

Pharmacodynamics of Selective Androgen Receptor Modulators

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ABSTRACT

The present study aimed to identify selective androgen receptor modulators (SARMs) with *in vivo* pharmacological activity. We examined the *in vitro* and *in vivo* pharmacological activity of four chiral, nonsteroidal SARMs synthesized in our laboratories. In the *in vitro* assays, these compounds demonstrated moderate to high androgen receptor (AR) binding affinity, with K_i values ranging from 4 to 37 nM, and three of the compounds efficaciously stimulated AR-mediated reporter gene expression. The compounds were then administered subcutaneously to castrated rats to appraise their *in vivo* pharmacological activity. Androgenic activity was evaluated by the ability of these compounds to maintain the weights of prostate and seminal vesicle, whereas levator ani muscle weight was used as a measure of anabolic activity. The maximal response (E_{max}) and dose for half-maximal effect (ED_{50}) were determined for each

compound and compared with that observed for testosterone propionate (TP). Compounds S-1 and S-4 demonstrated *in vivo* androgenic and anabolic activity, whereas compounds S-2 and S-3 did not. The activities of S-1 and S-4 were tissue-selective in that both compounds stimulated the anabolic organs more than the androgenic organs. These two compounds were less potent and efficacious than TP in androgenic activity, but their anabolic activity was similar to or greater than that of TP. Neither S-1 nor S-4 caused significant luteinizing hormone or follicle stimulating hormone suppression at doses near the ED_{50} value. Thus, compounds S-1 and S-4 were identified as SARMs with potent and tissue-selective *in vivo* pharmacological activity, and represent the first members of a new class of SARMs with selective anabolic effects.

Endogenous androgens play crucial physiological roles in establishing and maintaining the male phenotype (George and Wilson, 1986; Mooradian et al., 1987). Their actions are essential for the differentiation and growth of male reproductive organs, initiation and regulation of spermatogenesis, and control of male sexual behavior. In addition, androgens are important for the development of male characteristics in certain extragenital structures such as muscle, bone, hair, larynx, skin, lipid tissue, and kidney (Takeda et al., 1990). In females, the precise physiological roles of androgens are not completely understood, but the age-related decline in circulating androgen levels has been linked to symptoms such as decreased libido and sexuality, lack of vigor, diminished well

being, and loss of bone mineral density in postmenopausal women (Davis and Burger, 1996; Davis, 1999a,b).

Synthesized steroidal androgens, due to their ability to mimic the actions of their endogenous counterparts, have been used clinically as valuable therapeutic agents to target a variety of male and female disorders resulting from androgen deficiency. The principle clinical indication of androgens is as replacement therapy for hypogonadal men (Conway et al., 1988; Wu, 1992). Other documented clinical uses of androgens include delayed puberty in boys, anemias, primary osteoporosis, hereditary angioneurotic edema, endometriosis, estrogen receptor-positive breast cancer, and muscular diseases (Wu, 1992; Bagatell and Bremner, 1996; Nieschlag, 1996; Bhasin and Tenover, 1997). Also, androgens have been investigated as hormone replacement therapy for aging men and for regulation of male fertility (Wu, 1992; Tenover, 1997).

Since the discovery of the therapeutic benefits of testosterone in the 1930s, a variety of androgen preparations have been introduced and tested clinically. Unfortunately, virtu-

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ABBREVIATIONS: AR, androgen receptor; SARM, selective androgen receptor modulator; TP, testosterone propionate; PEG 300, polyethylene glycol 300; FSH, follicle stimulating hormone; LH, luteinizing hormone; ANOVA, analysis of variance; DHT, dihydrotestosterone; AST-SGOT, serum glutamicoxaloacetic transaminase; ALT-SGPT, serum glutamic pyruvic transaminase.

ally all currently available androgen preparations have severe limitations (Wu, 1992; Bhasin and Bremner, 1997). Unmodified testosterone is impractical for oral administration due to its low systematic bioavailability (Handelsman et al., 1990). Testosterone esters (e.g., testosterone propionate and testosterone enanthate) are presently the most widely used testosterone preparations, usually administered by intramuscular injection in oil vehicles (Snyder and Lawrence, 1980; Velazquez and Bellabarba Arata, 1998). A prolonged duration of action is achievable with these esters. However, they produce highly variable testosterone levels. 17α -Alkylated testosterone esters (e.g., methyltestosterone and oxandrolone) can be given orally. Nevertheless, they often cause unacceptable hepatotoxicity and are less efficacious; hence, they are not recommended for long-term androgen therapy (Heywood et al., 1977; Ishak and Zimmerman, 1987; Velazquez and Bellabarba Arata, 1998). Another common concern about steroidal androgens is the undesirable effects resulting from the cross-reactivity of the androgens or their in vivo metabolites with steroid receptors other than the androgen receptor (AR) (Wilson et al., 1980; Bhasin and Bremner, 1997).

