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ORIGINAL PAPER

A selective androgen receptor modulator with minimal prostate hypertrophic activity enhances lean body mass in male rats and stimulates sexual behavior in female rats

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Abstract Androgen receptor (AR) ligands with tissue selectivity (selective androgen receptor modulators, or SARMs) have potential for treating muscle wasting, hypogonadism of aging, osteoporosis, female sexual dysfunction, and other indications. JNJ-28330835 is a nonsteroidal AR ligand with mixed agonist and antagonist activity in androgen-responsive cell-based assays. It is an orally active SARM with muscle selectivity in orchidectomized rat models. It stimulated growth of the levator ani muscle, stimulating maximal growth at a dose of 10 mg/kg. At the same time, JNJ-28330835 reduced prostate weight in intact rats by a mean of 30% at 10 mg/kg, while having no inhibitory effect on muscle. Using magnetic resonance imaging (MRI) to monitor body composition, it prevented half of the loss of lean body mass associated with orchidectomy, and restored about 30% of lost lean mass to aged orchidectomized rats. It had agonist effects on markers of both osteoclast and osteoblast activity, suggesting that it reduces bone turnover. In a model of sexual behavior, JNJ-28330835 enhanced the preference of ovariectomized female rats for sexually intact male rats over nonsexual orchidectomized males. JNJ-28330835 is a prostate-sparing SARM with the potential for clinically beneficial effects in muscle-wasting diseases and sexual function disorders.

Keywords Selective androgen receptor modulator · Prostate · Lean body mass · Sexual function

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Introduction

Androgens control sexual function in males and are central to the anabolic processes that underlie the development of male sexual and physiological characteristics [1]. Serum androgen levels (primarily testosterone [T]) are low prior to puberty and climb exponentially during adolescence in the male. As men age, T levels decline during the so-called andropause [2].

Androgens have anabolic activity in prostate, bone, muscle, and hair follicles of the scalp and skin [3]. Normal levels of T are required for maintaining muscle mass and strength in adult males. Young hypogonadal males exhibit decreased muscle strength, decreased libido, sparse body hair, and, in severe cases, osteopenia and gynecomastia [4]. These patients respond well to exogenous T. The clearest benefits are seen on mood and body composition, with an increased lean body mass and decreased fat mass. Multiple exploratory trials of T therapy have been performed in older men [2], and the results suggest beneficial effects on body composition and strength, bone density, mood, sexual function, and quality of life. Potential risks of T therapy include increased hematocrit and the possibility of causing prostate cancer or accelerating latent disease.

Men lose bone density as they age, and fracture rates in men in their seventies and later approach those in women [5]. This is due to deterioration in both bone density and muscle strength; in other words, frailty. Androgens are anabolic in bone, and it appears that both estrogens and androgens directly contribute to bone health [6].

Androgens might be useful in both women and men. For example, a compound that strengthened bone and muscle—thereby reducing frailty—without virilizing effects on the skin, hair follicles, or vocal cords, or negative effects on cardiovascular health could be used to prevent bone

fractures in women. Similarly, a nonvirilizing androgen might be used to treat female sexual dysfunction, a condition that is known to respond to exogenous T [7, 8].

A handful of nonsteroidal SARMs with prostate-sparing agonist activity have been described [9–13]. All of these SARMs were orally bioavailable, and retained the muscle anabolic activity typical of androgens without a comparable stimulatory effect on prostate in rodent models. Several SARMs had positive effects on bone and sexual behavior. Bone mineral density or bone strength were increased in castrated male and female rats [10, 12, 14], and the number of mounts, emissions, and ejaculations in the presence of a responsive female was enhanced following the treatment of orchidectomized rats [12]. No studies have reported the effects of a SARM in animal models of female sexual function.

Our laboratory has identified several distinct chemical classes of nonsteroidal SARM [15–25]. These compounds were identified by screening in orchidectomized immature male rats. They were either partial androgen agonists or full androgen antagonists based on the degree of prostate hypertrophy observed in the absence or presence of exogenous testosterone. This report describes the pharmacology of the prostate-sparing SARM JNJ-28330835 in receptor binding and cell-based assays, and in rat models of muscle weight, lean body mass, and sexual function.

Results

In vitro properties

JNJ-28330835 is a pyrazoline derivative (Fig. 1), one of several lead series identified in an extended program to discover nonsteroidal, orally bioavailable small molecules with anabolic effects on muscle, and minimal androgenic effects on prostate [25]. JNJ-28330835 emerged from our structure–activity relationship studies on the pyrazoline analogs.

The in vitro properties of JNJ-28330835 were assessed in receptor binding and cell-based functional assays. Binding to steroid receptors was assessed using full-length recombinant receptors expressed in adenovirus–transduced Cos-7 cells or purified from baculovirus-transduced insect cells. The receptors were from rat (AR) or human (progesterone receptor [PR], glucocorticoid receptor [GR], estrogen receptor α [ERα], and estrogen receptor β [ERβ]). Binding data are summarized in Table 1. JNJ-28330835 bound to AR with a mean K_i of 630 nM (n = 3). It did not bind significantly to other steroid receptors.

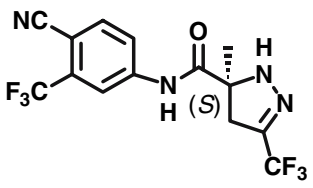

Fig. 1 Chemical structure of JNJ-28330835

Table 1 Binding of JNJ-28330835 to steroid receptors

Receptor	K_i (nM)[a]	
	Standard[b]	JNJ-28330835
AR	0.097	630
PR	5.5	>5000
GR	2.6	>20,000
ERα	6.5	50,000
ERβ	5.5	45,000

[a] Mean of 3 determinations

[b] R1881 (AR), R5020 (PR), dexamethasone (GR), 17β-estradiol (ERβ and ERβ)

In vitro androgen agonism and antagonism were measured using L929 mouse fibroblasts transduced with an androgen-responsive mouse mammary tumor virus-luciferase reporter [15, 26]. These cells possess low levels of functional AR that can be detected by adenovirus-mediated transduction of the reporter. JNJ-28330835 was tested as both an agonist and an antagonist in these cells. In the agonist format (Fig. 2A), JNJ-28330835 weakly (EC_{50} 15 μM) and partially (50% activation relative to maximal stimulation by the steroidal androgen R1881) stimulated the reporter. In the antagonist format (Fig. 2B), JNJ-28330835 lacked potency (IC_{50} 18 μM), but was fully effective (100% inhibition in the presence of 1 nM R1881).

