Original Article

Impaired Reproduction in Adult Male, but not Female, Rats Following Juvenile Treatment with the Aromatase Inhibitor, Exemestane

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BACKGROUND: Exemestane is an irreversible steroidal inhibitor of cytochrome-P450 aromatase required for estrogen synthesis. The safety of the drug in the pediatric population, particularly in males, has not previously been evaluated. Given the increased interest in treating children with aromatase inhibitors, we undertook a study in rats to assess the potential for exemestane to alter reproductive development and function when administered to juveniles. **METHODS:** Male and female rats were treated with exemestane at doses anticipated to produce exposures approximately 2- and 35-fold the expected clinical plasma exposure in young adult males during the period of reproductive maturation. After maturation, treated rats were mated to evaluate the potential impact on reproductive function. **RESULTS AND CONCLUSION:** There were no effects on sexual maturation in either sex or on female reproductive function. Treatment of juvenile male rats caused increased cohabitation time and decreased copulation rates; pregnancy rates and litter size were not affected in rats that mated. Decreased testis (10–15%) and epididymis (20–30%) weights, and decreased Sertoli cell numbers were noted at all doses. This indicates that exemestane can reduce Sertoli cell proliferation during maturation. The sensitive window for this effect is expected to be limited to the period of Sertoli cell proliferation, which is completed by around postnatal day 15 in rats and before puberty in humans. Treatment beginning at a later time relative to the window for Sertoli cell proliferation or for a longer duration is not expected to have additional adverse effect as the effect was not shown to be degenerative. *Birth Defects Res (Part B)* 92:304–313, 2011. © 2011 Wiley-Liss, Inc.

Key words: ***juvenile toxicity; rat; exemestane; aromatase inhibitor; puberty; reproduction***

INTRODUCTION

Exemestane is a synthetic steroid that inhibits the conversion of androgens (principally testosterone and androstenedione) to estrogen by irreversibly binding to aromatase, decreasing both ovarian aromatase activity and circulating estrogen levels in rats and humans (Giudici et al., 1988; Zaccheo et al., 1991). Exemestane and other aromatase inhibitors are approved for treatment of breast cancer; however, the role of estrogen in epiphyseal cartilage maturation has led to the use of aromatase inhibitors to promote growth and improve height in both boys and girls (Mauras et al., 2004, 2008; Kumar et al., 2009). Aromatase inhibitors have been used to enhance overall stature in children with various forms of short stature, including growth hormone deficiency (Mauras et al., 2008), Idiopathic Short Stature (Hero et al., 2005), Constiutional Delay of Puberty (Dunkel, 2006, 2009a), McCune-Albright syndrome (Feuillan et al., 2007) and in congenital adrenal hyperplasia (Merke et al., 2000; Hero et al., 2009; Santen et al., 2009; Dunkel, 2009b). Vertebral abnormalities have been reported, but no consistent safety signal has been seen. Lack of such safety concerns may lead to widespread and indiscriminate use in children. Previous reproductive toxicology studies with exemestane in adult rats showed effects consistent with the intended pharmacological effects of the drug, that is, inhibition of aromatase resulting in an increase in circulating androgens and a decrease in circulating estrogens (Beltrame et al., 2001). In adult male rats these effects included compromised mating behavior (decreased copulation rate) as indicated by a marked inability of the rats to produce copulatory plugs when

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mated with untreated females. In adult female rats, exemestane-induced suppression of estrogen secretion, perturbed estrous cyclicity, and adversely affected gestation and parturition, resulting in maternal deaths, abortions, and resorptions. Several studies have evaluated the potential for aromatase inhibition to alter growth and bone development when administered to peripubertal rats (exposure beginning on GD 26 or 31) (Bajpai et al., 2010; van Gool et al., 2010a). In peripubertal male rats, aromatase inhibition with exemestane or letrozole caused decreased growth and altered bone architecture (Bajpai et al., 2010; van Gool et al., 2010a). Peripubertal (postnatal day [PND] 26) female rats treated with exemestane for 3 weeks increased body weight gain, body length, and bone length, decrease trabecular number and thickness, and increased the number of ovarian cysts (van Gool et al., 2010b). These studies suggest that estrogen deprivation during the peri- and post pubertal period has gender-specific effects on growth and bone maturation, although it is not clear if these findings are different than observed in adult animals. To better understand the if aromatase inhibition in juveniles represents a specific concern based on stage of development relative to puberty, the targeted goal of this study was to assess the potential for exemestane to produce adverse effects on development of the reproductive system and reproductive capacity of mature animals when administered to immature (prepubertal) male and female Sprague-Dawley rats. The dosing period for male rats was for 44 days from PND 7 to 50 and that for female rats was 35 days from PND 7 to 41. Postnatal reproductive system development has been shown to occur in this window in female and male rats, respectively (Beckman and Feuston, 2003; Marty et al., 2003). As acute pharmacological effects were expected based on adult data, the focus of the current study was to identify effects that persisted into adulthood (i.e., permanent nonreversible effects) following exemestane administration to prepubertal rats. Therefore, assessments of reproductive competency and integrity of the reproductive system were performed after an approximately 1 month drug hiatus.