During studies with affinity ligands for the AR, our group discovered a group of nonsteroidal androgens that are structural derivatives of bicalutamide and hydroxyflutamide, two known antiandrogens (Dalton et al., 1998; Mukherjee et al., 1999). Other laboratories have also reported the identification of nonsteroidal compounds that possess androgen activity (Dalton et al., 1998; Hamann et al., 1999; Negro-Vilar, 1999). The discovery of these nonsteroidal androgens offers an opportunity for the development of a new generation of selective androgen receptor modulators (SARMs) superior to current steroidal androgens. Theoretically, SARMs are advantageous over their steroidal counterparts in that they can obtain better receptor selectivity and allow greater flexibility in structural modification. Thus, SARMs can potentially avoid the undesirable effects caused by receptor cross-reactivity and achieve superior pharmacokinetic properties.

Subsequent to our initial discovery of several nonsteroidal androgens, our laboratories designed and synthesized multiple series of nonsteroidal compounds, and explored the structure-activity relationships for androgenic and anabolic activities, both in vitro and in vivo (He et al., 2002; Yin et al., 2003a,b). According to results from these structure-activity relationship studies, we designed a group of novel nonsteroidal compounds (Fig. 1) that were structurally optimized. We report herein the results of our studies to examine the in vitro AR binding affinity and the androgenic and anabolic activities of these new compounds in an animal model. Two potent and tissue-selective SARMs were identified from these structurally similar compounds, and they are members of a promising new class of drug candidates for further development.

Materials and Methods

Materials. The *S*-isomers of compounds 1, 2, 3, and 4, and the *R*-isomer of compound 1 were synthesized in our laboratories (synthetic procedures will be reported separately). The purities of these compounds were greater than 99%, as determined by high-performance liquid chromatography. Testosterone propionate (TP), polyethylene glycol 300 (PEG 300, reagent grade), and dimethyl sulfoxide

(reagent grade) were purchased from Sigma-Aldrich (St. Louis, MO). Ethyl alcohol USP was purchased from Aaper Alcohol and Chemical (Shelbyville, KY). Alzet osmotic pumps (model 2002) were purchased from Alza (Palo Alto, CA).

In Vitro Pharmacological Activity. Cytosolic AR was prepared from ventral prostates of castrated male Sprague-Dawley rats (about 250 g). The binding affinity of compounds 1, 2, 3, and 4 to the AR preparation was determined and analyzed as described previously (Mukherjee et al., 1996, 1999). The ability of the compounds to influence AR-mediated transcriptional activation was examined using a cotransfection system, as described previously (Yin et al., 2003a). Transcriptional activation was measured using a single concentration (10 nM) of the indicated compound and reported as a percentage of the transcriptional activation observed for 1 nM DHT.

Animals. Male Sprague-Dawley rats, weighing 90 to 100 g, were purchased from Harlan Bioproducts for Science (Indianapolis, IN). The animals were maintained on a 12-h light/dark cycle with food and water available ad libitum. The animal protocol was reviewed and approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University.

Study Design. Animals were randomly distributed into 30 groups, with five rats per group. Treatment groups are described in Table 1. One day before the start of drug treatment, animals in groups 2 through 30 were surgically castrated. After 24 h of recovery, Alzet osmotic pumps (model 2002) prefilled with a designated solution (Table 1) were implanted subcutaneously in the scapular region of castrated animals. Drug solutions used to fill the osmotic pumps were prepared using aseptic techniques. For solutions of nonsteroidal compounds and low-dose (0.1 mg/day or lower) solutions of TP, drugs were first dissolved in minimal amounts of ethanol and then diluted to final concentrations with PEG 300 (this vehicle is designated as vehicle 1). Because higher doses of TP could not be completely solubilized in the above-mentioned vehicle, TP solutions for 0.3, 0.5, and 0.75 mg/day were prepared by dissolving the drug in a mixture of ethanol and dimethyl sulfoxide and adjusting with PEG 300 to the desired final volume (this vehicle was designated as

TABLE 1
Animal groups and experimental design

Group	Castration	Drug	Dose	No. of Animals
			mg/day	
1	No	None	None	5
2	Yes	None	Vehicle 1 only	5
3	Yes	Testosterone	0.03	5
4	Yes	Testosterone	0.05	5
5	Yes	Testosterone	0.1	5
6	Yes	Testosterone	0.3	5
7	Yes	Testosterone	0.5	5
8	Yes	Testosterone	0.75	5
9	Yes	R-1	1.0	5
10	Yes	S-1	0.1	5
11	Yes	S-1	0.3	5
12	Yes	S-1	0.5	5
13	Yes	S-1	0.75	5
14	Yes	S-1	1.0	5
15	Yes	S-2	0.1	5
16	Yes	S-2	0.3	5
17	Yes	S-2	0.5	5
18	Yes	S-2	0.75	5
19	Yes	S-2	1.0	5
20	Yes	S-3	0.1	5
21	Yes	S-3	0.3	5
22	Yes	S-3	0.5	5
23	Yes	S-3	0.75	5
24	Yes	S-3	1.0	5
25	Yes	S-4	0.1	5
26	Yes	S-4	0.3	5
27	Yes	S-4	0.5	5
28	Yes	S-4	0.75	5
29	Yes	S-4	1.0	5
30	Yes	None	Vehicle 2 only	5

vehicle 2). Due to the limited solubility of TP, two osmotic pumps were used in each animal to deliver TP at 0.5 and 0.75 mg/day. One osmotic pump was used in each animal for other groups.

