Fispemifene [Z-2-{2-[4-(4-Chloro-1,2-diphenylbut-1-enyl)phenoxy]ethoxy}-ethanol], a Novel Selective Estrogen Receptor Modulator, attenuates glandular inflammation in an animal model of chronic nonbacterial prostatitis

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ABSTRACT

The anti-inflammatory and antiestrogenic action of fispemifene [Z-2-{2-[4-(4-chloro-1,2-diphenylbut-1-enyl)phenoxy]ethoxy}-ethanol], a novel selective estrogen receptor modulator (SERM), was tested on the Noble rat model of chronic nonbacterial prostatic inflammation with cellular composition and inflammation patterns similar to those described in human prostatitis. Inflammation was assessed by counting perivascular and stromal infiltrates and the number of inflamed acini. Furthermore, the aggressiveness of inflammation was assessed on the basis of the relation of lymphocytes to the acinar epithelium. The immunohistochemical expression of progesterone receptor (PR) and Fos-related antigen 2 (Fra2), prolactin concentration in serum, and the weights of the seminal vesicles and pituitary glands were used as endpoints of estrogen action. Fispemifene significantly attenuated the glandular form of inflammation induced in the dorsolateral prostatic lobes (DLP) in the hormonal milieu of the decreased androgen/estrogen ratio. The anti-inflammatory action was seen in the decreased number of acini containing intraluminal neutrophils. As signs of antiestrogenic action, fispemifene blocked estrogen-induced expression of PR and Fra2 in the acinar epithelium of the DLP, and it decreased prolactin concentration in serum and the relative weights of the seminal vesicles and pituitary glands. Because fispemifene exhibited both antiestrogenic and anti-inflammatory action in the prostate, this experimental study suggests that SERMs could be considered as a new therapeutic option in the treatment and prevention of prostatic inflammation.

Nonbacterial prostatic inflammation after estradiol administration has been described in rat (Naslund et al., 1988; Robinette, 1988; Vykhovanets et al., 2006) and mouse (Bianco et al., 2002). Lymphocyte-predominant infiltrates and the inflammation pattern (perivascular, stromal/periglandular, and glandular) seen in animal studies are similar to those described in human chronic nonbacterial prostatitis (Nickel et al., 2001; Vykhovanets et al., 2007). As evidence for its autoimmune nature, estradiol-induced prostatic inflammation can be transferred to a naïve syngenic recipient by the adoptive transfer of CD3+ T cells (Keetch et al., 1994; Motrich et al., 2007). The autoimmune prostatitis leads to the development of pelvic pain, which is a distinguishing symptom of chronic prostatitis/chronic pelvic pain syndrome (Rudick et al., 2008). In testosterone-maintained Noble rats, the number of inflammatory infiltrates increased when the estradiol dose was increased and was decreased with an antiestrogen (ICI 182,780) treatment (Bernoulli et al., 2007). In addition to estrogen, androgen is needed for the induction of prostatic inflammation (Leav et al., 1989). Estradiol administration alone in castrated rats had no proinflammatory effects. Androgens were proinflammatory when administered at hypoandrogenic doses (judged on the basis of seminal vesicle weight gain) to estrogen-treated rats. Hyperandrogenic doses were required for anti-inflammatory action (E. Yatkin, J. Bernoulli, E.-M. Talvitie, and R. Santti, unpublished).

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ABBREVIATIONS: fispemifene, Z-2-{2-[4-chloro-1,2-diphenylbut-1-enyl]phenoxy}ethoxy)-ethanol; SERM, selective estrogen receptor modulator; T, testosterone; DHT, dihydrotestosterone; E2, 17β-estradiol; ER, estrogen receptor; Fis, fispemifene; H&E, hematoxylin and eosin; DLP, dorsolateral prostatic lobe(s); MPO, myeloperoxidase; PBS, phosphate-buffered saline; PR, progesterone receptor; Fra2, Fos-related antigen 2; Cast, castration; ICI 182,780, 7α-[9(4,4,5,5,5-pentafluoropentyl)sulfanyl]nonyl]-estra-1,3,5(10)-triene-3,17β-diol.
lished data). This implies that testosterone substitution in doses that may yield useful preventive effects on the prostatic inflammation may subject the sex accessory glands to more intense androgenic stimulation than is normal for the male. Thus, testosterone, an aromatizable androgen, may not be optimal for the prevention and treatment of estrogen-related prostatic inflammation.