MATERIALS AND METHODS

Animals and Treatment

The animal care and experimental procedures of this study were conducted in compliance with the US Animal Welfare Act and performed in accordance with the standards of the Institute of Laboratory Animal Resources (ILAR) Guide (1996). The Pfizer facility in which this study was conducted was accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. Exemestane (PNU-155971; Pfizer, New York, NY) was administered to juvenile male and female Sprague-Dawley rats from PND 7 to 50 and PND 7 to 41, respectively. Dose levels of 30, 100, and 3,000 mg/kg/day were selected as previous studies (unpublished) have shown these dose levels to produce systemic exposures (based on area under the concentration time curve; AUC) that were 2 to 40-fold those seen in young human subjects receiving a single 25 mg exemestane dose (Mauras et al., 2003) (which is believed to approximately result in efficacious exposures in pediatric patients). Exemestane was suspended in 0.5% Methylcellulose/0.4% Tween 80 and animals were dosed once daily by oral gavage at a dose volume of 10 ml/kg.

Forty-four timed-pregnant Sprague-Dawley rats [Crl:CD®(SD)] supplied by Charles River Breeding Laboratories (Kingston, NY) were used to deliver the male and female pups for the study. On PND 1 offspring were randomly distributed (cross-fostered) to form 20 litters of 8 pups each (4/sex) for the main study and an additional 16 litters of 8 pups each (4/sex) were formed for Toxicokinetic assessments. Each newly formed litter contained no more than 1 male and 1 female pup from the same birth litter. Within each newly formed litter pups were assigned to 4 groups of 20 animals per sex/group. In addition to these animals, sexually mature male and female rats of the same strain and source were used for mating with the treated animals. The dams and their litters were housed individually in polycarbonate shoebox cages with heat-treated wood chip contact bedding until weaning (PND 21). After weaning the offspring were housed by sex in groups of 4 until PND 28 at which time they were housed individually. During the mating segment, animals were pair-housed (1 male and 1 female). Food and water were available ad libitum and the room was on a 12-hour light/dark cycle.

Observations and Measurements

All animals were observed at least twice daily in their cages for moribundity, mortality, and clinical signs (any changes in appearance or behavior during the study). Body weights were measured. The onset of sexual maturation was evaluated by monitoring for the age at demonstration of vaginal patency or preputial separation for females or males, respectively. Estrous cycling was evaluated by monitoring the stage from a vaginal smears collected daily beginning on PND 63 and continuing through mating. Treated males and females were cohabitated with treatment naive sexually mature partners in a 1:1 ratio for a maximum of 2 weeks beginning on PND 77. Each morning of the cohabitation period, paired females were examined for evidence of copulation, that is, a sperm positive vaginal smear or observation of a copulatory plug. When positive signs of copulation were noted, the male and female were separated and returned to individual housing. The day that evidence of copulation was found was designated as GD 0. Females were euthanized on GD 14, the uterus and ovaries were removed, and the number of corpora lutea was recorded. The uterus was opened and the location and viability status of each implantation site was recorded. Uteri with no evidence of implantation were placed in a 10% ammonium sulfide solution for detection of early embryonic death. The hind legs were saved in 70% ethanol.

Males were euthanized following the female GD 14 evaluations. The accessory sex glands were weighed as a unit (prostate, seminal vesicles, and coagulating glands), and the weights of the left and right testes and left and right epididymides were recorded individually. A section of the right distal vas deferens was removed for evaluation of sperm motility. A section (~1 cm) of vas deferens was removed and placed in a culture vial containing 10 ml of Bovine serum albumin. The vial was

covered and placed in a warm water bath for at least 5 min. After 5 min, a sample of the diluted sperm was loaded into a glass cannula and the loaded cannula was placed into the computer-assisted sperm analysis system [Hamilton-Thorne Integrated Visual Optic System (IVOS)] for analysis. The seminal vesicles and prostate were fixed in 10% formalin and the left testis and epididymis were fixed in Modified Davidson's solution. The right epididymis and right testis were frozen for later evaluation of sperm count and spermatid head count, respectively. For sperm and spermatid head counts, the tissues were homogenized, mixed with a DNA-specific fluorescent dye, and fluorescent cells were counted using the Integrated Visual Optic System. Hind legs were retained in 70% ethanol. The seminal vesicles, prostate, left testis, and epididymis from the control and high dose males were evaluated using routine histopathology techniques; because no treatment-related effects were noted at the high dose, tissues from animals at the lower doses were not evaluated. Sertoli cells were counted in one section each prepared from the left testis from the control and the 30, 100, and 300 mg/kg dose groups. Sertoli cell numbers were estimated by three different approaches based on the Sertoli cell nucleus, Sertoli cell nucleolus, and seminiferous tubular length with number of Sertoli cells per tubular cross section. By using widely different methods, assumptions made in the calculation by any given method would not influence estimates by the other methods (Johnson et al., 2008).

Femoral length and width were measured using a digital caliper (Mutituyo Corp., Aurora, IL) for all animals. Additionally, three animals/sex/group were evaluated by X-ray radiography (Faxitron X-ray LLC, Wheeling, IL) and Micro-CT was used to visualize anatomy of the distal femur.

Plasma exemestane concentrations were determined at 0.5 hours postdose (HPD) following the first dose on PND 7 and at 0.5, 1, 2, 4, 8, and 24 HPD following administration of the last dose (PND 50 and PND 41 for male and female rats, respectively). The time after administration when the maximum plasma exemestane concentration was reached (T_{max}) in PND 7 rats was established as 0.5 HPD in a previous study (unpublished). Blood samples were collected into tubes containing K_3EDTA and plasma was separated from whole blood by centrifugation and stored frozen at $\sim 20^{\circ}C$ until analyzed using a validated HPLC method.

