

GnRH agonists and antagonists decrease the metastatic progression of human prostate cancer cell lines by inhibiting the plasminogen activator system

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Abstract. Prostate cancer (PCa) growth initially depends on circulating androgens. Gonadotropin-releasing hormone (GnRH) agonists are currently used for the treatment of PCa. However, after an initial responsiveness to hormonal deprivation, PCa progresses and metastasizes. Recently, also GnRH antagonists have been used for clinical trials in patients with PCa and the results seem promising. The components of the plasminogen activator (PA) system (urokinase-type PA, uPA; PA inhibitors, PAI-1/2; uPA receptor, uPAR) have been implicated in the local degradation of the extra-cellular matrix (ECM) and PCa progression. The aim of this study was to test the possible effects of the treatment with an agonist (Leuprolide, GnRH-A) and an antagonist (Cetorelix, GnRH-ANT) of GnRH on the expression and activity of uPA and PAI-1 in the conditioned media of DU145 and PC3, two PCa androgen-independent cell lines. The involvement of the PA system in the control of cellular migration was also investigated. The results obtained in DU145 and PC3 cells show that both GnRH-A and GnRH-ANT: i) inhibit cell proliferation; ii) significantly decrease the enzymatic activity and the secretion of uPA; iii) significantly increase the protein levels of PAI-1; iv) induce a significant decrease of the migratory and invasion PCa capabilities. This study suggest that GnRH analogues exhibit not only an antiproliferative effect, but also an anti-metastatic action exerted through the inhibition of the activity of PA system and might provide a rational basis for the development of clinical strategies for those tumours that progress towards an androgen-independent condition characterized by a higher metastatic potential.

Introduction

Prostate cancer (PCa) growth initially depends on circulating androgens (1). Gonadotropin-releasing hormone (GnRH) agonists, either alone, or in combination with antiandrogens, represent the most widely used current hormonal treatment. The antitumoural effect of GnRH agonists is based on their ability to suppress the activity of the pituitary-testicular axis, and therefore, to reduce testosterone production and secretion. However, GnRH agonists induce, at the beginning of treatment, an initial stimulation of LH and FSH (2). For this reason, GnRH antagonists have been developed. These compounds, while maintaining the ability to inhibit the activity of the pituitary-gonadal axis by binding to the pituitary GnRH receptors (GnRHR), do not induce the so-called 'flare-up' phenomenon, the intracellular cascade of events evoked by the natural hormone or GnRH agonists (3). The last generations of GnRH antagonists have already been successful in clinical trials of phase I and II.

More recently, GnRH has been shown to be produced also by endocrine-related tumours, where it acts as a growth inhibitory factor (4,5). In particular, it has been reported that a large portion of human PCa specimens as well as PCa cell lines express both mRNA and binding sites for GnRH (6). The activation of GnRHR by GnRH analogues induces a significant decrease of cancer cell proliferation either androgen-dependent (7) or androgen-independent (8), through signal transduction mechanisms different from those observed in pituitary cells (9,10).

However, after an initial responsiveness, advanced PCa almost invariably progresses to a condition of androgen-independence generally associated with a higher proliferation rate and an increased invasiveness, mainly at lymphnode and bone levels (11). Little information is available on the molecular mechanisms that are involved in the control of the metastatic properties of PCa cells.

The malignant process of tumour invasion and metastasis requires the extensive disruption of cell-cell and cell/extracellular matrix (ECM) contacts as well as the degradation of ECM components that represent the physical barrier in the direction of migration/invasion. Overexpression and/or inappropriate regulation of proteolytic activity often occur(s) in neoplasia. Moreover, invasion of cancer is supported by a

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controlled degradation of ECM by proteases released from cancer cells. Many findings strongly suggest that the plasminogen activators (PAs) are involved in PCa progression and invasion (12,13). In mammals, two molecular forms of PAs have been described: the urokinase-type PA (uPA) and the tissue-type PA (tPA). Both PAs catalyze the same reaction (the conversion of plasminogen to plasmin), but tPA is thought to be more important in vascular fibrinolysis, whereas uPA is considered to be involved in tissue remodelling. The serine protease uPA, initially secreted by malignant cells as an enzymatic inactive single chain pro-enzyme (pro-uPA), is cleaved into the active two chain 50-52 kDa form of uPA by binding with the cell-surface urokinase-type uPA receptor (uPAR). Hence receptor-bound, uPA promotes plasminogen conversion in plasmin, activating a cascade of proteinases culminating in the dissolution of the tumour matrix and the basement membrane (14). Overexpression of components of the uPA-system in non-metastatic cells enhances metastasis; inhibition of uPA and/or of the uPA/uPAR interaction prevents or reduces metastasis in animal models (15,16). Specific inhibitors such as the plasminogen activator inhibitor-1 (PAI-1) represent important regulators of the biology of the uPA system. PAI-1 is 52 kDa glycoprotein secreted by many cancer cells that binds uPA with the subsequent formation of an inactive complex (15). The PA system may also regulate activities that do not depend on the function of uPA as a proteinase. The complex uPA/uPAR may regulate cellular adhesion and migration by association to the extracellular domains of integrins, or by binding directly to vitronectin (17,18).