The aim of the present study was to test the anti-inflammatory and antiestrogenic action of fispemifene, a novel selective estrogen receptor modulator (SERM), in the Noble rat model of chronic nonbacterial prostatic inflammation (Bernoulli et al., 2007). Fispemifene acts via estrogen receptors and has tissue-selective estrogenic and/or antiestrogenic effects (L Kangas, J Bernoulli, P Härkönen, L Kallio, M Peräälä, J Reponen, R Santti, M Unkila, K Väänänen, E Yatkin, et al., unpublished data). The anti-inflammatory action of fispemifene was evaluated and compared with that of tamoxifen. Tamoxifen is the most widely studied estrogen antagonist in the male and has been shown to be anti-inflammatory in human and animal autoimmune diseases (Ackerman, 2006).

### Materials and Methods

#### Experimental Animals and Surgical Procedures

Adult male Noble rats (NBL/Cr) were obtained from Charles River Laboratories, Inc. (Wilmington, MA). All animals were housed (two rats per cage) under a 12-h light/12-h dark lighting cycle. They were handled in accordance with the institutional animal care policies of the University of Turku.

At the age of 10 to 12 weeks, the Noble rats in experiments I and II were castrated via the scrotal route by removing the epididymal fat pads with testes under general anesthesia. The mean body weight was between 290 and 330 g at the time of castration. Testosterone (T), dihydrotestosterone (DHT), and estradiol (E2) implants were used in the hormone-treated animals and the placebo animals per cage) under a 12-h light/12-h dark lighting cycle. They were handled in accordance with the institutional animal care policies of the University of Turku.

### Test Compounds

Fispemifene (synthesized by Hormos Medical, Oulu, Finland) is a novel SERM. Fispemifene binds to both ERα and ERβ receptors and has antiestrogenic or estrogenic actions or both, depending on the target cells and tissues. Fispemifene has either no or only slight effects on the safety pharmacology parameters studied, and it has been well tolerated in all in vivo toxicological studies performed (L. Kangas, J. Bernoulli, P. Härkönen, L. Kallio, M. Peräälä, J. Reponen, R. Santti, M. Unkila, K. Väänänen, E. Yatkin, et al., unpublished data). Fispemifene and tamoxifen (Sigma-Aldrich, St. Louis, MO) were dissolved in corn oil (Fluka, Buchs, Switzerland) and administered daily by gavage. Fispemifene (Fis) was given in three different doses (3, 10, and 30 mg/kg/day). The dose of tamoxifen was 1 mg/kg/day (Fitts et al., 2004). The control animals received an equal volume of the vehicle (corn oil).

#### Treatment Protocols for Studies I to V

**I: Antiestrogenicity Study: 3-Week Study of Castrated Rats Treated with E2 (n = 48).** In the first 3-week study, the pellets released 5 μg/day E2 (0.1 mg/21-day releasing pellet). E2- and placebo-implanted castrated rats were divided into four groups (n = 6 in each group), which were treated with different doses (3, 10, or 30 mg/kg) of the test compound, fispemifene, or with vehicle alone for 3 weeks. Thereafter, the animals were sacrificed, and serum and tissue samples were removed for further analysis (for studies I to V, see Table 1).

**II: Short-Term Anti-Inflammatory Study: 3-Week Study of Castrated Rats Treated with DHT + E2 (n = 70).** In the second 3-week study, implants released 70 μg/day E2 (1.5 mg/21-day releasing implants) and 140 μg/day DHT (3 mg/21-day releasing implants). Castrated Noble rats were divided into 10 groups (n = 7 in each group). The rats were treated with DHT and either with E2 or the test compounds fispemifene (3, 10, or 30 mg/kg) or tamoxifen (1 mg/kg) or with vehicle alone for 3 weeks.

In a previous study, this treatment was shown to result in the maximum number of perivascular and stromal inflammatory infiltrates within 3 weeks (L. Kangas, J. Bernoulli, P. Härkönen, L. Kallio, M. Peräälä, J. Reponen, R. Santti, M. Unkila, K. Väänänen, E. Yatkin, et al., unpublished data). The weight increase of the seminal vesicles indicated hypostrogenic stimulation.

**III: Establishment of the Inflammation for Long-Term Studies: 13-Week Study of Intact Rats Treated with T + E2 (n = 7).** The daily released amounts of E2 and T were 83 and 830 μg/day, respectively (5 mg/60-day releasing implant and 50 mg/60-day releasing implant). The pellets were replaced by new pellets on day 45 of the experiment.