Statistical Evaluation

Body weight data was evaluated using the Welch trend test; vaginal opening and preputial separation, laparotomy data (numbers of corpora lutea, implantations sites, live fetuses, dead fetuses, early resorptions, late resorptions), estrous cycle data, duration of cohabitation, and sperm data were evaluated using Jonckheere test; copulation and pregnancy rates were evaluated using the Exact trend test for proportions; and organ weight and Sertoli cell data were evaluated using Dunnett's test.

RESULTS

Growth and General Development

Exemestane was well tolerated. Male body weights were similar among groups during the treatment period; however, at 300 mg/kg/day a transient decrease in male body weight was noted following the end of dosing (PND 52–PND 63 body weight were 0.90–0.94 × control) that resolved by PND 66, after which male body weights were similar to controls (Fig. 1A). An increase in female body weights were noted at 100 and 300 mg/kg/day beginning on PND 31 that persisted through the end of the study (Fig. 1B). There were no treatment-related differences in the length or width of the femur (Table 1) and no anatomical differences noted by micro-CT evaluation (data not provided).

Reproductive Development and Function

There were no effects on the age at preputial separation or vaginal patency (Table 2). An increase in the number of days cohabitated before mating (3.1 and 5.2 days for the control and 300 mg/kg/day groups, respectively) and a decrease in the number of males with evidence of copulation (18 copulated out of 19 cohabitated in the control group compared to 15 copulated out of 19 cohabitated at 300 mg/kg/day) was noted for the 300 mg/kg/day males cohabitated with untreated females (Table 3). Although these differences were not statistically significant, the combination of endpoints and a decrease in overall fecundity (number of pregnancies/number of pairs cohabitated; 89, 90, 85, and 68% at 0, 30, 100, and 300 mg/kg/day, respectively) supports a treatment-related decrease in male reproductive function at 300 mg/kg/day (Table 3). There were no effects on estrous cyclicity or on mating and fertility parameters for exemestane-treated females cohabitated with untreated males (Table 4). There were no treatment-related changes in cesarean section observations for treated males mated to untreated females or for treated females mated to untreated males (Table 5).

Treatment-related decreases in the weights of the epididymis, testis, and accessory sex gland were noted for all treatment groups; the differences from control were not statistically significant at 30 mg/kg and sporadically statistically significant at 100 and 300 mg/kg/day, but given the lack of meaningful differences in weights between dose levels the decreases at all dose levels were considered treatment-related and likely represents a flat dose–response (Fig. 2). Expressing the organ weights as a ratio of body weight did not resolve the differences between the control and treated groups (data not shown).

There were no treatment-related effects on sperm motility, epididymal sperm concentration, or testicular spermatid count (Table 6) and no treatment-related microscopic findings (data not shown). A treatment-related reduction in the number of Sertoli cells per testis was noted at all dose levels (Table 7). The number of Sertoli cells per testis was significantly reduced in each of the three treatment groups as determined from each of the three methods of calculation: (1) based on volume densities of Sertoli cell nuclei; (2) based on volume densities of Sertoli cell nucleoli; or (3) based on length of seminiferous tubules. The estimates calculating absolute volume of Sertoli cells per testis based on nucleoli was higher than the other methods, but the relative comparison still indicated a reduced number of Sertoli cells in each of the exemestane-treated groups.

