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TWO AROMATASE INHIBITORS INHIBIT THE ABILITY OF A THIRD TO PROMOTE MATING IN MALE RATS

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Abstract

Aromatase, the enzyme that aromatizes androstenedione (A) to estrone and testosterone (T) to estradiol (E), affects androgen control of male sex behavior in many vertebrates. In male monkeys, rats and quail, E mimics the ability of T to promote mating, and aromatase inhibitors block mating induced by T but not E. Aromatase inhibitors include androgens with different A-rings than T and A, *e.g.*, 1,4,6-androstatriene-3,17-dione (ATD), azoles, *e.g.*, fadrozole, and androgens α-halogenated at carbon 6, *e.g.*, 6α-bromoA, 6α-fluoroA and 6α-fluoroT. 6α-FluoroT is the only 6α-halogenated androgen studied in regard to mating. It promotes mating in male rats and quail and was studied, before it was known to inhibit aromatase, because it can not be aromatized yet has the same A-ring as T. 6α-FluoroT might promote mating by binding estrogen receptors (ER) directly, *i.e.*, unassisted, or by metabolism to an androgen that binds ER. Since neither process would require aromatase, this study tested both hypotheses by determining how mating induced in castrated male rats by 6α-fluoroT is affected by ATD and fadrozole. Both aromatase inhibitors inhibited the effects of 6α-fluoroT on mating. Thus, 6α-fluoroT does not promote mating by direct ER binding or metabolism to another androgen. Since aromatase underlies a process in which 6α-fluoroT, unlike most nonaromatizable androgens, mimics T effects on male sex behavior, the process must involve a feature that 6α-fluoroT shares with T but not other nonaromatizable androgens. A-ring structure is a candidate. A hypothesis is also offered for how aromatase may participate without aromatizing the androgen.

Keywords

aromatase; male sex behavior; 6α-fluorotestosterone; 6α-halogenated androgen; 1,4,6-androstatriene-3,17-dione; fadrozole

Introduction

Aromatase is unique among cytochrome P450 enzymes in its selectivity for androgen substrates and is the only vertebrate enzyme known to convert androgens to estrogens, such

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as androstenedione (A) to estrone and testosterone (T) to estradiol (E). How it effects the last step in this reaction, *i.e*., A-ring aromatization, is unclear, as is the basis of its androgen selectivity. Thus aromatase is studied to understand its structure-function relationships (Ghosh et al., 2009). It is also studied to identify inhibitors for use in treating cancers. Aromatase inhibitors include androgens with different A-rings than A and T, *e.g*., 1,4,6-androstatriene-3,17-dione (ATD), azoles, *e.g*., fadrozole, and androgens α-halogenated at carbon 6 (C6), *e.g*., 6α-bromoA (Osawa et al., 1987), 6α-fluoroA (Rowlands et al., 1987) and 6α-fluoroT (Kellis and Vickery, 1990). When A is in the aromatase catalytic cleft, C6 is near the access channel (Ghosh et al., 2012).

Aromatase is abundant in tissues that secrete estrogens, such as ovary and placenta, so they are used to assay androgen aromatizability and identify inhibitors. But aromatase is also found in tissues where the estrogen produced acts locally, such as breast and the preoptic area (POA) of the brain. The POA is a site where T acts to stimulate male sex behavior, particularly mounting, and E formed there mediates at least some effects of T. Like T, E promotes mounting, and this effect of T, but not E, is blocked by ATD and fadrozole in male monkeys, rats and quail (Morali et al., 1977; Adkins et al., 1980; Bonsall et al., 1992; Zumpe et al., 1993; Foidart et al., 1995; Vagell and McGinnis, 1997).

The only 6α-halogenated androgen studied in regard to mating, 6α-fluoroT, was studied, before it was known to inhibit aromatase, because it is nonaromatizable (Gual et al., 1962) yet has the same A-ring as T; thus, it dissociates features commonly confounded in nonaromatizable androgens like 5α-dihydroT (DHT; Thompson and Siiteri, 1974) that do not support mating.

6α-FluoroT promotes mating in male rats (Yahr and Gerling, 1978; Nordeen and Yahr, 1981) and quail (Adkins et al., 1980). How it does this is unclear, but it does not simply become T *in vivo* as it is only half as potent as T on seminal vesicles and for defeminizing sex behavior. It also activates hypothalamic-POA nuclear estrogen receptors (ER) more slowly than T does. A second possibility considered (Nordeen and Yahr, 1981) was that 6α-fluoroT may act like 5α–androstane-3β,17β-diol (3β-Diol), that is, by binding ER directly, *i.e*., unassisted (Vreeburg et al., 1975), or by being metabolized to an androgen that does.

