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Effects of Triptorelin, a Gonadotropin-Releasing Hormone Agonist, on the Human Prostatic Cell Lines PC3 and LNCaP

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ABSTRACT: Some analogues of gonadotropin-releasing hormone (GnRH) influence the in vitro proliferation of cultured human cells by complex interactions that are only partially understood. This study explored the effect of Triptorelin, a GnRH agonist, on the LNCaP and PC3 prostatic cell lines, which are, respectively, responsive and unresponsive to androgen stimulation. The toxicity and cell cycle modifications induced by the drug were investigated by FACScan analysis; the effect on cell proliferation in different culture conditions was determined by counting in a Burker chamber; and the expression of binding sites for ^{125}I-Triptorelin was revealed by displacement experiments. PC3 cell growth was completely unaffected by Triptorelin. The drug caused a double stimulatory-inhibitory action on the growth of actively proliferating LNCaP cells, depending upon the dose and environment. A significant inhibitory effect on proliferation, ranging from 25% to 65% compared with controls, was observed at a high dose (10^{-4} M) according to the culture conditions; and a proliferative effect (42% compared with controls) was observed at a lower dose (10^{-7}M) only in fetal bovine serum–supplemented medium. Displacement experiments revealed the expression of moderately high affinity and low affinity binding sites in LNCaP cells ($K_d = 2.6 \times 10^{-8}$ and 7.7×10^{-6} M) but only low affinity binding sites in PC3 cells ($K_d = 2.7 \times 10^{-6}$ M), which suggests that the expression of binding sites with different affinity could be associated with a biological response to the drug. Proliferation studies in the presence of Cetrorelix, a GnRH antagonist, confirmed the different sensitivity of the 2 cell lines to GnRH analogues and showed that the proliferative effect of Triptorelin on LNCaP cells can be inhibited by the antagonist. Data confirm the cell specificity of Triptorelin's action and the peculiarity of its effects on prostatic cell proliferation in our experimental conditions.

Key words: GnRH agonist, proliferation, GnRH receptor, Cetrorelix.

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Gonadotropin-releasing hormone (GnRH) agonists have therapeutic effects on sex steroid–dependent prostate and breast carcinomas (Bare and Torti, 1998; von Minckwitz and Kaufmann, 1998). These peptides are believed to act primarily by inhibiting the pituitary-gonadal axis, thereby reducing testosterone and estradiol levels (Redding and Schally, 1990). Extrapituitary peripheral tissues, including ovary, testis, brain, and placenta express binding sites for GnRH analogues (Chamson-Reig et al, 1999; Kakar et al, 1994; Jennes et al, 1997; Boyle et al,1998) and some breast, prostate, ovarian, and pancreatic tumors have GnRH-binding sites (Qayum et al, 1990; Kottler et al, 1997; Chamson-Reig et al, 1999; Szende et al, 1994). Some patients with these tumors have shown clinical improvement after treatment with GnRH analogues. Several authors have demonstrated that tumor cell growth inhibition may be the part of a mechanism that does not depend on pituitary gonadotropin release, since GnRH analogues influence the in vitro proliferation of various tumor cells (Dondi et al, 1994; Kim JH et al, 1999; Kim JW et al, 1999). Triptorelin is a Gly^6 substituted synthetic agonist of GnRH. It has been shown to inhibit the proliferation of endometrial cancer cells CUME-1 (Kim JW et al, 1999), epithelial ovarian cancer cells OV-1063 (Yano et al, 1994), OVCAR-3 and SKOV-3 (Kim JH et al, 1999), and estrogen-stimulated human breast cancer cells MCF-7 and CG-5 (Marini L et al, 1994). On the other hand, it has not been shown to inhibit insulin-like growth factor–stimulated MCF-7 cells (Hershkovitz et al, 1993). The events that lead to Triptorelin's biological effects on tumor cell proliferation are gradually being elucidated. Recent studies have reported that it interacts with the signal transduction of growth factors (Emons et al, 1998).

In an attempt to broaden our knowledge of Triptorelin's direct effect on prostate tissue, we studied its action at different doses on the LNCaP and PC3 cell lines. LNCaP cells, derived from a lymph node metastasis of prostatic carcinoma, maintain some characteristics of primary human prostate carcinoma in that they produce acid phosphatase and are androgen-dependent. Proliferation is almost entirely inhibited in a medium supplemented with

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Table 1. *Mortality of LNCaP cells induced by Triptorelin in different culture conditions**

Variable	FBS	DCC	R1881	EGF
9 days of treatment				
Control	1	1	1	1
Triptorelin (M)				
10^{-4}	1.2	1.1	0.8	1
10^{-5}	1.3	0.9	0.8	0.9
10^{-6}	1	1.1	0.9	0.9
10^{-7}	1	1.3	0.8	1
10^{-8}	0.8	1.1	0.8	1
10^{-9}	0.8	1.1	1	0.8
10^{-10}	1.1	1.1	0.9	0.8
10^{-11}	1.1	0.9	0.9	0.8
4 days of treatment				
Control	1	1	1	1
Triptorelin (M)				
10^{-4}	1.1	1	0.9	1.1
10^{-6}	1	1.2	0.8	0.9
10^{-8}	0.9	1	1	1.2
10^{-10}	1.1	0.9	0.9	0.9

* Data were analyzed by FACScan and expressed as fold induction of dead cells in treated samples compared with controls. Data are representative of 3 independent experiments with comparable results.

fetal bovine serum (FBS) deprived of androgens (DCCFBS) unless the cells are stimulated by androgens, estrogens, or other growth factors such as epidermal growth factor (EGF; Horoszewicz et al, 1983; Schuurmans et al, 1991; Yang et al, 1998). PC3 cells, derived from bone metastasis of prostate cancer, fail to respond to androgens and other growth factors (Kaighn et al, 1979; Tang et al,1998).

