EXPERIMENTAL STUDY

Effects of a new steroidal aromatase inhibitor, TZA-2237, and/or chlormadinone acetate on hormone-induced and spontaneous canine benign prostatic hyperplasia

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Abstract

Objective: It has been known for many years that human benign prostatic hyperplasia (BPH) is composed predominantly of hyperplastic stromal cells rather than epithelial cells. In the present study the effects of a new steroidal aromatase inhibitor on hormone-induced and spontaneous canine BPH were investigated.

Methods: (1) *Effects of TZA-2237 on hormone-induced canine BPH.* Ten castrated beagles were administered testosterone and androstenedione 6 days/week for 8 months, and divided randomly into three groups after 2 months of treatment as follows. Group I served as controls, Group II was given 0.5 and Group III was given 2.5 mg/kg/day TZA-2237 5 days/week for 6 months. (2) *Effects of TZA-2237 on spontaneous canine BPH.* Twenty aged beagles with BPH were divided into five groups, Group IV was untreated, Group V was treated with 1 and Group VI with 5 mg/kg/day TZA-2237 5 days/week for 31 weeks. Group VII was treated with 5 mg/kg/day Atamestane and Group VIII was treated with 0.3 mg/kg/day chlormadinone acetate (CMA) 5 days/week. (3) *Effects of TZA-2237 combined with CMA on spontaneous canine BPH.* Three aged beagles with BPH were treated with 1 mg/kg/day TZA-2237 and 0.03 mg/kg/day CMA 5 days/week for 20 weeks (Group IX) and a further three aged beagles with BPH were treated with 0.3 mg/kg/day CMA alone 5 days/week (Group X).

Results: Hormone-induced prostatic growth was significantly suppressed in group III compared with that in other groups. In Group III, the intraprostatic aromatase activity, estradiol level and androgen receptor content decreased significantly in comparison with the values in Group I. The prostatic weights in Groups V, VI and VII increased significantly in comparison with the weight in Group IV. Serum LH and testosterone levels in Groups V, VI, and VII increased significantly in comparison with the level in Group IV. The prostatic weight in Group IX was decreased only slightly, but the smooth muscle component was decreased significantly.

Conclusions: TZA-2237 is a new, unique and effective aromatase inhibitor that causes inhibition of both epithelial and stromal compartments in hormone-induced canine BPH. Dual inhibition of androgen and estrogen resulted in inhibition of smooth muscle growth, and should prove effective as a new method of treatment given the atrophic effects on not only the epithelium but also the stroma in human BPH.

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Introduction

Benign prostatic hyperplasia (BPH) can be demonstrated histologically in 50% of 60-year-old men, and this percentage increases to 90% in 85-year-old men (1). Androgens are widely acknowledged to be central to the pathogenesis of BPH. However, BPH increases in prevalence at the life stage during which plasma androgens are decreasing. The serum and intraprostatic estradiol (E₂) to testosterone (T) ratio and the level of plasma sex hormone-binding globulin (SHBG) increase

with age, and the resulting estrogen-dominant environment has long been suspected to be important for the pathogenesis of BPH (2–5). The evidence for estradiol production in the rat, canine and human prostate was demonstrated using the estrogen formation assay and/or H₂O release assay (6). Seppelt demonstrated a significant correlation between the individual physiological estradiol levels and the amount of prostate stroma (7). Suzuki *et al.* reported that the serum E₂/T ratio was correlated with the prostatic volume (8) and Shibata *et al.* demonstrated that the E₂/dihydrotestosterone

(DHT) ratio in the transitional zone of human BPH was positively correlated with the prostatic volume, the proportion of stroma (%) and age (9). Recently, a novel estrogen receptor (ER) cDNA was cloned from the rat prostate, and the putative polypeptide encoded thereby was termed ER- β due to its high homology with the classical ER- α . Fukabori *et al.* demonstrated that ER- β was localized in human prostatic epithelial cells, which contrasts with the stromal localization of ER- α in the human prostate (10). Recently, Yeh *et al.* demonstrated the new E₂-androgen receptor (AR)-ARA70 pathway in human prostate cancer cells, and concluded that E₂ may represent an essential ligand of AR that plays an important role in the development and/or functioning of the male reproductive system (11). However, the hypothesis of a non-genomic pathway was proposed in which estrogen, mediated by the SHBG-receptor complex, combines with androgen in setting the pace of prostate growth and function (12). It has been reported that estradiol causes a marked, dose-dependent increase in stromal cytosolic cAMP in the presence of receptor-bound SHBG (13). In addition, it appears that the tyrosine kinase pathway mediated by cAMP is capable of activating IGF-I synthesis and paracrine secretion of IGF-I to the epithelial IGF-I receptors, thereby shifting cells from the G1 to the S phase, and causing epithelial proliferation (12).

Thus, estrogen has many roles, and it is of great interest to determine the effect of estrogen deprivation therapy alone and with combined androgen deprivation in prostate physiology and morphology. One aromatase inhibitor, Atamestane, was not an effective treatment for symptomatic BPH in a prospective randomized double-blind trial (14). However, since that failure resulted in a counter-regulatory increase in androgens, one cannot exclude the possible therapeutic usefulness of estrogen deprivation for BPH.