The specificity of JNJ-28330835 was tested using functional assays of progestin and estrogen activity (data not shown). In T47D human breast cancer cells, JNJ-28330835 was a weak progestin agonist with an EC_{50} of approximately 50 μM; it stimulated 90% of progestin-dependent alkaline phosphatase activity at 100 μM. It had little or no progestin antagonist activity (17% inhibition at 100 μM). In Ishikawa human endometrial cells, which possess detectable levels of ERα, but not of ERβ, JNJ-28330835 had no estrogen agonist activity (EC_{50} > 100 μM) and very weak estrogen antagonist activity (IC_{50} 50 μM).

Rat tissue weights

The hypertrophic effects of JNJ-28330835 on prostate and its anabolic effects on muscle were assessed in mature rats using the Hershberger assay [27]. Orchidectomized rats were treated for 2 weeks with orally administered JNJ-28330835. The wet weights of ventral prostate and levator

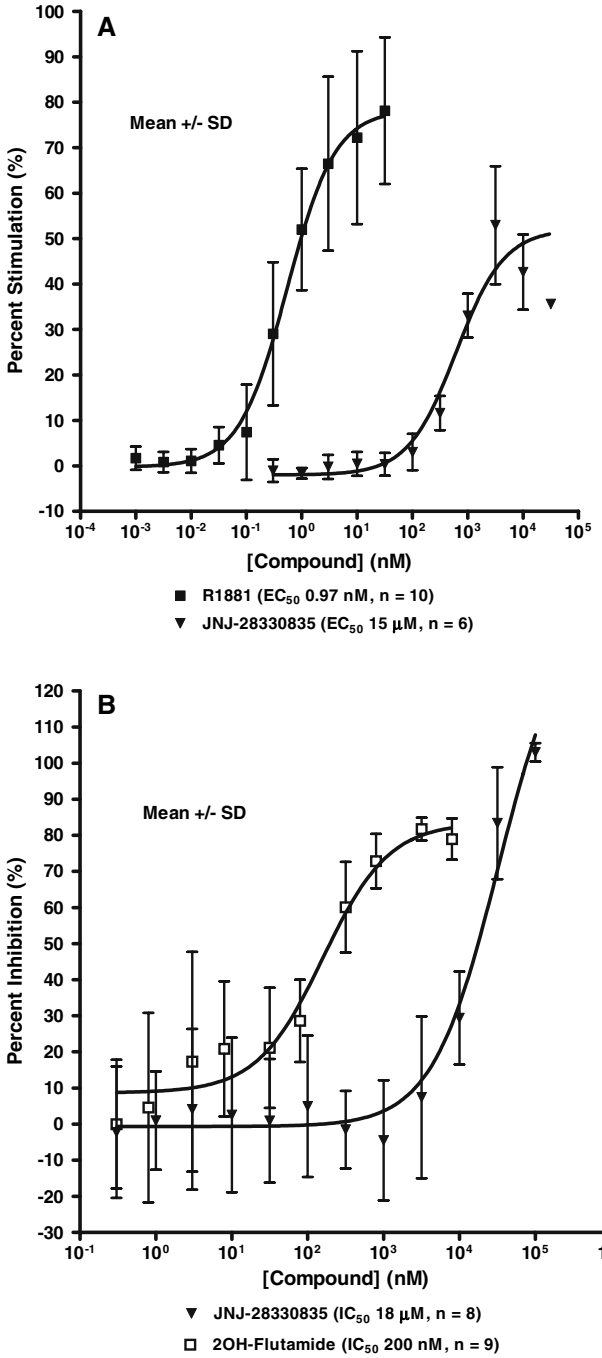

Fig. 2 Androgen functional activity in L929 cells. (**A**) agonist format with the compounds alone. (**B**), antagonist format with the compounds in the presence of 1 nM R1881. Each data point is the mean ± SD of at least six determinations

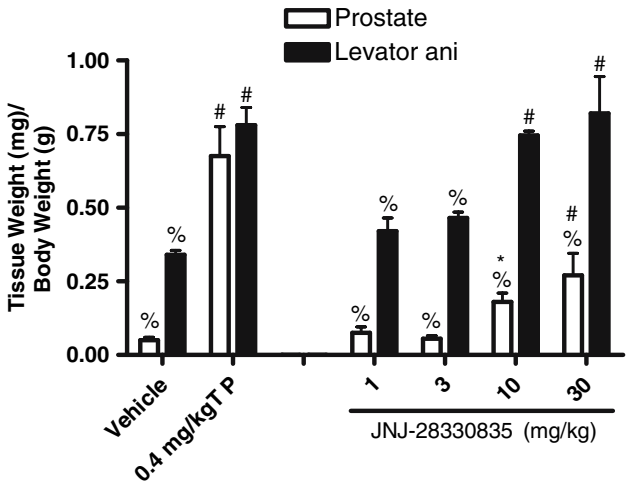

Fig. 3 Anabolic and androgenic activity in orchidectomized rats. Mature rats were treated with JNJ-28330835 for 2 weeks and tissue wet weights were measured. Tissue weights were normalized to body weight at necropsy. Mean ± SD, $n = 3$ per group. Statistically significant difference from the controls (calculated by ANOVA) is shown for each group: #, $P < 0.01$ and *, $P < 0.05$ relative to vehicle control; %, $P < 0.01$ relative to TP control

