Rapid Suppression of Plasma Testosterone Levels and Tumor Growth in the Dunning Rat Model Treated with Degarelix, a New Gonadotropin-Releasing Hormone Antagonist

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ABSTRACT

Degarelix (FE 200486) is a member of a new class of water-soluble (<50 mg/ml) gonadotropin-releasing hormone (GnRH) antagonists in clinical development for prostate cancer. Upon subcutaneous administration, degarelix forms a gel that results in a sustained release of the compound into the circulation, immediately blocking GnRH receptors in the pituitary and inducing a fast and sustained suppression of gonadotrophin secretion in rats and primates. One of the few animal models of prostate adenocarcinoma is the Dunning R-3327H rat carcinoma transplanted into Copenhagen rats. The growth of the Dunning tumor can be inhibited by various treatments reported to be effective in the clinic, such as GnRH superagonists, antiandrogens, 5-alpha-reductase inhibitors, tyrosine kinase inhibitors, and surgical castration. We report in this study that degarelix produces a fast and sustained suppression of the pituitary gonadal axis in rats and a similar inhibition of tumor growth compared with surgical castration in the Dunning R-3327H rat carcinoma model. First, degarelix has been compared with D-Trp6-luteinizing hormone-releasing hormone and surgical castration on a short-term study (2 months); and second, degarelix has been compared with leuprolide and surgical castration on a long-term study (12 months). In both studies, degarelix demonstrated a sustained inhibition of tumor growth at least comparable with surgical castration. These data provide a convincing profile of degarelix as a potential candidate for the clinical management of sex steroid-dependent pathologies, such as prostate cancer, where long-term reversible chemical castration is required.

Prostate cancer is a leading cause of mortality and morbidity of men in the industrialized world, second only to lung cancer. In these countries, where the incidence is highest, a man has a 10% chance of developing prostate cancer and a 3 to 4% chance of dying from the disease (Cook and Sheridan, 2000). In the European Community and in the United States, where both the prevalence and incidence of prostate cancer are increasing, more than 500,000 new cases are diagnosed each year, with an estimated 50,000 deaths (Kirkels and Rietbergen, 1997; Jemal et al., 2004). The number of patients with prostate cancer is expected to increase steadily over the next decade, because the average age of men is increasing and the incidence of prostate cancer increases more rapidly with age than the incidence of other cancers (Jemal et al., 2004).

A widely recognized feature of prostate cancer is its high sensitivity to testosterone deprivation. The current medical approach in the treatment of androgen-dependent prostate tumors is to suppress testosterone production using agonist analogs of the hypothalamic gonadotrophin-releasing hormone (GnRH, also known as LHRH) that control the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary gland. Chronic administration of GnRH superagonists eventually leads to a desensitization of pituitary GnRH receptors and a corresponding inhibition of gonadotrophin and testosterone secretion (Rivier et al., 1978; Nett et al., 1981; Heber et al., 1982). However, a major drawback of agonist therapy is that it initially stimulates gonadotrophin and testosterone secretion, resulting in a surge in LH and testosterone levels for 2 to 3 weeks before castration is completed and a subsequent delay in therapeutic benefit. This temporary increase in testosterone levels can exacerbate the hormone-sensitive cancer, as well as patient symptoms, such as sudden paraplegia due to spinal cord compression by epidural metastases, ex-
acervation of bone pain, and urinary retention (Kahan et al., 1984). Therefore, antiandrogens are often coadministered in the initial phase of GnRH receptor agonist treatment to minimize the stimulatory effects of the testosterone surge on the prostate cancer cells (Dupont et al., 1988). Furthermore, “depot” injections may result in small bursts of androgens (acute on chronic phenomenon), which may overstimulate growth of tumors that have adapted (through gene mutations) to low levels of androgens by overactivity of androgen receptors and associated transcription factors (Langel et al., 1993; Montgomery et al., 2001; Suzuki et al., 2003).