In this context, we hypothesized that modulation of peripheral estrogen production by inhibitors of aromatization would have a palliative effect on BPH. At first, we investigated the effects and mechanisms of action of a new steroidal aromatase inhibitor TZA-2237 on hormone-induced canine BPH. The inhibitory effects of this compound on prostatic growth were evaluated by

transrectal ultrasonography (TRUS) and prostatic weight, and the inhibitory mechanisms of TZA-2237 were also evaluated by endocrinological measurements, including measurement of the levels of estrogen and androgen products, steroid receptor contents and 5 α -reductase activity. Then, as a pre-clinical study, we also investigated the effect of TZA-2237 with or without CMA on spontaneous canine BPH, and acquired important knowledge that should be useful for the future studies on the clinical applicability of this therapy.

Materials and methods

Materials

Testosterone, 4-androstene-3,17-dione (A-dione) and triolein were purchased from Sigma Chemical Co. (St Louis, MO, USA). TZA-2237, 7 α -mercapto-D-homo-17-oxa-androsta-1,4-diene-3,17 α -dione (Fig. 1), Atamestane, 1-methyl-androsta-1,4-diene-3,17-dione and CMA were synthesized at Teikoku Hormone Manufacturing Co. Ltd (Kawasaki, Japan). Radiolabeled [1- β -³H]androst-4-ene-3,17-dione (specific activity 15 Ci/ mmol) and 17- β -[³H]estradiol (specific activity 96.5 Ci/ mmol) for receptor binding assays were purchased from New England Nuclear Research Products (Boston, MA, USA). NADPH was purchased from Kohjin Co. Ltd (Tokyo, Japan).

The characteristics of TZA-2237

Androgenic activity Three-week-old male rats were castrated under general anesthesia, and 2 weeks later TZA-2237 was administered orally, or testosterone was injected s.c. as a positive control. The animals were killed by prolonged exposure to ether anesthesia 72 h after a single administration, and the ventral prostate was weighed. Oral treatment with TZA-2237 did not increase the weight of the ventral prostate.

Estrogenic activity TZA-2237 was orally administered, or estradiol was injected s.c. as a positive control for 3 successive days in 3-week-old female rats. On the day following the final administration, each animal was

7 α -mercapto-D-homo-17-oxa-androsta-1,4-diene-3,17 α -dione

Figure 1 The structure of TZA-2237.

weighed and killed by prolonged exposure to ether anesthesia, the uterus was excised and weighed. Oral treatment with TZA-2237 did not increase the weight of the uterus.

Progestational activity (Clauberg's test) Immature female rabbits (aged 8–10 days) were primed by s.c. injection of estradiol benzoate at 2 µg/rabbit for 5 successive days. For 5 successive days after that, TZA-2237 and reference compounds (norethisterone and medroxyprogesterone acetate) were orally administered. Each animal was weighed on the day following the final administration, and killed by injecting a fatal dose of pentobarbital Na. The uterus was excised and weighed. A uterine horn was fixed in 10% formalin buffered solution. After fixation, the uterus was sectioned perpendicular to the long axis, and hematoxylin–eosin-stained sections were prepared. Progestational activity was measured by taking the growth of the endometrium as a parameter (the McPhail index) (15). The minimum effective dose was taken to be the minimum dose required to induce endometrial hyperplasia in all animals in the group. TZA-2237 showed no progestational activity in that experiment.

5 α -Reductase inhibition activity TZA-2237 showed no inhibitory effects of 5 α -reductase activity in the *in vitro* study using the rat ventral prostate tissues.

Canine studies

A total of 10 young male beagles (2–3 years old) and 20 aged beagles (5–6 years old) were obtained. The animals were kept in a controlled environment (temperature 20 °C, light–dark: 12 h). All surgery and examinations were performed under pentobarbital anesthesia. Animals were castrated via the scrotal route 1 week before the start of the studies.

Effects of TZA-2237 on hormone-induced canine BPH

On day 0, all animals in the hormone-induced

BPH groups that were castrated 1 week before were injected i.m. with 30 mg testosterone and 7.5 mg androstenedione in 2 ml triolein 6 times a week for 8 months, and allocated at random into three groups during the last 6 months of androgen treatment as follows. Group I served as a control. Group II was given 0.5 and Group III was given 2.5 mg/kg of TZA-2237 orally 5 times a week (Table 1).

Effects of TZA-2237 on spontaneous canine BPH

Twenty aged beagles with BPH were divided into 5 groups and not treated (Group IV), treated with 1 (Group V) or 5 mg/kg/day TZA-2237 (Group VI), treated with 5 mg/kg/day Atamestane (Group VII), or treated with 0.3 mg/kg/day Chlormadinone acetate (CMA) (Group VIII) (Table 1).

Effects of TZA-2237 combined with CMA on spontaneous canine BPH

Six aged beagles with BPH were divided into two groups, and treated with 1 mg/kg/day TZA-2237 and 0.03 mg/kg/day CMA (Group IX), or treated with 0.03 mg/kg/day CMA alone (Group X).