PCa cells vary in terms of net PA activity (19). This variability positively correlates with the *in vitro* invasive activity of PCa cell lines and with their *in vivo* metastatic potential (20,21). The two human androgen-independent PCa cell lines DU145 and PC3, express high levels of uPA, while the androgen-dependent PCa cell line LNCaP is devoid of detectable uPA (22).

The aim of this study was to examine whether a GnRH agonist (Leuprolide, GnRH-A) as well as a GnRH antagonist (Cetorelix, GnRH-ANT) may interfere with the proteolytic activity of the PA proteases released from DU145 and PC3 cells. To this purpose, uPA and PAI-1 activities and levels have been evaluated in the cell culture media of the two human androgen-independent PCa cell lines. Moreover, it has also been analysed whether both the GnRH analogues might affect not only the proliferation, but also the migration and invasiveness of PCa cells.

Materials and methods

GnRH analogues. The GnRH agonist Leuprolide [(pGlu¹-His²-Trp³-Ser⁴-Tyr⁵-D-Leu⁶-Leu⁷-Arg⁸-Pro⁹-NHC₂H₅) GnRH; GnRH-A] was kindly provided by Takeda (Osaka, Japan); the GnRH antagonist Cetorelix [SB-75; (ac-D-Nal¹-D-Cpa²-D-Pal³-Ser⁴-Tyr⁵-D-Cit⁶-Leu⁷-Arg⁸-Pro⁹-D-Ala¹⁰-NH₂) GnRH; GnRH-ANT] was kindly provided by Zentaris (Frankfurt, Germany). GnRH analogues were dissolved in serum-free RPMI-1640 medium (Biochrom KG, Berlin, Germany).

Cell culture. The PCa androgen-independent cells DU145 and PC3 were obtained from the American Type Culture

Collection (Rockville, MD). Cells were routinely grown in RPMI-1640 medium (Biochrom KG), supplemented with 5% fetal bovine serum (FBS) that was obtained from Gibco BRL, Grand Island, NY), glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and geneticin (25 µg/ml) in a humidified atmosphere of 5% CO₂/95% air at 37°C.

Cell growth studies. DU145 and PC3 cells were plated at a density of 500 cells/cm² in 10-mm dishes in RPMI-1640 medium supplemented with 5% FBS; cells were allowed to attach and start growing for 2 days and the seeding media were then changed. Cells were daily treated (for 4 days) with graded doses (10⁻¹⁰-10⁻⁶ M) of the GnRH agonist (Leuprolide, GnRH-A) or the GnRH antagonist (Cetorelix, GnRH-ANT). At the end of the treatments, cells were harvested and counted using a hemocytometer; cell viability was determined by trypan blue dye exclusion after staining for 5 min at room temperature. Similar treatments were used to analyse whether GnRH analogues might influence the migration and the invasiveness of the PCa DU145 and PC3 cells.

The results of three separate experiments are presented as the mean ± SD. Each experimental group was performed in triplicate. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparison tests. P<0.05 was considered statistically significant.

PA assay and uPA enzymatic immuno assays (EIA). Plasminogen activator assays were carried out in 96-well flat-bottomed multi-well plates (NUNC) using H-D-Val-Leu-Lys-pNA (S2251, Sigma) as substrate of plasmin as described previously (22). Briefly, 24 h conditioned media of DU145 and PC3 cultures, grown onto 24-well plates in the presence or in the absence of graded doses (10⁻⁸-10⁻⁶ M) of GnRH-A or GnRH-ANT, were incubated in assay buffer (100 mM Tri-HCl, pH 8.8, 0.5% Triton X-100) with plasminogen (2.8 µg) for 4 h at room temperature. Plasmin activity assay was started by the addition of substrate (S2251, 50 µg) in a final volume of 100 µl. The change in A405 nm was monitored with a Titertek multiscan reader (Bio-Rad). Enzymatic activities were determined following subtraction of values found for conditioned media incubated without plasminogen. Results are presented as Plough Units, determined using a standard plasmin or urokinase preparation (Sigma). Plasmin inhibitor (D-Val-Phe-Lys CMK, Sigma) was used to confirm PA dependence of substrate degradation.

Previous studies performed in our laboratories demonstrated that the *in vitro* uPA secretion from DU145 and PC3 cells follows an exponential kinetic curve between 8 and 24 h, reaching a plateau at 48 h; for this reason, we studied the uPA secretion in the cell culture media after 8 and 24 h from the treatments. Enzymatic activity of uPA was measured by Enzymatic immuno-adsorbent assay by coating 96 flat bottomed microtiter plates with 20 mg/ml of anti-uPA antibody as previously described (22). The uPA activity was measured in 8- and 24-h conditioned media of DU145 and PC3 cultures grown onto 24-well plates in the presence or absence of graded doses (10⁻⁸-10⁻⁶ M) of GnRH-A or GnRH-ANT. Enzymatic activity was measured (uPA-bound to the antibody-coated plates) using both plasmin and urokinase colorimetric substrate (Calbiochem, EMD Bioscience Inc., La Jolla, CA, USA).

