in G_0-G_1 have the same DNA content; (*b*) all of the cells in the G_2 + M phase have twice the DNA content of G_0-G_1; and (*c*) the cells in the S phase have a DNA content ranging between the G_1 and G_2 + M values. Our results show that after 36 and 48 h of exposure to 0.1 nM DHT, a decrease of the G_0-G_1 fraction occurs to the benefit of the S as well as G_2 + M fractions, thus explaining the stimulatory effect on cell proliferation. This observation is in agreement with the results obtained on the stimulation of breast cancer cell proliferation by estrogens (24, 36).

In LNCaP cells, stimulation of cell kinetic parameters is restricted to 36 and 48 h, since at 72 and 144 h (data not shown), no clear stimulatory effect of DHT is obtained. Moreover, doses of DHT higher than or equal to 1 nM concentration, which induced no change in cell proliferation stimulation when compared to control values, dramatically increased the G_0-G_1 fraction at the expense of the S phase and more particularly of the G_2 + M fraction. As discussed by Steel (37), the growth of a cell results from the interaction of several parameters which control cell division and cell death and/or differentiation. The difference between these two main processes determines a growing, a steady-state, or a decreasing cell population. Thus, after 12 days of hormonal treatment, the number of cells counted in a plate is the result of all of the above-mentioned processes. In the present study, control plates and plates treated with high concentrations of DHT, although containing approximately the same number of cells, contained cells with completely different cell kinetic patterns. Thus, for the first time, it is clearly established that the inhibitory effect of high doses of DHT on LNCaP cell proliferation is not the result of the unsticking of the cells from the plates due to a cytotoxic effect but is associated with a change in cell cycle kinetic parameters.

The technique of continuous incubation with an analogue of a deoxyribonucleotide, *i.e.*, 5′-bromodeoxyuridine, introduced by Rabinovitch (27), permits one to follow the percentage of cells that enter into the cell cycle, *i.e.*, proliferating cells as opposed to quiescent cells (G_0 cells). Treatment with 10 μM BrdUrd, however, induces a displacement of the maximal effect on cell proliferation by one order of magnitude when compared to cells treated with hormones in the absence of BrdUrd. The maximal effect of 1 nM DHT, observed in the present study after 72 h in the presence of BrdUrd, is a time interval which corresponds to an incubation with DHT for 6 days, since cells had already been exposed to DHT for 3 days before addition of BrdUrd. Longer incubation periods with BrdUrd are cytotoxic since the stimulatory effect of 1 nM DHT decreases compared to that observed at 3 days. This finding results from a BrdUrd blockade of the cell cycle (38). When looking at the percentage of cells that have incorporated the nucleotide analogue at early time intervals, the value of BrdUrd-positive cells increased more than 85%, as early as 48 h, after addition of 1 nM DHT. In contrast, not more than 45% of control cells have gone through the S phase after 144 h, thus indicating that more than half of control cells are quiescent. A high concentration of DHT (100 nM) induced a return of this parameter to the control level, thus indicating that high concentrations of DHT block the cells that enter the S phase.

The most efficient treatment of human prostatic carcinoma includes an antiandrogen associated with medical or surgical castration (10–14). The benefits of the antiandrogen are not limited to prevention of spread of the disease during the first days of treatment with a luteinizing hormone-releasing hormone agonist but are secondary to inhibition by the antiandrogen of the action of the adrenal androgen (10–13).

Unfortunately, the growth of LNCaP cells, which represent the only androgen-sensitive human prostatic cancer line, are stimulated by antiandrogens such as flutamide (15, 16). As recently reported by Tilley *et al.* (39), LNCaP cells contain high levels of androgen receptor protein and mRNA, whereas the two other well known human prostatic cell lines, namely DU-145 and PC-3, do not express androgen receptor protein or mRNA. More recently, Veldscholte *et al.* (16) discovered in the LNCaP androgen receptor a single point mutation, thus changing the steroid-binding domain. Following expression of the mutated androgen receptor by transfection, the pattern of stimulation by androgens, progestagens, estrogens, and antiandrogens was similar to the effects observed in intact LNCaP cells (16).

The present data show that a new pure steroidal antiestrogen, EM-139, synthesized in our laboratory (22) and already shown to be a potent inhibitor of cell proliferation and cell cycle kinetic parameters of human breast cancer cells (23, 24), not only markedly inhibits the stimulation of proliferation induced by DHT in LNCaP cells but also inhibits basal cell proliferation. This effect on cell proliferation is confirmed by the BrdUrd

incorporation studies. Since the LNCaP cell line does not contain detectable estrogen receptors (5), the present results suggest that EM-139 acts on cell proliferation via pathways other than the classical mechanisms of binding to the androgen and estrogen receptors. Although estrogens have previously been shown to stimulate the level of androgen receptors in the human prostate (20), we have previously reported that DHT and testosterone as well as 17β-estradiol and progesterone stimulate cell proliferation of human hyperplasic prostatic tissue in organ culture, as assessed by tritiated thymidine incorporation (40). Although the mechanisms involved in the action of EM-139 are not yet apparent, the observations indicating a role of estrogens in androgen action (17–21) suggest that the new antiestrogen EM-139 could interfere at some step(s) involved in the modulation of steroid receptor action (41).

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