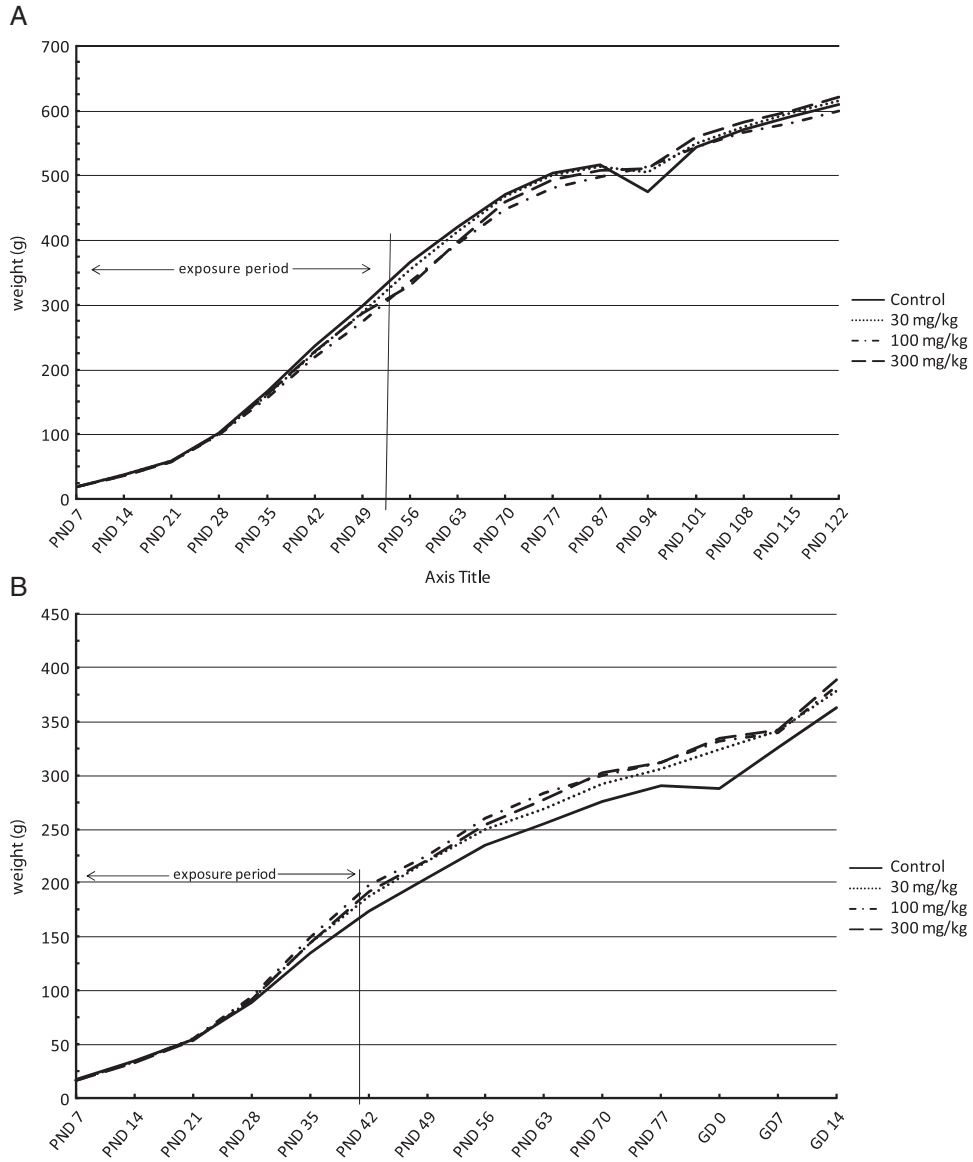

Fig. 1. (**A**) Body weigths of exemestane treated male rats. (**B**) Body weigths of exemestane treated female rats. (A) Male body weights were similar among groups during the treatment period; however, at 300 mg/kg/day a transient statistically significant ($P \leq 0.01$) decrease in male body weight was noted from PND 52 to 63. (B) A statistically significant ($P \leq 0.05$) increase in female body weights was noted at 100 and 300 mg/kg/day beginning on PND 31 that persisted through the end of the study.

Exemestane Exposure

Systemic exposure to exemestane, assessed by Cmax and AUC(0–24) values in males and females on PND 41 and PND 50, respectively, increased dose proportionally (Table 8). However, there was a ~96 to 98-fold decrease in concentration of exemestane (assessed at 0.5-hour time point, previously defined as T_{max}) between PND 7 and PND 41 of 50 at 30 and 100 mg/kg and ~224-fold decrease at 300 mg/kg. The observed age-related change in exposure could be due to several factors, including maturation of metabolism pathways, age-related differences in absorption and distribution, and decreased systemic exposure as exemestane induces an increase in its own metabolism with repeated dosing in rodents (Vignati et al., 2004).

DISCUSSION

Exemestane was well tolerated in immature rats treated with doses up to 300 mg/kg/day treated from PND 7 to 50 and PND 7 to 41 for males and females, respectively. Male body weights were similar among groups during the treatment period; however, a transient decrease in male body weight was noted following the end of dosing that resolved by PND 66. This is consistent with the observation in adult male rats treated with exemestane (Beltrame et al., 2001) and adult male rats treated with the aromatase inhibitor anastrozole (Turner et al., 2000) where body weight were unaltered by chronic administration. However, peripubertal rats treated with exemestane (100 mg/kg/week slow release from PND 26 to 68) or with the aromatase inhibitor

letrozole (1 mg/kg/day from PND 30 to 90) had reduced body weight gain (Bajpai et al., 2010; van Gool et al., 2010a), whereas prepubertal male mice treated letrozole (2 mg/kg/day from PND 21 to 31) showed increased body weight gain (Eshet et al., 2004). These findings may indicate a sensitive period of estrogen-induced growth in rats sometime following onset of puberty, typically between PND 42 and PND 46 (Marty et al., 2003). Increased female body weights were noted beginning on PND 31 that persisted through the end of the study. Similar increases in weight gain have been noted in peripubertal and adult female rats treated with exemestane and were attributed to an anabolic effect secondary to the intended pharmacological effect of exemestane (Beltrame et al., 2001; van Gool et al., 2010b).

There were no effects on femur length, width, or structure in male or female rats as assessed by measuring the length and width of the femur and by Micro-CT evaluation of the femur. This differs from studies showing altered bone growth and architecture in male and female rats treated with aromatase inhibitors (Vanderschueren et al., 1997; Bajpai et al., 2010; van Gool et al., 2010a,b). Treatment of peripubertal male rats with letrozole (1 mg/kg/day from PND 30 to 90) caused reduced bone length and thickness without impacting trabecular volume, although the authors suggest that severe decrease in overall growth may have accounted for this decrease (body weight of letrozole-treated male rats at euthanasia was 46% less than control) (Bajpai et al., 2010). The likelihood that the decrease in bone length noted in letrozole-treated rats is secondary to generalized growth retardation is supported by a study in peripubertal rats treated with exemestane (100 mg/kg/week slow release from PND 26 to 68) showed only small decreases in growth with no effects on bone length (van Gool et al., 2010a). Although the study by van Gool et al. showed no effect on bone length, they did report a decrease in trabecular volume length (van Gool et al., 2010a). Similar effects on trabecular volume were noted in adult male rats treated with the aromatase inhibitor vorozole (17 mg/kg/day from PND 60 for 18 weeks) (Vanderschueren et al., 1997). The consistency in findings between studies in peripubertal and adult male rats indicates that the decrease in trabecular volume is not indicative of a special sensitivity to juvenile rats based on the developmental state of the skeletal system but is a generalized effect on bone in male rats. It is also possible that the discrepancy in effects on trabecular volume may be due to the timing of the bone evaluations relative to exposure, immediately at the end of exemestane treatment compared to 90 days after letrozole treatment, suggesting that any alterations in trabecular volume may have recovered during the posttreatment period. The lack of effect on bone length in our study is consistent with the minimal effect on overall growth, and the lack of effects on bone architecture may be due to the timing of exposure (mainly prepubertal compared to mainly postpubertal) or reflect recovery of