All dogs were weighed before and after treatment. At the conclusion of the experiment, dogs were killed with an overdose of pentobarbital. The prostate was dissected and weighed, part of it was frozen in liquid nitrogen for biochemical assays and part of it in liquid propane for immunocytochemistry. The remainder of the tissue was fixed with 10% formaldehyde for histopathological studies. Trunk blood was collected, and the serum fraction was stored at –70 °C for estradiol and LH RIA, and testosterone gas chromatography-mass spectrometry (GC-MS).

Prostatic volume measurements

Before the start of treatment and every 2 months thereafter, the prostatic volume was measured by the method of Berry *et al.* (16). Transrectal ultrasonography (TRUS) was performed with Toshiba SAL-77B

Table 1 Scheme of treatment.

Group	No. of dogs	Treatment ^a
I	4	Castration: testosterone (30 mg) and androstenedione (7.5 mg) for 8 months
II	3	Castration: testosterone (30 mg) and androstenedione (7.5 mg) for 2 months and for a further 6 months along with aromatase inhibitor TZA-2237 (0.5 mg/kg/day)
III	3	Castration: testosterone (30 mg) and androstenedione (7.5 mg) for 2 months and for a further 6 months along with TZA-2237 (2.5 mg/kg/day)
IV	4	None (untreated control)
V	4	TZA-2237 (1 mg/kg/day) for 31 weeks
VI	4	TZA-2237 (5 mg/kg/day) for 31 weeks
VII	4	Aromatase inhibitor Atamestane (5 mg/kg/day) for 31 weeks
VIII	4	Antiandrogen CMA (0.3 mg/kg/day) for 31 weeks
IX	3	TZA-2237 (1 mg/kg/day) and CMA (0.03 mg/kg/day) for 20 weeks
X	3	CMA (0.03 mg/kg/day) for 20 weeks

^a Testosterone and androstenedione were given 6 times a week intramuscularly. The other substances were given orally 5 times a week.

and PVL-516S transducers (Toshiba Medical Co., Tokyo, Japan). The prostate was scanned along the sagittal and transaxial planes. The maximum-sized prostate image was freeze-framed, and the length (in cm) and the height of the prostate (in cm) were measured in the sagittal plane and the width of the prostate (in cm) was measured in the transaxial image. The prostate volume was calculated using the following formula:

Prostate volume = $0.62 \times \text{length} \times \text{height} \times \text{width} + 1.16$.

Determination of serum LH level

Serum concentrations of LH were determined by RIA as reported previously by Nett *et al.* (17), using porcine LH (LER-778) for radioiodination and anti-porcine LH serum. Purified canine LH (LER-1685) was used as a reference standard.

Determination of serum testosterone

T-d₃ (5ng) was added as an internal standard to each volume of serum (1 ml), and 1 ml H₂O was added to the serum which was then applied to a Bond Elut C18 cartridge column (3 ml) that had been prewashed with two volumes each of methanol and distilled water. The columns were then washed successively with 3 ml each of 30% acetonitrile/70% H₂O. The steroid fraction was eluted with 2.5 ml 70% acetonitrile/30% H₂O and dried with the centrifugal evaporator. The extract was dissolved in 3 ml benzene and applied to a Bond Elut CN cartridge column (3 ml) that had been prewashed with 10 ml benzene. The column was washed with 2.5 ml each of benzene and 2% ethyl acetate/98% benzene, and then the testosterone fraction was eluted with 3 ml 10% ethyl acetate/90% benzene. The fraction was dried under nitrogen in a water bath at 45 °C. The testosterone concentrations were quantified by GC-MS.

Determination of testosterone, DHT and estradiol in the prostate

The frozen tissue (200–500 mg) was pulverized in liquid nitrogen and homogenized in 10 ml of 0.1 M phosphate buffer (pH 7.0) using an Ultra-Turrax homogenizer (Junke and Kunkel) in an ice bath. T-d₃ (5ng), [³H]DHT (2000 d.p.m.) or [³H]E₂ was added as an internal standard to each piece of homogenized tissue, and then 20 ml ethylacetate/n-hexane (3:1) was added to the samples and the mixture was shaken for 10 min. Each organic layer was transferred to a test tube. The organic solvent was washed successively with 5% NaHCO₃/95% H₂O and distilled water, and dried over anhydrous Na₂SO₄. After evaporation of the organic solvent, the extract was dissolved in 3 ml 30% acetonitrile/70% H₂O in a warm bath, and then applied to a Bond Elut C18 cartridge column (3 ml) that had

been prewashed with two volumes each of methanol and distilled water. The columns were then washed successively with 3 ml each of distilled water and 30% acetonitrile/70% H₂O. The steroid fraction was eluted with 2.5 ml 70% acetonitrile/30% H₂O and dried under nitrogen in a water bath at 45 °C. The extract was dissolved in 1 ml benzene and applied to a Bond Elut CN cartridge column (3 ml) that had been prewashed with 10 ml benzene. The column was washed with 1 ml benzene and 2% ethyl acetate/98% benzene, and then the testosterone fraction was eluted with 3 ml 10% ethyl acetate/90% benzene, the DHT fraction was eluted with 3.5 ml 2% ethyl acetate/98% benzene, and the estradiol fraction was eluted with 5 ml 10% ethyl acetate/90% benzene. Each fraction was dried under nitrogen in a water bath at 45 °C. The testosterone concentrations were quantified by GC-MS and the DHT and estradiol concentrations were determined by RIA corrected for the recovery of radioactivity.

