Identification of Anabolic Selective Androgen Receptor Modulators with Reduced Activities in Reproductive Tissues and Sebaceous Glands

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Androgen replacement therapy is a promising strategy for the treatment of frailty; however, androgens pose risks for unwanted effects including virilization and hypertrophy of reproductive organs. Selective Androgen Receptor Modulators (SARMs) retain the anabolic properties of androgens in bone and muscle while having reduced effects in other tissues. We describe two structurally similar 4-aza-steroidal ligands, CI-4AS-1, a full agonist, and TFM-4AS-1, which is a SARM. TFM-4AS-1 is a potent AR ligand (IC_{50}, 38 nM) that partially activates an AR-dependent MMTV promoter (55% of maximal response) while antagonizing the N-terminal/C-terminal interaction within AR that is required for full receptor activation. Microarray analyses of MDA-MB-453 cells show that whereas CI-4AS-1 behaves like 5α-dihydrotestosterone (DHT), TFM-4AS-1 acts as a gene-selective agonist, inducing some genes as effectively as DHT and others to a lesser extent or not at all. This gene-selective agonism manifests as tissue-selectivity: in ovariectomized rats, CI-4AS-1 mimics DHT while TFM-4AS-1 promotes the accrual of bone and muscle mass while having reduced effects on reproductive organs and sebaceous glands. Moreover, TFM-4AS-1 does not promote prostate growth and antagonizes DHT in seminal vesicles. To confirm that the biochemical properties of TFM-4AS-1 confer tissue selectivity, we identified a structurally unrelated compound, FTBU-1, with partial agonist activity coupled with antagonism of the N-terminal/C-terminal interaction and found that it also behaves as a SARM. TFM-4AS-1 and FTBU-1 represent two new classes of SARMs and will allow for comparative studies aimed at understanding the biophysical and physiological basis of tissue-selective effects of nuclear receptor ligands.

Androgens, primarily testosterone (T) and its more potent derivative, 5α-dihydrotestosterone (DHT), induce male reproductive physiology and secondary sexual traits such as facial hair and deepened voice. Additionally, in both genders androgens regulate bone and muscle anabolism, adipose mass, lipoprotein metabolism, and behavior (1–3). Androgens decline with age in both men and women (4), which contributes to age-related bone and muscle loss and increases in fat mass (5). Several studies report low testosterone as a risk factor for age-related diseases including osteoporosis (6), sarcopenia (7), atherosclerosis (8), type 2 diabetes/metabolic syndrome and obesity (9), cognitive impairment (10), and depression (11). Restoring androgens to youthful levels could thus slow unfavorable changes in body composition and improve mood, motivation, and general health. Unfortunately, current androgens induce male secondary sexual traits such as acne and hirsutism, an effect known as virilization, (12) and pose concerns related to unwanted effects in the prostate and other reproductive organs (13–15). Therefore, androgens are limited by concerns over safety and tolerability.

Androgens exert their physiological effects by activating the androgen receptor (AR), a nuclear receptor expressed in reproductive tissues and other organs. Once bound by ligand, AR dissociates from chaperones in the cytoplasm and translocates to the nucleus where it induces target genes by binding to DNA sequences called androgen response elements (AREs) present in promoter/enhancer regions of responsive genes (16, 17). AR represses transcription by binding and inhibiting certain transcription factors (18–21). Based on precedent with the estrogen receptor α (ERα), for which tissue-selective estrogen receptor modulators (SERMs) have been developed into effective medicines, there has been an effort to discover tissue-selective androgen receptor modulators (SARMs). Ideally, SARMs would produce anabolism in bone and muscle with limited effects on uterus, skin, and prostate (22, 23). The properties of several experimental SARMs such as BMS-565929, S-40503,

The abbreviations used are: T, testosterone; SARM, selective androgen receptor modulator; SERM, selective estrogen receptor modulator; AR, androgen receptor; DHT, 5α-dihydrotestosterone; GST, glutathione S-transferase; LBD, ligand binding domain; ANOVA, analysis of variance; BFR, bone formation rate; ORX, orchidectomy; sc, subcutaneously.
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S-1, S-4, Ostarine, LGD-2226, and LGD-2941, have been reviewed (23). All of these compounds exhibit different dose-response curves in androgen-responsive tissues relative to a full agonist, they vary in their in vitro and in vivo properties, and it remains to be determined which will be suitable for therapeutic use. Furthermore, the molecular mechanisms by which tissue selectivity occurs are not known, impairing rational design of improved SARMs.