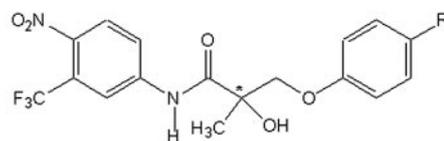
After 14 days of drug treatment, rats were weighed, anesthetized, and sacrificed. Blood samples were collected by venipuncture of the abdominal aorta. For each animal, a whole blood sample and a serum sample were used for complete blood count and chemistry profile analyses, and a portion of the blood was centrifuged to prepare plasma. Plasma samples from selected groups were analyzed for FSH, LH, GH, AST-SGOT, ALT-SGPT, cholesterol, high-density lipoprotein, and triglyceride. Plasma samples for these analyses were collected from a separate group of animals in the case of the TP-treated groups, using dose rates of 0.1, 0.3, 0.5, 0.75, and 1 mg/day. The ventral prostates, seminal vesicles, levator ani muscle, liver, kidneys, spleen, lungs, and heart were removed; cleared of extraneous tissue; weighed; and placed in vials containing 10% neutral buffered formalin. Preserved tissues were subjected to histopathological analysis. Osmotic pumps were removed from animals to check for correct pump operation.

Data Analyses. The weights of all organs were normalized to body weight, and analyzed for any statistically significant differences between groups using single-factor ANOVA with the α value set a priori at $p < 0.05$. The weights of prostates and seminal vesicles were used as indices for evaluation of androgenic activity, and the levator ani muscle weight was used to evaluate the anabolic activity. Statistical analyses of parameters from complete blood count or serum chemical profiling, wherever applicable, were performed by single-factor ANOVA with the α value set a priori at $p < 0.05$. For compounds demonstrating full-range dose-response relationships in any of the measured parameters, the maximal response produced by the compound (E_{max}) and the dose rate that induced 50% of the maximal response (ED_{50}) were obtained by nonlinear regression analysis using WinNonlin (version 3.1; Pharsight Corporation, Mountain View, CA) and the sigmoid E_{max} model. The E_{max} value indicated the efficacy of each compound, whereas the ED_{50} indicated its potency. The relative efficacy of each compound to TP was defined as the ratio of (E_{max} of the compound) to (E_{max} of TP). The relative potency was defined as the ratio of (ED_{50} of TP) to (ED_{50} of the compound).

Results

The *in vitro* AR binding of the *R*-isomer of compound 1 (designated as *R*-1) and the *S*-isomers of compounds 1, 2, 3, and 4 (designated as *S*-1, *S*-2, *S*-3, and *S*-4, respectively) was examined with a radioligand competitive binding assay. *R*-1 demonstrated poor AR binding affinity ($K_i = 225 \pm 15$ nM), whereas *S*-1, *S*-2, *S*-3, and *S*-4 bound to the AR with moderate to high affinity, with K_i values ranging from 4 to 37 nM (Fig. 1). Next, the ability of these compounds to stimulate AR-mediated transcription was determined in an *in vitro* cotransfection system. At a concentration of 10 nM, compounds *S*-3 and *S*-4 stimulated AR-mediated transcription to 75 and 93%, respectively, of that observed for 1 nM DHT, whereas compounds *S*-1 and *S*-2 demonstrated lesser stimulation (i.e., 43 and 9.7%, respectively). Given previous studies in our laboratories demonstrating that *in vitro* cotransfection models poorly predict *in vivo* pharmacological activity (Yin et al., 2003a), we then examined the androgenic and anabolic activities of these nonsteroidal compounds in a castrated rat model after 14 days of drug administration. *R*-1 was included as a negative control. TP, at increasing doses, was used as the positive control for anabolic and androgenic effects.

In accordance with literature reports (Saksena and Chaudhury, 1970; Teutsch et al., 1994; Battmann et al.,



Name	Isomer	R	K_i (nM)	Activation (% of 1 nM DHT)
R-1	R	F	225 ± 15	N.D.
S-1	S	F	6.1 ± 0.2	43 ± 2.6
S-2	S	COCH ₃	37 ± 2.4	9.7 ± 1.5
S-3	S	COC ₂ H ₅	6.1 ± 0.1	75 ± 11
S-4	S	NHCOCH ₃	4.0 ± 0.7	93 ± 7.0

Fig. 1. Chemical structures and *in vitro* pharmacological activity of nonsteroidal AR ligands. Cytosolic AR was prepared from ventral prostates of castrated male Sprague-Dawley rats. The AR binding affinity was determined and analyzed as described previously (Mukherjee et al., 1996, 1999). The ability of the compounds to influence AR-mediated transcriptional activation was examined using a cotransfection system, as described previously (Dalton et al., 1998). Transcriptional activation was measured using a single concentration (10 nM) of the indicated compound and reported as a percentage of the transcriptional activation observed for 1 nM DHT.