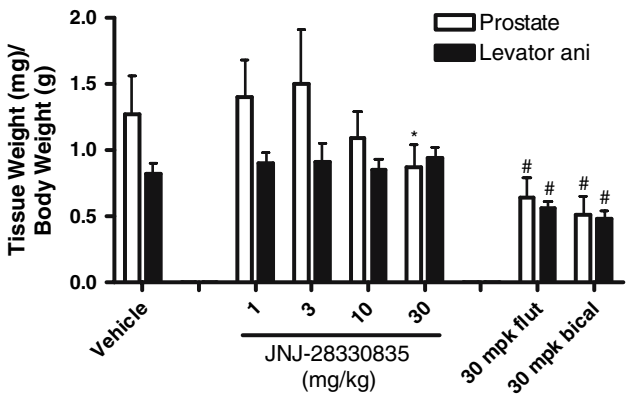

Fig. 4 Androgen antagonist activity in intact rats. Mature rats were treated with JNJ-28330835 for 6 weeks and tissue wet weights were measured. Tissue weights were normalized to body weight at necropsy. Mean ± SD, $n = 3$ per group. Statistically significant difference from the vehicle control (calculated by ANOVA) is shown for each group: #, $P < 0.01$ and *, $P < 0.05$ relative to vehicle control. flut = flutamide, bical = bicalutamide, mpk = mg/kg

ani were then measured (Fig. 3). As a control, testosterone propionate (TP) was administered subcutaneously (s.c.) at a dose (0.4 mg/kg) that stimulated prostate and levator ani to 60% to 100%, respectively, of their weights in age-matched intact animals (compare the TP control in Fig. 3 with the vehicle control in Fig. 4).

JNJ-28330835 stimulated levator ani weight maximally—i.e., to the mean weight of the tissue in age-matched intact rats—at a dose of 10 mg/kg. It had an ED_{50} on levator ani of 3.8 mg/kg (mean of two experiments). At the same time, it stimulated ventral prostate weight to a mean of 26% of intact weight at 10 mg/kg, and had an ED_{50} on this tissue of greater than 29 mg/kg. Thus, JNJ-28330835 selectively stimulated levator ani over prostate and met the definition of a SARM. In contrast, TP stimulated both tissues equally well at 0.4 mg/kg. In a separate

experiment (not shown), TP had an ED_{50} on prostate of 0.25 mg/kg, and on levator ani of 0.17 mg/kg.

The ability of JNJ-28330835 to counteract the androgenic and anabolic activity of endogenous T—i.e., to act as an androgen antagonist—was assessed by treating intact male rats for 6 weeks with the test compound. Tissue weights were then measured as in the Hershberger assay (Fig. 4). As a control, the androgen antagonists flutamide and bicalutamide were included.

JNJ-28330835 reduced the weight of ventral prostate in a dose-dependent manner, decreasing the weight of this tissue to half of its initial size at the maximum dose tested (30 mg/kg). At the same time, it had no effect on levator ani weight. At 10 mg/kg, the concentration at which it was maximally anabolic in orchidectomized rats, JNJ-28330835 reduced prostate weight in intact rats by 30% ± 15% (mean ± SD, $n = 3$). Flutamide and bicalutamide acted as pure antagonists, reducing the weights of both tissues by more than 50% at 30 mg/kg. Combining the results of the experiments in intact and orchidectomized rats, JNJ-28330835 was a mixed agonist and antagonist on prostate, and a pure agonist on levator ani.

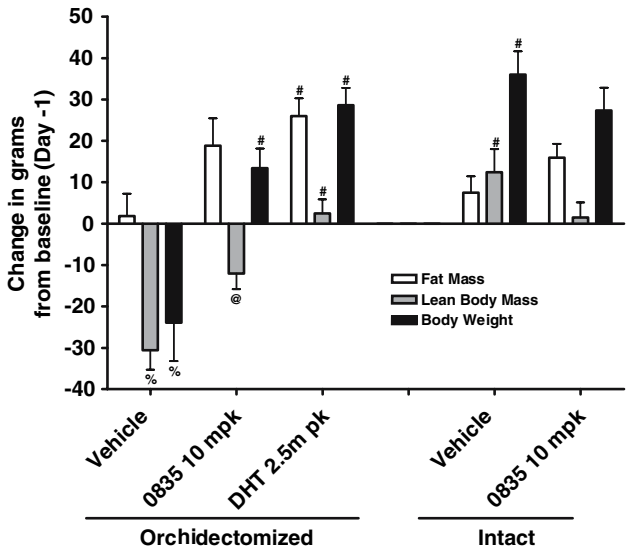

Fig. 5 Prevention of body composition changes in aged rats. Rats ($n = 10$ per group) were orchidectomized on the first day of an 8-week daily dosing regimen. Body composition was analyzed weekly by MRI. The mean ± SEM change from baseline in lean mass, fat mass, or body weight is shown. Statistically significant difference from the controls (calculated by ANOVA) is shown for each group: %, $P < 0.01$ relative to intact vehicle control; #, $P < 0.01$ and @, $P < 0.05$ relative to orchidectomized vehicle control. 0835 = JNJ-28330835, mpk = mg/kg

Lean body mass

The effects of JNJ-28330835 on body composition in aged male rats were assessed using magnetic resonance imaging (MRI). The compound was tested in two models. In the prevention model, 6-month-old rats were orchidectomized immediately prior to the first dose, then treated with test compound for 8 weeks to determine if it could prevent the loss of lean body mass that follows castration. As a control, orchidectomized rats were treated with dihydrotestosterone (DHT), and intact rats were dosed in parallel. In the restoration model, 2-month-old rats were orchidectomized, and aged to 6 months. The aged rats were treated with test compound for 2.5 months to determine if it could restore lost lean body mass in the animals. As a control, orchidectomized rats were treated with TP, and intact rats were dosed in parallel.

Eight weeks after orchidectomy, aged rats lost approximately 40 g of lean body mass and 60 g of total body weight as compared to control intact rats (Fig. 5). These losses corresponded to approximately 10% each of the lean mass and body weight of intact rats. Fat mass was not affected by orchidectomy. Treatment with 10 mg/kg JNJ-28330835 prevented the orchidectomy-induced loss of a mean of 18 g (45%) of lean body mass and 34 g (57%) of body weight. At 2.5 mg/kg, DHT prevented the loss of a mean of 33 g (82%) of lean body mass and 52 g (87%) of body weight. Interestingly, both JNJ-28330835 and DHT appeared to increase fat mass in orchidectomized rats relative to the vehicle-treated orchidectomized control. JNJ-28330835 had no significant effects on lean mass, fat mass, or total body weight in intact rats.