We hypothesized that all AR ligands capable of significant transactivation would produce anabolism, but SARMs would create a unique receptor conformation that could be detected in other biochemical experiments. AR has a unique feature in which full activation requires physical interaction between its N-terminal and C-terminal domains (N/C interaction). Human mutations associated with partial androgen insensitivity reduce this interaction (24–31). Because these individuals are often incompletely virilized, we hypothesized that those AR ligands that do not promote the conformation required for the N/C interaction would have reduced virilizing effects. Using this approach, we identified two new distinct anabolic SARMs, TFM-4AS-1 and FTBU-1, which display agonism in bone and muscle while having reduced effects on reproductive organs and sebaceous glands. We provide evidence suggesting that the molecular basis of these differential responses is gene-selective agonism.

MATERIALS AND METHODS

Reagents and Animals—All reagents were from Sigma unless noted. Rats were Sprague-Dawley from Taconic Farms (Tarrytown, NY) and were individually housed with ad libitum access to food and water. All procedures were in accordance with Institutional Care and Use Committee guidance. The compounds TFM-4AS-1 [(4aR,6aS,7S,11aR)-1,4a,6a-trimethyl-2-oxo-N-[2-(trifluoromethyl)phenyl]-2,4a,4b,5,6a,7,8,9,9b,10,11,11a-tetradecahydro-1H-indenone[5,4-f]quinoline-7-carboxamide], FTBU-1 [(4aR,6aS,7S,11aR)-N-(2-chlorophenyl)-1,4a,6a-trimethyl-2-oxo-N-[2-(trifluoromethyl)phenyl]-2,4a,4b,5,6a,7,8,9,9b,10,11,11a-tetradecahydro-1H-indenone[5,4-f]quinoline-7-carboxamide], Cl-4AS-1 [(4aR,6aS,7S,11aR)-N-(2-chlorophenyl)-1,4a,6a-trimethyl-2-oxo-N-[2-(trifluoromethyl)phenyl]-2,4a,4b,5,6a,7,8,9,9b,10,11,11a-tetradecahydro-1H-indenone[5,4-f]quinoline-7-carboxamide], and Ostarine, LGD-2226, and LGD-2941, have been reviewed (23).

Animal Studies—Animal procedures were performed as described (38–40). Briefly, bone, body composition, and uterine studies were performed in ovariectomized (OVX) or sham-operated rats at age 6–9 months, 3 months post-surgery. Animals were also treated with the bone resorption inhibitor alendronate unless noted (5.6 μg/kg/week). Animals were randomized into groups of equal weights (μ = 10–16) and compounds in 3% benzyl alcohol in sesame oil (vehicle) were given by subcutaneous (sc) injection for 24 days. Uteri were dissected at the cervix and weighed. Sebaceous gland area in dorsal skin sections was measured using BioQuant. Femur and brand lean body mass composition changes were assessed by dual energy x-ray absorptiometry (DEXA). Statistical analysis was performed by Kruskal-Wallis non-parametric ANOVA followed by Student-Newman-Keuls post-hoc testing for intergroup differences.

Microarray and RNA Analyses—Prostate microarray studies were performed as described (38). For cell culture studies, total RNA was extracted using TRIzol (Invitrogen, Grand Island, NY) from duplicate 10-cm dishes of MDA-MB-453 cells treated 18 h. Microarray analysis was performed on 5 μg of total RNA (38, 41). Data were normalized to achieve identical median fluorescence intensity of each array. For a transcript to be considered DHT-regulated, the probe must have corresponded to a gene annotated at Entrez Gene, and the hybridization signals must have tested different from vehicle control (p < 0.05, Rosetta error model (42) and differed from vehicle controls by ≥1.5-fold in both duplicate 200 nM DHT samples. Gene expres-
sion data are the mean of the duplicates ± S.D. For quantitative RT-PCR, total RNA was collected and analyzed as described after 18 h of treatment (41). For studies involving cycloheximide, MDA-MB-453 cells in 10-cm dishes were pretreated for 30 min with 10 μg/ml cycloheximide, and the AR ligand was added at indicated concentrations for an additional 6 h.