A third was that 6α-fluoroT may be aromatized after all, or in brain if not placenta. This possibility was raised because the aromatizability of 6α-fluoroT had been evaluated with a colorimetric test (Gual et al., 1962) that could have lacked sensitivity. But this hypothesis is no longer viable. 6α-FluoroT is as ineffective a substrate for aromatase in rat ovary and human placental microsomes as DHT and other androgens deemed nonaromatizable (Kellis and Vickery, 1990). Radioimmunological and fluorometric tests that detected 1% of the estrogen formed from T detected none from 6α-fluoroT. Also, in rats, aromatase activity in ovary and the male POA reflects an enzyme encoded by the same RNA transcript (Roselli et al., 1998).

This research tested the remaining hypothesis. Since neither direct ER binding nor metabolism to another androgen requires aromatase, this study asked if either ATD or fadrozole would affect the ability of 6α-fluoroT to promote mating in castrated male rats.

Androgens were given as their propionate esters, TP and 6α-fluoroTP, but de-esterification occurs rapidly *in vivo* (Keating and Tcholakian, 1983). Treatment began at castration using half the dose needed for TP to maintain mating (Nordeen and Yahr, 1981) to optimize chances of detecting inhibitory effects of fadrozole or ATD, if present. When mating declined, the androgen doses were increased as more androgen is needed to reinstate mating than to maintain it.

Materials and methods

Animals

Long-Evans male and Sprague-Dawley-derived female rats (Simonsen Labs; Gilroy, CA) were housed individually (males) or in groups (females) under a 14:10-hr light:dark cycle. Food and water were freely available. Females were ovariectomized and injected subcutaneously (sc) with 20 μg E benzoate 2 days before use and 400 μg progesterone 3 hr before use. Steroids were in 0.1 ml safflower oil unless noted. For all surgeries, rats were anesthetized with intraperitoneal injections of ketamine (100 mg/kg) and xylazine (10 mg/kg). All procedures were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Behavioral testing

Males were pretested under red light in early scotophase at 1- to 3-day intervals to identify those that would copulate reliably, *i.e.*, ejaculate in three of 3–5 tests. Each male was placed in a Plexiglas arena (32–42 cm diam) with sawdust on the floor. After 5 min, a female was introduced. The male was given 30 min to intromit. If he did not do so in 15 min, a new female was used for the rest of the test. If he intromitted, he was given 30 min to ejaculate.

Later tests were like pretests except males had 15 min after ejaculation to intromit again. The number of mounts (with pelvic thrusting) and intromissions were recorded as were latencies from introduction of the first female to the first mount and intromission, from first intromission to ejaculation, and from ejaculation to the next intromission (post-ejaculatory interval; PEI). Intromission and ejaculation latencies and the PEI were used only if the male showed the prerequisite behavior (mount, intromission or ejaculation, respectively). If he did not show a behavior but had shown its prerequisite, he was given the shortest latency for the omitted behavior consistent with his observed behavior (15 min for PEI; 30 min for others). Mount rate was computed as the number of acts involving a mount (mounts, intromissions, ejaculation) divided by test length, excluding the PEI. Mount efficiency (the percent of mounts resulting in intromission) was computed by dividing the number of acts with intromission (intromissions, ejaculation) by the number involving a mount, excluding the PEI, and multiplying by 100.

Experimental design and treatments

Reliable copulators (N = 51) were weighed, castrated and randomly assigned to two sets of three groups each. Starting the day after castration, one set was injected sc daily with TP and the other with 6α-fluoroTP (Steraloids, Newport RI) at a dose of 25 μg/day. That was

increased to 50 μg after week 5, 100 μg after week 6, 200 μg 4 days after week 7, and 400 μg after week 8.

In each androgen-treatment set, one group (N = 8) was injected sc with 15 mg ATD (Steraloids) in 0.2 ml oil just before each androgen injection. Another (N = 9–10) was implanted sc at castration with osmotic pumps (Alzet, Cupertino CA) filled with fadrozole (Novartis, Basel) in saline (8.33 mg/ml). The third group (N = 8) was a control. ATD-treated males and controls were given empty Silastic capsules sc at castration.

More males were assigned to fadrozole groups in case any pumps malfunctioned. Pumps were replaced every 2 wk and monitored per manufacturer's instructions to verify the release rate (0.5 μl/hr). They performed properly, exposing males to 0.25 mg/kg/day fadrozole, but four males did not wake up after one replacement surgery. This reduced the N for fadrozole groups to 7 (TP) and 8 (6α-fluoroTP) for weeks 6–9. Also, one male given TP + ATD died after week 4, reducing the N for that group to 7 for later tests.

Starting 9 days after castration, males were tested weekly for 9 weeks, except in week 4. The day after the last test, males were weighed and euthanized. Seminal vesicles were removed and stored in Bouin's fluid until they were cleaned, ruptured, blotted dry and weighed.