In the search for alternatives to superagonist therapy, researchers have been developing GnRH antagonists that suppress the release of gonadotrophins by binding competitively to pituitary GnRH receptors. GnRH antagonists do not induce the LH and testosterone surge; instead, they work directly to reduce the release of gonadotrophin and testosterone within hours. Despite the advantages of GnRH antagonists over agonists in suppressing serum gonadal steroids, many of the peptide GnRH antagonists investigated so far show histamine-releasing properties and/or solubility limitations that affect their clinical usefulness or even preclude their development as drugs (Hocart et al., 1987; Flouret et al., 1992; Bagatell et al., 1993, 1995).

Degarelix (FE 200486) is a member of a new class of water-soluble (>50 mg/ml) GnRH antagonists forming a subcutaneous gel that has only very weak histamine-releasing potential compared with other GnRH antagonists (Broqua et al., 2002; Schwach et al., 2003, 2004; Tornoe et al., 2004). When administered subcutaneously in rats and primates, degarelix immediately blocks GnRH receptors in the pituitary, resulting in fast and sustained suppression of gonadotrophin secretion with the absence of initial stimulation of the gonadotrophic axis (Broqua et al., 2002).

The present study compares the pharmacological activity of a new GnRH antagonist, degarelix, to two widely and currently used GnRH superagonists (D-Trp6-LHRH and leuprolide) in an experimental model of prostate adenocarcinoma in both short- and long-term studies. The Dunning R-3327H rat carcinoma transplanted into Copenhagen rats (Block et al., 1977; Claflin et al., 1977; Redding and Schally, 1981) is of dorsolateral prostatic origin, developed spontaneously in an aged animal, has histological and biochemical similarities to the human prostatic carcinoma, and is androgen-dependent (Hierowski et al., 1983; Daehlin and Damber, 1986; Daehlin et al., 1987). The growth of the Dunning tumor can be inhibited by various treatments reported to be effective in the clinic, such as GnRH superagonists, antiandrogens, 5-αphareductase inhibitors, tyrosine kinase inhibitors, and surgical castration (Ichikawa et al., 1988; Pinski et al., 1994; Pressnell et al., 1998; George et al., 1999; Tennant et al., 2000).

Blockage of the GnRH receptor by GnRH antagonists produces a fast, profound, and sustained suppression of gonadotrophin release and, therefore, testosterone secretion in human males (Jockenhovel et al., 1988; Salameh et al., 1991; Bagatell et al., 1993, 1995; Klingmuller et al., 1993; Pinski et al., 1994). Consequently, antagonists such as degarelix have been recognized as potential drugs for the management of sex steroid-dependent pathologies, such as prostate cancer.

Materials and Methods

**Animals.** In vivo studies were performed by Oncodesign (I’Arbresle, France). Male Copenhagen/Hsd rats, 7 to 8 weeks old and weighing 100 to 125 g, were obtained from Harlan (Indianapolis, IN). Animals were observed for 7 days in a specific-pathogen-free animal care unit before tumor implantation. The animal care unit (Institut National de la Recherche Agronomique, Dijon, France) is authorized by the French Ministry of Agriculture and Research (Agreement No. A21100). All animal experiments were performed according to ethical guidelines of animal experimentation. Animals were maintained in rooms under controlled conditions of temperature (25 ± 1°C, humidity (55 ± 1%), photoperiod (12-h light/12-h dark), and air exchange. The experimentation room is maintained at a positive pressure to prevent outside contamination into the rat colony. Animals were housed in polycarbonate cages (UAR, Epinay sur Orge, France) with sterile wood shavings (UAR) as bedding, which were replaced twice a week. Diet (controlled and sterilized granules; AO4) was purchased from UAR. Food and water were provided ad libitum. Water bottles were cleaned, sterilized, and replaced once a week. The water was sterilized by filtration using 0.2-μm absolute filters (Millipore Corporation, Billerica, MA).