Assay for aromatase activity in the prostate

Aromatase activity was measured by an ³H₂O-release assay. The frozen tissue was pulverized in liquid nitrogen and homogenized in four volumes of 0.1 M phosphate buffer (pH 7.5) with an Ultra-Turrax homogenizer in ice water. The homogenate was centrifuged at 900 *g* for 10 min, and the supernatant was used as an enzyme source. The reaction mixture containing [³H]androstenedione (50 µCi), NADPH (1 mg), homogenate, and 0.1 M phosphate buffer in a final volume of 1 ml was incubated at 37 °C for 45 min. The reaction mixture was stopped by chilling on ice, and supplemented with 1 ml H₂O and 3 ml CH₃Cl. This solution was vortexed and centrifuged at 1500 *g* for 10 min. Aliquots of the aqueous phase (1.0 ml) were analyzed for ³H content using a liquid scintillation counter (LSC 3500, Aloca). The aromatase activity was expressed as fmol/h/mg protein. The protein content was determined by the methods of Lowry (18).

Assay for 5α-reductase activity in the prostate

The pulverized dog prostate tissue was homogenized in ten volumes of 0.1 M phosphate buffer with an Ultra-Turrax homogenizer in ice water. The homogenate was centrifuged at 100 *g* for 10 min to sediment the cellular debris. The mixture containing [¹⁴C]testosterone (0.1 µCi), NADPH (1 µM), homogenate and 0.1 M phosphate buffer in a final volume of 1 ml at pH 5.5 was incubated at 37 °C for 45 min. Under these incubation conditions, the production of the 5α-reduced metabolite was linear for at least 60 min. The reaction was stopped by the addition of 0.1 ml of 2 N HCl. The internal standards [³H]DHT (500 000 d.p.m.) and [³H]androstenediol (73 000 d.p.m.) were added to the incubation mixture. The mixture was applied to a Bond Elut C 18 column (3 ml) that had been prewashed with two volumes of

methanol followed by two volumes of distilled water. After washing the loaded column with 3 ml distilled water, the metabolite fraction was eluted with 2 ml methanol. The eluant was evaporated at 40 °C under vacuum, and the residues were taken up in 100 µl 50% methanol/50% chloroform. The solution was applied to a ready-made silica gel sheet on a plastic plate (Kiesel Gel ⁶⁰F₂₅₄, Merck), and the plate was developed twice using a solution of benzene/methanol (15:1) as the developer solvent. The plate was dried and used to expose x-ray film for 7 days. Separated androgens corresponding to DHT and androstenediol on the chromatogram were scraped from the plate, and the radioactivity of each was counted in a liquid scintillation counter (LSC 3500, Aloca). 5α-Reductase activity was calculated from the percentage of recovered radioactivity converted to DHT plus androstenediol and was recorded in terms of pmol/h/mg protein.

Estrogen receptor assay

The nuclear and cytosolic estrogen receptors (ERs) were assayed according to the method of Leake *et al.* (19). Approximately 700 mg tissue was homogenized with an Ultra-Turrax homogenizer in seven volumes of ice-cold TEDG buffer (10 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, 10% glycerol and 1 mM dithiothreitol) using three 5 s periods, with 2 min cooling intervals between bursts. The homogenate was centrifuged at 900 *g* for 10 min to prepare a crude nuclear pellet. The supernatant was added to sodium molybdate (1 mM) and then centrifuged at 105 000 *g* for 60 min to yield the cytosol fraction. The nuclear pellet was washed in two volumes of TEDG buffer. The crude nuclear fraction was suspended in TEDK buffer (10 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, 0.6 M KCl, 1 mM dithiothreitol) and stirred gently at 4 °C for 1 h, and the solution was centrifuged at 105 000 *g* for 60 min. The supernatant was used for the nuclear receptor assay. Each fraction was incubated with 1–16 nM of [³H]estradiol at 4 °C for 24 h in the presence or absence of 3 µM DES for determination of nonspecific binding. Bound steroid was separated from free steroid using the dextran-coated charcoal (DCC) technique. All determinations were done in duplicate. The receptor concentrations were then obtained using Scatchard plots after subtraction of nonspecific bindings, and specific binding was expressed in fmol/mg DNA for nuclear ER and in fmol/mg protein for cytosolic ER. Prostatic DNA content was determined by the method of Burton using calf thymus DNA as a standard (20).

Histopathological study and immunohistochemistry

Prostate tissues were placed in 10% neutral buffered formalin. Histological preparations of paraffin-embedded samples were sectioned at 6 µm, stained

with Hematoxylin and Eosin and examined by light microscopy.

Immunohistochemistry of AR was carried out according to the modified method reported previously (21). Frozen sections of prostates were incubated overnight at 4 °C with 1500-fold-diluted anti-AR antibody (NH27) that was produced and supplied by Dr A Mizogami (Department of Urology, University of Occupational and Environmental Health, Fukuoka, Japan) (22). The anti-AR antibody produced polyclonal antibodies against human AR (hAR) by means of immunizing a rabbit with hAR fusion protein that was expressed in *E. coli*. The indirect ABC method was performed with an avidin-biotin peroxidase kit (DAKO LSAB2 kit, DAKO Co., Carpinteria, CA, USA), using diaminobenzidine tetrachloride as a chromagen. The sections were stained with Methyl Green as a nuclear counterstain.