Statistical analyses

Mean scores for each male for weeks 1–5 were analyzed within androgen-treatment set by 1-way analysis of variance followed by orthogonal contrasts using Stata/IC software (College Station, TX). Fisher exact probabilities (2-tailed) were used to compare, within set, the number of males that mounted in weeks 8–9.

Results

Mating declined after castration while males were exposed to TP and 6α-fluoroTP at a dose below the threshold needed for TP to maintain mating. Raising the doses to that threshold did not stop the behavioral decline once it had begun. During this phase of the study, males given 6α-fluoroTP differed in mount latency [$F(2,23) = 3.93$, $p < 0.04$], mount rate [$F(2,23) = 3.67$, $p < 0.05$] and PEI [$F(2,19) = 4.54$, $p < 0.025$]. This reflected the effects of fadrozole. 6α-FluoroTP-treated males given fadrozole took longer to initiate mounting [$F(1,23) = 7.13$, $p < 0.015$], mounted at a slower pace [$F(1,23) = 7.10$, $p < 0.015$] and had longer PEIs [$F(1,19) = 8.49$, $p < 0.01$] than controls and those given ATD. PEI was also affected in TP-treated males [overall $F(2,22) = 14.37$, $p < 0.0002$; fadrozole vs. other groups $F(1,22) = 26.08$, $p < 0.0001$]. Control and ATD groups did not differ in either androgen-treatment set, and groups did not differ overall on any other measure in either set. These data are shown in Figure 1 and Table 1.

Higher androgen doses restored mounting in the control groups, and both fadrozole and ATD blocked this (see Fig. 1). In weeks 8–9, 8 of 8 TP controls mounted versus 5 of 14 males given ATD or fadrozole [Fisher exact $p = 0.0055$]. Among males given 6α-fluoroTP, 7 of 8 controls mounted versus 0 of 16 males given ATD or fadrozole [Fisher exact $p < 0.0001$].

All but five males gained weight during the study. Those five had been given ATD (3 TP, 2 6α-fluoroTP) and lost 3–15 g. Among TP-treated males, controls gained more weight than other groups [TP: $F(1,19) = 22.22$, $p < 0.0003$; overall [$F(2,19) = 14.96$, $p = 0.0001$], and males given fadrozole gained more than those given ATD [$F(1,19) = 7.70$, $p < 0.02$]. In 6α-fluoroTP-treated males, weight gain did not vary significantly overall, but controls gained more than other groups [$F(1,23) = 4.33$, $p = 0.05$]. Seminal vesicles were twice as large in controls given TP versus 6α-fluoroTP (Table 1). ATD and fadrozole did not affect this measure.

Discussion

The data presented here show that two aromatase inhibitors, ATD and fadrozole, inhibit the ability of 6α-fluoroT to promote mating in male rats. This rules out the hypothesis that 6α-fluoroT stimulates mating by binding ER directly, *i.e.*, unassisted, or by being metabolized to another androgen since neither process requires aromatase activity, *i.e.*, A-ring aromatization and estrogen synthesis. But explaining how aromatase mediates the action of a nonaromatizable androgen aromatase inhibitor is a challenge.

Part of the process may involve a specific androgen feature. Since 6α-fluoroT, unlike other nonaromatizable androgens, mimics T effects on male sex behavior, the feature involved is presumably one that 6α-fluoroT shares with T but not other nonaromatizable androgens. A-ring structure is a candidate. As for how aromatase may use androgens with an appropriate A-ring without aromatizing them, I offer this highly speculative hypothesis.

Just as dopamine can activate the progesterone receptor and phosphorylation can activate ERβ without ligand (Power et al., 1991; Sanchez et al., 2010), aromatase may be able to activate an adjacent ER by transferring directly to it an androgen, which has undergone a temporary shape change as a result of having been in the catalytic cleft, as it exits the access channel. Since aromatase would not normally encounter halogenated androgens, I must postulate that it can also act this way with T. Since fadrozole and ATD block 6α-fluoroT effects on male sex behavior, they presumably block the hypothesized transfer function as well as aromatization. If aromatase does act in more than one way, 6α-halogenated androgens may be useful for dissociating them.

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Highlights

- Aromatase inhibitor 6α-fluorotestosterone (6α-fluoro T) promotes male sex behavior.
- Aromatase inhibitors fadrozole and ATD block mating induced by 6α-fluoroT.
- Thus, 6α-fluoroT does not promote mating by unassisted estrogen receptor binding.
- And, 6α-fluoroT does not promote mating via metabolism to another androgen.
- Aromatase underlies the action of a nonaromatizable androgen aromatase inhibitor.