In the restoration model, 8.5 months after orchidectomy, aged rats had lost a mean of 120–150 g (20–25%) of their lean mass and total body weight as compared to intact rats (Fig. 6). Fat mass was relatively unchanged (data not shown). After 2.5 months of dosing, 10 mg/kg JNJ-28330835 restored about one-third of the lost lean mass and half of the lost body weight. Surprisingly, a higher dose of JNJ-28330835 (30 mg/kg) was less effective. The effect of the compound on lean mass and body weight was not maximal at 2.5 months. The positive control (TP) restored about half of the lost lean mass and body weight; its effect on lean mass was at its maximum after 1 month of dosing. As was the case with the prevention model, fat mass was unaffected by orchidectomy, but JNJ-28330835 modestly increased fat mass in orchidectomized rats relative to the vehicle-treated orchidectomized controls; also, JNJ-28330835 had no effect on body composition in intact rats (data not shown).

Hormone levels

The effect of chronic exposure to JNJ-28330835 on plasma hormone levels was examined in intact and

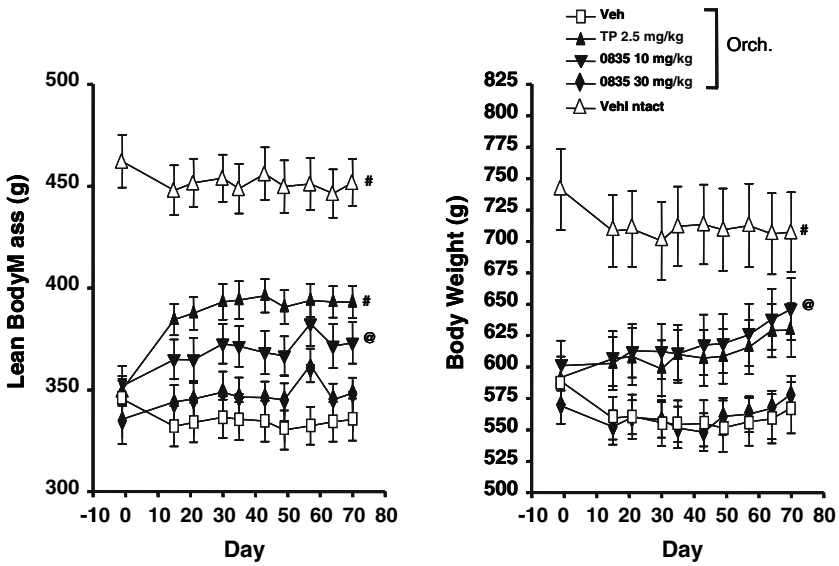

Fig. 6 Restoration of lean mass to aged rats. Two-month-old rats (n = 10 per group) were orchidectomized, aged to 6 months, and then dosed daily for 2.5 months. Body composition was analyzed weekly by MRI. Mean ± SEM lean mass and body weight are shown. Statistically significant difference from control (calculated by ANOVA) is shown for each group: #, $P < 0.01$ and @, $P < 0.05$ relative to the orchidectomized vehicle control. Veh = vehicle, 0835 = JNJ-28330835, Orch. = orchidectomized, mpk = mg/kg

orchidectomized rats. Plasma was prepared from cardiac blood taken at necropsy—i.e., one day after the final dose—for the experiments shown in Figs. 3 and 4. In orchidectomized rats, plasma follicle-stimulating hormone (FSH) levels were elevated 4-fold as compared to the level in intact rats (compare Fig. 7A, and B). JNJ-28330835 dose-dependently inhibited the elevation in FSH levels. Inhibition was statistically significant at 10 and 30 mg/kg. In intact rats, JNJ-28330835 had no effect on plasma FSH levels (Fig. 7B), while it tended to reduce plasma T levels—albeit without statistical significance—relative to the vehicle control (Fig. 7C). JNJ-28330835 also tended to dose-dependently reduce plasma androstenedione levels in intact rats (data not shown). For comparison, the androgen antagonist flutamide increased intact levels of both FSH (3-fold) and T (10-fold).

Bone turnover markers

Following the final dose of the lean body mass prevention experiment shown in Fig. 5, urine was collected for deoxypyridinoline (dPD) assay and blood was collected for plasma osteocalcin assay. These end points serve as markers of osteoclast-dependent bone turnover (dPD) and osteoblast-dependent bone turnover (osteocalcin). The levels of both of these markers increased following orchidectomy (Fig. 8). JNJ-28330835 at 10 mg/kg prevented the increase in dPD levels, and tended to prevent the increase in osteocalcin levels. JNJ-28330835 had no statistically significant effect on bone markers in intact rats. Control DHT prevented the increase in dPD levels but not in the osteocalcin levels. The androgen antagonist bicalutamide had no effect on bone marker levels in orchidectomized rats.

Female sexual behavior

Ovariectomized adult female Long-Evans rats were tested in a partner preference paradigm to assess preference ("sexual motivation") for a sexually intact male or an orchidectomized nonsexual male. In this model, vehicle-treated, progesterone-primed ovariectomized female rats had no preference for testis-intact or orchidectomized male rats (Fig. 9). In contrast, progesterone-primed females that had been treated with JNJ-28330835 for seven days had a dose-dependent preference for intact rats. A maximum effect was seen at 10 mg/kg. Likewise, rats treated with 2.4 mg/kg TP preferred intact males. Prior to preference testing, 66% of TP-treated females showed a lordosis reflex in a brief pair-test with a sexually experienced intact male; no JNJ-28330835-treated females showed lordosis.

Discussion

JNJ-28330835 is a selective AR ligand with a submicromolar K_i for AR, and little or no apparent affinity for other steroid receptors. It acted as a mixed agonist and antagonist on the endogenous AR of L929 cells. In mature male rats, JNJ-28330835 acted as a pure muscle agonist and a mixed prostate agonist and antagonist.