We investigated toxicity and cell cycle modifications induced by Triptorelin by using FACScan analysis and propidium iodide (PI), and, using a Burker chamber, determined the drug's effects on cell proliferation under various culture conditions. Finally, in order to evaluate the specificity of Triptorelin's action, we examined the binding characteristics to both cell lines and compared the effects on cell proliferation with those induced by Cetrorelix, the GnRH antagonist.

Materials and Methods

Reagents

We obtained Triptorelin acetate (pGlu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH_2CH_3COOH) from Ipsen S.p.A. (Milan, Italy) and dissolved it in water according to solubility characteristics supplied by the manufacturer. The antagonist, Cetrorelix ([Ac-D-Nal(2)[1], D-Phe(4Cl)[2], D-Pal(3)[3] D-Cit[6], D-Ala[10]] LHRH), was supplied by Asta Medica (Frankfurt, Germany); ^{125}I-triptorelin was obtained from Lasa Laboratorios (Barcelona, Spain); and the synthetic androgen, R1881 (17α-methyltrienolone), was obtained from New England Nuclear (Milan, Italy). All other reagents were of analytical grade.

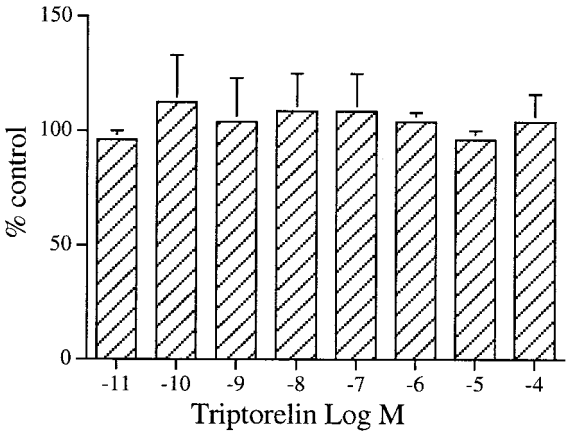

Figure 1. Effect of Triptorelin on PC3 cell proliferation after 4 days of treatment. PC3 cells were plated at a density of 10^3 cells/cm^2 in 60 mm dishes in 10% FBS supplemented medium. The following day the medium was changed and cells were treated with increasing doses of Triptorelin (10^{-11}—10^{-4} M). Data represent the mean ± standard error of the mean of 3 experiments with at least 2 replicates each and are expressed as percent of untreated control culture. The 100% value assigned to the control culture is not shown. Treatment with Triptorelin did not affect cell proliferation at any of the doses tested.

Cell Cultures

PC3 cells were provided by Dr ME Kaighn (Pasadena Foundation for Medical Research, Pasadena, Calif) and we obtained LNCaP cells from American Type Culture Collection (Rockville, Md). The cells were routinely grown, respectively, in Dulbecco's modified Eagle's medium (D-MEM) and in RPMI-1640 medium (Flow Laboratories, Ayrshire, Scotland) supplemented with 10% heat-inactivated FBS (Flow Laboratories), glutamine (1 mM), and antibiotics (100 units/mL of penicillin and 100 mg/mL of streptomycin) in a humidified atmosphere of 5% CO_2 and 95% air.

Analysis of Cell Mortality and Cell Cycle

To measure cell mortality, we assessed the degree of PI incorporation by dead cells and used cytofluorimetric analysis (FACScan; Becton Dickinson, Milpitas, Calif). We seeded 6×10^4 PC3 cells and 3×10^4 LNCaP cells in 60-mm dishes. The next day, we began treating the cells with increasing doses of Triptorelin ($10^{-11} - 10^{-4}$ M). We repeated the treatments daily and changed the medium every 3 days. After treating the PC3 cells for 4 days and the LNCaP cells for 9 days, they were harvested, washed once with phosphate buffered saline (PBS), resuspended at 1×10^6 cells/mL in the same buffer, and stained with PI to a final concentration of 10 mg/mL immediately before being analyzed with the FACScan. Samples were analyzed using an argon laser at 488 nm and a standard filter configuration. Using red and green fluorescence, we counted dead cells and expressed the number as the percentage that showed positive (red) fluoresence in 10 000 events. We repeated these counts 3 times and obtained similar results.

To perform cell cycle analysis, cells that had been treated with Triptorelin for different lengths of time were centrifuged (200 × *g*), fixed in 2 mL of cold 70% ethanol at 4° for 30 minutes, washed in 1 mL of PBS, resuspended in 0.2 mL of an RNAse solution (1 mg/mL in PBS) and, after gentle mixing, again resuspended in 0.2 mL of a PI solution (100 mg/mL). Cells were then mixed, incubated in the dark at room temperature for 15