**IV: Long-Term Anti-Inflammatory Study: 18-Week Study of Intact Rats Treated with T + E2 (n = 22).** The daily released amounts of E2 and T were 83 and 830 μg/day, respectively (5 mg/60-day releasing implant and 50 mg/60-day releasing implant). The pellets were replaced on days 45 and 90 of the treatment by new ones to ensure constant hormone release (in total, replaced twice during 18-week study; total length, 45 + 45 + 36 days). After treatment for 13 weeks, E2- and T-treated animals were divided into two groups. Fispemifene (30 mg/kg) was administered daily by gavage to the rats (n = 11) for 5 more weeks (T + E2 + Fis). Control animals (T + E2 + oil) received an equal volume of vehicle (corn oil) (n = 11).

### Table 1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Intact/Castrated</th>
<th>Treatment Time</th>
<th>E2</th>
<th>T or DHT</th>
<th>Doses of Fispemifene and Tamoxifen</th>
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<tbody>
<tr>
<td>I</td>
<td>Castrated</td>
<td>3 weeks</td>
<td>5</td>
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<td>3, 10, or 30 mg/kg Fispemifene</td>
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<tr>
<td>II</td>
<td>Castrated</td>
<td>3 weeks</td>
<td>70</td>
<td>DHT (140)</td>
<td>3, 10, or 30 mg/kg Fispemifene</td>
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<td>III</td>
<td>Intact</td>
<td>13 weeks</td>
<td>83</td>
<td>T (830)</td>
<td>Fispemifene 3 mg/kg tamoxifen</td>
</tr>
<tr>
<td>IV</td>
<td>Intact</td>
<td>18 weeks</td>
<td>83</td>
<td>T (830)</td>
<td>30 mg/kg Fispemifene</td>
</tr>
<tr>
<td>V</td>
<td>Intact</td>
<td>15 + 3 weeks</td>
<td>83</td>
<td>T (830)</td>
<td>Fispemifene 3 mg/kg tamoxifen</td>
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</table>
V: The Role of Estrogen in the Inflammation: Removal of E2 Implants after T + E2 Treatment for 15 Weeks (n = 5). Intact rats were treated with E2 (83 µg/day) and T (830 µg/day) for 15 weeks (5 mg/60-day releasing implant and 50 mg/60-day releasing implant). E2 implants were removed at 15 weeks, and T (830 µg/day) treatment was continued for 3 more weeks until the experiment was terminated at 18 weeks.

Serum Hormone Measurements
Serum samples were stored at −70°C until the measurement of hormone concentrations. To measure the concentrations of unconjugated estradiol and testosterone, samples were extracted twice with diethyl ether, and the remaining organic solution was evaporated under nitrous gas in a warm water bath. The hormones were dissolved in zero-standard serum and concentrated when necessary. Thereafter, an enzyme immunoassay for the in vitro diagnostic quantitative determination of 17β-estradiol and testosterone was conducted according to the instructions given by the manufacturer (17β-estradiol enzyme-linked immunosorbent assay for human serum and plasma, testosterone enzyme-linked immunosorbent assay for human serum and plasma; IBL, Hamburg, Germany). Serum prolactin was measured with a rat prolactin enzyme immunoassay kit without pretreatment (rat prolactin ELISA kit; Spi-Bio, Bertin Group, Montigny le Bretonneux, France). The detection ranges for 17β-estradiol, testosterone, and prolactin were 25 to 2000 pg/ml, 0.2 to 16 ng/ml, and 0.39 to 50 ng/ml, respectively. All samples were analyzed in duplicate.

Preparation of the Histological Sections
At the end of the treatment period, the animals were weighed and sacrificed by CO2 suffocation, and blood was collected by heart puncture before neck dislocation. Seminal vesicles (after removal of the seminal vesicle ducts), and pituitary gland were rapidly excised and weighed. Thereafter, an enzyme immunoassay for the in vitro diagnostic quantitative determination of 17β-estradiol and testosterone was conducted according to the instructions given by the manufacturer (17β-estradiol enzyme-linked immunosorbent assay for human serum and plasma, testosterone enzyme-linked immunosorbent assay for human serum and plasma; IBL, Hamburg, Germany). Serum prolactin was measured with a rat prolactin enzyme immunoassay kit without pretreatment (rat prolactin ELISA kit; Spi-Bio, Bertin Group, Montigny le Bretonneux, France). The detection ranges for 17β-estradiol, testosterone, and prolactin were 25 to 2000 pg/ml, 0.2 to 16 ng/ml, and 0.39 to 50 ng/ml, respectively. All samples were analyzed in duplicate.