Table 1
Femur Length and Width

	Femur length (mm)		Femur width (mm)	
	Right	Left	Right	Left
Male				
Control	41.3±1.04	41.3±1.09	5.0±0.22	5.0±0.21
30 mg/kg	41.4±0.70	41.6±0.83	5.0±0.23	5.1±0.19
100 mg/kg	41.0±0.94	41.3±0.99	5.0±0.17	5.1±0.25
300 mg/kg	41.2±1.14	41.5±1.17	5.0±0.24	5.0±0.28
Female				
Control	35.9±1.25	35.9±0.78	4.2±0.24	4.2±0.21
30 mg/kg	35.7±1.19	35.9±1.00	4.3±0.28	4.3±0.27
100 mg/kg	36.2±1.23	36.1±1.10	4.4±0.18	4.4±0.12
300 mg/kg	36.3±0.70	36.2±0.87	4.2±0.27	4.3±0.23

Data presented as mean±standard deviation. There were no statistically significant or biologically meaningful changes in femur length or width; $n = 20$ per group.

Table 2
Age at Onset of Preputial Separation, and Vaginal Patency

	Age at preputial separation	Age at vaginal opening
Control	39.2±1.9	33.3±1.3
30 mg/kg	39.6±2.2	33.7±1.8
100 mg/kg	39.3±2.2	33.6±1.7
300 mg/kg	38.6±1.6	32.3±2.0

Data presented as mean postnatal day±standard deviation. There were no statistically significant or biologically meaningful ages at onset of preputial separation or vaginal opening. Number of males evaluated = 19, 20, 20, and 19 for the control, 30, 100 and 300 mg/kg groups, respectively; and number of females evaluated = 18, 20, 19, and 20 for the control, 30, 100 and 300 mg/kg groups, respectively.

Table 3
Mating and Fertility Evaluation of Male Rats Treated with Exemestane

	Days cohabitated[a]	Copulation rate[b]	Pregnancy rate[c]	Fecundity rate[d]
Control	3.1±3.0	95% (18/19)	94% (17/18)	89% (17/19)
30 mg/kg	3.7±4.1	90% (18/20)	94% (17/18)	85% (17/20)
100 mg/kg	4.2±3.8	90% (18/20)	94% (17/18)	85% (17/20)
300 mg/kg	5.2±4.9	79% (15/19)	87% (13/15)	68% (13/19)

There were no statistically significant differences in days cohabitated, or copulation, pregnancy and fertility rates. However, the increase in days cohabited and decrease in copulation and fecundity rates was considered to indicate an adverse effect of exemestane treatment.
[a]Days cohabitated presented as mean±standard deviation.
[b]Copulation rate is the number of animals copulating/number of animals paired for mating.
[c]Pregnancy rate is the number of animals pregnant/number of the animals that copulated.
[d]Fecundity rate is the number of animals pregnant/number of animals paired for mating.

Table 4
Mating and Fertility Evaluation of Female Rats Treated with Exemestane

	Estrous cycle length (days)	Days cohabitated[a]	Copulation rate[b]	Pregnancy rate[c]	Fecundity rate[d]
Control	4.2±0.3	2.4±1.2	100% (18/18)	100% (18/18)	100% (18/18)
30 mg/kg	4.5±0.7	2.2±1.1	100% (20/20)	100% (20/20)	100% (20/20)
100 mg/kg	4.7±1.2	2.0±1.1	100% (19/19)	100% (19/19)	100% (19/19)
300 mg/kg	4.1±0.7	2.6±1.3	100% (20/20)	95% (19/20)	95% (19/20)

There were no statistically significant or biologically meaningful differences in estrous cycles, days cohabitated, or copulation, pregnancy, and fecundity rates.
[a]Days cohabitated presented as mean±standard deviation.
[b]Copulation rate is the number of animals copulating/number of animals paired for mating.
[c]Pregnancy rate is the number of animals pregnant/number of the animals that copulated.
[d]Fecundity rate is the number of animals pregnant/number of animals paired for mating.

Table 5
GD 15 Cesarean Section Data

	Control	30 mg/kg	100 mg/kg	300 mg/kg
Exemestane-treated females mated to untreated males				
Number of animals	18	20	19	19
Corpora lutea	15.7±1.7	16.1±1.8	16.6±2.0	16.4±1.9
Impanation sites	15.1±1.8	15.6±1.8	16.2±2.1	15.0±2.5
Early resorptions	0.9±1.9	2.2±3.5	1.7±3.6	1.6±3.4
Viable embryos	14.2±1.7	13.4±4.4	15.4±4.2	13.4±4.2
Untreated females mated with exemestane-treated males				
Number of animals	17	17	17	13
Corpora lutea	15.4±1.6	15.3±2.8	16.1±2.3	15.2±1.8
Impanation sites	14.9±2.4	14.1±4.0	14.2±4.8	14.3±2.6
Early resorptions	0.5±0.6	1.2±1.4	0.6±0.9	0.4±0.5
Viable embryos	14.4±2.5	12.9±4.9	13.6±4.7	13.9±2.5

Data presented as mean±standard deviation. There were no statistically significant or biologically meaningful differences.