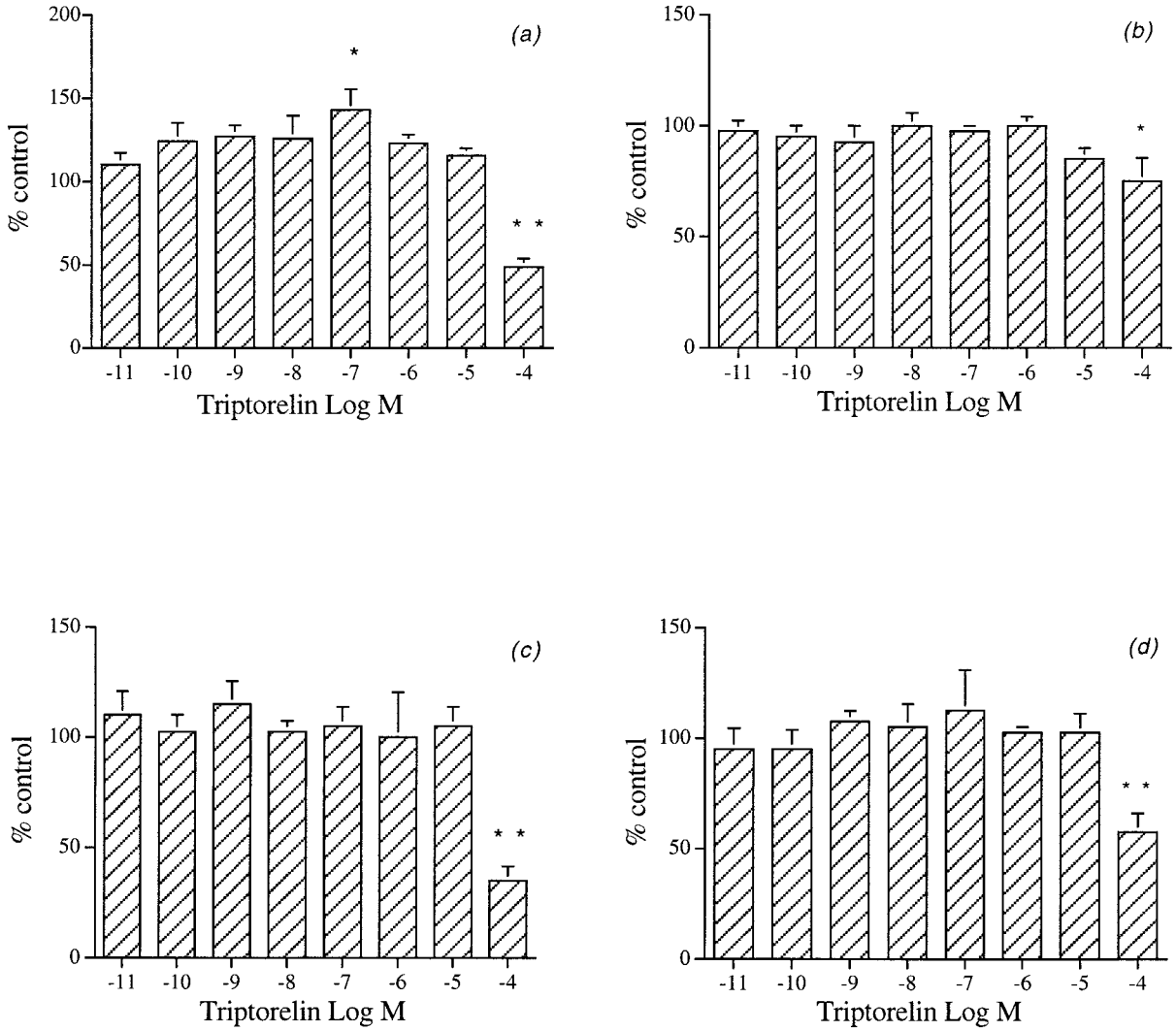

Figure 2. Effect of Triptorelin on LNCaP cell proliferation after 9 days of treatment in different culture conditions. LNCaP cells were plated at a density of 10^6 cells/cm^2 in 60-mm dishes in 10% dextran-coated charcoal-stripped fetal bovine serum (DCCFBS)–supplemented RPMI 1640 medium. the seeding medium was changed 3 days later to experimental media. **(a)** 10% FBS-supplemented medium, **(b)** 10% DCCFBS-supplemented medium, **(c)** 10% DCCFBS-supplemented medium + R1881 0.1 nM, **(d)** 10% DCCFBS-supplemented medium + EGF 0.1 ng/mL. Cells were treated with increasing doses of Triptorelin ($10^{-11} - 10^{-4}$ M). Data represent the mean ± standard error of the mean of 3 experiments with at least two replicates each and are expressed as **(a**,**b)** percent of untreated control culture, **(c)** percent of R1881-treated culture considered as control, **(d)** percent of EGF-treated culture considered as control. The 100% value assigned to the control culture is not shown. Triptorelin caused a significant antiproliferative effect at the dose of 10^{-4} M in all culture conditions. In FBS supplemented medium only, a slight but significant proliferative effect was also observed.
*$P < .05$ versus control.
†$P < .01$ versus control.

minutes, and kept in the dark at 4°C until they were used. Cell cycle analysis was performed on a FACScan using Sum of Broadened Rectangles (SOBR) software. We performed 4 experiments for each culture condition.

Cell Proliferation Studies

To compare proliferation rates, we plated slow-growing LNCaP cells and fast-growing PC3 cells at a density of 10^3/cm^2 and 10^6 cells/cm^2, respectively, in 60-mm dishes. Cells were allowed to attach for 24 hours and after 24, 48, 72, and 96 hours for PC3 cells; and 48, 96, 144, and 192 hours for LNCaP cells, they were trypsinized and counted using a Burker chamber.