Analytical methods to estimate the proportions of the epithelium and stroma

The proportions (%) of the histological components were calculated in Azan-Mallory-stained specimens using the modified method of Shibata *et al.* (9). Microscopic images of each tissue were captured on a Macintosh computer at a magnification of ×200. The captured images were analyzed for the area of four components, glandular epithelium, smooth muscle, fibrous tissue and glandular lumen using a microscopic pattern-measuring system (ATTO Co., Japan). At least eight randomly chosen test areas were analyzed for each tissue specimen, to exclude the effects of heterogeneity. At first, the total area of the images was calculated. Next, the proportions of three components, glandular epithelium, fibrous tissue and glandular lumen, were calculated. Then the proportions of the area of smooth muscle were obtained by removing the other three compartments from the total area.

Statistics

Statistical significance was determined by Student's *t*-test. Differences were considered significant when *P* was smaller than 0.05. Values are expressed as the median and 25th and 75th percentiles unless otherwise indicated.

Results

Effects of TZA-2237 on hormone-induced canine BPH

The change in prostate volume, expressed as a percentage of the prostate volume at 2 months after the start of testosterone and A-dione treatment, is presented in Fig. 2. Treatment with 2.5 mg/kg TZA-2237 reduced prostate growth from the fourth month onward compared with the control or with 0.5 mg/kg

Figure 2 Sequential change of mean prostate volume estimated by transrectal ultrasonography during treatment with TZA-2237 on hormone-induced canine BPH.

TZA-2237 treatment. There was no difference in the final body weight or final to initial body weight ratio among the three groups at necropsy. Treatment with 2.5 mg/kg TZA-2237 reduced normalized (per kg body weight) adrenal and prostatic weights compared with control or 0.5 mg/kg TZA-2237 treatment (Table 2). There was no difference in the other organ weights among the three groups.

Prostatic DHT and estradiol concentrations in each group are shown in Table 3. Treatment with 2.5 mg/kg TZA-2237 induced a significant decrease in intraprostatic estradiol concentration, but induced a significant increase in intraprostatic DHT levels compared with controls. Prostatic 5 α -reductase activity in the 2.5 mg/kg TZA-2237 group was significantly higher than that in the control group (Table 4). There were no significant differences in the cytosolic or nuclear ER contents among the 3 groups (Table 5). Scatchard plot analysis demonstrated only one binding site, and the ER measured in this study were type I.

Figure 3 shows an immunohistochemical demonstration of AR in each group. The treatment with testosterone and A-dione (control group) induced a

pronounced stimulation of AR content exclusively within the epithelial compartment, and this stimulation was completely antagonized by the treatment with 2.5 mg/kg TZA-2237. The percentage of AR-positive nuclei was 74 and 22% in the control group and the 2.5 mg/kg TZA-2237 treated group respectively.

Effects of TZA-2237 on spontaneous canine BPH

The change in prostate volume, expressed as a percentage of the prostate volume at the start of treatment, is presented in Fig. 4. The prostatic weights increased significantly from 18 weeks onward in the aromatase inhibition groups (Groups V, VI and VII) compared with the controls (Group IV). TZA-2237 and Atamestane treatment increased normalized (per kg body weight) prostatic weights compared with the controls (Table 2). There were no differences in the other organ weights among the three groups.

The mean serum LH concentrations in controls, aromatase inhibition and antiandrogen-treated dogs are illustrated in Fig. 5A. In control dogs, the serum LH

Table 2 Final body weight and excised organ weight.

Group	Body weight (kg)	Prostate (g)	Adrenal gland (g)
I	12.3 $\pm$ 1.0	26.0 $\pm$ 12.1	1.02 $\pm$ 0.08
II	13.5 $\pm$ 0.4	25.9 $\pm$ 8.8	1.12 $\pm$ 0.08
III	13.2 $\pm$ 0.5	15.8 $\pm$ 0.6	0.88 $\pm$ 0.08
IV	14.8 $\pm$ 2.1	23.6 $\pm$ 11.8	1.27 $\pm$ 0.22
V	16.0 $\pm$ 1.4	41.1 $\pm$ 17.3	1.44 $\pm$ 0.10
VI	14.8 $\pm$ 2.2	37.0 $\pm$ 11.2	1.08 $\pm$ 0.20
VII	14.6 $\pm$ 1.6	31.8 $\pm$ 1.7	1.18 $\pm$ 0.37
VIII	15.5 $\pm$ 1.0	6.6 $\pm$ 0.8	0.59 $\pm$ 0.09
IX	15.3 $\pm$ 4.9	16.6 $\pm$ 9.2	1.38 $\pm$ 0.03
X	15.8 $\pm$ 0.3	15.5 $\pm$ 2.5	1.44 $\pm$ 0.17

Table 3 The concentrations of testosterone, DHT and estradiol in the prostate.