Histopathological Assessment of the Inflammation
The paraffin-embedded urethroprostatic complex was cut in serial cross-sections (5-µm thickness) to cover the whole prostate. Fifteen to 20 sections at 200 to 500-µm intervals were obtained depending on the prostate size. Four to six representative sections that contained dorsolateral prostatic lobes (DLP) were then further selected from the serial sections for the assessment of inflammation in each animal. The sections were selected carefully to represent the DLP to their full extent at each level of cutting. In general, one section can give a good indication of the inflammation status, but additional sections were selected to improve the confidence of inflammation assessment. From each prostate section, the whole dorsolateral prostatic area was included in inflammation assessment. Sections were screened by two independent observers who were blinded to the treatment groups. A standardized histopathological classification method developed for the human prostate (Nickel et al., 2001) was used with some modifications as described previously (Bernoulli et al., 2007). The DLP of the rodents was chosen for the assessment of inflammatory response because the DLP is known to be the site of inflammation. It can be used successfully in the assessment of inflammation after hormonal treatments of 3 to 6 weeks (Bernoulli et al., 2007). In experiment II, inflammatory foci were divided into three different categories: perivascular (inflammatory cells tightly around the blood capillaries), stromal and periglandular (inflammatory cells in the stroma and in the intraepithelial space), and glandular (inflammatory cells inside the lumen of the glands) and counted in six sections. Sections were selected carefully to represent the DLP to their full extent at each level of cutting.

In experiments III to V, it was not possible to count individual perivascular and stromal infiltrates because the inflammatory cells were distributed over wider stromal areas and did not form infiltrates of high cell density with clear borders. Therefore, in these experiments, the aggressiveness of inflammation and the number of inflamed acini were determined from four sections of each animal. The aggressiveness of inflammation was analyzed according to the method developed for human prostate sections (Irani et al., 1997; Sciarra et al., 2007). In brief, inflammation was analyzed on a four-point grading scale on the basis of its relation to the epithelium. Grade 0 meant no contact between inflammatory cells and epithelium; grade 1, some contact; grade 2, periglandular infiltrates adjacent to partially destroyed epithelium; and grade 3, the number of these acini was more than 25%. The number of inflamed acini was counted for the whole DLP by using the same sections. Glandular inflammation was confirmed by MPO (neutrophil) staining, but counting was done based on H&E-stained sections.

Immunohistochemical Staining of Progesterone Receptor, Fos-Related Antigen 2, ERα, CD3+ and CD8+ Cells, Neutrophils, and Macrophages
Tissue sections were deparaffinized and rehydrated, and antigens were retrieved by incubating the sections in a microwave oven by using 10 mM sodium citrate buffer, pH 6.0, for 15 min. The sections were allowed to cool down and rinsed with PBS buffer; subsequently, the sections were incubated with primary antibodies overnight at +4°C. The following primary antibodies were used: progesterone receptor (PK; polyclonal rabbit anti-human; Dako Denmark A/S, Glostrup, Denmark), ERα (monoclonal mouse anti-human; Dako Denmark A/S), Fos-related antigen 2 (Fra2; rabbit polyclonal anti-Fra2; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), T-lymphocyte (CD3; monoclonal mouse anti-rat; Caltag Laboratories, Burlingame, CA), cytotoxic T-lymphocyte (CD8; monoclonal mouse anti-rat; Caltag Laboratories), neutrophil (MPO; polyclonal rabbit anti-human; Hyclut Biotech, Uden, The Netherlands), and macrophage (CD68, monoclonal mouse anti-rat, clone ED1; AbD Serotec, Oxford, UK). Primary antibodies were diluted in PBS/3% bovine serum albumin + 0.05% Tween as follows: PR, 1:400; ERα, 1:50; Fra2, 1:400; CD3, 1:100; CD8, 1:100; MPO, 1:400 (without Tween; normal goat serum added); and macrophages, 1:2000. The following day, the sections were washed with PBS and incubated at room temperature for 30 min with secondary antibody (horseradish peroxidase-conjugated antimouse secondary antibody for ERα, CD3, CD8 and macrophage antibodies and horseradish peroxidase-conjugated anti-rabbit secondary antibody for PK, Fra2, and MPO; DAKO EnVision Systems; Dako Denmark A/S). The slides were rinsed with PBS. Color was developed with diaminobenzidine substrate (Dako EnVision System). The sections were then slightly counterstained with Mayer’s hematoxylin, dehydrated, and mounted.