Proliferation studies with Triptorelin were carried out on the androgen-independent PC3 cells in 10% FBS-supplemented medium and on the androgen-dependent LNCaP cells in medium supplemented with 10% dextran-coated charcoal-stripped serum (DCCFBS), in the presence and absence of the synthetic androgen, R1881 (0.1 nM) and with EGF (1 ng/mL). The day after the cells were plated, the seeding medium was changed to experimental media and cells were treated with increasing doses of Triptorelin ($10^{-11} - 10^{-4}$ M). The pH of the culture medium was checked in all experimental conditions before trypsinization. When the DCCFBS-supplemented medium was used, cells were grown for 3 days before starting Triptorelin treatment. The drug

was added to the medium every day and was changed every 3 days. The length of the different treatments was established by taking into account the different growth rate of the cell lines in FBS-supplemented medium. On the basis of Trypan blue exclusion and, using a Burker chamber, viable PC3 cells were counted after 4 days after the beginning of treatment, whereas LNCaP cells were counted after 9 days.

In each experiment we assessed the number of spontaneously detached cells floating in the medium and those that had been stained blue with Trypan (dead cells). We performed 3 experiments for each culture condition with at least 2 replicates each.

In the experiments with Cetrorelix, the GnRH antagonist was tested at concentrations of 10^{-9}, 10^{-7}, and 10^{-5} M on both cell lines for 4 and 9 days, respectively. We selected the $10-7$ M-dose for the experiments with Triptorelin cotreatment.

GnRH Receptor Studies

LNCaP and PC3 cells were grown, respectively, in RPMI-1640 and D-MEM media supplemented with 10% DCCFBS. The binding assay of Triptorelin was carried out on confluent cells in Costar 24 multiwell plates. The cell monolayers were washed twice with Hank's balanced salt solution (HBSS) without Ca^{++} and Mg^{++} and the binding reaction was performed in HBSS with Ca^{++} and Mg^{++} containing 0.1% bovine serum albumin (BSA) and 20 mM of Hepes buffer. The cells were incubated with 100 000 cpm ^{125}I-Triptorelin (1500 Ci/mmol) in the presence or absence of increasing concentrations ($10^{-10} - 10^{-4}$ M) of unlabeled peptide in a total volume of 250 mL in triplicate. After 2 hours of incubation at 4°C, the binding reaction was stopped and the monolayers were washed twice with ice-cold HBSS containing 0.1% BSA. The cells were detached with 0.7 mL of trypsin/EDTA solution and 0.6 mL of the sample solution was transferred into tubes for counting in an automatic γ counter. Representative wells were used to determine cell number. Nonspecific binding was assessed in the presence of 10^{-4} M of unlabeled Triptorelin. Three binding experiments were performed for each cell line.

Statistical Analysis

The data from experiments to examine cell proliferation were analyzed using the Dunnett's test (Dunnett, 1955) after one-way analysis of variance. To examine cell cycle experiments, we used Student's *t* test for paired data. We used the ligand computerized curve-fitting program described by Munson and Rodbard (1980) to determine the dissociation constant (K_d) and the maximal binding capacity of receptors (B_{max}).

Results

Cytotoxicity Determined by FACScan Analysis

Table 1 shows the effect of treatment with different doses of Triptorelin ($10^{-11} - 10^{-4}$ M) on cell mortality on PC3 and LNCaP cells after 4 and 9 days, respectively. Mortality was not modified in either cell line in any of the tested doses. These results agreed with Trypan blue staining determinations (data not shown).

Cell Proliferation Studies

Growth rate studies on PC3 and LNCaP cells in FBS-supplemented medium indicated a population doubling time of approximately 24 hours for PC3 cells and 48 hours for LNCaP cells (data not shown). Figure 1 shows the effect of the 4-day treatment with different doses of Triptorelin ($10^{-11} - 10^{-4}$ M) on the growth of PC3 cells and reveals that the GnRH analogue did not significantly affect cell proliferation at any of the doses tested.

Figure 2 shows the effect on LNCaP cells of a 9-day treatment with the same doses of Triptorelin that were used in the PC3 cell experiments. The concentrations of R1881 (0.1 nM) and EGF (1 ng/mL) were selected either on the basis of our previous results or on the basis of data published by other authors (Ravenna et al, 1995; Schuurmans et al, 1991). Cellular growth rates differed according to the medium used. After 9 days of growth, the cell number increased 1.2-fold in DCCFBS-supplemented medium and 10-fold in FBS-supplemented medium, whereas cells stimulated with R1881 or EGF in DCCFBS-supplemented medium showed an increase in cell numbers of 5.1-fold and 1.8-fold, respectively (data not shown). When cells were actively proliferating (FBS-supplemented medium, DCCFBS-supplemented medium in the presence of R1881 or EGF) Triptorelin caused a strong antiproliferative effect at a dose of 10^{-4} M (52%, 65%, and 43%, inhibition, respectively, compared with controls). In almost quiescent cells (DCCFBS-supplemented medium), we observed a slight but still significant antiproliferative action (25% inhibition compared with controls). In none of the tests was the decrease in cell number after Triptorelin treatment due to cell mortality induced by the drug, as shown by FACScan analysis with PI (Table 1). Moreover, variations in culture medium pH were not observed at any of the tested doses (data not shown).

In FBS-supplemented medium, we observed a slight but significant proliferative effect at the 10^{-7} M dose, which was not significantly present in the other experimental conditions.