JNJ-28330835 is a weak SARM in vitro: Its mean K_i for the rat AR transduced in Cos-7 cells was three to four orders of magnitude higher than the mean K_i of the high-affinity steroid R1881. In L929 cells, it was a weak mixed agonist and antagonist. Modest in vitro potencies are typical for nonsteroidal AR ligands [28, 29]. Potent nonsteroidal SARM ligands have only recently begun to

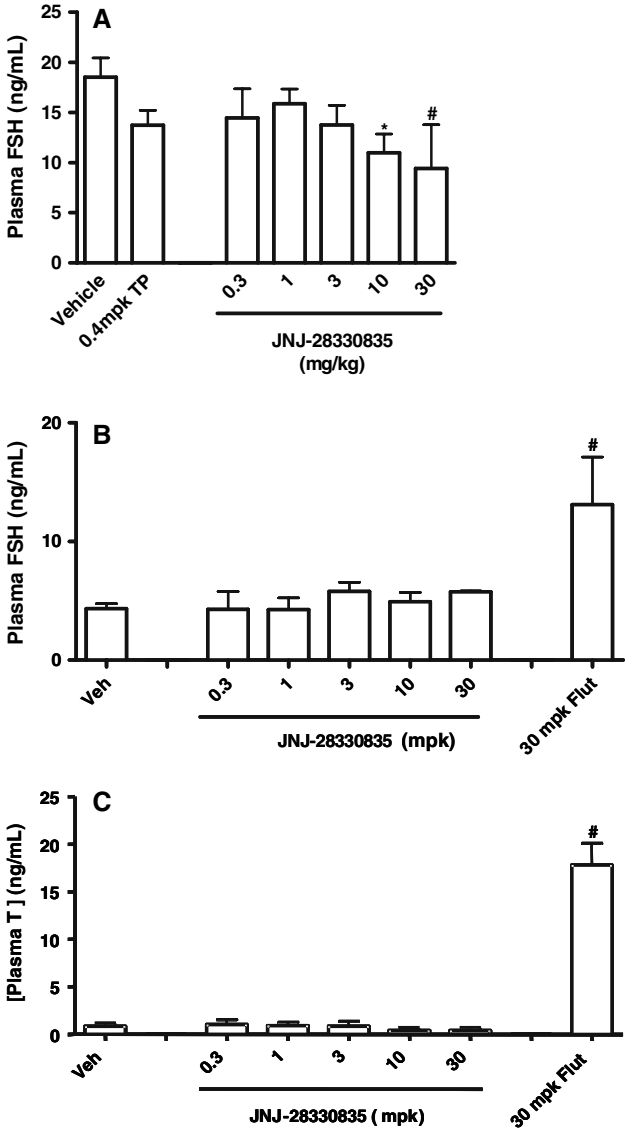

Fig. 7 Plasma hormone levels. Hormone levels (mean ± SD, $n = 3$ per group) were measured following 2-week treatment of orchidectomized rats or 6-week treatment of mature rats. (**A**) Plasma FSH levels in orchidectomized rats. (**B**) Plasma FSH levels in intact rats. (**C**) Plasma T levels in intact rats. Statistically significant difference from the vehicle control (calculated by ANOVA) is shown for each group: #, $P < 0.01$, *, $P < 0.05$. Veh = vehicle, Flut = flutamide, mpk = mg/kg

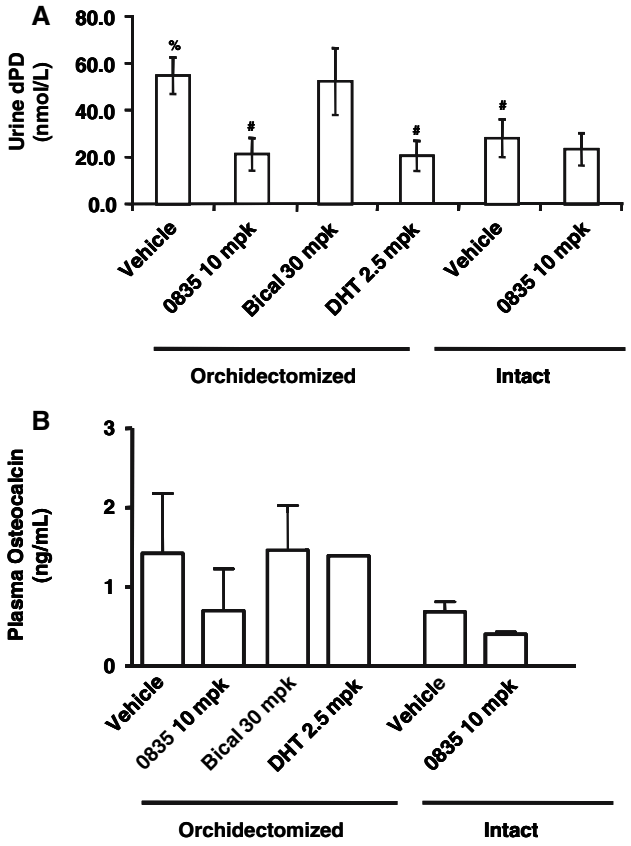

Fig. 8 Markers of bone turnover. Urine and plasma was collected from aged rats ($n = 3$ per group) following 8 weeks of treatment with test compound. (**A**) Urine dPD. (**B**) Plasma osteocalcin. Three rats per group (mean ± SD). Statistically significant difference from the vehicle control (calculated by ANOVA) is shown for each group: #, $P < 0.01$ relative to the orchidectomized vehicle control; %, $P < 0.01$ relative to the intact vehicle control. Veh = vehicle, 0835 = JNJ-28330835, Bical = bicalutamide, Orch = orchidectomized, Int = intact, mpk = mg/kg

emerge from several laboratories [11–13, 30–32], including our own ([21]; Allan and Ng, manuscript submitted).