Analysis of PR and Fra2 Expression in the DLP
The number of the cells with a PR- and Fra2-positive nucleus in the DLP epithelium was analyzed by a semiquantitative grading system. Immunohistochemically stained sections were analyzed under a 10 × objective. Values from 0 to 4 were given depending on the...
number of positive nuclei. Value 0 was given when there were no cells with a positive nucleus, whereas value 4 was given when all nuclei were positive. Values 1 to 3 were given according to the number of positive cells found in the DLP. The sections were screened by two blinded independent observers.

Statistical Analysis

The Shapiro Wilk normality test was performed for normal distribution of the data. In experiments I and II, the statistical analyses were carried out using SAS software (version 9.1, SAS Institute, Cary, NC). In normally distributed data, one-way analysis of variance with Dunnett’s post hoc test was performed. If data were not normally distributed, the Kruskal-Wallis test was performed. In experiments III and IV, the analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software Inc., San Diego, CA). In normally distributed data, one-way analysis of variance and Tukey’s post hoc test were used. If the data were not normally distributed, the Kruskal-Wallis test was used. All data presented as group means ± S.D. p Values less than 0.05 were considered to be statistically significant.

Results

I: Antiestrogenicity Study: 3-Week Treatment of Castrated Rats with E2

Hormone Concentrations and Organ Weights. Estradiol concentration was below the detection limit in untreated castrated rats, and, as expected, estradiol treatment alone significantly increased serum estradiol concentration. Fispemifene had no statistically significant effect on estradiol concentration (Fig. 1A). Estradiol also increased prolactin concentration in serum. Coadministration of fispemifene with estradiol dose-dependently decreased the prolactin concentrations in serum (Fig. 1B). Fispemifene, when given alone, significantly increased the prolactin concentration at the 3 and 10 mg/kg dose levels (Fig. 1B).

Estradiol treatment alone significantly increased the weight of the seminal vesicles. This was reversed dose-dependently by fispemifene to the level of castrated controls (Fig. 1C). Fispemifene administered alone had no effect on the weight of the seminal vesicles (Fig. 1C). Estradiol treatment alone increased the weight of the pituitary gland. This was reversed dose-dependently by fispemifene to the level of castrated controls (Fig. 1D).

Immunohistochemical Staining of PR and Fra2. There were no cells with a PR- or Fra2-positive nucleus in the DLP lobes of castrated rats (Fig. 2, A and E). On occasion, a small number of Fra2-positive nuclei was found in castrated animals (Fig. 2H). When castrated animals were implanted with estradiol, the number of cells with PR- or Fra2-positive nuclei were increased as scored with values from 0 to 4 (Fig. 2, D and H). The PR- and Fra2-positive nuclei were predominantly localized in the acinar epithelium of the lateral prostate (Fig. 2, B and F). Some stromal cells in the lateral prostate were positive for both.
PR and Fra2. Fispemifene decreased the number of cells with a PR- and Fra2-positive nucleus. The highest dose of fispemifene (30 mg/kg) completely blocked PR staining (Fig. 2, C and D) and significantly decreased Fra2 staining (Fig. 2, G and H). Fispemifene itself induced no positive cells. There was no perivascular or stromal accumulation of lymphocytes in any animal treated with estradiol or fispemifene (in three different doses) or with both estradiol and fispemifene.

II: Short-Term Anti-Inflammatory Study: 3-Week Study of Castrated Rats Treated with DHT + E2

Hormone Concentrations and Organ Weights. Estradiol treatment significantly increased serum estradiol concentration in all rats above the detection limit (Fig. 3A). Fispemifene had no significant effects on estradiol concentration at any dose level. In contrast, tamoxifen significantly increased estradiol concentration compared with the control estradiol group (Fig. 3A). Estradiol increased serum prolactin concentrations. This was reversed dose-dependently by both tamoxifen and fispemifene (Fig. 3C). Fispemifene and tamoxifen administered alone had no effect on pituitary weight.

Assessment of Inflammation. The total number and distribution of inflammatory infiltrates into different categories are given in Fig. 4. Estradiol treatment in the presence of DHT increased the number of the infiltrates, which were predominantly perivascular and stromal. The infiltrates were located mostly in the lateral lobe. A small number of inflamed acini were seen after the 3-week treatment of castrated rats with DHT + E2. Fispemifene caused a slight dose-related decline in the total number of the infiltrates, but the differences between the dose groups were not statistically significant. When fispemifene was administered alone to DHT-maintained animals, there was no significant increase in the number of inflammatory infiltrates. In contrast, tamoxifen significantly increased the total number of perivascular and stromal inflammatory infiltrates and had no significant anti-inflammatory effect in the presence of estradiol (Fig. 4).