Experiments with Cetrorelix were performed to compare the effects of Triptorelin on cell proliferation to those induced by a GnRH antagonist. Figures 3a and 3b illustrate the effect of the GnRH antagonist on PC3 and LNCaP cells, respectively. In PC3 cells, we observed no significant antiproliferative activity at the tested doses. LNCaP cells were much more sensitive to the drug and showed a clear dose-related inhibition on proliferation, beginning with the 10^{-7} M concentration. To evaluate whether the stimulatory action of Triptorelin on cell growth could be counteracted by the antagonist, LNCaP cells were simultaneously treated with 10^{-7} M of both Triptorelin and Cetrorelix. The proliferative effect of the agonist was shown to be completely counteracted by the antagonist, with the inhibitory action of the Cetrorelix showing prevalence (Figure 3c).

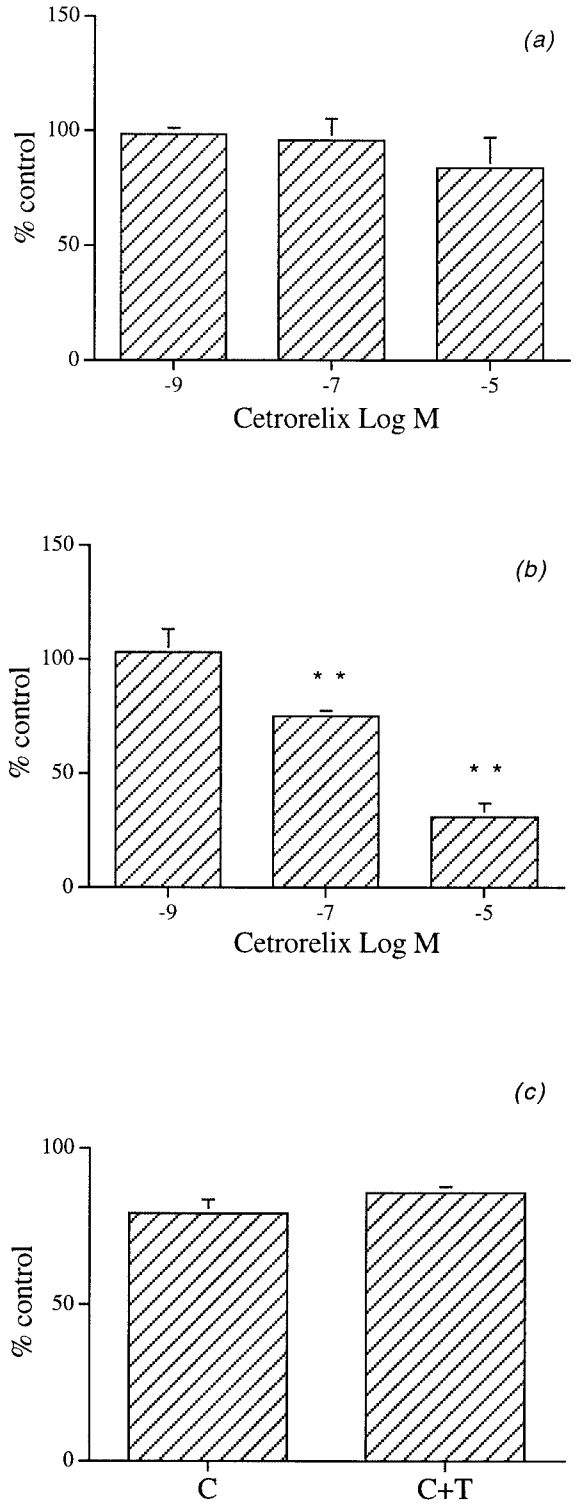

Figure 3. Effects of Cetrorelix ($10^{-9} - 10^{-5}$ M) on PC3 and LNCaP cell proliferation in 10% FBS-supplemented medium. PC3 and LNCaP were plated as shown in Figures 1 and 2, respectively. Cetrorelix was added to the medium daily and was changed every 3 days. At the end of the treatment, viable cells were counted as shown in Figures1 and 2. **(a)** Effect of Cetrorelix on PC3 cell proliferation after 4 days of treatment; **(b)** effect of Cetrorelix on LNCaP cell proliferation after 9 days of treatment; **(c)** effect of Cetrorelix alone (C) or in combination with Triptorelin (C + T) on LNCaP cell proliferation after 9 days of treatment. Both analogues were tested at a dose of 10^{-7} M. Data represent the mean ± standard error of the mean of 3 experiments with at least 2 replicates each and are expressed as a percent of untreated control culture. The 100% value assigned to the control cultures is not shown. **(a)** In PC3 cells, no significant antiproliferative activity was observed at the doses tested. **(b)** LNCaP were sensitive to the drug and showed a dose-related inhibition of proliferation starting from a concentration of 10^{-7} M. **(c)** In the same cell line, the proliferative effect of Triptorelin was completely counteracted by Cetrorelix. (**$P < .01$ versus control.)

Cell Cycle Analysis

Table 2 illustrates the distribution of LNCaP cells in the different compartments of the cell cycle after 12 hours of culture with or without 10^{-4} M Triptorelin. In FBS-supplemented medium and in DCC-supplemented medium + R 1881, the drug produced an accumulation of cells in the G_0/G_1 resting phase compared with untreated control cultures (140% and 123% of controls, respectively), accompanied by a concomitant and statistically significant decrease in the percentage of cells in S phase (22% and 58% of controls, respectively). Also, in DCC-supplemented medium and in DCC-supplemented medium + EGF, experimental results suggest that Triptorelin at the 10^{-4} M dose induces an accumulation of cells in the G_0/G_1 phase and a decrease in the percentage of cells in S phase. In these culture conditions, the differences were not statistically significant, which is probably a consequence of the cells' low growth rate.