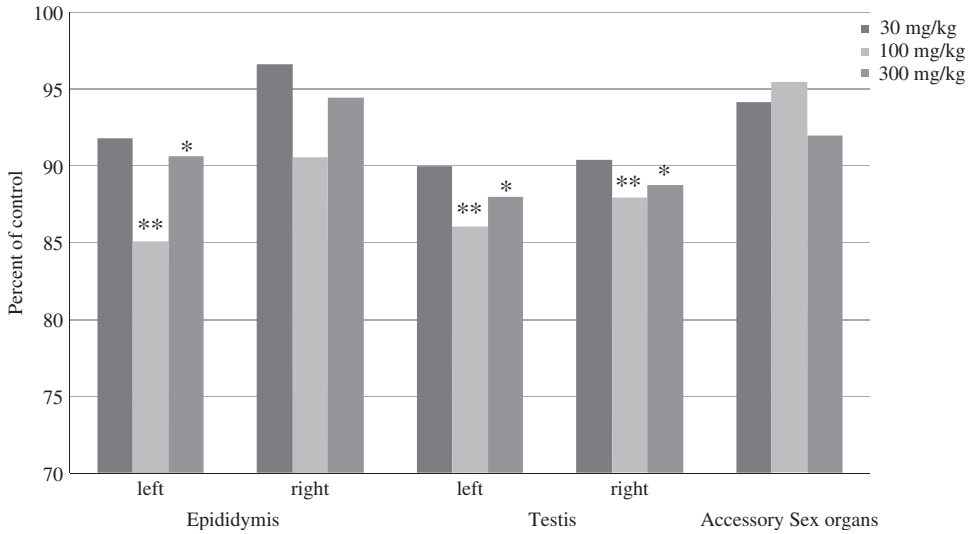

Fig. 2. Male reproductive organ weights. Male rats treated with exemestane during postnatal development showed decreases in the weights of the epididymis, testis, and accessory sex gland at all doses evaluated. Although the differences from control were not statistically significant at 30 mg/kg and only sporadically statistically significant at 100 and 300 mg/kg, given the lack of meaningful differences between dose levels the decreases at all dose levels were considered treatment-related. $^{*}p<0.05$; $^{**}p<0.01$.

any alterations between the end of exposure and evaluation (~2 months).

Treatment of juvenile female rats with exemestane from PND 7 to 41 had no effect on reproductive development or function. This is consistent with a study showing that treatment of juvenile female rats with fadrozole from PND 21 to 80 produced no alterations in reproductive function when evaluated following a

Table 6
Sperm Parameters for Exemestane-Treated Male Rats

Dose (mg/kg)	Control	30 mg/kg	100 mg/kg	300 mg/kg
Sperm motility [a]	89.0±22	90±21	95±2	95±3
Sperm counts[b]	691±227	651±182	711±171	707±135
Spermatid head counts[c]	93±29	102±31	96±28	104±21

There were no statistically significant or biologically meaningful changes in sperm parameters. Data presented as mean±standard deviation. Number of animals evaluated was 19, 20, 20, and 19 in the control, 30, 100, and 300 mg/kg groups, respectively.
[a]Percent motile cells.
[b]Million sperm/gram cauda weight.
[c]Million sperm/gram testis weight.

Table 7
Number of Sertoli Cells per Testis in Male Rats Treated with Exemestane

	0 mg/kg	30 mg/kg	100 mg/kg	300 mg/kg
Number of sertoli cells per testis based on nuclei	34.8±1.7	28.4±1.1*	27.8±1.2*	27.8±1.2*
Number of sertoli cells per testis based on nucleoli	120.7±6.8	81.8±3.0*	85.2±4.7*	99.2±5.8*
Number of sertoli cells per testis based on seminiferous tubule length and number per cross-section	51.6±1.7	36.5±1.4*	32.7±1.0*	38.4±0.9*

The number of Sertoli cells per testis was reduced at all dose levels. The number of Sertoli cells per testis was significantly reduced in each of the three treatment groups as determined from each of the three methods of calculation: (1) based on volume densities of Sertoli cell nuclei; (2) based on volume densities of Sertoli cell nucleoli; or (3) based on length of seminiferous tubules. *$p<0.05$. Data presented as mean number of Sertoli cells ($\times 10^6$)±standard deviation.

Table 8
Exemestane Exposure Data

	PND 7 (0.5 HPD)		PND 41 (0.5 HPD)	PND 50 (0.5 HPD)	$AUC_{0-24\,hr}$ (ng × hr/ml)	
	Female	Male	Female	Male	PND 41 female	PND 50 Male
30 mg/kg	358±358 (4)	374±374 (4)	26±7.9 (4)	22.7±6.3(4)	186	76.4
100 mg/kg	5,940±885 (4)	6,790±2,240 (4)	68.4±18.4 (4)	59.6±9.1 (3)	490	606
300 mg/kg	26,900±22,200 (2)	14,100±3,650 (3)	102±29.4 (4)	69.7±2.5 (4)	1,370	1,270

Data presented as mean±standard deviation (number of samples). 0.5 HPD was previously determined to be Tmax for exemestane in PND 7 rats and was confirmed as Tmax for mature rats in this study. For comparison, C_{max} and $AUC_{0-24\,hr}$ following a single oral dose of 25 mg exemestane to adolescent males were 16.1±8.0 ng/ml and 36.4±8.8 ng × hr/ml, respectively (Mauras et al., 2003). Concentration at 0.5 HPD expressed as ng/ml; $AUC_{0-24\,hr}$ expressed as ng × hr/ml.