Perivascular and stromal inflammation consisted predominantly of CD3+ cells. A few CD8+ cells, neutrophils, macrophages, and mast cells were seen in the stroma. No cells with ERα- or PR-positive nuclei were seen in the acinar epithelium or interacinar stroma in any treatment group (data not shown).
III and IV: 13- and 18-Week Treatments of Intact Rats with T/H₁₁₀₀₁₁_E₂ and Removal of E₂ Implants at 15 Weeks

Hormone Concentrations and Organ Weights. Serum testosterone, estradiol, and prolactin concentrations were significantly increased at 13 and 18 weeks in hormone-treated animals compared with placebo animals (Fig. 5, A–C). Testosterone concentrations were significantly lower at 18 weeks than at 13 weeks in all groups. The relative weights of the seminal vesicles and the pituitary glands (Fig. 5, D and E) followed the testosterone and prolactin concentrations, correspondingly.

In the animals in which the estradiol implant was removed at 15 weeks for the following 3-week period (18 weeks of T/H₁₁₀₀₁₁_E₂), testosterone concentration was comparable, but estradiol and prolactin concentrations were significantly decreased compared with animals treated with T + E₂ + oil for 18 weeks (Fig. 5, A–C). Correspondingly, no changes were seen in relative seminal vesicle weights, but removal of the estradiol implant significantly decreased the relative weight of the pituitary gland (Fig. 5, D and E).

Fispemifene had no effect on testosterone or estradiol concentrations when compared with animals treated with T + E₂ + oil for 18 weeks. However, it significantly decreased prolactin concentration, which was followed by the decreased relative weight of the pituitary gland (Fig. 5, C and E). No changes were seen in relative seminal vesicle weight between the fispemifene and control groups.

Assessment of the Inflammation. The inflammation in the stroma varied from solitary infiltrates to confluent areas (Fig. 6, A–F). All animals had inflamed acini that contained mainly neutrophils (Fig. 6, C–H). Two main types of inflamed...
acini were observed; the majority consisted of acini with intact epithelium and a varying number of neutrophils in the lumen. No macrophages or CD8+ cells were present in these acini. There was no contact between inflammatory cells and epithelium (value 0 in the assessment of the aggressiveness of inflammation). In the other type of inflamed acini, the epithelium was partially destroyed and surrounded by lymphocytes (value 1 or more in the aggressiveness assessment). CD8+ cells were seen intraepithelially, and the lumina of these acini contained neutrophils, macrophages, and shedded epithelial cells (Fig. 6F). Because there were various intermediate forms of glandular inflammation types, no preferential effects on different glandular forms were detected between the fispemifene- and placebo-treated animals.

At 18 weeks, inflammation was significantly more aggressive than at 13 weeks (2.0 versus 1.4; p < 0.001), and there were significantly more inflamed acini at 18 weeks than at 13 weeks (Fig. 7). Fispemifene (30 mg/kg/day) showed only a
3 weeks
Cast + DHT + E2

Fig. 6. Representative histological sections and immunohistological stainings from 3-, 13-, and 18-week treatments. A and B, H&E- and CD3-immunostained sections from the 3-week treatment with DHT + E2 (magnification, 10×). Perivascular inflammatory infiltrates consisting of CD3+ cells were often observed in the DLP (inset magnification, 40×). C and D, H&E- and neutrophil-immunostained sections from the 13-week treatment with T + E2 (magnification, 40×). Perivascular and glandular inflammation consisting mainly neutrophils were observed especially in the lateral prostate. E and F, H&E- and neutrophil-immunostained sections from the 18-week treatment with T + E2 + oil (magnification, 40×). Longer hormone exposure induced more stromal and periglandular inflammatory infiltrates and more inflamed acini. Inset, CD68 (macrophage) staining (magnification, 40×). G and H, H&E- and neutrophil-immunostained sections from the 18-week treatment with T + E2 treatment and the last 5 weeks simultaneous oil treatment; 18 weeks T + E2 + oil, 18-week combined T + E2 treatment and the last 5 weeks simultaneous oil treatment; 18 weeks T + E2 + Fis, 18-week combined T + E2 treatment and the last 5 weeks simultaneous fispemifene (30 mg/kg/day) treatment.