Corresponding cells were collected and counted in a Neubauer chamber (Hausser, Blu Bell, PA, USA) to normalize the enzymatic activity of uPA of the conditioned media; the results are expressed as ng/mg protein.

Zymography for plasminogen activators. Samples of conditioned media from DU145 and PC3 cells treated for 8 or 24 h with graded doses (10^{-8} - 10^{-6} M) of GnRH-A or GnRH-ANT were separated by electrophoresis under non-reducing conditions using 7.5% SDS-PAGE co-polymerized with 0.1% lactose-free casein and 15 μ g/ml human plasminogen. Following electrophoresis, gels were washed three times for 5 min in a solution of 50 mM Tris pH 7.5 containing 2% Triton X-100 and rinsed three times in 50 mM Tris pH 7.5. Gels were incubated in a buffer containing 50 mM Tris, 0.1% Triton X-100 and 0.02% NaN_3 , pH 7.5 for 4 h at 37°C in a humidified environment. Enzyme activity was detected as a negatively stained region following staining in 0.1% solution of coomassie blue in a mixture of methanol:acetic acid:water (3:1:6) and de-stained in the same solution without dye. Controls were performed using SDS-PAGE co-polymerized with casein without plasminogen and/or using serine protease inhibitors added in the incubation buffer. For uPA analysis, we used a goat polyclonal antibody against uPA (C20, Santa Cruz) diluted 1:400. Corresponding cells were collected and counted in a Neubauer chamber (Hausser, Blu Bell) to normalize the enzymatic activity of the conditioned media. The zymography allows the direct and simultaneous detection of the major plasminogen activator form (uPA, 52-50 kDa) with its enzymatic activity; the uPA activity in this blot is shown by clear zones of proteolysis on a dark background of non-degraded stained casein.

Immunoblot analysis. Samples of conditioned media from DU145 and PC3 cultures treated for 8 or 24 h with graded doses (10^{-8} - 10^{-6} M) of GnRH-A or GnRH-ANT were separated by electrophoresis under non-reducing conditions and transferred to Hybond paper. Non-specific binding sites were blocked for 1 h in 5% non-fat dried milk in a Tris-buffer containing 20 mM Tris and 137 mM NaCl (pH 7.6). Blots were then incubated with goat polyclonal antibody against PAI-1 (C20, Santa Cruz) diluted 1:100 for 1 h. The staining was visualised by a secondary anti-goat IgG antibody linked to horseradish peroxidase (Amersham International, Aylesbury, UK).

Migration and invasion assay. Briefly, cell migration assay was performed using a 48 well-Boyden chamber (Neuroprobe, Inc., Concorezzo, Milan, Italy) containing 8 μ m polycarbonate filters. Filters were coated on one side with 50 μ g/ml laminin, rinsed once with PBS, and then plated in contact with the lower chamber containing 200 μ l of RPMI-1640 medium. DU145 and PC3 cells pre-treated for 4 days with GnRH-A (10^{-6} M) or GnRH-ANT (10^{-6} M) were collected, washed twice with PBS, rinsed in complete medium and incubated at 37°C for 30 min to reconstitute the membrane structures. Cells were then added in aliquots (75,000 cells/ 50 μ l) to the top of each chamber and allowed to migrate through coated filters for 4 h. At the end of the incubation, the chamber was disassembled and non-migrated cells on the upper membrane surface were removed with a

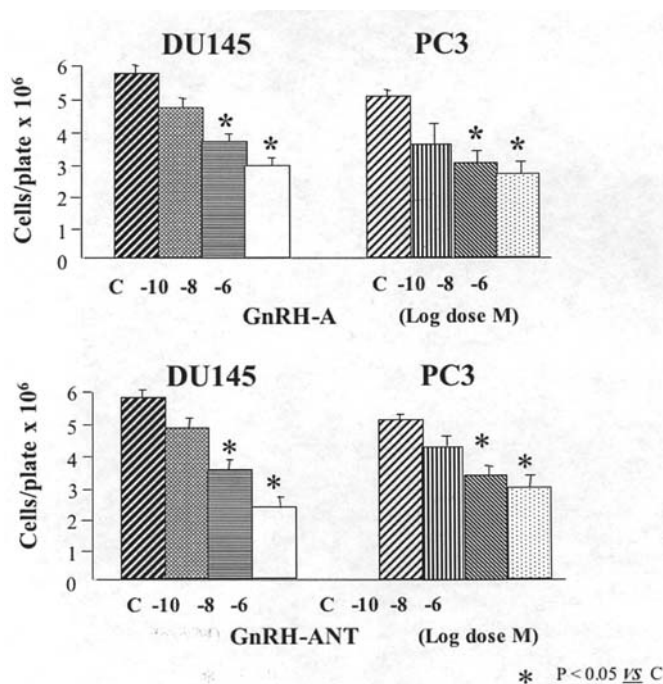


Figure 1. Effect of the treatment with GnRH-A or GnRH-ANT on the proliferation of DU145 and PC3 cells. Cells were treated for 4 days with graded doses (10^{-10} - 10^{-6} M) of GnRH-A or GnRH-ANT. At the end of the treatment, cells were collected and counted by haemocytometer. The results are averages (\pm SD, bars) from three independent experiments. C, control cells.

cotton swab. The migrated cells attached on the lower membrane surfaces were fixed and stained with Diffquik (Biomap, Italy) for 20 min at room temperature. Migrated cells were counted at a x40 magnification in standard optical microscopy.