1998), we observed significant decreases in the weights of prostate, seminal vesicles, and levator ani muscle in castrated, vehicle-treated rats (Figs. 2-5). The weights of the

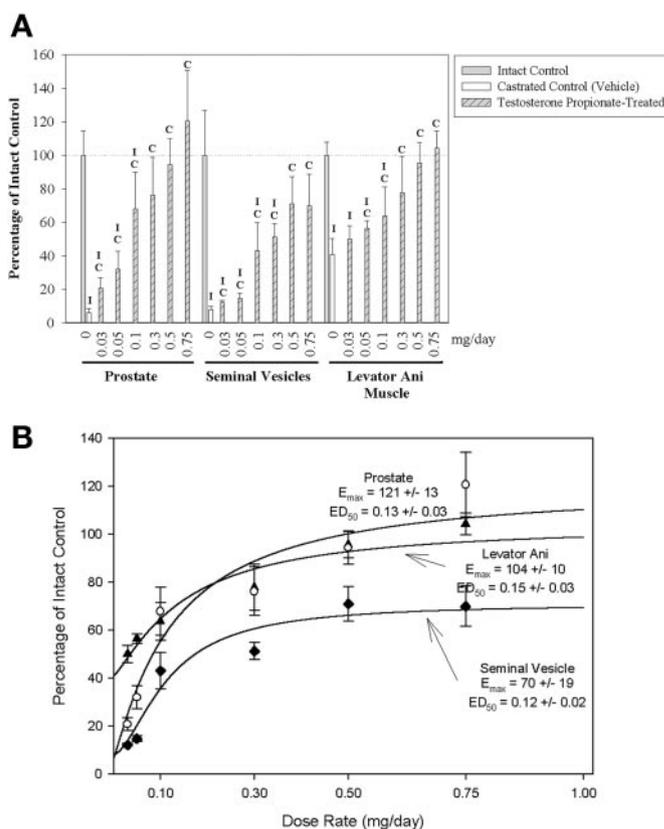


Fig. 2. A, assay for androgenic and anabolic activity of TP in castrated immature rats. One day after castration, immature rats received 1 mg/day of TP via Alzet osmotic pumps for 14 days. All weights were corrected for 100 g of body weight and were converted to the percentage of the weights in the intact control group. Values represent the mean \pm standard deviation ($n = 5$ /group). The letters "I" and "C" above the error bars indicate a significant difference between the group and the intact control group or castrated control group, respectively, as tested by single-factor ANOVA ($p < 0.05$). B, dose-response relationships of TP. E_{max} and ED_{50} values for the levator ani (triangles), prostate (open circles), and seminal vesicles (diamonds) were obtained by nonlinear regression analysis. Curves were obtained by fitting the data into sigmoid E_{max} model.

prostate, seminal vesicles, and levator ani muscle in castrated rats were 6.2, 8.1, and 40.9%, respectively, of those in intact animals. The reduction in masses of these androgen-targeted organs in castrated animals is the result of ablation of endogenous androgen production (Saksena and Chaudhury, 1970). Exogenous administration of TP, an androgenic and anabolic steroid, increased weights of the prostate, seminal vesicles, and levator ani muscle in castrated rats (Fig. 2). The increases in organ weights induced by TP were dose rate-dependent.

Figure 3 shows that compound *S*-1 had no significant effect on prostate, seminal vesicles, and levator ani muscle in castrated animals at 0.1 and 0.3 mg/day, but significantly stimulated the growth of these organs at higher doses. The weights of prostate, seminal vesicles, and levator ani muscle were maximally restored by *S*-1 to 14.9, 13.4, and 74.3%, respectively, of those in intact animals. The ED_{50} values of *S*-1 in prostate, seminal vesicle, and levator ani muscle, as obtained by nonlinear regression analysis of dose-response relationships, were 0.42 ± 0.04 , 0.38 ± 0.26 , and 0.44 ± 0.01 mg/day, respectively (Fig. 3B; Table 2), corresponding to 1.63, 1.47, and 1.70 mg/kg, respectively, based on the mean body weight of *S*-1-treated animals at the end of the study. The