Group	Testosterone (ng/g tissue weight)	DHT (ng/g tissue weight)	Estradiol (pg/g tissue weight)
I	n.d.	14.04 ± 3.34	1332 ± 267
II	n.d.	13.02 ± 1.87	2332 ± 1434
III	n.d.	22.04 ± 7.68 ^b	797 ± 189 ^a
IV	1.22 ± 0.90	9.64 ± 2.98	117 ± 34
V	1.27 ± 0.53	4.77 ± 1.76 ^c	62 ± 25
VI	0.65 ± 0.30	6.03 ± 2.12	61 ± 14 ^c
VII	1.66 ± 0.77	13.66 ± 3.55	121 ± 33
VIII	0.58 ± 0.25	2.08 ± 0.84 ^c	58 ± 24 ^c
IX	0.21 ± 0.03	8.80 ± 3.24	14 ± 3
X	0.36 ± 0.19	4.61 ± 1.23	47 ± 35

n.d.: not determined. ^a $P < 0.05$ vs. Group I. ^b $P < 0.1$ vs. Group I, $P < 0.05$ vs. Group II. ^c $P < 0.05$ vs. Group IV.

concentration averaged 0.71 ng/ml over a 31 week treatment period and ranged from 0.48 to 1.42 ng/ml. Following 13 weeks of treatment with 5 mg/kg TZA-2237, the serum LH concentration increased ($P < 0.05$) approximately 2-fold, to 2.27 ng/ml, and then further increased at 26 weeks of treatment to 4.40 ng/ml. Following 13 weeks of low (1 mg/kg)-dose TZA-2237 treatment, the serum LH concentration was increased ($P < 0.05$) approximately 2-fold, to 1.02 ng/ml, and then further increased at 26 weeks of treatment to 3.74 ng/ml. The LH concentration of CMA-treated dogs was slightly higher than that of control dogs from 8 weeks onward. At the 31 week treatment period, the serum LH was 0.84 and 1.38 ng/ml in dogs treated with 5 and 1 mg/kg TZA-2237, respectively, and statistical analysis indicated no significant increase over controls.

The mean serum testosterone concentrations in controls, aromatase inhibitor- and antiandrogen-treated dogs are illustrated in Fig. 5B. Testosterone concentrations were similar in control and treated dogs in the pretreatment period. In control dogs, the testosterone concentration averaged 1.23 ng/ml over the 31 week treatment period, ranging from 0.73 to 2.5 ng/ml. Following 8 weeks of treatment, the testosterone concentration increased ($P < 0.05$) approximately 4.5-fold, to 7.91 ng/ml in dogs treated with 5 mg/kg TZA-2237 and then further increased at 26 weeks

of treatment to 10.33 ng/ml. In the group treated with 1 mg/kg TZA-2237, the testosterone concentration increased ($P < 0.01$) approximately 13-fold, to 10.23 ng/ml, at 26 weeks of treatment. Over the 31 week treatment period, the serum testosterone averaged 6.40 ng/ml and 3.74 ng/ml in dogs treated with 5 and 1 mg/kg TZA-2237, respectively, and statistical analysis indicated a significant ($P < 0.05$) increase over the control level. The mean ($\pm$ standard deviation) prostatic testosterone, DHT and estradiol concentrations in each group are shown in Table 3. The mean testosterone concentrations were not significantly different among the groups, but the mean DHT concentrations decreased significantly in the groups treated with 1 mg/kg TZA-2237 and CMA in comparison with the control group. Treatment with 5 mg/kg TZA-2237 induced a significant decrease in the intraprostatic estradiol concentration compared with controls. There was no significant difference in aromatase activity or 5 α -reductase activity among the five groups (Table 4).

Effects of TZA-2237 combined with CMA on spontaneous canine BPH

The prostate volume, expressed as a percentage of the prostate volume at the start of treatment was 106, 96, 88 and 101% in the combination treatment group and 77, 74, 70 and 66% in the CMA-treated group at 5, 10, 15 and 20 weeks of treatment, respectively. The mean

Table 4 The activity of 5 α -reductase and aromatase in the prostate.

Group	5 α -reductase activity (pmol/min/mg protein)	Aromatase activity (fmol/h/mg protein)
I	1.56 ± 0.29	n.d.
II	1.07 ± 0.52	n.d.
III	2.70 ± 0.41 ^a	n.d.
IV	1.52 ± 0.68	0.29 ± 0.14
V	1.55 ± 0.40	0.39 ± 0.16
VI	1.92 ± 0.61	0.26 ± 0.15
VII	2.40 ± 1.24	0.98 ± 1.00
VIII	1.43 ± 0.40	0.53 ± 0.22

n.d.: not determined. ^a $P < 0.01$ vs. Group I.

Table 5 Estrogen receptor content in the prostate.

Group	Estrogen receptor	
	Nuclear fraction (fmol/mg DNA)	Cytosol fraction (fmol/mg protein)
I	579.1 ± 123.2	59.1 ± 10.1
II	756.1 ± 230.1	37.3 ± 15.7
III	715.2 ± 218.1	64.9 ± 26.7

Figure 3 Immunohistochemical demonstration of androgen receptors in the prostate after 6 months of treatment with TZA-2237. (A) Control. (B) High-dose TZA-2237 group (original $\times 400$).

($\pm$ standard deviation) prostatic testosterone, DHT and estradiol concentrations in each group are shown in Table 3. Combination treatment with TZA-2237 and CMA induced a significant decrease in the intraprostatic estradiol concentration compared with all other groups. The testosterone and DHT concentrations in the combined treatment group (Group IX) were not increased relative to those in the control group.