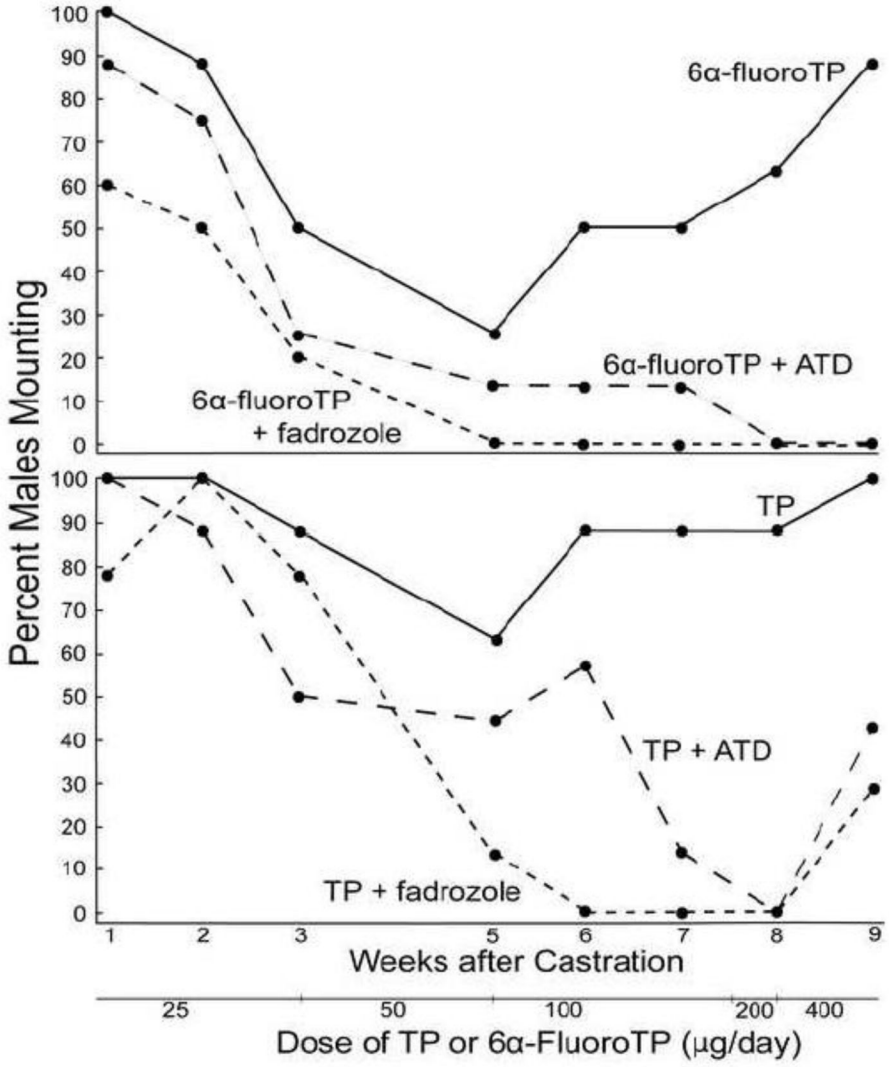

Fig. 1.

Effects of ATD and fadrozole on the mounting behavior of castrated male rats given TP or 6α-fluoroTP. Weekly testing began nine days after castration. Fadrozole exposure began at castration. All other treatments began the next day.

Table 1

Parameters (mean ± SEM) of male sex behavior over the first five weeks after castration and body and seminal vesicle weights at the end of the study for male rats given 6α-fluoroTP or TP alone (control) or with ATD or fadrozole. The PEI was computed only for tests in which a male ejaculated (mts = mounts; intro = intromission).

	Mount rate (mounts per min)	Mount latency (min)	Mount efficiency (% mts with intro)	PEI (min)	Body weight gain (g)	Seminal vesicle weight (mg/100g)
6α-FluoroTP						
Control	1.0 ± 0.3	13.9 ± 2.3	71 ± 5	6.9 ± 0.2	49 ± 10[a]	41 ± 6
ATD	1.2 ± 0.3	16.9 ± 2.6	74 ± 5	7.6 ± 0.7	22 ± 8	64 ± 22
Fadrozole	0.4 ± 0.1[a]	22.9 ± 1.8[a]	65 ± 8	9.9 ± 1.2[a]	31 ± 8	82 ± 17
TP						
Control	2.1 ± 0.4	5.9 ± 2.5	73 ± 3	6.1 ± 0.3	55 ± 4[a]	89 ± 11
ATD	1.3 ± 0.4	12.0 ± 3.1	71 ± 3	7.3 ± 0.3	7 ± 7[b]	64 ± 5
Fadrozole	1.0 ± 0.3	13.2 ± 2.9	72 ± 5	9.8 ± 0.7[a]	32 ± 8	100 ± 21

[a] Group differs from others given the same androgen.

[b] Group differs from the one given fadrozole and the same androgen.