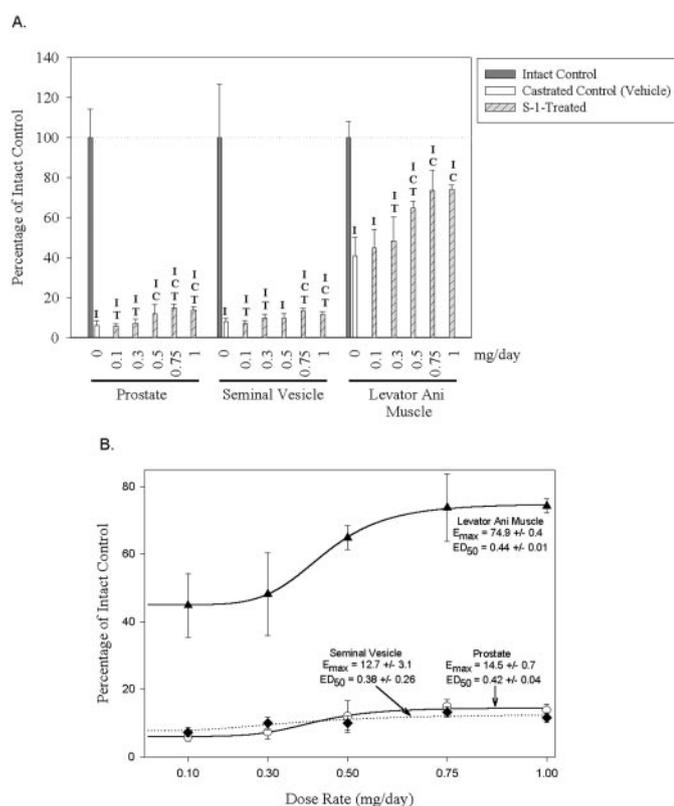


Fig. 3. A, assay for androgenic and anabolic activity of *S*-1 in castrated immature rats. One day after castration, immature rats received the indicated dose rates of *S*-1 via Alzet osmotic pumps for 14 days. All weights were corrected for 100 g of body weight and were converted to the percentage of the weights in the intact control group. Values represent the mean \pm standard deviation ($n = 5$ /group). The letters "I", "C", and "T" above the error bars indicate a significant difference between the group and the intact control group, castrated control group, or corresponding TP group, respectively, as tested by single-factor ANOVA ($p < 0.05$); B, dose-response relationships of *S*-1. E_{max} and ED_{50} values for the levator ani (triangles), prostate (open circles), and seminal vesicles (diamonds) were obtained by nonlinear regression analysis. Curves were obtained by fitting the data into sigmoid E_{max} model.

elevations in organ weights by *S*-1 demonstrated its androgenic and anabolic activities in animals. In comparison to TP, corresponding dose rates of *S*-1 induced significantly smaller increases in the weight of the prostate and seminal vesicles but a similar degree of increase in levator ani muscle weight (compare Fig. 2A with 3A). This result denoted the tissue selective androgenic and anabolic activity of *S*-1 in rats. The selectivity was also demonstrated by its relative efficacy compared with TP (Table 2). The relative efficacy in maintaining levator ani muscle weight was 0.72, much higher than the relative efficacies in maintaining prostate and seminal vesicle weights, which were less than 0.20.

Despite their high AR binding affinity, compounds *S*-2 and *S*-3 failed to exert any significant effect on the weights of prostate, seminal vesicles, and levator ani muscle in castrated animals, with dose rates up to 1 mg/day (Fig. 4). This suggests that rapid metabolism or clearance of these compounds led to lower plasma concentrations of these drugs, and thus no pharmacological activity. Likewise, compound *R*-1 (the stereoisomer of *S*-1), at 1 mg/day, produced no apparent effect on the weights of prostate, seminal vesicles, and levator ani muscle in castrated animals, demonstrating the stereoselective pharmacological action of these compounds.

Compound *S*-4 (Fig. 5) caused dose-dependent stimulation of growth in prostate, seminal vesicles, and levator ani muscle, with their weights in castrated animals being maximally promoted to 33.8, 28.2, and 101% of intact controls, respectively. Nonlinear regression analysis of dose-response relationships showed that the ED_{50} values of *S*-4 were 0.43 ± 0.01 , 0.55 ± 0.02 , and 0.14 ± 0.01 mg/day in prostate, seminal vesicles, and levator ani muscle, respectively (Fig. 5B; Table 2), corresponding to 1.62, 2.07, and 0.53 mg/kg, respectively, based on the mean body weight of *S*-4-treated animals at the end of the study. These results clearly revealed the androgenic and anabolic activities of *S*-4 in animals. In particular, *S*-4 exhibited potent and efficacious anabolic activity, as indicated by its ability to fully maintain the levator ani muscle weight in castrated animals at the same level as intact controls, at a dose rate as low as 0.3 mg/day (Fig. 5A). The relative potency and efficacy of *S*-4 in androgenic tissues were less than 0.3 and 0.4, respectively, compared with 1 for TP, whereas its relative potency and efficacy in the levator ani muscle was 1.07 and 0.97, respectively, compared with 1 for TP (Table 2).

Table 2 compares the androgenic and anabolic activities of *S*-1 and *S*-4, two compounds that exhibited *in vivo* functional activity in the present study, with those of TP. The efficacy for androgenic activity of *S*-4 (as indicated by relative efficacies in prostate and seminal vesicle) was about twice that of *S*-1, but the potency for androgenic activity (as indicated by relative potencies in prostate and seminal vesicle) was similar between these two compounds. As to anabolic activity, *S*-4 displayed much higher efficacy (as indicated by relative efficacy in levator ani muscle) and 2-fold greater potency (as indicated by relative potency in levator ani muscle) than *S*-1. These results suggest the greater selectivity of *S*-4 toward the anabolic target organ.