GnRH Receptor Studies

Figures 4 and 5 show that binding sites for ^{125}I-Triptorelin are present on the surface of both LNCaP and PC3 cells. In PC3 cells, the ligand program revealed the presence of a single class of binding sites and showed a low affinity for the ligand used ($K_d = 2.7 \times 10^{-6}$ M) and a high capacity (2.4×10^7 sites/cell).

In LNCaP cells, computer analysis of binding data revealed the existence of 2 different classes of binding sites. One showed a moderately high binding affinity ($K_d = 2.6 \times 10^{-8}$ M) and low capacity (5.7×10^4 sites/cell); the other showed a low binding affinity ($K_d = 7.7 \times 10^{-6}$ M) and high capacity (3.3×10^7 sites/cell).

Discussion

The mechanism of action of GnRH analogues on tumor cells is not homogeneous because individual response patterns exist in different cancer cell lines (Dondi et al, 1994; Yano et al, 1994; Sica et al, 1999; Kim JH et al, 1999) and even in different subclones of the same cell line (Mullen et al, 1991). Our study sheds additional light on the effects of Triptorelin on prostatic tumor cells and stresses

Table 2. *Percentage of LNCaP cells present in the 3 major phases of the cell cycle**

Culture conditions	Phase of cell cycle					
	G_0/G_1, %		S, %		G_2/M, %	
	C	T	C	T	C	T
FBS	40 ± 1	56 ± 4†	50 ± 2	39 ± 3†	10 ± 1	5 ± 1
DCC	69 ± 3	76 ± 1	20 ± 1	16 ± 2	11 ± 3	9 ± 3
R1881	56 ± 2	69 ± 3†	30 ± 3	14 ± 1†	14 ± 1	17 ± 3
EGF	59 ± 3	63 ± 1	36 ± 1	31 ± 1	4 ± 1	6 ± 1

* C indicates control cells; T, 10^{-4} M Triptorelin. Cells were treated with Triptorelin for 12-hours. Data are means ± standard error of the mean for 4 experiments.
† $P < .05$ vs control.

the peculiarity of its effects on cell specificity, range of active concentrations, and different culture media.

We chose to test the effects of Triptorelin on 2 tumor cell lines of prostate origin that mimic the initial and advanced phases of prostate carcinoma: the highly malignant and androgen-insensitive PC3 cells and the androgen-sensitive, with markedly less invasive potential, LNCaP cells. Triptorelin treatment did not cause any biological effects on androgen-independent PC3 cells. Cell cycle and proliferation studies showed that the drug influences cell growth only on hormone-dependent LNCaP cells, which highlights the cell specificity of the analogue action. Experiments with Cetrorelix confirmed the different sensitivity of the 2 cell lines to GnRH analogues in vitro. Cetrorelix reduced LNCaP cell proliferation at lower doses than Triptorelin, but like Triptorelin, it was ineffective on PC3 cells.

We performed binding studies with ^{125}I-Triptorelin to determine whether specific receptors could mediate the effects on proliferation observed in LNCaP cells and to study the different behavior of the 2 prostatic cell lines following Triptorelin treatment. In our experimental conditions, LNCaP revealed 2 classes of binding sites with moderately high and low affinity for the ligand; whereas PC3 cells showed low-affinity binding sites only. This observation suggests that the absence of high-affinity binding sites could be related to the lack of responsiveness of PC3 cells following Triptorelin and Cetrorelix treatment. Other authors have described the effects on proliferation caused by different GnRH agonists on prostatic cancer cells that express only low-affinity receptors (Dondi et al, 1994). These reports do not necessarily contrast with this hypothesis if we consider that the drugs and binding conditions were different. The cascade of events leading to the biological effects of GnRH analogues on cancer cells in vitro is only poorly understood and may differ according to cell line (Moretti et al, 1996). Furthermore, local production of GnRH or GnRH-like peptides that are active in modulating cell proliferation has been well-established for LNCaP cells (Limonta et al, 1993). Currently, no data are available on the expression of a GnRH-like system by PC3 cells. Differences in the expression of GnRH-like peptides or in cellular sensitivity to these molecules could be responsible for the different sensitivity of the 2 cell lines to the drug. Furthermore, some intracellular signals elicited by GnRH receptor activation, both through the GnRH agonist and antagonist, could be different in PC3 cells than it is in LNCaP cells.

The effect of Triptorelin on LNCaP cell proliferation was studied under various experimental conditions. The drug exerted a double inhibitory-stimulatory action on cell growth depending upon the dose and environmental conditions. In FBS-supplemented medium, the drug exerted a proliferative effect, which was especially evident at the 10^{-7} M dose, whereas it strongly inhibited cell growth at a dose of 10^{-4} M. In all the other culture conditions tested, only the antiproliferative effect, at high doses, was observed. A similar biphasic effect on proliferation was observed by other authors with another GnRH agonist, Buserelin, although under different culture conditions (Qayum et al, 1990). Both the extent of antiproliferative activity and the presence of the proliferative effect are linked to the composition of the culture media, which stresses the importance of the different growth factors in modulating the action of the drug. Binding studies and experiments with Cetrorelix support the interpretation that proliferative effect could depend upon the interaction of the drug with its receptors. In fact, the affinity of the binding sites for labeled Triptorelin is in the range of the active dose and the antagonist completely counteracts the proliferative effect of Triptorelin, if they are tested at the same concentrations. On the other hand, the decrease in the proliferation rate observed at a high dose, in all culture conditions, was not the result of a cytotoxic effect of the drug. Moreover, reports from other authors indicate that the drug is stable in cell culture medium (Emons et al,1993) and no variations in pH of the culture media were observed at any of the doses tested. The early decrease in the percentage of cells in S phase and the related increase of cells in G_0/G_1 phase observed in the cell cycle studies appear to suggest that the antiproliferative action of the drug involves modifying the cellular regulatory events, which are necessary to progress from the resting phase to DNA synthesis.