JNJ-28330835 was selective for AR based on binding and cell-based assays for progestin, glucocorticoid, and estrogen activity. Its K_i for these receptors was 10–1,000 times higher than its K_i for AR. Consistent with these results, JNJ-28330835 had weak or no progestin or estrogen agonist and antagonist activity in vitro. Since the compound also had low potency in L929 cells, it will be important to assess cross-reactivity with other steroid pathways in vivo.

Despite its weak in vitro activity, JNJ-28330835 was an effective and moderately potent SARM in vivo. When JNJ-28330835 was administered to intact rats at its maximal stimulatory dose on levator ani (10 mg/kg), it reduced prostate weight by 30%, while not affecting levator ani weight. These results suggest that, in hypogonadal men, JNJ-28330835 at doses required to increase muscle mass might have a much reduced stimulatory effect on prostate relative to steroidal androgens; while in eugonadal men, JNJ-28330835 might maintain muscle mass, while potentially reducing prostate size. This is an important feature of the preclinical profile of JNJ-28330835, and it is one that has not been associated with most other published SARM agonists. Chen et al. [30] described a compound, C-6, that stimulated levator ani, while having mixed agonist and antagonist activity on prostate; it reduced prostate weight in intact rats by 50%, apparently by inhibition of gonadotropin-regulated T synthesis. In contrast, the

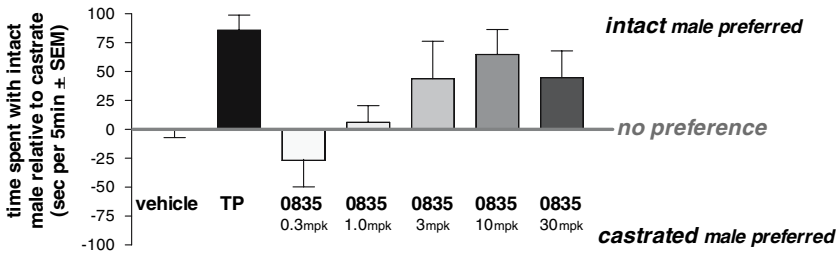

Fig. 9 Partner preference in female rats. Ovariectomized females were treated with test compound for seven days, followed by priming with progesterone 4 h before testing. Females that exhibited a lordosis response in the presence of an active male were next tested for partner preference by giving them a choice of interaction with an intact male or an orchidectomized male. The data show the time spent (mean ± SEM, $n = 3$ females per group) with each male rat over a 5-min testing period. Control TP was administered at a dose of 2.4 mg/kg. 0835 = JNJ-28330835, mpk = mg/kg

tetrahydroquinolin derivative described by Hanada et al. [11] stimulated prostate weight in castrated rats, but did not reduce prostate weight in intact animals. Other investigators did not report assays in intact rats, so it is not clear how common is this property of JNJ-28330835. We have also observed mixed prostate agonism and antagonism with a trifluoroethyl-benzimidazole SARM ([21]; Allan and Ng, manuscript submitted).

Two mechanisms are possible for antagonism at the prostate in intact rats: incomplete ligand-dependent coactivator recruitment or removal of corepressor from the receptor in prostate cells; or inhibition of gonadotropin secretion from the pituitary, reducing the testicular synthesis of T. The SARM C-6 mentioned above appeared to reduce prostate weight by the latter mechanism, since it decreased levels of FSH and LH in orchidectomized rats, and those of T in intact rats [30]. Moreover, C-6 reduced testis weight by about 40% and inhibited spermatogenesis in rats. Although JNJ-28330835 decreased FSH in orchidectomized rats and T levels in intact rats (Fig. 7), it was much less effective at reducing the levels of these hormones than was C-6. In addition, we did not observe a strong inhibitory effect on LH levels, and testis weight was unchanged up to 30 mg/kg (data not shown). In orchidectomized immature rats, JNJ-28330835 acted as a prostate agonist by itself and inhibited TP-dependent increases in prostate weight (data not shown). Finally, JNJ-28330835 had mixed activity in L929 cells, while C-6 was a pure androgen agonist in vitro. For these reasons, JNJ-28330835 appears to work as a partial antagonist predominately at the level of the prostate cell, possibly with some contribution from systemic reduction of the T level. The lack of a strong effect of JNJ-28330835 treatment on FSH and LH levels, and on testis size suggests that the compound will not have contraceptive effects in males, but this has not been tested directly.

The strong stimulatory effect of JNJ-28330835 on levator ani weight is suggestive of an anabolic effect on muscle. The levator ani is used as a physiological marker of anabolism by most investigators [9–13]. However, the responsiveness of the levator ani to T is more reminiscent of an androgenic response than an anabolic one, and there is direct evidence from the pharmacological characterization of the androgen antagonist JNJ-26146900 that an isolated effect on levator ani is not predictive of a global effect on lean body mass [15]. Therefore, assays of anabolic potential on muscle throughout the body—i.e., assays measuring lean body mass—are required, though they are infrequently performed. One publication [10] has described the use of dual-energy X-ray absorptiometry to characterize the effects of a propionamide SARM (S-4) on body composition. In the current work, MRI was used to further characterize the anabolic potential of JNJ-28330835; the results showed that the compound both prevented the loss of lean body mass and body weight that follows orchidectomy, and restored lean body mass and body weight to previously orchidectomized animals. At 10 mg/kg, JNJ-28330835 was only slightly less effective than steroidal androgens in the prevention and restoration of body composition changes. In the restoration study, the effect of JNJ-28330835 on lean mass had not reached a plateau after 2.5 months of dosing, while the effect of TP reached a maximum at 1 month. Note that, relative to the intact control, TP restored only about half of the lost lean body mass to the orchidectomized rats. JNJ-28330835 may have additional restorative benefits with longer-term treatment. These results demonstrate the potential usefulness of JNJ-28330835 in treating the muscle loss associated with hypogonadism, certain cancers, kidney disease, acquired immune deficiency syndrome, and aging.

Unexpectedly, a higher dose of JNJ-28330835 (30 mg/kg) was less effective than 10 mg/kg in the restoration study. It is unclear why this was the case. The compound had a dose-responsive effect on levator ani weight up to 30 mg/kg (Fig. 3), and had linear pharmacokinetics up to 100 mg/kg after oral administration (data not shown). The pharmacodynamics of JNJ-28330835 on lean body mass may differ from the pharmacodynamics on levator ani in ways that we have yet to understand.