30-day recovery period (Nunez et al., 1996). Fadrozole suppressed estrous cyclicity during the treatment period, but normal cycling initiated within several days after treatment was discontinued. Although not monitored during the treatment period in our study, it is also likely that exemestane treatment suppressed estrous cyclicity as has been previously shown in adult females (Beltrame et al., 2001). Exemestane-treated female rats (100 mg/kg/week slow release formulation from PND 26 to 47) showed decreased ovarian weight and an increased incidence of ovarian cysts (van Gool et al., 2010b). The presence of ovarian cysts in adult females rats treated with exemestane has been noted to occur following as little as 8 days of treatment (Mirsky et al., 2011); therefore, the occurrence of ovarian cysts in female rats treated from PND 26 to 47 aligns with the adult female findigns and is not suggestive of a special sensitivity for peripubertal females.

Administration of exemestane (300 mg/kg/day) to immature (prepubertal) male rats from PND 7 to PND 50 caused alterations in male reproductive capability that were manifested by altered mating behavior, reduced male reproductive organ weights, and decreased numbers of Sertoli cells. Exemestane-treated juvenile male rats showed an increase in duration of cohabitation before mating, and a decrease in the number of males copulating. Estrogen plays a significant role in the regulation of sexual behavior in adult male rats (Sharpe, 1998) and administration of the aromatase inhibitors anastrozole and exemestane to adult male rats inhibited mating behavior (Turner et al., 2000; Beltrame et al., 2001). Therefore, it is possible that the effects on mating behavior could be due to residual effects of decreased estrogen in the brain. It is also possible that the alterations in mating behavior could reflect longer-lasting changes in reproductive function. The development of sexually dimorphic behaviors involves both estrogens and androgens, and the manipulation of hormones can impact adult reproductive behaviors (Dohler, 1991, 1998; Wilson and Davies, 2007). Male rats exposed to the aromatase inhibitor letrozole in utero near term (gestation day 21 and 22) showed decreased ability to produce

pregnancy and had altered mating behavior (mounting, intromission and ejaculation) as adults (Gerardin and Pereira, 2002; Gerardin et al., 2008). However, these males also showed impaired fertility as a decrease in the number of fetuses produced per pregnancy. Similarly, boars exposed to letrozole from 1 week of age showed reduced sperm fertilizing ability (McCarthy et al., 2006). In contrast, adult male rats exposed to exemestane as juveniles that copulated produced successful pregnancies and the number of viable fetuses was normal, indicating normal fertility in those animals that mated. This is similar to adult male rats with anastrozole-mediated alterations in mating behavior but, when treated with estrogen to restore mating behavior, had normal fertility (Turner et al., 2000). Additionally, juvenile male rats treated with the aromatase inhibitor fadrozole from PND 21 to 80 showed no effects on mating or fertility following a 30-day recovery period (Nunez et al., 1996). Taken together, this data suggest that that the alteration in mating behavior seen with exemestane treatment during postnatal development is likely due to residual effects of decreased estrogen in the brain and would likely resolve as estrogen levels recover. It is likely that the impaired fertility and permanent effects on male reproductive behavior produced by in utero letrozole exposure is due to the earlier exposure window, consistent with the testosterone spike and establishment of sexually dimorphic that occurs around birth in rodents (Weisz and Ward, 1980; Negri-Cesi et al., 2008).

In addition to the alterations in reproductive behaviors, weights of the testis and right epididymis were reduced in all treated groups. There were no histological lesions in the testis; however, the number of Sertoli cells was decreased in all dose groups, and similar to the decrement in testis weight the decrease in Sertoli cell number did not increase with increasing dose. Decreases in testis weight without histological correlate were also noted in rats treated with exemestane from PND 26 to 48, although the number of Sertoli cells was not evaluated (van Gool et al., 2010a). Maturation of Sertoli cells occurs around puberty and involves loss of proliferative ability; therefore, as only immature Sertoli cells proliferate the number of Sertoli cells is determined before adulthood (Sharpe et al., 2003). In rats, Sertoli cells divide mostly prenatally with division tapering off postsnatally and ceasing about PND 15 (Sharpe et al., 2003). As the number of Sertoli cells established during the prenatal and early postnatal period determines the final testis size and sperm output (Sharpe et al., 2003; Walker, 2003), inhibition of Sertoli cell proliferation may have resulted in decreased testicular weights in the study. Estrogen receptors are present in Sertoli cells and in vitro estrogen promotes proliferation of Sertoli cells cultured from immature rats (Lucas et al., 2008). It has been proposed that estrogen has a stimulatory effect on Sertoli cell division and that the source of estrogen is via FSH-induced aromatase activity (Dorrington et al., 1993; O'Donnell et al., 2001). In contrast to the effects on Sertoli cell and testis weights produced by exposure to juvenile rats, chronic (19 weeks) treatment of adult male rats with anastrozole did not alter Sertoli cell number (Turner et al., 2000). This supports the hypothesis that the decrease in Sertoli cells in adult male rats exposed to exemestane during postnatal development is due to inhibited proliferation during a sensitive window of development; as Sertoli cell proliferation is completed by about PND 15 in rat (Orth, 1982; Wang et al., 1989) exposure after that period would not be expected to alter the number of Sertoli cells.