Several lines of evidence suggest that the GnRH system in human cancer cells interacts with the expression of

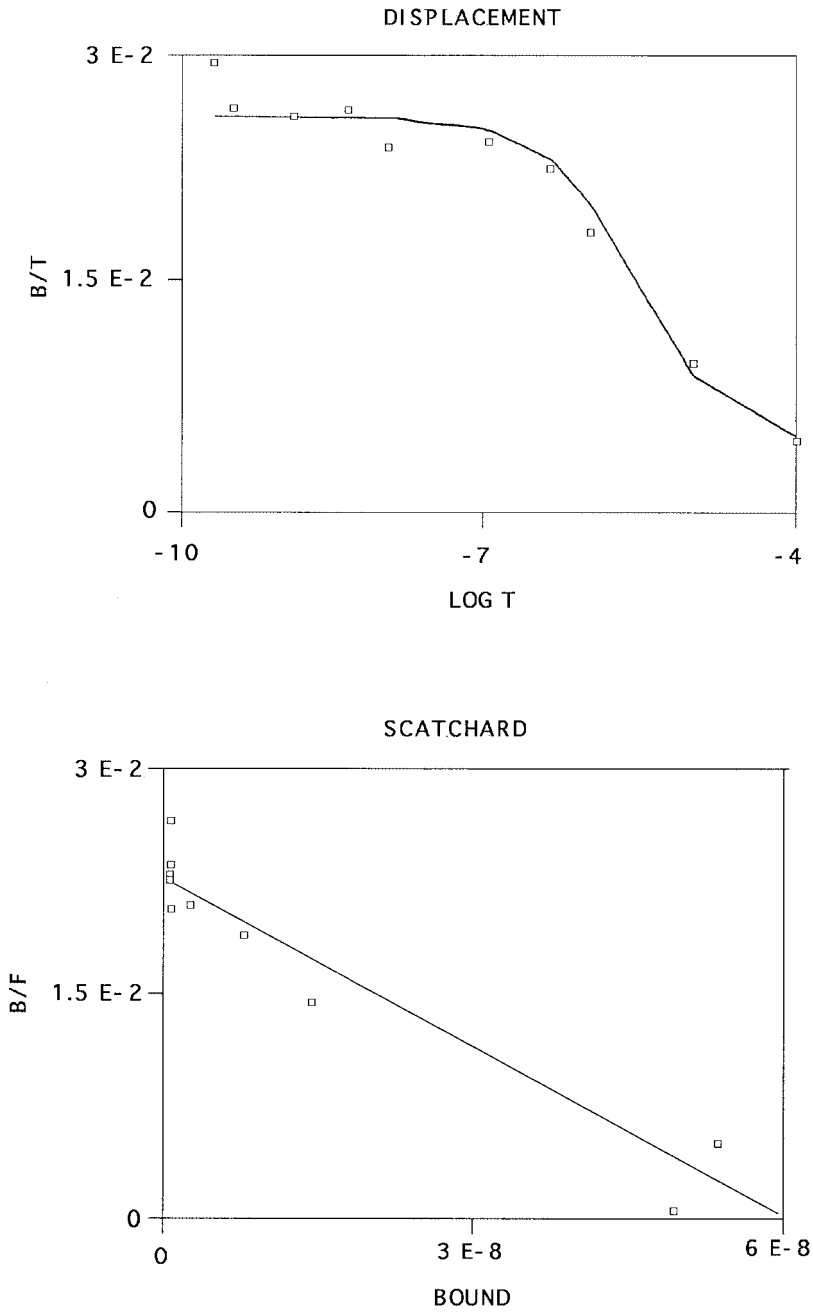

Figure 4. Displacement of ^{125}I-Triptorelin from PC3 cells and Scatchard transformation. Binding analysis was carried out in Costar 24 multiwell plates. The cell monolayers were incubated with 100 000 cpm ^{125}I-Triptorelin (1500 Ci/mmol) in the presence or absence of increasing concentrations (10^{-10} – 10^{-4} M) of unlabeled peptide in a total volume of 250 μL in triplicate. After 2 hours of incubation at 4°C, cells were detached with trypsin/EDTA solution and the sample was counted in an automatic γ counter. Representative wells were used to determine cell number. Nonspecific binding was assessed in the presence of 10^{-4} M of unlabeled Triptorelin. The graph represents 3 independent experiments, each performed in triplicate, with comparable results. Analyses of the binding data by a ligand program were consistent with 1 class of binding site and show a low affinity for the ligand used ($K_d = 2.7 \times 10^{-6}$ M) and high capacity (2.4×10^7 sites/cell).

growth factor receptors and their signaling pathways (Moretti et al, 1996; Emons et al, 1998). Because androgens and EGF are both involved in controlling LNCaP cell line proliferation (Schuurmans et al, 1991), we tested Triptorelin in different culture conditions to establish whether the drug could inhibit cell proliferation by interfering with the activity of androgens or a specific growth factor such as EGF. Our data indicate that the stimulatory action of both EGF and R1881 is significantly counteracted by the analogue. These results are similar to data from other reports that showed a reduction in the number of EGF receptors and inhibition of the intracellular mechanism activated by EGF