JNJ-28330835 increased fat mass in both the restoration and prevention models (Fig. 5 and data not shown). Likewise, DHT increased fat mass in the prevention study. This was despite the fact that orchidectomy alone had no effect on fat mass in either model. In a clinical setting, steroidal androgens decrease fat mass, while increasing muscle mass [3]. This suggests that the relationship between androgen treatment and fat mass is not the same in humans and rats. Using dual-energy X-ray absorptiometry, S-4 was shown to have no apparent effect on fat mass in rats [10]. However, the fat content of S-4-treated rats (10–20 g) at end point was comparable to those of JNJ-28330835–treated rats in our experiment. The fat mass of our vehicle-treated animals was lower than those of Gao et al. [10]. It is possible that the adipose tissue of rats with high fat levels at baseline responds less to SARM treatment than does the adipose tissue of lean rats. JNJ-28330835 had no effects on fat or lean mass in intact (eugonadal) rats.

JNJ-28330835 reduced osteoclast-dependent bone turnover as assessed by measuring the levels of dPD in urine. It also appeared to reduce turnover due to osteoblast activity as determined by osteocalcin levels in plasma. These results suggest that JNJ-28330835 acts as an androgen agonist in bone. The influence of JNJ-28330835 on bone density and bone strength remains to be determined. For other SARMs [10, 12], there was a good correlation between effects on marker levels and positive effects on the density or strength of bone.

In a partner preference model, JNJ-28330835 dose dependently increased the preference of treated, ovariectomized females for sexually intact male rats, suggesting that the compound could have efficacy as a therapy for female sexual dysfunction. The maximally effective agonist dose in this model of female sexual motivation [33] corresponded to the ED_{max} on levator ani. Female sexual behavior was also assessed using a copulation-pair test. In this model, ovariectomized, estrogen- and progesterone-primed, adult Long-Evans rats were tested in a sexual facilitation pair-test paradigm to assess proceptivity ("sexual motivation") with an intact, sexually experienced adult Long-Evans male [34, 35]. In a preliminary study (data not shown), JNJ-28330835 increased the number of sexual solicitations (hop darts, ear wiggles, and positional orientation) [36] made by a female in the presence of an intact male more than 100-fold over females primed only with estrogen and progesterone. The compound was maximally effective at a dose 30 times less than its ED_{max}. This strengthens the possibility that the compound will have efficacy as a therapy for female sexual dysfunction. Overall, JNJ-28330835 specifically increased precopulatory behaviors associated with sexual motivation and desire. In contrast, compound treatment did not affect the rodent-specific receptive reflex response of lordosis [37], which is mediated by a separate central mechanism. To our knowledge, this is the first published evidence that a SARM has efficacy in a female sexual function model. Previously, LGD2226 was shown to increase the sexual activity of male rats [12].

In conclusion, JNJ-28330835 is a prostate-sparing SARM with favorable effects on muscle weight, lean body mass, and bone turnover markers in male rats, which suggest that it could be an effective anabolic agent in men. Unlike most other SARMs, JNJ-28330835 has partial antagonist activity at the prostate that may give it a safety advantage in eugonadal men. In addition, it has strong stimulatory effects on sexual performance in female rats that could translate into a beneficial therapy for female sexual dysfunction.

Materials and methods

In vitro assays

Whole-cell AR binding assays were performed as previously described [15]. Fluorescence polarization binding assays for human PR, GR, ERα, and ERβ were performed using recombinant receptors, fluorescence-labeled ligands, and assay buffers from Invitrogen (Carlsbad, CA). Competition assays were performed in Microflour 2 Black plates (Dynex; Chantilly, VA) by incubation of each receptor with its fluorescent ligand, and with test compound in the dark for 1 h at room temperature. The completed assay was analyzed on an LJL Analyst fluorescence polarization plate reader (Molecular Devices; Sunnyvale, CA). Binding data (counts per minute or fluorescence polarization units) were converted to percentages of inhibition relative to no-competitor and maximum-competitor controls, and IC_{50}s were determined from the 50% intersection with the nonlinear regression curve. The IC_{50} was converted to a K_i using the Cheng and Prusoff formula [38].

L929 cell functional assays of androgen agonism and antagonism [15] and T47D cell assays of progesterone agonism and antagonism [39] were performed as previously described. Ishikawa human endometrium cell (American Type Culture Collection; Manassas, VA) assays of estrogen agonism and antagonism were performed as follows: Cells were transferred to 96-well Dynatech Microlite plates (ThermoLabsystems; Franklin, MA) at 5,000 cells per well in phenol red-free DMEM/F12 medium (Invitrogen) with 2%(v/v) charcoal-stripped calf serum (Hyclone; Logan, UT); beginning the following day, the cells were incubated for three days at 37°C with test compound alone (agonist format), or along with 10 nM 17β-estradiol (antagonist format); finally, estrogen-induced

cell alkaline phosphatase activity was measured using a SEAP kit (Clontech; Mountain View, CA) and a Luminoskan Ascent reader (Thermo Electron; Waltham, MA). Functional data (luminescence units) were converted to percentages of stimulation (agonist format) relative to vehicle and maximum-stimulation controls, or to percentages of inhibition (antagonist format) relative to no-inhibition and maximum-inhibition controls. All EC_{50}s and IC_{50}s were determined from the 50% intersection with the stimulation and inhibition nonlinear regression curves, respectively.

For hormone assays, rat blood collected at necropsy was centrifuged to obtain plasma. Plasma samples were analyzed for T, androstenedione, or FSH using enzyme immunoassay (EIA) kits from American Laboratory Products Co. (ALPCO; Salem, NH) following the manufacturer's directions.

Urine deoxypyridinoline (dPD) assays were performed using a Metra EIA kit (Quidel; San Diego, CA) following the manufacturer's directions. Creatinine levels were used for normalization. Plasma osteocalcin assays were performed using an EIA kit from Biomedical Technologies (Stoughton, MA) following the manufacturer's directions.