**Tumor Preparation.** Cryopreserved R-3327H tumor trocar pieces were obtained from Dr. J. T. Isaacs (John Hopkins Oncology Center, Baltimore, MD), and they were kept in liquid nitrogen. Before implantation, the trocar pieces were thawed by immersing the vial in a 37°C water bath, and tumors were washed in RPMI 1640 medium. Surgical operations were performed under isoflurane anesthesia. The hairs from the flank region of rats were shaved, and a skin incision was made. Fresh tumor trocar pieces of R-3327H tumor were serially implanted subcutaneously into the right flank of the rats (one tumor/rat). Before implantation, the mean weight of 10 tumor fragments was 48.5 ± 2.6 mg.

**Test Substance.** Degarelix [N-acetyl-3-(naphtalen-2-yl)-D-alanyl-4-chloro-D-phenylalan-3-yl-(pyridin-3-yl)-D-alanyl-l-seryl-4-[[4-(4-(carbamoylamino)-D-phenylalanyl-L-leucyl-N-[(1-methylthyl)-L-lysyl-l-prolyl-D-alaninamide), FE 200486 acetate salt (batch no. 2010-042-01; mol. wt., 1632.3; peptide content, 88.2%; purity, 98.8%) was supplied by Ferring Research Institute (San Diego, CA) as a white powder. It was stored at −20°C. The manipulation of compound was performed under laminar flow conditions. The vehicle of degarelix was 5% mannitol diluted in sterile distilled water. The degarelix solutions were prepared just before administration to animals.

Leuprolide-depot (Enantone; batch no. S306) was supplied by Oncodesign from Takeda (Puteaux, France). The vehicle for leuprolide was composed of cellulose and D-mannitol diluted in distilled water just before administration to rats.

**Treatment Doses and Regimens.** Treatments started at D0 when the tumor size reached approximately 300 mm³. Before treatment, the mean tumor size of each group at randomization was not statistically different. For the short-term study, d-Trp⁶-LHRH was administered subcutaneously at 0.5 mg base/kg, daily for 62 days (n = 10). At this dose, d-Trp⁶-LHRH induced a complete suppression of testosterone after the flare-up period to levels published in the literature (Redding and Schally, 1981, 1990; Schally et al., 1986). Degarelix was administered subcutaneously at 1.0 mg base/kg, monthly (n = 10). The pharmacological profile of degarelix has been described recently (Broqua et al., 2002; Agero et al., 2003). At this dose, degarelix induced complete and rapid suppression of testosterone. One control group received a monthly subcutaneous injection of 5% mannitol (n = 10), and another control group was castrated and injected subcutaneously with 5% mannitol once a month (n = 10).
The blood samples were collected at D0, D1, D2, D3, D4, D5, D6, D7, D10, D14, D21, D28, D35, D42, D49, and D62. For the group treated with D-Trp^6-LHRH, blood samples were also taken 2 h after treatment. For the long-term study, leuprolide-depot was administered subcutaneously at 1.5 mg base/kg, every 3 weeks until day 288 (n = 11). At this dose, leuprolide-depot induced a complete suppression of testosterone after the flare-up period to levels published in the literature (Okada et al., 1991). Degarelix was administered subcutaneously at 1.0 mg base/kg, monthly (n = 11) (Broqua et al., 2002; Agero et al., 2003). One control group received a monthly subcutaneous injection of 5% mannitol (n = 11), and another control group was castrated and injected subcutaneously with 5% mannitol once a month (n = 11). Blood samples were collected at D0, D2, and every month thereafter until the end of the study (D343).

Blood Sample Collection and Testosterone Assay. Blood samples (approximately 500 μl) were taken 2 h after treatment from the caudal vein into tubes containing lithium heparin and immediately centrifuged at 1000g for 10 min at 4°C to obtain plasma samples. The samples were collected in propylene tubes and stored at −80°C. Plasma testosterone was assayed by radioimmunoassay following the manufacturer’s instructions (Diagnostic System Laboratories, Webster, TX).