The volume densities of various histological components evaluated by quantitative morphometric analysis in each group are shown in Fig. 6. The proportions of the glandular lumen, glandular epithelium, connective tissue and smooth muscle were 19.5, 48.0, 5.0 and 27.5%, respectively, in the controls, 14.0, 44.0, 6.0 and 36.0% in the group treated with 0.03 mg CMA and 12.4, 45.8, 26.6 and 15.6% in the combined treatment group. The proportion of smooth muscle compartment

in the combined treatment group was significantly decreased compared with the proportion in the group treated with 0.03 mg CMA and the control group.

Discussion

Androgens are widely acknowledged to be central to the pathogenesis of BPH. In this respect, the treatment with 5α -reductase inhibitor and androgen ablation therapy have achieved some success in decreasing prostate size, and improving symptoms and urinary flow rates (23–25). However, the development of BPH is considered to be attributed to a benign proliferative process of the stromal and epithelial elements of the prostate. Therefore, it might be expected that the poor response to hormonal treatment was caused by the accumulation of

Figure 4 Sequential change of mean prostate volume in cases of spontaneous canine BPH estimated by transrectal ultrasonography during treatment with aromatase inhibitor or antiandrogen.

Figure 5 Mean serum LH (A) and testosterone (B) concentrations (ng/ml) in controls, aromatase inhibitor-, and antiandrogen-treated dogs.

connective tissue in the hyperplastic human prostate. Several authors reported that an absolute increase in the stromal elements of the prostate, rather than glandular hyperplasia, was the most important morphological finding in human BPH (26–29). The correlation of the extent of clinical symptoms with BPH size, pathologic type and location of the lesions, as well as smooth muscle and neurologic control of prostate tone, remain to be elucidated, and it is well known that the severity of clinical symptoms of BPH is not related to the size of the hyperplastic prostate (30). The bladder outlet obstruction in symptomatic BPH appears to be affected not only by mechanical obstruction due to the enlarging adenoma but also to the tone of the prostate smooth muscle (31). Several studies have demonstrated that one of the endocrinological factors in the pathogenesis of BPH is estrogen stimulation of stromal

growth. Animal experiments have emphasized the potential role of estrogens in the pathogenesis of BPH (32–35). Habenicht *et al.* first demonstrated the effect of aromatase inhibitor, 4-hydroxy-4-androstene-3,17-dione, on the experimentally induced BPH in the dog (36). They suggested that estrogen-related effects could be clearly antagonized by simultaneous treatment with the aromatase inhibitor. Recently, Krieg *et al.* demonstrated an age-dependent decrease in the DHT level in the epithelium and a concomitant increase of estrone and estradiol levels in stroma. This, in turn, could be of pathogenic importance for BPH development if in fact a balanced estrogen/androgen synergism is necessary for integrity and normal growth of the prostate (4). Recently, the highest expression of ER- β mRNA was found in the prostate among the various tissues tested, and this suggests that the prostate may be an important target in which to explore ER- β protein expression once antibodies or ligands of sufficient specificity and quality become available (37, 38). Nakano *et al.* demonstrated by an immunohistochemical study that ER- β protein is expressed specifically in the human prostatic epithelium and ER- α in the stroma (11). Therefore, decreased estrogen stimulation of the prostatic stroma in patients treated with an aromatase inhibitor should be a promising novel treatment of BPH.

The findings of the present study clearly indicated that 2.5 mg/kg TZA-2237 significantly suppressed hormone-induced prostatic growth. We investigated the inhibitory mechanism of this compound using biochemical and immunohistological analyses. In the prostate, a much lower estradiol concentration, but much a higher DHT level and 5 α -reductase activity was demonstrated in the group treated with 2.5 mg/kg

Figure 6 The proportions of the glandular lumen, glandular epithelium, connective tissue, and smooth muscle in controls, CMA, TZA-2237 and CMA groups.

TZA-2237 than in the control group. The reason for the higher DHT level and 5α -reductase activity is unclear, but the consumption of the substrate was lowered by aromatase inhibitor administration, and the 5α -reductase activity was secondarily activated, and then the concentration of DHT in the prostate increased. Moreover, 2.5 mg/kg TZA-2237 caused a significant decrease in androgen receptor content in comparison with that of the other groups as shown in the immunohistochemical study, although the estrogen receptor content showed no significant difference among the three groups in the biochemical study. It is expected that this inhibitory effect in the group treated with 2.5 mg/kg TZA-2237 was exerted via a significant decrease in the intraprostatic estradiol level and epithelial androgen receptor concentration. Estrogens in combination with androgens increase the prostatic nuclear androgen receptor content of the canine prostate (33). Moreover, it has been demonstrated that estrogen, mediated by the SHBG-receptor complex, participates with androgen in setting the pace of prostate growth and function (10). It has also been demonstrated that estradiol and 5α -androstane- $3\alpha,17\beta$ -diol causes a marked dose-dependent increase in stromal cytosolic cAMP in the presence of receptor-bound SHBG (11, 34). In addition, it would appear that the estrogen not only directs stromal proliferation and secretion, but also, through IGF-I synthesis mediated by cAMP, conditions the response of the epithelium to androgen (10). Estrogen also has indirect effects on the prostate, including estrogen's ability to cause a marked increase in the release of prolactin from the anterior pituitary. Prolactin has been shown to stimulate the citric acid production in the rat lateral prostate (39, 40). Lloyd *et al.* demonstrated that prolactin alters androgen uptake and metabolism in the prostate (41). Recent studies have also shown that the permeability of the prostatic cell membrane to androgen in patients with BPH may be altered in response to prolactin (42). From these perspectives, it is possible that a significant decrease of estradiol in the prostate suppresses a protein kinase pathway in the stroma, and this biochemical change inhibits stromal proliferation directly and epithelial proliferation indirectly through IGF-I and androgen receptor suppression. It is also expected that a decrease of prolactin suppresses prostatic growth. Moreover, Yeh *et al.* (11) demonstrated that ARA70, a steroid receptor coactivator 1, can induce AR transcriptional activity >30-fold in the presence of 10 nM 17β -estradiol. E_2 represents another important natural ligand for AR that may play an essential role in the AR function and the development of the male reproductive system. Therefore, this new pathway for E_2 may play an important role in the prostate.