Mature rat tissue weight and hormone assays

All rodent studies were performed according to regulations governed by the local animal care and use committee. Animals were housed under a 12-h light and dark cycle with a casein-based diet and water ad libitum. Mature (2-month-old) orchidectomized Sprague Dawley rats (Charles River; Wilmington, MA) were dosed once daily by gavage (p.o.) for 2 weeks with test compound suspended in 20%(w/v) hydroxypropyl-β-d-cyclodextrin (HPBCD) (Cargil; Cedar Rapids, IA); control animals were dosed p.o. with vehicle (HPBCD) alone, or s.c. with 0.4 mg/kg testosterone propionate (TP) dissolved in sesame oil (Sigma; St. Louis, MO). Alternatively, mature (2-month-old) testis-intact male Sprague Dawley rats were dosed once daily p.o. for 6 weeks with test compound suspended in 20%(w/v) HPBCD; control animals were dosed p.o. with vehicle (HPBCD) alone, or with 30 mg/kg flutamide (Sigma) or bicalutamide (purified in-house from 50-mg Casodex® pills [Astra Zeneca; Wilmington, DE]). Both flutamide and bicalutamide were suspended in 20%(w/v) HPBCD for dosing. The day following the final dose, rats were euthanized by asphyxiation in carbon dioxide, body weights were measured, and blood was taken by cardiac puncture using Monovette collection tubes (Sarstedt; Newton, NC). Ventral prostates and levator ani were removed, and their wet weights were determined.

Tissue wet weights (in milligrams) were normalized to body weight (in grams). For the agonist format, normalized tissue weights were converted to percent stimulation relative to the vehicle control (0% stimulation), and to the known tissue weight ratios in age-matched intact rats ("100% stimulation"). For the antagonist format, normalized tissue weights were converted to percent inhibition relative to the intact vehicle control (0% inhibition) and vehicle-treated orchidectomized rats ("100% inhibition"). For the 100% stimulation and 100% inhibition controls, mean tissue weight ratios from numerous previous experiments (e.g., [15]) were used for calculation. All ED_{50}s were determined from the 50% intersection with the stimulation or inhibition curve determined by nonlinear regression. The maximally efficacious dose (ED_{max}) was the dose that stimulated levator ani weight to a maximal (100%) level in orchidectomized rats; i.e., to a level equivalent to that in intact rats.

Aged rat models

For the prevention model, aged (6-month-old) male Sprague Dawley rats were used. Half of the animals were orchidectomized 6 h prior to the first dose of the study; the remainder of the animals was left intact. Dosing continued once daily p.o. for 8 weeks with test compound suspended in 20%(w/v) HPBCD. Control animals were dosed with vehicle (HPBCD) p.o. or with 2.5 mg/kg DHT (Sigma) s.c. (dissolved in sesame oil and administered three times per week). Body composition and body weight were measured in conscious animals the day before the first dose of the study (Day –1), and once per week thereafter. Body composition was analyzed by MRI using an EchoMRI Body Composition Analyzer (Echo Medical Systems; Houston, TX). Body weight was measured using an electronic scale. After the final dose of the study, the animals were housed in metabolism cages overnight for collection of urine; they were housed with water ad libitum and without chow. The next day the rats were euthanized. Blood was collected for plasma preparation as described above. End-of-study changes in body mass parameters (lean mass, fat mass, or total body weight) were calculated using Day –1 parameters for each animal as a baseline. All statistical analyses were done using one-way analysis of variance (ANOVA) with Dunnett's post test.

For the restoration model, 2-month-old male Sprague Dawley rats were orchidectomized or left intact, then aged to 6 months. Dosing began at 6 months and was performed as in the prevention model, except it continued for 2.5 additional months, and TP was substituted for DHT. Body composition and body weight were measured once weekly as above.

Female rat sexuality models

Ovariectomized adult Long-Evans rats were tested in a partner preference paradigm to assess preference ("sexual motivation") for a sexually intact male or an orchidectomized nonsexual male [33]. Females were ovariectomized by the vendor, and were tested for a minimum of 3 weeks after surgery. Size-matched males were tested beginning a minimum of 2 months after surgery or after arrival in the animal colony. Females were dosed for seven days prior to the first day of testing, and on the test day itself, with 20%(w/v) HPBCD (p.o.), TP in sesame oil (2.4 mg/kg s.c.), or test compound in HPBCD (p.o.). The male rats were not dosed. Four hours before behavioral testing and 2 h after the last oral or TP dose, all test females received progesterone (0.1 mg/kg s.c.; Sigma), which is necessary to facilitate the full repertoire of sexual behavior in ovariectomized rats [36]. Progesterone treatment in the absence of estrogen or TP yields no sexual behavior [36], or male preference [33] in female rats.

Ten minutes before partner preference testing, a female rat was placed in a sexually experienced, intact male's cage for a 3-min sexual pair test to assess lordosis and facilitate female sexual interest. The partner preference test was then conducted in a computerized test environment using hardware and custom-written software from Colbourn Instruments (Allentown, PA). A female rat was placed in a neutral octagonal hub (10.5-inch diameter) with two plexiglass runways (each 29 inches long) projecting in opposite directions. An orchidectomized or intact male was placed in a small cage (9 × 12 inches) at the end of each runway, physically separated from the runway by an olfactory- and auditory-permeable mesh screen. A fan blew from the back of each male's cage toward the runway. Females were free to spend their time in the neutral hub or in either runway in noncontact proximity to either male. Animals were habituated to the testing apparatus during three 30-min sessions over three days. During testing, females were placed in the neutral hub 3 min before the test began. Guillotine doors automatically opened the runways at the start of each 5-min test. Graphic State software (Colbourn) calculated the frequency and duration of stay of the rat in three locations (neutral hub, orchidectomized male runway, or intact male runway) via photocell receptors placed in the neutral hub and the runway doorways. Hubs and runways were sprayed with disinfectant between tests. The dependent measure was the amount of time (in seconds) the female spent in the intact male's runway relative to the orchidectomized male's runway per 5-min test.

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