Invasion assay was performed in invasion chambers (Becton-Dickinson, Bedford, MA) contain a membrane coated with Matrigel™, a layer of extracellular matrix. The digestion of Matrigel allowed the migration of cancer cells. Briefly, DU145 and PC3 cells pre-treated for 4 days with GnRH-A (10^{-6} M) or GnRH-ANT (10^{-6} M) were collected, and 500 μ l of cell suspension (75,000 cells) were added to trans-well inserts with an 8 μ m pore size coated with Matrigel. In the lower compartment of the invasion chamber, 5% FBS contained medium was added as chemo-attractant. After a 22-h incubation period at 37°C, cells that passed through the filter into the bottom wells were fixed, stained with Diffquik (Biomap, Italy) and counted at a x40 magnification in standard optical microscopy.

Each individual experiment of migration and invasion was performed in triplicate. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparison tests. $P < 0.05$ was considered statistically significant. The results are expressed as a percentage of migrated cells vs control cells.

Results

GnRH analogues decrease cell proliferation in PCa cells. This experiment has been performed to clarify whether, in our experimental conditions, Leuprolide (GnRH-A) and

Table I. Effect of the treatment with GnRH-A or GnRH-ANT on the total PA activity in DU145 and PC3 cells.

	Total PA (mU/mg protein)	
	DU145	PC3
Controls	4.1±0.5	8.4±0.2
GnRH-A (10 ⁻⁸ M)	2.7±0.4	6.3±0.6
GnRH-A (10 ⁻⁶ M)	1.8±0.2 ^a	5.4±0.2 ^a
GnRH-ANT (10 ⁻⁸ M)	3.1±0.4	5.3±0.2 ^a
GnRH-ANT (10 ⁻⁶ M)	2.1±0.1 ^a	4.7±0.3 ^a

The results are averages (± SD, bars) from three independent experiments. ^aP<0.05 vs controls.

Cetorelix (GnRH-ANT) might affect the proliferation of DU145 and PC3 cells.

Cells were treated daily (for 4 days) with graded doses of GnRH-A or GnRH-ANT. The treatment of PCa cells with both GnRH-A and GnRH-ANT resulted in a dose-dependent inhibition of tumour cell growth (Fig. 1). Both GnRH analogues were significantly effective from 10⁻⁸ to 10⁻⁶ M. It is of interest to note that, while the treatment with the highest dose (10⁻⁶ M) of GnRH-A seems to produce a quite similar inhibitory effect on the proliferation of both DU145 (49±6% of inhibition) and PC3 (45±8% of inhibition) cells, the treatment with the same dose of GnRH-ANT appears to be more effective in DU145 (57±8% of inhibition) with respect to PC3 cells (42±8% of inhibition).

GnRH analogues inhibit the PA system activity. To determine the effectiveness of the treatment with GnRH analogues on the PA system activity, we evaluated the effect of GnRH-A and GnRH-ANT on the total enzymatic activity of plasminogen

(PA) by using a chromogenic assay for plasmin and S2251 as substrate. The assay was performed on conditioned media of DU145 and PC3 cells treated for 24 h in the presence or in the absence of graded doses of GnRH-A or GnRH-ANT. PA secreted from both DU145 and PC3 cells was significantly decreased following the treatment with the highest dose (10⁻⁶ M) of both GnRH-A and GnRH-ANT (Table I). The dose of 10⁻⁸ M of GnRH-ANT decreased the secretion of PA only from PC3 cells, being the same dose ineffective on DU145 (Table I).

To recognize which form of PA is more influenced by the treatment with GnRH analogues, we studied, by enzymatic immuno-absorbant assay (EIA), the presence of the urokinase plasminogen activator (uPA) enzyme in the conditioned media of DU145 and PC3 cells treated for 8 and 24 h in the presence or in the absence of graded doses of GnRH-A or GnRH-ANT. Interestingly, both the PCa cells released the uPA protein (measured as ng/mg protein) into the culture medium, even if the basal levels of secreted uPA in DU145 cells were significantly lower than those observed in PC3 cells at the same time interval (Fig. 2).

The treatment with the dose of 10⁻⁶ M of GnRH-A (Fig. 2) significantly reduced, at the times considered (8 and 24 h), the uPA secretion in both the PCa cells. The lower dose (10⁻⁸ M) of the GnRH agonist was also efficacious in inhibiting the 24 h secretion of uPA in PC3 cells, while it was ineffective at any time considered on the secretion of uPA from DU145 cells.