RESULTS

TFM-4AS-1 Displays Partial Agonism in Transcriptional Assays—To identify AR ligands with distinct transcriptional properties, we mined the Merck chemical library for compounds with binding affinities lower than 300 nM for AR and counterscreened them for binding glucocorticoid, progesterone, mineralocorticoid (GR, PR, MR), and both ERs. Compounds selective for AR were tested for MMTV promoter transactivation mediated by endogenous AR in MDA-MB-453 cells. This cell line was selected because agonists (DHT, testosterone, R1881, mibolerone, and danazol) and antagonists (cyproterone acetate, hydroxyflutamide, and bicalutamide) exhibited activities that matched their actions in human and animal studies. The MMTV promoter screen produced two hits we selected for further study. The structurally related compounds CI-4AS-1 and TFM-4AS-1 had potent AR binding activities in radioligand binding assays with IC50 values of 12 and 30 nM, respectively (43); IC50 values for GR, PR, MR, or ER were greater than 5000 nM. Both molecules were synthesized during the development of finasteride, a 5α-reductase type I inhibitor, so in addition they are also inhibitors of rat 5α-reductase enzymes type I (6 and 2 nM, respectively) and type II (10 and 3 nM, respectively) (Ref. 43 and Fig. 1A).

In binding studies, DHT exhibited similar apparent affinity (Kd values of 0.3–0.46 nM) for AR that is natively expressed in MDA-MB-453 cells and to the rhAR LBD that was expressed as GST fusion protein in yeast (Fig. 1B). In contrast, TFM-4AS-1 had a 40-fold reduction in the affinity to the rhAR LBD relative to the AR aporeceptor natively expressed in MDA-MB-453 cells. This change in potency was not related to species differences because TFM-4AS-1 had the expected binding affinity of ~30 nM to full-length rhAR transfected into cells (data not shown). Therefore, unlike DHT, TFM-4AS-1 requires a mature AR aporeceptor complex for high affinity binding.

In transactivation assays using the MMTV promoter, CI-4AS-1 maximal fold stimulation was similar to the full agonist R1881 (Fig. 2A). Its maximal transactivation relative to 100 nM R1881 reached an agonistic activity of 135 ± 23% (mean of n = 7 S.E.) with an inflection point (IP) value of 2.8 ± 2.1 nM compared with 0.3 nM for R1881. In contrast, TFM-4AS-1 produced only partial stimulation of the MMTV promoter that was maintained through 2–3 log concentrations at ~50% (Fig. 2A). Relative to 100 nM R1881, its maximal transactivation was 55 ± 11% with an IP value of 28.4 ± 19.9 nM (average of n = 244). TFM-4AS-1 antagonizes 1 nM R1881 as does the anti-androgen bicalutamide, but unlike bicalutamide, TFM-4AS-1’s antagonist activity did not exceed ~50% up to 1 μM (Fig. 2A). These data established that the partial activation of the MMTV promoter by TFM-4AS-1 represents a true submaximal effect that stems from functional activity of the ligand on the receptor and confirms that TFM-4AS-1 is an AR ligand with mixed agonist and antagonist activities.

We next examined AR-dependent repression of the phorbol ester-activated MMP-1 promoter in 22Rv1 human prostate cancer cells. CI-4AS-1 suppressed promoter activity much like DHT (Fig. 2B). In contrast, TFM-4AS-1 behaved like AR antagonists such as hydroxylutamide and did not decrease MMP-1 promoter activity. Thus, in contrast to MMTV promoter transactivation, TFM-4AS-1 did not suppress the MMP-1 promoter where transrepression is mediated via AP-1 binding.

To compare the ability of these compounds to induce the AR N/C interaction, a mammalian two-hybrid assay in CV1 cells was employed. The Gal4-DNA binding domain (DBD) was fused with the LBD of rhAR and transfected with a VP16 construct containing the rhAR NTD and a Gal4-DBD luciferase reporter. Upon agonist binding to the LBD, a conformational change occurs and the NTD/VP16 fusion protein is recruited, resulting in luciferase transcription. CI-4AS-1 (10 μM) effectively promoted the AR N/C interaction (Fig. 2C), with an average (n = 184) maximal activity of 35.3% ± 5.3% relative to 1 nM R1881. In contrast, TFM-4AS-1 (10 μM) showed an activity that
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FIGURE 2. In vitro activities of CL-4AS-1 and TFM-4AS-1. A, TFM-4AS-1 is a partial agonist in the MMTV-luciferase transactivation assay in AR⁺ MDA-MB-453 cells. Left, dose response curves of R1881, TFM-4AS-1, and Cl-4AS-1 reveal the partial agonism of TFM-4AS-1. Right, TFM-4AS-1 partially antagonizes the effect of 1 nM R1881 relative to the antagonist bicalutamide. B, MMP-1 promoter repression assay in 22RV1 cells. DHT and Cl-4AS-1 repress promoter activity whereas hydroxyflutamide and TFM-4AS-1 do not. C, AR N-terminal domain/C-terminal domain mammalian two-hybrid assay in CV1 cells. Left, in an agonist mode, the graph shows the maximal activities of hydroxyflutamide, bicalutamide Cl-4AS-1 and TFM-4AS-1 at doses of 1 μM relative to 1 nM R1881; right, antagonist action of TFM-4AS-1, the graph expresses the maximum fold induction relative to DMSO. Note that TFM-4AS-1 antagonizes the effects of Cl-4AS-1. All error bars represent the S.D. of >3 measurements; data are representative of >3 experiments.