Castration. A 0.8-cm incision was made transversally in the abdomen, just above the pubis. The anterior abdominal muscles and skin were incised, and the testes were delivered into the surgical field and removed from animals. The incisions were closed separately using Dexon needle and suture thread (reference 58419; Sherwood, Bondoufle, France).

Sacrifice of Animals. The animals were sacrificed under anesthesia with isoflurane by cervical dislocation. The R-3327H tumors, seminal vesicles, prostates, and testes of each rat were removed, cleaned, and weighed. Animals reaching the maximal tumor size defined by the welfare of the animal were sacrificed.

Tumor Volume Determination. The length and width of the tumor were measured once a week with calipers, and the volume of the tumor was estimated by the formula (length × width^2)/2.

### Statistical Analysis.
The statistical tests were performed using StatView software (Abacus Concepts, Berkeley, CA). The following parameters were compared: the plasma testosterone level at each sampling time; the mean body weight change; the weight of tumors, seminal vesicles, testes, and prostates at the end of the experiment; and the tumor volumes on each day of measurement. Statistical analysis of the treatment was performed using an analysis of variance followed by Bonferroni/Dunn multiple comparison procedures or by unpaired t-tests. A p value < 0.05 was considered significant.

### Results

#### Short-Term Effects of Degarelix, D-Trp^6-LHRH, and Surgical Castration on the Growth of the Dunning R-3327H

**Effects of Degarelix and D-Trp^6-LHRH on Plasma Testosterone Levels.** In rats treated with 0.5 mg base/kg, daily, D-Trp^6-LHRH (Fig. 1), testosterone levels were initially raised to more than 20 ng/ml (flare-up effect). Testosterone levels then decreased to a low level at D5 (80 pg/ml) and thereon slowly to castrate levels at D28. Thereafter, castration levels (below detection limit (<25 pg/ml) were maintained to the end of the study (D62). In rats treated with 1 mg/kg monthly degarelix, the initial flare-up was not observed; moreover, plasma testosterone quickly reached castrated levels at D3 (below detection limit (<25 pg/ml)) and remained at this level throughout the time course of the experiment, with a pattern similar to castration. In control animals, testosterone level is within normal range (Fig. 1).

**Effects of Degarelix and D-Trp^6-LHRH on Tissues Weights.** Testes weights at the end of the study (D62) represent an integrated measure of the pituitary-gonadal axis activity over time. Testes weights was significantly lower (p < 0.01) in rats treated with degarelix (410 ± 40 mg) compared with rats treated with D-Trp^6-LHRH (900 ± 120 mg). Control animals showed normal testes weights (2650 ± 250 mg) compared with the two treated groups (Table 1).

However, no statistically relevant differences were observed for the weight of the seminal vesicles and prostates of the animals treated with D-Trp^6-LHRH or degarelix (40 ± 10 and 30 ± 20 mg, respectively) (Table 1). Prostates from castrated animals were too small to be weighted accurately.

**Effects of Degarelix, D-Trp^6-LHRH, and Surgical Castration on Tumor Volume.** Degarelix and surgical castration produced an immediate suppression of tumor growth that lasted throughout the study period (Fig. 2). Tumor volumes in rats treated with degarelix were significantly smaller than those in control rats at D21. This difference lasted until the end of the study at D62. For the D-Trp^6-LHRH group, there is no apparent suppression of the tumor.
growth until D38 compared with control animals. A significant difference \((p < 0.01)\) between \(\text{d-Trp}^6\)-LHRH-treated rats and controls occurred at D49, and it lasted until the end of the study at D62 (Fig. 2; Table 1).