We also determined the serum levels of LH and FSH in animals that received *S*-1 and *S*-4, and compared them with the levels of these hormones observed in the intact, castrated, or TP-treated animals. As shown in Table 3, castration led to a significant elevation in FSH and LH levels, compared with

TABLE 2

Comparison of androgenic and anabolic activities of S-1 and S-4 to TP

Organs	Treatment	E_{max} (Percentage of Intact Control)	Relative Efficacy	ED ₅₀ mg/day	Relative Potency	
Androgenic	Prostate	TP	1.00	0.13 ± 0.03	1.00	
		S-1	14.5 ± 0.7	0.12	0.42 ± 0.04	0.31
		S-4	35.2 ± 0.4	0.29	0.43 ± 0.01	0.30
	Seminal vesicle	TP	70.0 ± 18.8	1.00	0.12 ± 0.02	1.00
		S-1	12.7 ± 3.1	0.18	0.38 ± 0.26	0.32
		S-4	28.5 ± 0.8	0.40	0.55 ± 0.02	0.22
Anabolic	Levator ani muscle	TP	104.2 ± 10.1	1.00	0.15 ± 0.03	1.00
		S-1	74.9 ± 0.4	0.72	0.44 ± 0.01	0.34
		S-4	101.0 ± 1.0	0.97	0.14 ± 0.01	1.07

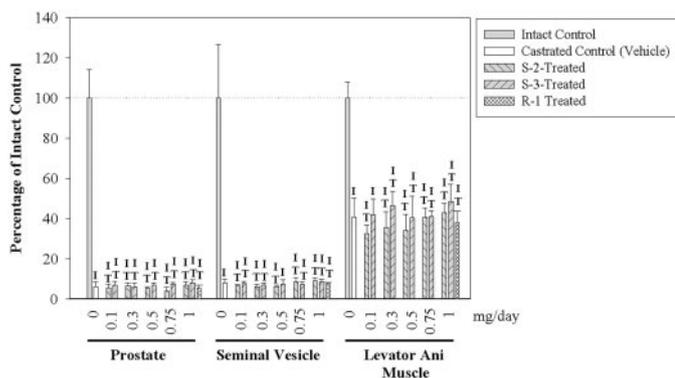


Fig. 4. Assay for androgenic and anabolic activity of S-2, S-3, and R-1 in castrated immature rats. One day after castration, immature rats received the indicated dose rates of S-2, S-3, or R-1 via Alzet osmotic pumps for 14 days. All weights were corrected for 100 g of body weight and were converted to the percentage of the weights in the intact control group. Values represent the mean ± standard deviation ($n = 5/\text{group}$). The letters "I", "C", and "T" above the error bars indicate a significant difference between the group and the intact control group, castrated control group, or corresponding TP group, respectively, as tested by single-factor ANOVA ($p < 0.05$).

intact animals. TP showed no dose-dependent effect on castration-induced change in FSH, but partially inhibited the castration-induced increase in LH levels at higher doses. The activities of S-4 on LH and FSH were similar to those produced by TP. S-1 and S-4 partially suppressed LH production at dose rates of 0.5 mg/day or higher. However, it is important to note that S-1 and S-4 did not suppress LH production at the dose levels needed to produce the desired pharmacological effects in the levator ani muscle or prostate. Interestingly, S-1 also partially suppressed FSH production at dose rates of 0.5 mg/day or higher. The FSH suppression noted at higher doses of S-1 suggested that this compound might interact with other steroid receptors, most probably progesterone receptors, in addition to the AR. Although statistically significant differences were noted in some instances, GH, AST-SGOT, ALT-SGPT, and serum lipids (including cholesterol, high-density lipoprotein, and triglyceride) were all within normal ranges for drug-treated animals. No drug- or dose-related changes in these indices were observed.

We also examined the effects of all compounds on total body weight and the weights of a variety of nonreproductive organs, including liver, heart, kidney, spleen, and lungs of treated animals. None of the compounds led to a dose-related change in these weights (data not shown). To further check for any signs of acute toxicity in animals from the studied