Discussion

The anti-inflammatory and antiestrogenic actions of fispemifene, a novel SERM, were studied in an animal model of chronic nonbacterial prostatic inflammation. The model is well documented and based on the combined treatment of adult rats with estrogen and androgen (Robinette, 1988; Bosland et al., 1995; Thompson et al., 2002; Vykhovanets et al., 2006; Bernoulli et al., 2007). The present study showed that fispemifene decreased the number of inflamed acini but did not influence the number of perivascular and stromal inflammatory infiltrates in the DLP of the Noble rat. Fispemifene exhibited antiestrogenic action as evidenced by decreased serum prolactin concentration and pituitary weight and PR and Fra2 expression in the acinar epithelium, suggesting that the attenuation of glandular inflammation may be due to the antiestrogenicity of the compound. The amounts of estradiol and testosterone released daily from the s.c. implants were chosen to achieve an elevated estrogen concentration and a decreased androgen/estrogen ratio in serum (Bernoulli et al., 2007, 2008). The seminal vesicle and pituitary weights correspondingly followed the alterations of testosterone and estradiol concentration in serum. The estradiol concentration was higher in long-term experiments in testosterone-treated animals, even though similar estradiol implants were used in short- and long-term experiments. It is possible that the aromatization of exogenous estradiol significantly contributed to estradiol concentration.

The perivascular and stromal inflammation infiltrates developed within 3 weeks in the DLP, consisting mainly of CD3+ cells. Fispemifene had no significant effect on the estrogen-induced accumulation of T cells in the perivascular space and stroma. The dose of DHT in the 3-week experiment was chosen to induce a maximal extravasation of T cells at the 70 μg/day dose level of estradiol (E. Yatkin, J. Bernoulli, E.-M. Talvitie, and R. Santti, unpublished data). The lack of the anti-inflammatory effect was surprising. The role of estrogen in the control of the lymphocyte migration through the vascular wall is well established, even though the sites and mechanisms of estrogen action remain open (Straub, 2007). Inhibition of lymphocyte accumulation by ICI 182,780, a pure antiestrogen, was consistent with the ER-mediated mecha-
Fispemifene blocked the expression of two estrogen-responsive genes (PR and Fra2) in the acinar epithelium of the DLP. The expression of these genes was also blocked by DHT (shown in experiment II). It is possible that these estrogen-induced, androgen-antagonized events may be activated with the changes in the ratio between the estrogen and androgen. The same mechanism could account for down-regulation of the expression of multiple other estrogen-responsive genes and proteins in the prostate (West et al., 1990; Risbridger et al., 2001; Nellermann et al., 2005). In general, the antigenic proteins would be sequestered or suppressed, but an increased estrogen/androgen ratio of would expose them or induce their expression. The inflammatory response may thus represent a host response to the acinar epithelium that is antigenically distinct from normal epithelium (Blumenfeld et al., 1992).

The estrogen-induced inflammation of the DLP has been associated with the increase of prolactin release and pituitary size (Tangbanuelak and Robinette, 1993). The present findings match earlier results and are consistent with the hypothesis that prolactin contributes to inflammation in the prostate. Fispemifene decreased, in a dose-dependent manner, prolactin concentration and pituitary size in the 3-week study but displayed only a marginal, nonsignificant inhibition of prostatic inflammation consisting mostly of perivascular and stromal inflammatory infiltrates (Thorat et al., 1993). This implies that the extravasation of lymphocytes in the DLP of the Noble rat may not be dependent on estrogen-induced increased prolactin secretion. This confirms earlier findings in transgenic mice (McPherson et al., 2001; Kindblom et al., 2003). The inhibition of prolactin release by fispemifene at 18 weeks, which showed attenuation of glandular inflammation, was at most partial. The possibility remains that prolactin is involved in the development of glandular inflammation.

There are some differences between fispemifene and tamoxifen action. Neither fispemifene nor tamoxifen differentiate between the two ER subtypes. Both tamoxifen (1 mg/kg) and fispemifene (3–30 mg/kg) decreased the pituitary weight and serum prolactin concentration induced by estradiol in the present study (Lyle et al., 1984). In contrast to fispemifene, tamoxifen treatment of castrated rats significantly increased the number of perivascular and stromal infiltrates and had no significant anti-inflammatory effect in the 3-week experiment. These findings are consistent with the generally accepted idea that tamoxifen exhibits significant estrogenic effects on the prostate when administered perinatally or alone to adult males (Orgebin-Crist et al., 1983; Lyle et al., 1984; Taguchi, 1987; Karlsson, 2006). Due to this estrogenic and proinflammatory action, tamoxifen was not tested in the 18-week experiment. Fispemifene itself was devoid of estrogenic effects in the prostate, and no inflammatory effects were seen in the DLP when fispemifene was administered alone or together with DHT.