An inhibition of uPA production was also observed when the PCa cells were treated with the GnRH-ANT (Fig. 3). The highest dose (10⁻⁶ M) of GnRH-ANT significantly inhibited the uPA secretion in both the PCa cells; on the other hand, only DU145 and not PC3 cells responded to the inhibitory effect of the lowest dose (10⁻⁸ M) of GnRH-ANT at 24 h (Fig. 3).

To further characterize the uPA enzymatic activity in PCa cells, we performed zymogram analysis on the same

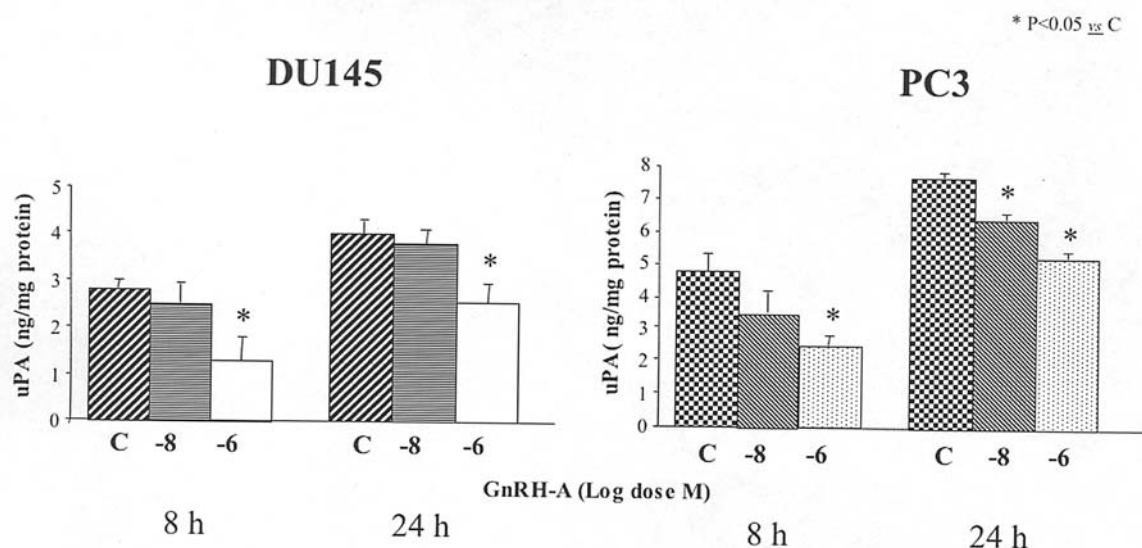


Figure 2. Effect of GnRH-A on the uPA production. The assay was performed on conditioned media collected from DU145 and PC3 cells either untreated (C) or treated (for 8 and 24 h) with graded doses (10⁻⁸-10⁻⁶ M) of GnRH-A. The evaluation of the presence of uPA was performed by using an enzymatic immuno-absorbant assay (EIA).

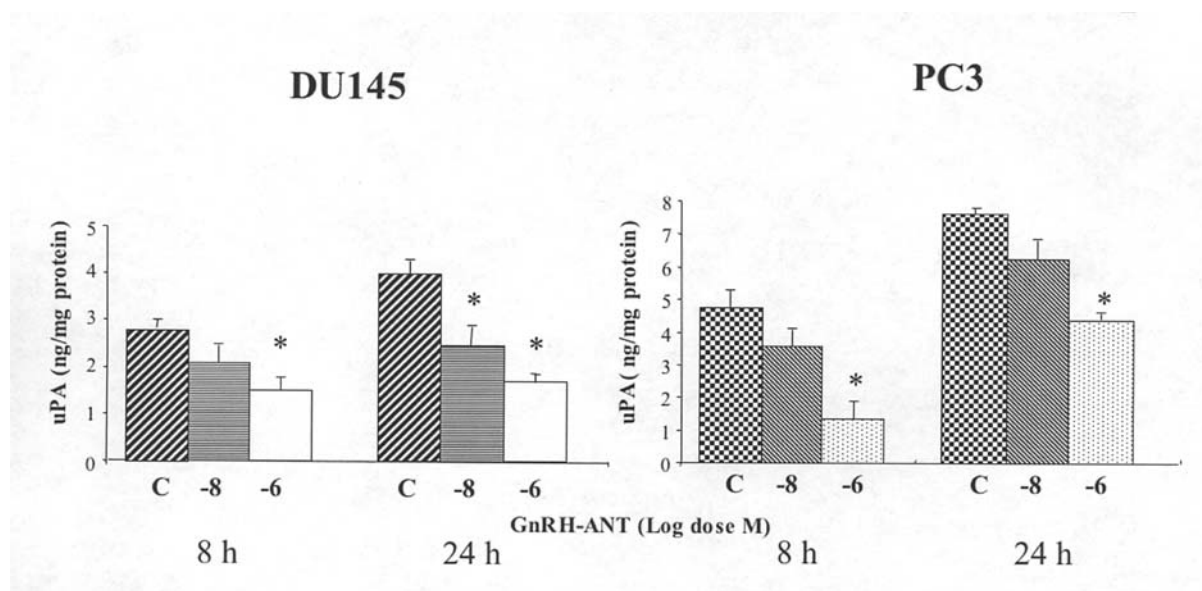


Figure 3. Effect of GnRH-ANT on the uPA production. The assay was performed on conditioned media collected from DU145 and PC3 cells either untreated (C) or treated (for 8 and 24 h) with graded doses (10^{-8} - 10^{-6} M) of GnRH-ANT. The evaluation of the presence of uPA was performed by using an enzymatic immuno-absorbent assay (EIA).