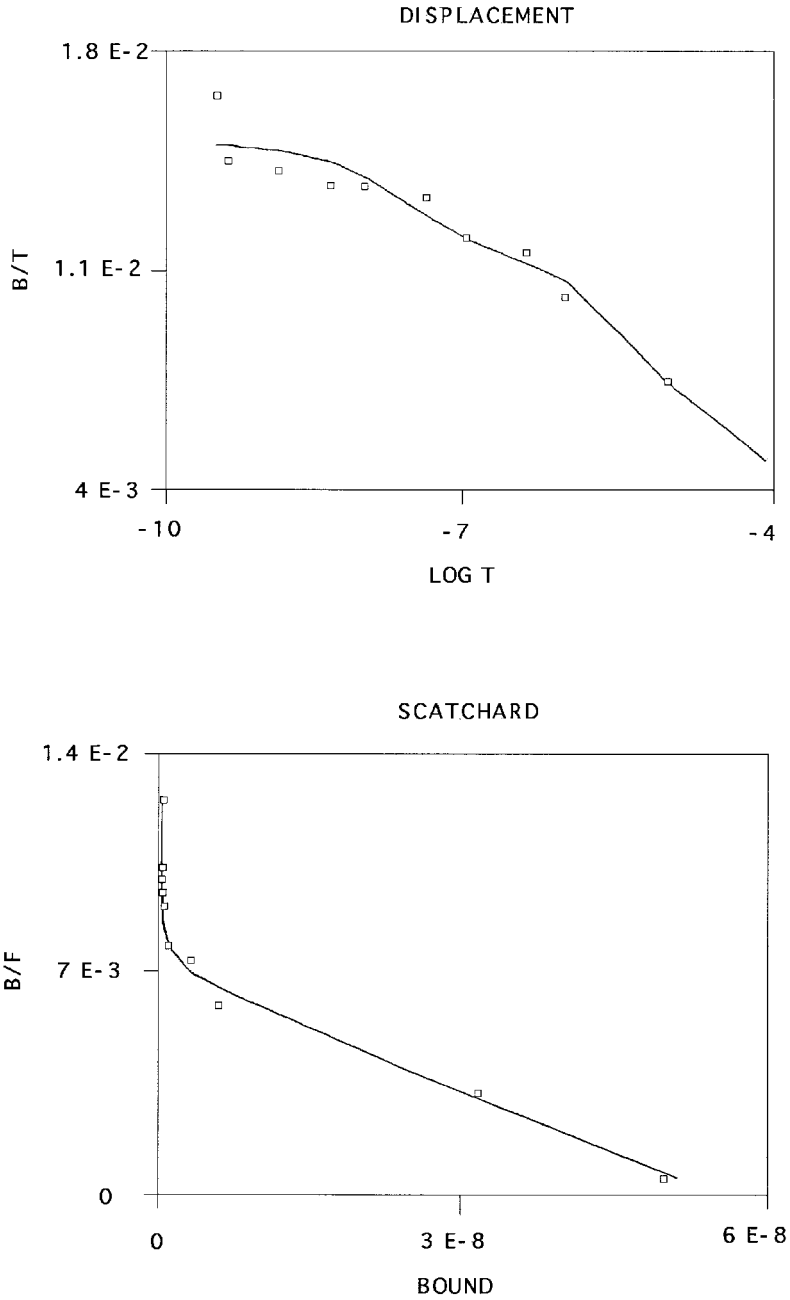

Figure 5. Displacement of ^{125}I- Triptorelin from LNCaP cells and Scatchard transformation. Graph prepared as in Figure 4. The analyses of the binding data by a ligand program were consistent with 2 different classes of binding sites. One showed a moderately high binding affinity ($K_d = 2.6 \times 10^{-4}$ M) and low capacity (5.7×10^4 sites/cell); the other showed a low binding affinity ($K_d = 7.7 \times 10^{-6}$ M) and high capacity.

following treatment with the GnRH agonist, Zoladex (Moretti et al, 1996). Moreover, androgen-induced proliferation appears to be related to the stimulatory action of EGF receptors (Ravenna et al, 1995). Overall, these observations suggest that Triptorelin can interfere with the proliferation-promoting effects exerted by the local EGF/TGFα loop, as indicated by other agonists (Moretti et al, 1996) and for cell lines with different origins (Emons et al, 1998).

Furthermore, the observation that Triptorelin also abolishes low residual growth in DCCFBS-supplemented medium indicates that the drug probably interferes with the pathway of growth factors other than EGF and androgens, which are present at a very low concentrations in charcoal-stripped serum.

Recent studies indicate that the dichotomy between GnRH agonists and GnRH antagonists, which is already well accepted for the pituitary system, is not valid for the GnRH system in cancer cells (Emons et al, 1998). Further-

more, the physiological effect of GnRH on prostatic cells is not yet fully understood. The lack of uniformity in the action of Triptorelin observed on LNCaP and PC3 cells is related to this complex scenario and stresses the role being played by the experimental conditions and by the cell lines tested in evaluating the biological effects of GnRH analogues in vitro.

In summary, from our results, we can make the following conclusions: 1) the effects of Triptorelin on prostatic cell proliferation in vitro are cell-specific; 2) LNCaP cells are responsive and PC3 cells are unresponsive to the analogue and express binding sites for the drug that have different affinities for the ligand; and 3) Triptorelin exerts a double proliferative-antiproliferative action on LNCaP cells, depending upon the culture conditions and the dose used.

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