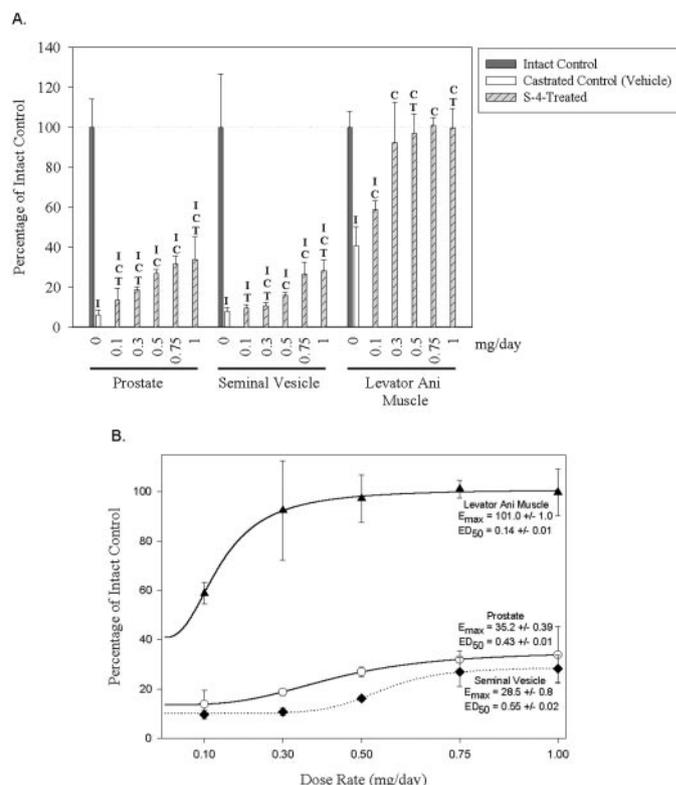


Fig. 5. A, assay for androgenic and anabolic activity of S-4 in castrated immature rats. One day after castration, immature rats received the indicated dose rates of S-4 via Alzet osmotic pumps for 14 days. All weights were corrected for 100 g of body weight and were converted to the percentage of the weights in the intact control group. Values represent the mean ± standard deviation ($n = 5/\text{group}$). The letters "I", "C", and "T" above the error bars indicate a significant difference between the group and the intact control group, castrated control group, or corresponding TP group, respectively, as tested by single-factor ANOVA ($p < 0.05$). B, dose-response relationships of S-4. E_{max} and ED₅₀ values for the levator ani (triangles), prostate (open circles), and seminal vesicles (diamonds) were obtained by nonlinear regression analysis. Curves were obtained by fitting the data into sigmoid E_{max} model.

compounds, complete diagnostic hematology studies of compound-treated animals were also performed. No drug- or dose-related changes were observed in any of the hematology diagnostic indices. These data suggest that all compounds manifested no acute toxicity during treatment.

Discussion

With *in vitro* AR binding and transcription activation assays, our laboratories previously identified a group of potent

TABLE 3
Effects of *S*-1 and *S*-4 on serum LH and FSH levels

Treatment	FSH	LH
	ng/ml	ng/ml
Normal Range	5.6–20	0.15–0.7
None (intact)	7.1 ± 1.2	0.27 ± 0.08
None (castrated)	30 ± 3 ^I	5 ± 2 ^I
TP (mg/day)		
0.1	26 ± 6 ^I	4 ± 4 ^I
0.3	23 ± 6 ^I	2 ± 1 ^{I,C}
0.5	30 ± 7 ^I	5 ± 2 ^I
0.75	22 ± 5 ^{I,C}	1.4 ± 0.7 ^{I,C}
1.0	24 ± 8 ^I	2 ± 1 ^{I,C}
<i>S</i> -1 (mg/day)		
0.1	28 ± 7 ^I	5 ± 2 ^I
0.3	27 ± 5 ^I	3 ± 1 ^I
0.5	22 ± 3 ^{I,C}	1.7 ± 0.3 ^{I,C,T}
0.75	20 ± 5 ^{I,C}	1.0 ± 0.7 ^C
1	23 ± 3 ^{I,C}	1.6 ± 0.7 ^{I,C}
<i>S</i> -4 (mg/day)		
0.1	27 ± 5 ^I	4 ± 1 ^I
0.3	26 ± 1 ^I	3.7 ± 0.9 ^I
0.5	26 ± 8 ^I	2.4 ± 0.9 ^{I,C}
0.75	31 ± 5 ^{I,T}	3 ± 1 ^I
34 ± 5 ^{I,T}	2.4 ± 0.4 ^{I,C}	

I, C, and T: significantly different ($p < 0.05$) from intact, castrated, and corresponding dose rate of TP-treated animals, respectively, as analyzed by single-factor ANOVA.

and efficacious nonsteroidal androgens that are structurally related to antiandrogen pharmacophores (Yin et al., 2003a). However, in vivo studies in a rat model with one of these nonsteroidal androgens, acetothiolutamide, failed to show androgenic activity (Yin et al., 2003b). Subsequent pharmacokinetic and metabolism studies in rats demonstrated that the lack of in vivo androgenic activity of acetothiolutamide in the pharmacology study was caused by its insufficient plasma exposure, which in turn resulted from its extensive hepatic degradation. Also, we found that oxidation at the sulfur linkage position was one major metabolic pathway for acetothiolutamide in rats, and that this oxidation likely produced deactivated or even antagonizing metabolites (Yin et al., 2003b). Considering these facts, we proposed to modify the linkage sulfur atom to block the oxidation at this position, thereby reducing the overall hepatic metabolism. As a result, a series of novel molecules that carry an ether linkage instead of a thio linkage in the structure were designed and synthesized. The present studies demonstrated that two of these ether-bearing molecules, *S*-1 and *S*-4, were androgen receptor modulators with tissue-selective activity in animals.