Each Sertoli cell supports a finite number of germ cells; therefore, the number of Sertoli cells is key to sperm production (Russell and Peterson, 1984; Orth et al., 1988; Johnson et al., 2008). Although exemestane exposure to juvenile male rats reduced the number of Sertoli cells, sperm measures were normal (i.e., no effects on sperm motility, epididymal sperm concentration, and testicular spermatid concentration). Additionally, for animals that copulated there was no reduction in fertility. Therefore, the decreased testicular and epididymal weights, and reduced Sertoli cell number had no detectable effects on testicular function or reproductive outcomes. This is likely due to the decrease in Sertoli cell numbers being quite slight, and that each individual rat Sertoli cell can sustain a large number of germ cells (Russell and Peterson, 1984). In addition, the lack of an effect on sperm parameters and fertility is consistent with a study showing that juvenile male rats treated with the aromatase inhibitor fadrozole from PND 21 to 80 showed no effects on mating or fertility following a 30-day recovery period (Nunez et al., 1996), although Sertoli cell numbers were not evaluated in that study.

The lack on effects on sperm parameters following postnatal exposure to exemestane or fadrozole differs from studies where in utero exposure decreased the number of spermatozoa and reduced daily sperm production (Gerardin and Pereira, 2002; Gerardin et al., 2008). Although the numbers of Sertoli cells were not determined in the studies showing decreased spermatozoa and sperm production, fetal baboons exposed to letrozole during the second half of gestation did not show a decrease in Sertoli cell number when evaluated at term (Albrecht et al., 2009). In all species evaluated, it appears that Sertoli cell proliferation occurs during two periods of life, in the fetal/neonatal period and in the peripubertal period, and these periods of proliferation are separated by a quiescent period (Russell and Peterson, 1984; Cortes et al., 1987; Wang et al., 1989; Sharpe, 1994; Marshall and Plant, 1996; Sharpe et al., 2003) In species such as rat that have short duration between the neonatal and peripubertal period, there is little to distinguish these two periods of Sertoli cell proliferation, whereas in humans the separation in age is large and there are two clearly distinguishable periods. It appears that different factors control proliferation during these two periods (Sharpe et al., 2003). Therefore, the discrepancy between findings following in utero and postnatal exposure may reflect a different role for estrogen on testicular development at different stages of maturation.

There also appears to be some cross-species differences in the role for estrogen in Sertoli cell development, most notably, letrozole administered to boars during postnatal development produced increased testis weight and increased the number of Sertoli cells (At-Taras et al., 2006; Berger et al., 2008). In addition, these animals showed histological evidence of delayed testicular maturation and sperm production was increased. Although this clearly shows species differences in the

response to estrogen deprivation during testicular maturation, the boar is unusual in that they have much greater concentrations of estrogen than males of other species (Overpeck et al., 1978; Ford, 1983). Other differences in mechanism involved in testicular maturation have been noted between rodent and pigs (Panno et al., 1995; Klobucar et al., 2003), suggesting that the pig may not be a representative model for male reproductive development (Ramesh et al., 2007).

Treatment of male rats with the aromatase inhibitor letrozole from PND 30 to 90 caused focal prostatic hyperplasia that was evident 90 days after the end of treatment (Bajpai et al., 2010). The lack of a similar effect on prostate histology in our study is likely due to the younger age of the animals at time of exposure (PND 7–50).

CONCLUSION

Administration of exemestane to immature (prepubertal) Sprague-Dawley rats (PND 7–41 or 50 for females and males, respectively) had no effect on female reproductive development but did impact male testicular development by inhibiting Sertoli cell proliferation with a subsequent reduction in testis weight, and reproductive function. The systemic exposure for the lowest dose that these effects were noted at was only slightly higher than the exposure produced following a single oral dose of 25 mg exemestane to adolescent boys (Mauras et al., 2003). In rats and humans Sertoli cell proliferation continues into the prepubertal period (Sharpe, 1994; Marshall and Plant, 1996; Sharpe et al., 2000) and maturation of Sertoli cells involves loss of proliferative ability; therefore, as only immature Sertoli cells proliferate the number of Sertoli cells is determined before adulthood (Sharpe et al., 2003). Our findings are consistent with a hypothesis that reduced estrogen, via inhibition of aromatase activity by exemestane, caused suppression of Sertoli cell proliferation that was subsequently reflected by reduced testis weight. Although the decrease in Sertoli cells had no functional correlate in rats, this may not extrapolate to humans. Each Sertoli cell supports a finite number of germ cells; therefore, the number of Sertoli cells is key to sperm production (Russell and Peterson, 1984; Orth et al., 1988; Johnson et al., 2008). Because Sertoli cell proliferation is normally complete by ages 12 to 15 in humans (Cortes et al., 1987), treatment with exemestane after that age would not be expected to impact Sertoli cell number, but may be detrimental if used before this age and stage of development. The role of estrogen in epiphyseal cartilage maturation has led to the use of aromatase inhibitors in children with growth disorders where AI's are intended to interfere with epiphyseal plate closure, and thus prolong the time available for growth. The success of such treatments, combined with the lack of a consistent safety signal may lead to indiscriminate and inappropriate use of AI's in children. Vertebral body and intervertebral disc abnormalities have been reported in children, and are a reason for caution. We are unaware of reports of boys with Sertoli cell impairment, but appropriate studies will not easily be done in humans. We therefore suggest that our findings of reduced Sertoli cell number in rats be viewed as further reason for caution in the use of aromatase inhibitors in boys under the age of 12 to 15 years.

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