**Effects of Degarelix, \(\text{d-Trp}^6\)-LHRH, and Surgical Castration on Tumor Weight.** At the end of the study (D62), tumors were excised and weighed. Tumors in the degarelix-treated and surgically castrated groups had similar weights (310 ± 150 and 370 ± 120 mg, respectively). Tumors in the degarelix-treated group were significantly smaller \((p < 0.01)\) than tumors in the \(\text{d-Trp}^6\)-LHRH-treated group (370 ± 120 versus 1340 ± 750 mg) (Table 1).

**Long-Term Effects of Degarelix, Leuprolide-Depot, and Surgical Castration on the Growth of the Dunning R-3327H**

**Effects of Degarelix, Surgical Castration, and Leuprolide-Depot on Plasma Testosterone Levels.** Degarelix suppressed testosterone to castration levels within 2 days of treatment initiation, and it maintained castration levels throughout the course of the study. Onset of testosterone suppression by leuprolide-depot was delayed after the flare-up period, with castration levels achieved within a month. Thereafter, testosterone remained suppressed until treatment was stopped. Control animals stayed within normal range \((\sim 2 \text{ ng/ml})\) throughout the study (Fig. 3). Data from animals of the control and leuprolide-depot groups were discarded after 223 days (animals reaching the maximal size of the tumor were sacrificed). However, data from castrated and degarelix-treated groups were continued until the end of the study (D343).

**Effects of Degarelix, Surgical Castration, and Leuprolide-Depot on Tumor Volume.** Degarelix profoundly suppressed tumor growth immediately after administration and with an efficacy similar to surgical castration. In contrast, similar to the short-term study during the first month, there was no obvious suppression of tumor growth for the leuprolide-depot compared with the control group (Fig. 4). Moreover, escape from androgen deprivation occurred sooner in rats treated with leuprolide-depot. Data from control groups or those treated with leuprolide-depot were discarded after D223 due to low animal numbers remaining (animals reaching the maximal size of the tumor were sacrificed; Table 2). At D223, the mean tumor volume for the leuprolide-depot group \((2489 ± 1063 \text{ mm}^3)\) was higher than that for degarelix and castration groups \((666 ± 291 \text{ and } 820 ± 552 \text{ mm}^3, \text{ respectively})\). Degarelix treatment inhibited tumor growth throughout the experiment with efficacy at least identical to surgical castration until D343 \((2140 ± 1165 \text{ and } 1897 ± 1391 \text{ mm}^3, \text{ respectively})\); Fig. 4; Table 2).

**Discussion**

Our interest in developing an GnRH antagonist was stimulated by the need for improved therapeutics for the management of sex steroid-dependent pathologies, including uterine leiomyoma, endometriosis, and sex steroid-dependent cancer, such as prostate cancer. Prostate cancer is currently managed with long-acting (1- or 3-month) formulations of GnRH superagonists that initially stimulate pituitary LH and FSH release as well as gonadal steroids; and then, after 2 to 4 weeks, they desensitize the gonadotrophin cells, leading to suppression of gonadotrophin and sex steroid secretion. An alternative pharmacological approach is the use a GnRH antagonist that immediately blocks GnRH
Effects of degarelix (1 mg/kg s.c., monthly), leuprolide-depot (1.5 mg/kg s.c., every 3 weeks), and surgical castration on tumor volume in rats bearing a Dunning R-3327H prostate tumor. Results are the mean ± S.D. of 10 to 11 rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 0</th>
<th>Day 223</th>
<th>Day 343</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of Animals</td>
<td>Tumor Volume</td>
<td>No. of Animals</td>
</tr>
<tr>
<td>5% Mannitol</td>
<td>11</td>
<td>247 ± 61</td>
<td>5</td>
</tr>
<tr>
<td>Castration</td>
<td>11</td>
<td>260 ± 71</td>
<td>11</td>
</tr>
<tr>
<td>Leuprolide</td>
<td>11</td>
<td>277 ± 87</td>
<td>7</td>
</tr>
<tr>
<td>Degarelix</td>
<td>10</td>
<td>257 ± 76</td>
<td>10</td>
</tr>
</tbody>
</table>

**p < 0.01 versus 5% mannitol.
††p < 0.01 versus D-Trp6-LHRH.
*Animals reaching the maximal size of the tumor were sacrificed.
One animal died before the start of the treatment.
One animal was sacrificed before the end of the study.