Despite structural similarities, this series of ether-carrying compounds exhibited diverse in vitro and in vivo activity profiles. The *S*-isomers of compounds 1, 2, 3, and 4 displayed moderate to high binding affinity for the AR, whereas the *R*-isomer of compound 1 had poor receptor binding. This finding was consistent with our previous observation regarding the stereoselective AR binding of nonsteroidal ligands (Mukherjee et al., 1996, 1999). Compounds *S*-1, *S*-3, and *S*-4 were further characterized as AR agonists with the in vitro cotransfection assay. The failure of *S*-2, a moderate AR binder, to stimulate AR-mediated gene transcription confirmed our previous finding that high receptor binding affinity is a prerequisite for agonist activity (Yin et al., 2003a). When tested in the castrated rat model, *S*-1 and *S*-4 demonstrated potent in vivo functional activity, and compounds *S*-2 and *S*-3 were inactive. Specifically, *S*-4 produced the greatest

androgenic and anabolic activity in animals, with anabolic activity greater than that of TP. *S*-1 had a similar degree of anabolic activity as TP, but had much less androgenic activity. Interestingly, the ED₅₀ values for *S*-1 in prostate, seminal vesicle, and levator ani muscle were approximately the same (i.e., about 0.4 mg/day), whereas *S*-4 demonstrated more than 2-fold greater potency in levator ani muscle compared with prostate and seminal vesicle, as indicated by the ED₅₀ values (Table 2). The distinction in functional activities in vivo among the four structurally related compounds could be caused by difference in any of numerous factors, including intrinsic activity, in vivo disposition and metabolism, or intracellular signaling pathway. Further studies to explore the physicochemical, physiological, and cellular/molecular determinants for nonsteroidal androgenic and anabolic activity will lead to insights into the mechanism of action of these nonsteroidal agents, and thereby provide a basis for future structural optimization.

The in vitro cotransfection assay is generally regarded as a valuable tool for screening of nonsteroidal AR ligands. With this assay, compounds *S*-1, *S*-3, and *S*-4 were successfully identified as potential AR agonists. However, as demonstrated in the animal study, *S*-3 did not show any measurable in vivo functional activity. Thus, the observation of in vitro agonist activity in the cotransfection assay can be but is not always predictive of in vivo activity. The pharmacological activity in vivo is determined not only by the ability of the compound to interact with the receptor, but also limited by complicated factors governing the accessibility of the compound to the effect site, such as disposition and metabolism. To fully predict the in vivo behavior and understand the structure-activity relationships, it is necessary to perform further studies examining the pharmacokinetics and metabolism of the compound. As a result of these and other studies, we abandoned use of the in vitro cotransfection assay in favor of in vivo pharmacologic assessment for discovery of SARMs.

The tissue-selective anabolic activity exhibited by *S*-1 and *S*-4 validated the feasibility of developing SARMs as a new generation of androgens. The possible mechanisms underlying the tissue-selectivity of these agents could be tissue-specific recruitment of cofactors/corepressors during the AR signaling pathway, or very likely for our nonsteroidal ligands, their distinct in vivo disposition from testosterone and its ester derivatives. The effects of testosterone in certain tissues, including most accessory reproductive organs and skin, are amplified through local conversion to DHT, the more potent bioactive form, by 5 α -reductase (Mooradian et al., 1987). Nevertheless, testosterone exerts direct effects in the testis, skeletal muscles, and bone (Mukherjee et al., 1996). For nonsteroidal ligands, their actions in accessory reproductive organs such as prostate would not be amplified as they are for testosterone; therefore, such a nonsteroidal androgen with equivalent activity to testosterone on bone and muscle would likely have less activity on prostate or other accessory reproductive organs than testosterone.

Compounds *S*-1 and *S*-4 are the first nonsteroidal androgens with in vivo functional activity among our series of compounds. More significantly, the discovery of these two in vivo functional drug candidates represents a major progress toward the development of therapeutically useful SARMs. SARMs, like the clinically available selective estrogen receptor modulators, would offer unique therapeutic advantages

over their steroidal counterparts. The tissue selectivity of these agents offers an exciting opportunity to differentially regulate the androgen effects in various target tissues, thus minimizing the interference to normal physiological processes while targeting desirable therapeutic goals. For example, SARMs with potent anabolic activity but minimal androgenic activity would be ideal for the treatment of patients who bear muscular diseases (such as sarcopenia or trauma-induced muscle wasting) but are contraindicated for androgenic stimuli (such as for aging population or prostate cancer patients). In perspective, not only could SARMs be used as superior alternatives to current steroidal androgens in therapy of male hypogonadism but also they could expand the scope of androgen therapy to include wasting syndromes, aging-related disorders due to declined androgen levels, male fertility regulation, and other androgen deficiency-related diseases.

In summary, the present studies examined the *in vitro* and *in vivo* activity profiles of a series of novel nonsteroidal AR ligands, among which two were identified as *in vivo* functional androgens with selective anabolic activity. These SARMs, with many advantages over current steroidal androgen preparations, implicate potential therapeutic significance in a scope of androgen-deficiency related disorders. Continued studies in our laboratories will focus on preclinical and clinical development of identified SARMs and further optimization of chemical structures based on understanding the mechanisms underlying nonsteroidal androgenic and anabolic activities.

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