was only slightly higher than that of hydroxyflutamide or bicalutamide (Fig. 2C). On the average, its maximal activity was only 5.7 ± 1.9% (n = 179) of 1 nM R1881. Moreover, 1 μM TFM-4AS-1 antagonized the N-C interaction induced by 10 nM CL-4AS-1 (Fig. 2C). These data suggest that Cl-4AS-1 activates the N-C interaction much like an agonist (albeit to a lesser degree), but that TFM-4AS-1 does not.

TFM-4AS-1 Differentially Modulates the Expression of Native Androgen-responsive Genes—We then examined native gene regulation by TFM-4AS-1 in MDA-MB-453 cells. Cells were treated for 18 h and using microarrays, 294 DHT-responsive genes were identified (supplemental Table S1). The results were displayed on a heatmap where the order of experiments is fixed on the y-axis, and genes are clustered on the x-axis by correlation coefficient (Fig. 3A). Next, for each gene the effect of 200 nM DHT was set to 100%, and all other conditions were normalized to this value to calculate potency and allow statistical comparisons. DHT dose-dependently altered the expression of genes with an average EC₅₀ of 0.97 nM (Fig. 3B). Cl-4AS-1 (1 μM) behaved like DHT (Fig. 3, A and B), with transcript changes on average equaling 90.6 ± 37.1% of 200 nM DHT. TFM-4AS-1 also regulated many genes similarly to DHT (Fig. 3A) with an EC₅₀ of 10.85 nM, close to its half-maximal effects in binding assays and MMTV transactivation (Fig. 3B). However, at 1 μM, ~100-fold greater than its EC₅₀, TFM-4AS-1 transcript changes were on average 74.6 ± 45.5% the effect of
200 nM DHT, which was significantly less than either 20 nM
DHT or 1 μM Cl-4AS-1 (p = 1 × 10^{-13} and 1 × 10^{-6} respec-
tively, ANOVA).

This analysis indicated that TFM-4AS-1 was less effective
than DHT and Cl-4AS-1 at inducing this gene set. To assess
whether this sub-effective response was due to all genes being
inefficiently induced, or rather to some genes responding fully
and others not responding, we compared the variation in gene
response between treatments. We first compared the effect of 1
μM Cl-4AS-1 to 20 nM DHT (20 nM was used because 200 nM
values were set to 100%, precluding analysis of variation). 49% of
gene expression values were within one S.D. of DHT mean values,
and 26% were 3 S.D. values lower than DHT. This analysis suggests
that in the presence of TFM-4AS-1 ~25% of AR-responsive genes respond as if DHT
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In contrast for UGT2B7, TFM-4AS-1 only induces the RNA FGF18, the response to the three ligands was indistinguishable. (Fig. 3 illustrate this result, we selected the genes UGT2B7 and FGF18 effects on reproductive organs.

Distinct Effects of TFM-4AS-1 and CL-4AS-1 on Bone Formation and Uterus Weight—We used double calcein labeling to quantify changes in BFR in the periosteum, the external bone surface that in rats is highly androgen-sensitive. Alendronate was included to isolate the anabolic effect of andro-

gens by eliminating any periosteal bone formation occurring secondary to catabolic increases in bone resorption as these processes are coupled. BFR measurements in the femora revealed dose-dependent stimulatory effects of both compounds on the periosteum. TFM-4AS-1 dosed sc for 24 days at 10 mg/kg/day increased the periosteal double-labeled surface, mineral apposition rate, and bone formation rate to levels similar to 3 mg/kg/day sc DHT, the lowest fully effective DHT dose as determined in pilot experiments (Fig. 4, A–C).

In rats, DHT has a pronounced trophic effect on uterus. Whereas DHT increased uterine weight, TFM-4AS-1 produced little or no effect at doses that fully induced bone formation (Fig. 4D). In contrast, CL-4AS-1 produced significant increases in uterine weight at all the doses that stimulated bone formation, indicating TFM-4AS-1 has tissue-selective effects in vivo.