Some investigators have reported effects of other aromatase inhibitors, 1-methyl-1,4-androstadiene-3,17-dione (Atamestane) (43–45), and 7-mercapto-D-homo-17-oxa-androsta-1,4-dien-3-one (TZA-2209)

(46), in the treatment of BPH. Haebenicht & Etreby demonstrated the inhibitory effects of Atamestane on androstenedione-induced hyperplastic effects in the prostates of castrated dogs (43). In addition, they also demonstrated that treatment of intact cynomolgus monkeys with androstenedione resulted in estrogen-related changes, particularly in the stroma of the prostate, and these effects were antagonized by simultaneous treatment with Atamestane (44). However, the possibility that the suppression of estrogen resulted in a counter-regulatory increase in androgen, and thereby evoked epithelial growth, must be considered. Using morphometric analysis, Suzuki *et al.* demonstrated that the administration of TZA-2209 led to a significant decrease in stroma and a significant increase in the canine prostate (46). Juniewicz *et al.* reported a change in the endocrine environment of aromatase inhibitor, 4-(5,6,7,8-tetrahydroimidazo [1,5a] pyridin-5-yl) benzonitrile hydrochloride, for intact male dogs (47). They demonstrated that inhibition of estrogen biosynthesis results in increased serum LH and testosterone concentrations as well as increased capacity of the testis to secrete androgens in response to LH. The findings of the present study clearly indicated that the treatment of spontaneous canine BPH with aromatase inhibitor increased serum LH and testosterone concentrations. Thus, it is not surprising that the findings of the present study clearly indicate that an aromatase inhibitor significantly increased prostatic growth in dogs with spontaneous BPH. Moreover, a recent placebo-controlled double-blind study demonstrated that Atamestane is not an effective treatment for symptomatic BPH (14). The possibility that a counter-regulatory increase in androgens may counterbalance any positive effect of the decrease in estrogens to preserve intraprostatic homeostasis was discussed relative to that finding. However, since this failure resulted in a counter-regulatory increase in androgens, one cannot exclude the therapeutic possibility of estrogen deprivation for BPH. The change in the endocrine environment of serum LH and testosterone resulting from the administration of aromatase inhibitor was similar between dogs and humans in the present study.

Recently, we assessed the effect of dual inhibition of 5α -reductase and aromatase on spontaneously developed canine prostatic hypertrophy (48). Administration of a 5α -reductase inhibitor, finasteride, and an aromatase inhibitor, arimidex, resulted in a significant increase in prostate volume, accompanied by a 3- to 10-fold increase in serum testosterone levels and a significant increase in testicular volume. In connection with the synergistic effects of Atamestane and anti-androgen cyproterone acetate (CPA), it would appear that CPA in combination with Atamestane induced a selective and complete inhibition of the androgenic effects at the level of the prostate, while CPA did not negatively influence the function of the testis, the

epididymidis or the pituitary (45). These experimental findings demonstrated that estrogen deprivation with antiandrogen might represent a useful treatment for human BPH. We investigated the synergistic effect of TZA-2237 and CMA on spontaneous canine BPH. Combination treatment with CMA and TZA-2237 induced a significant decrease in the intraprostatic estradiol concentration, and a significant decrease in the smooth muscle component. The decrease in smooth muscle which is related to the neurologic control of prostate tone may improve the clinical symptoms of BPH. However, these combination treatments did not decrease the prostate volume and the relative volumes of glandular epithelium and lumen, and the intraprostatic DHT concentration of the combination treatment group increased to the level similar to the control group. There were clear endocrinological and morphological changes in the prostates of dogs treated with aromatase inhibitor and antiandrogen. It is still necessary to determine the most effective dose of aromatase inhibitor and antiandrogen, and to investigate various factors such as urethral compliance, α_1 -receptor content, residual urine volume, ER- β expression and apoptosis. It is hoped that these studies will give us definitive information in the near future. In addition, it is important to investigate the role of the SHBG-steroid-responsive second messenger system, ER β and ARA70, in the prostate, and to understand how estrogen modulates the autocrine relationship within the stroma and the paracrine relationship between stroma and epithelium.

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