When the testosterone and estradiol treatment time was extended to 13 and 18 weeks, stromal infiltrates occupied an increasing stromal proportion of the DLP. The periacinar accumulation of lymphocytes and the number of inflamed acini simultaneously increased. Indeed, at 18 weeks, there were significantly more inflamed acini, and the inflammation was significantly more aggressive than at 13 weeks. The effect on fispemifene on aggressiveness was not significant; however, fispemifene significantly decreased the total number of inflamed acini. Two main types of inflamed acini were identified. Intact epithelium and luminal neutrophils were characteristic for the first type of inflamed acini. There were no lymphocytes immediately adjacent to the epithelium. These acini formed the noticeable majority of inflamed acini; also, various intermediate types were observed. The presence of luminal macrophages was associated with partially destroyed acinar epithelium in the second type. These acini had intraepithelial CD8+ cells and periglandular accumulation of lymphocytes and resembled the segregated inflamed glands described in the human prostate. Periglandular inflammation (called periacinar in that communication) probably represents the resolving phase or residuals of segregated glandular inflammation (Kohnen and Drach, 1979).

The presence of multiple intermediate forms of inflamed acini showing various numbers of neutrophils, macrophages, and lymphocytes and varying degrees of epithelial alterations may indicate that the acute acinar inflammation gradually progresses to chronic inflammation.

Counting of inflamed acini indicated that fispemifene inhibited migration of neutrophils into the acinar lumina. A role for estrogen in the recruitment of neutrophils into inflamed sites has also become evident in studies using the hypogonadal mouse, which features complete postnatal deficiency of pituitary gonadotropins, and subsequently of sex steroids (Bianco et al., 2002). Coagulating glands were most sensitive to estradiol and showed the most severe epithelial changes. Neutrophils seemed to migrate toward the acinar epithelium. A strong recruitment of neutrophils and macrophages (but not lymphocytes) into the mouse uterus after the treatment of the mouse with estradiol at pregnancy levels offers another example of these organ-specific estrogenic effects on neutrophils (Tibbetts et al., 1999). The mechanism of estrogen action is not known but might be found in the control of chemotactic factors and their receptors (Straub, 2007). Epithelial cells may also be directly involved in the recruitment of inflammatory cells. When the insulting stimulus is eliminated, mainly via granulocyte-dependent mechanisms, the acute inflammation resolves. However, chronic inflammation can supervene the acute inflammation if the stimulus cannot be removed. Continuous relative androgen deficiency combined with increased estrogen and prolactin concentrations would maintain the insult in epithelium. Fispemifene would, as an antiestrogen, have a “healing” effect and eliminate estrogen-induced inflammatory factors or exposed antigenic protein(s).

Chronic pelvic pain syndrome, a nonbacterial category of prostatitis, accounts for 90% of all chronic prostatitis and is the most common urological diagnosis in men over 50 years of age in the United States (Collins et al., 2002). The cause of nonbacterial prostatic inflammation is not known, but autoimmunity, intraprostatic reflux, and hormonal imbalance are recognized as some of the potential risk factors in its etiology.
Treatment consists of antibiotics, anti-inflammatories, and prostate-specific medications such as α-blockers and 5α-reductase inhibitors. However, these and all the other treatments employed to treat chronic “nonbacterial prostatitis” and “prostatodynia” had never been evaluated or proven to be effective in properly designed clinical trials (Curtis Nickel et al., 2007). Numerous animal models of chronic prostatitis/chronic pelvic pain syndrome have been developed that use spontaneous, infectious, immune-mediated, and hormone-associated methodology to induce prostatitis (Vykhovanets et al., 2007). The hormonally induced prostatic inflammation (lymphocyte-predominant infiltrates and perivascular, stromal/periglandular, and glandular inflammatory pattern) described in this report has several similarities to both spontaneously developing rat prostatitis and human chronic nonbacterial prostatitis (Nickel et al., 2001; Vykhovanets et al., 2007). Thus, this model may offer insights into the pathogenesis of prostatitis and may also serve as a model for the prevention and treatment of this disease. The anti-inflammatory action of fispemifene in this study suggests that SERMs could be considered as a new therapeutic option for the treatment and prevention of prostatic inflammation.

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References


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