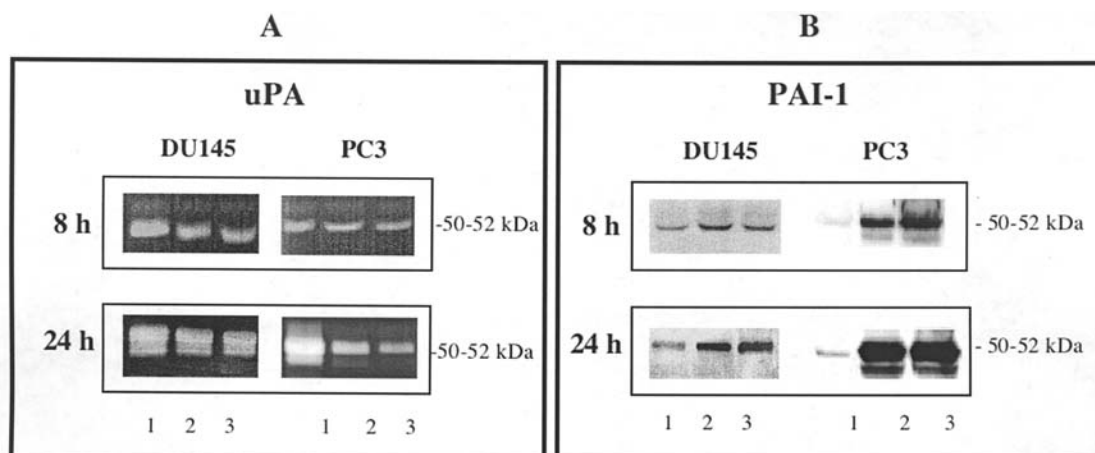


Figure 4. Representative gelatin zymography of uPA (panel A) and Western blot for PAI-1 (panel B) performed on the conditioned media of DU145 and PC3 cells untreated (lane 1) or treated for 8 and 24 h with graded doses of GnRH-A (10^{-8} M, lane 2; 10^{-6} M, lane 3).

conditioned media tested for the enzymatic activity. Fig. 4 (panel A) clearly shows that in both DU145 and PC3 cells, GnRH-A induced a marked and dose-dependent decrease of the proteolysis associated to the 52-50 kDa isoform of uPA, which is more evident after 24 h of incubation (lanes 2 and 3). To fully characterize the effects of GnRH-A on the uPA system in PCa cells, we analysed, in the same samples, the presence of the uPA inhibitor PAI-1 by Western blot (Fig. 4, panel B). PAI-1 is co-secreted with the uPA by both PCa cell lines. After GnRH-A treatment (lanes 2 and 3) there is, at the times considered (8 and 24 h), a clear and dose-dependent induction of the PAI-1 in both DU145 and PC3 cells.

The effects of GnRH-ANT on uPA in both DU145 and PC3 cells demonstrated (Fig. 5, panel A) that the antagonist decreased in a dose-dependent manner the proteolysis

associated to uPA at all the time considered (lanes 2 and 3); at the same time, the GnRH-ANT increased the PAI-1 (lanes 2 and 3) in both DU145 and PC3 cells (Fig. 5, panel B).

GnRH analogues inhibit the migration and invasiveness of PCa cells. The PA system is known to be deeply implicated in the process of invasiveness and metastasis of PCa cells. Therefore, it was of interest to study whether the inhibitory effects of the two GnRH analogues on the PA system activity previously observed, might interfere with the ability of DU145 and PC3 cells to migrate and invade. For the following experiments, the dose of 10^{-6} M was selected because of its efficacy on both PCa cell types in every experiment performed and at each time interval considered. DU145 and PC3 cells, pre-treated for 4 days with GnRH-A