In conclusion, degarelix is a new GnRH antagonist immediately blocking GnRH receptors in the pituitary, resulting in a fast and sustained suppression of gonadotrophin secretion and allowing an immediate inhibition of tumor growth and avoiding other side effects of the flare. Such a rapid antitumor response cannot be achieved by a GnRH superagonist. In the long-term studies, superiority of degarelix over a GnRH agonist is demonstrated; escape to androgen independence occurred sooner in animals treated with the GnRH superagonist leuprolide. These data provide a convincing profile of degarelix (GnRH antagonist) as a more effective GnRH blocker than the superagonists to inhibit the growth of the Dunning tumor, possibly due a better short-term and long-term testosterone control, and they provide a convincing profile of degarelix as a potential candidate for the clinical management of sex steroid-dependent pathologies where long-term chemical castration is required.

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Receptors in the pituitary, resulting in fast and sustained suppression of gonadotrophin secretion together with a tight control over the testosterone level (below castration level), which could translate into significant clinical advantages. In the first study, we investigated the short-term effects of degarelix on tumor growth over a 2-month period. We have demonstrated that suppression of the pituitary-gonadal axis occurred sooner in rats treated with degarelix than in rats treated with D-Trp6-LHRH as evidenced by the shorter time to castration levels of testosterone. Differences in the mechanisms of pituitary-gonadal suppression produced by GnRH superagonists and GnRH antagonists can explain this different onset of action. D-Trp6-LHRH will first stimulate LH release (flare-up effect) from the pituitary and then testosterone secretion from the testes before suppressing the pituitary-gonadal axis by a "down-regulation" mechanism. In contrast, degarelix will immediately bind to the pituitary GnRH receptor, block it, and preclude substantial occupation and stimulation by endogenous GnRH and, therefore, the release of LH/FSH from the pituitary. The flare of testosterone initially produced by D-Trp6-LHRH translates into a significant delay in onset of tumor inhibition compared with degarelix and surgical castration. The data are consistent with a correlation between the time to castrate and the time to suppress the tumor growth. Over a 2-month period, this delay has significant consequences on the overall antitumor efficacy as indicated by the measurement of tumor weight. More importantly, in the second study (long-term effect), tumor growth started to escape androgen deprivation after approximately 5 months of treatment with leuprolide-depot.

In contrast, degarelix induced a rapid suppression of plasma testosterone levels and sustained inhibition of tumor growth over the whole study period. Two hypotheses can be proposed to explain the premature tumor growth from androgen deprivation in rats treated with leuprolide-depot. First, there are reports suggesting that low concentrations of GnRH superagonists can stimulate the proliferation of human prostate cancer cell lines by acting through tumor GnRH receptors (Qayum et al., 1990; Bahk et al., 1998; Limonta et al., 1999). The Dunning tumor expresses GnRH receptors; it is therefore possible that long-term exposure to leuprolide may stimulate proliferative pathways or promote adaptation to androgen-deprivation. Second, it has been suggested that escape from androgen deprivation occurs when the tumor has adapted to low levels of androgens. In tumors escaping from androgen deprivation, androgen receptors and downstream pathways for control of androgen-dependent gene expression are still functional. Escape from androgen deprivation could result from mutation of androgen receptors leading to ligand-independent activation, or from supersensitivity of the androgen receptors and/or activation of alternative transcription factors. In that situation even very low levels of androgens will be sufficient to maintain tumor survival and expansion.

Tier 2
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