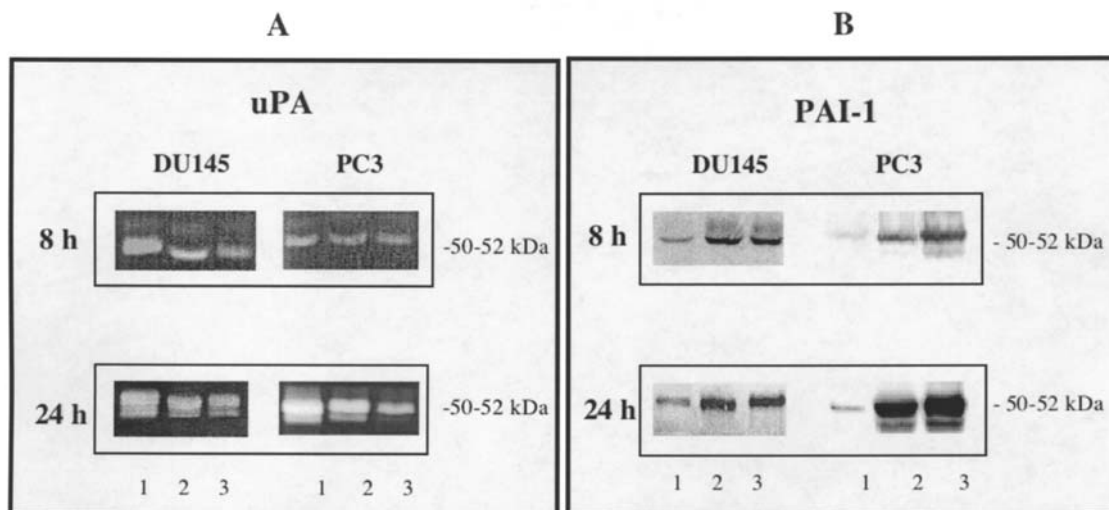


Figure 5. Representative gelatin zymography of uPA (panel A) and Western blot for PAI-1 (panel B) performed on the conditioned media of DU145 and PC3 cells untreated (lane 1) or treated for 8 and 24 h with graded doses of GnRH-ANT (10⁻⁸ M, lane 2; 10⁻⁶ M, lane 3).

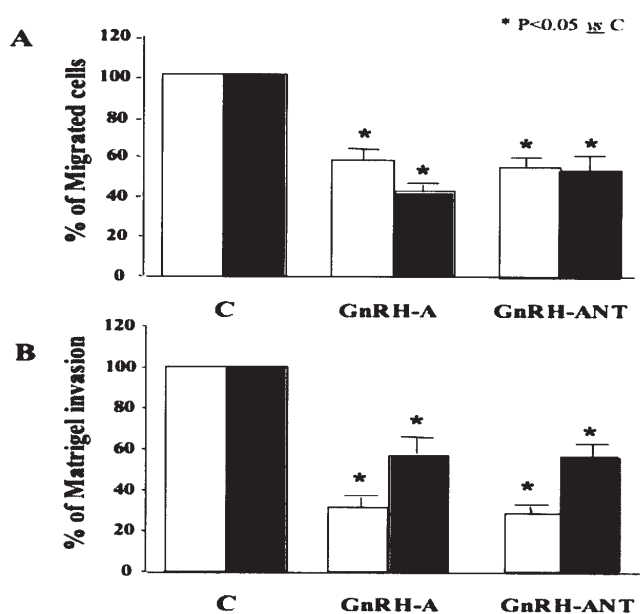


Figure 6. Effect of the treatment with GnRH-A (10⁻⁶ M) or GnRH-ANT (10⁻⁶ M) on the haptotaxis induced by laminin (panel A) and on Matrigel invasion (panel B) in DU145 (□) and PC3 (■) cells. Cells were pre-treated for 4 days with GnRH-A or GnRH-ANT. At the end of the treatment, cells were harvested, counted and seeded in Boyden's chamber (A) or in Matrigel invasion chamber (B). The results are expressed as percentage (\pm SD, bars) from three independent experiments. C, control cells.

or GnRH-ANT were then collected and transferred to the migration and invasion assays. Fig. 6 (panel A) shows that PCa cells responded to both the GnRH analogues decreasing their migration through membranes coated with laminin in a Boyden chamber analysis. In particular, GnRH-A exposure significantly reduced the migratory activity of DU145 and PC3 from 100% down to $58\pm6\%$ and $42\pm4\%$ respectively, while GnRH-ANT significantly inhibited the two PCa cells from 100% down to $55\pm5\%$ and $53\pm8\%$, respectively (Fig. 6, panel A).

The effects of GnRH analogues have then been evaluated on the ability of DU145 and PC3 cells to invade a Matrigel matrix. PCa pre-treated cells (4 days) with GnRH-A (10⁻⁶ M) or GnRH-ANT (10⁻⁶ M) were transferred to Matrigel invasion chambers. Fig. 6 (panel B) indicates that the GnRH-A exposure significantly reduced the invasiveness of both DU145 (from 100% down to $31\pm6\%$) and PC3 (from 100% down to $58\pm7\%$) sublines through the Matrigel barrier. Also GnRH-ANT (10⁻⁶ M) significantly inhibited the invasiveness of DU145 ($28\pm5\%$) and PC3 ($57\pm6\%$) cells.

Discussion

The present study shows that the treatment with both the GnRH agonist Leuprolide (GnRH-A), and the GnRH antagonist Cetrorelix (GnRH-ANT), inhibits, as expected, the proliferation of the androgen-independent PCa cells DU145 and PC3; moreover, the treatment with both GnRH-A and GnRH-ANT decreases the expression and the activity of uPA and, at the same time, increases PAI-1 in the conditioned media of DU145 and PC3 cells. Both the GnRH analogues reduce the metastatic potential of PCa cell lines.

The GnRH-ANT exerts a marked antiproliferative activity on PCa cell lines, indicating that this compound might behave as antagonist at pituitary level and as agonist at the level of the tumour. In addition, when administered simultaneously, GnRH-ANT did not antagonise the inhibitory action of GnRH-A and no summation or potentiation of the inhibitory effect exerted separately by each compound was observed in PCa cells (data not shown), as already reported previously (23). The mechanisms by which GnRH-ANT produces the same antiproliferative effect than the agonist, observed in a number of human malignant tumours such as breast, ovary and endometrium (24), however, remain unknown. It might be related to different molecular characteristics between the GnRH present at pituitary level, where the receptor is coupled to the Gq/11-PLC intracellular system of events (9,10), and that expressed at the tumour

level, where the receptor is coupled to the Gi protein (25,26). A second plausible explanation might lie in the possible, but not yet detected, existence of a GnRH type II receptor in PCa tissues; this second hypothesis is proposed by Gründker *et al* (24) for endometrial and ovarian cancers. However, further studies are needed to clarify this issue in PCa cells.

The novel finding of this study, observed to our knowledge for the first time in PCa cells, resides in the inhibitory effect exerted by both GnRH agonists and antagonists on the activity of the PA system, one of the most important protease networks controlling degradation of ECM matrix (12,13).

We observed that both GnRH-A and GnRH-ANT significantly decrease the secretion as well as the enzymatic activity of the serino-protease uPA in the conditioned media of androgen-independent PCa cells. Concomitantly, we demonstrated that both the GnRH analogues induce a marked increase of the protein levels of PAI-1, the inhibitor that inactivates uPA through the formation of a complex (15). It is well known that high uPA levels indicate a poorer prognosis (27) and that the inhibition of uPA and/or of the uPA/uPAR interaction prevents or reduces metastasis (15,16). Little information is available on the interactions between the GnRH analogues and the uPA system in cancer cells. McDonnell and Murdoch (28) described that GnRH did not affect the secretion of uPA by SKOV-3 ovarian cancer cells; however, this discrepancy might depend on the experimental conditions adopted (native GnRH vs GnRH analogues, type of cancer cell lines utilized, etc.).

The activation of the PA system is also known to promote diverse processes that require cell migration *in vivo*, which is a prerequisite for invasion and metastasis. The present observation that GnRH analogues, besides modulating the PA system activity, antagonise the metastatic behaviour of both DU145 and PC3 cells by decreasing their capacity to invade a reconstituted matrix and to migrate in response to a haptotactic stimulus gives support to the involvement of the PA system on the anti-metastatic effect of these drugs. Yates and colleagues (29) have shown that, in DU145 cells, Cetrorelix increases the cell-cell adhesion complex molecules E-cadherin, α and β catenin and p120 through a down-regulation of the EGFR signalling. In addition, the interaction between GnRH and PA system has already been reported in other experimental models: GnRH modulates the invasive capacity of human trophoblastic cells and human decidual stromal cells through the control of the activity of uPA and PAI-1 (30,31).

To date, the mechanism(s) by which GnRH analogues regulate uPA/PAI-1 expression has not been determined. One possible mechanism is through the GnRH-mediated increase of crucial transcription factor such as AP-1 (32). Multiple AP-1 binding sites have been detected in the promoter regions of both the uPA and PAI-1 human genes (33). The activation of transcription factors such as AP-1 might be induced by mitogen-activated protein kinases (MAPKs). Preliminary experiments performed in our laboratory showed that both GnRH-A and GnRH-ANT induce the phosphorylation of ERK1/2 in DU145 and PC3 cells; this effect seems to be specific, because it is prevented by a pre-treatment with the MAPK/ERK kinase inhibitor PD98059 (Piccolella *et al*, unpublished data). The phosphorylation of ERK1/2 might be

responsible for the activation of several nuclear transcription factors involved in cellular proliferation, migration and invasion processes. To this regard, GnRH agonists are reported to induce apoptosis through both JNK and ERK MAPKs in the nucleus of DU145 cells (34). Recently, Kim and co-workers suggest that the activation of ERK1/2 by GnRH-II subsequently phosphorylates Elk-1 as a down-stream pathway in ovarian cancer cells (35).

In conclusion, the present study indicates that both GnRH agonists and antagonists induce, in addition to their anti-proliferative effect, an inhibition of the activity and efficacy of the PA system in PCa cells. These effects are obtained through the inhibition of the production and the activity of uPA concomitantly with the increase of its inhibitor, PAI-1. The two GnRH analogues are also able to decrease the migratory capabilities and the invasiveness of the PCa cells.

The fact that both GnRH agonists and antagonists are effective in inhibiting the production of proteases controlling the degradation of cell-cell and cell-matrix interactions in some steroid-unresponsive cancer cell lines might provide a rational basis for the development of clinical strategies for those tumours that progress towards an androgen-independent condition characterized by a high metastatic potential.

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