Effects of TFM-4AS-1 on Sebaceous Gland Formation—To evaluate the potential of TFM-4AS-1 to stimulate the pilosebaceous unit, histomorphometric measurements of sebaceous gland area in dorsal skin were performed in aged OVX female rats after 24 days of treatment with the maximally anabolic doses of 10 mg/kg/day for TFM-4AS-1 and 3 mg/kg/day DHT. In pilot experiments in this OVX rat model, DHT increased sebaceous gland area with a maximal response occurring within 4 days, whereas the anti-androgen cyproterone acetate produced little or no increase in sebaceous gland size after 28 days.8

was the ligand, while another ~25% are less responsive. To illustrate this result, we selected the genes UGT2B7 and FGF18 (Fig. 3D). Both genes were induced ~10-fold by DHT. For FGF18, the response to the three ligands was indistinguishable. In contrast for UGT2B7, TFM-4AS-1 only induces the RNA 2.7-fold at 1000 µM. The responses of UGT2B7 and FGF18 to DHT were insensitive to cycloheximide, which blocks transcriptional effects requiring translation of another gene product (Fig. 3E) but were blocked by bicalutamide, suggesting direct AR involvement (Fig. 3E). Sequence analysis detected potential AREs in the 5′-regions of both genes (data not shown). Thus FGF18 and UGT2B7 are potentially both direct transcriptional targets of AR, supporting the hypothesis that TFM-4AS-1 has selective effects on native AR target genes within a uniform cellular context depending on promoter context.

Distinct Effects of TFM-4AS-1 and CL-4AS-1 on Bone Formation and Uterus Weight—We used double calcein labeling to quantify changes in BFR in the periosteum, the external bone surface that in rats is highly androgen-sensitive. Alendronate was included to isolate the anabolic effect of andro-

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FIGURE 4. TFM-4AS-1, but not Cl-4AS-1 exhibits SARM activities in OVX rats. OVX rats were treated with AR ligands at the indicated doses (mpk) plus alendronate (5.6 µg/wk) and subjected to double-calcin labeling. A, periosteal bone formation measured as bone formation rate (percent double-labeled surface/total bone surface). B, mineral apposition rate measuring rate of bone growth in double-labeled regions (microns per day). C, calculated annual bone formation rate (mm2/mm/year). D, uterine wet weight as a measure of androgen effects on reproductive organs. E, anabolic effects of DHT and TFM-4AS-1 in muscle. F, effects of DHR and TFM-4AS-1 on adiposity. Lean and fat mass was measured in OVX rats. Animals were scanned by dual x-ray absorptiometry before and after 6 weeks of dosing with 3 mpk DHT or 10 mpk TFM-4AS-1. Values for lean mass (E) and fat mass (F) are expressed as mean change from baseline. Uterine wet weight was determined at the end of the experiment. All values are ± S.E., n = 10–16. *, different from ovariectomy (OVX) alone (p < 0.05, Kruskal-Wallis).
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FIGURE 5. Anabolic doses of TFM-4AS-1 have reduced effects on the formation of sebaceous glands in skin and the growth of prostate and seminal vesicles. OVX rats were dosed for 24 days with DHT or TFM-4AS-1 at fully anabolic exposures: A, bone formation rate determined from double-calcine labeling; B, mean sebaceous gland area determined by quantitative histomorphometry of dorsal back skin (n=3 fields per specimen, 10–16 per group); and C, uterine wet weight. All values are ± S.E., n = 10–16. *, different from vehicle (p < 0.05, Kruskal-Wallis). D, effects of Cl-4AS-1 and TFM-4AS-1 on rat prostate. Intact or castrated (ORX) rats treated for 7 days with the indicated compound (10 mpk) and prostate wet weights were measured and expressed as percent of body weight (mean of 8 animals, ± S.E.). Note that Cl-4AS-1 is less effective at reducing prostate wet weight, and more effective at restoring prostate weight in ORX rats than is TFM-4AS-1. E, TFM-4AS-1 and bicalutamide antagonize the stimulations of SV growth by DHT. Seminal vesicle weight in ORX or mock-castrated (SHAM) animals treated for 14 days with a DHT pellet designed to provide DHT at a constant level regardless of gonadotropins or 5α-reductase inhibition. Bicalutamide fully inhibited the effects of DHT whereas TFM-4AS-1 partially inhibited DHT at 30 mg/kg/day. All values are ± S.E., n = 9, *, indicates different from control (p < 0.05, Kruskal-Wallis). Control represents vehicle-treated ORX animals with only DHT pellets.

Similar to the previous experiment (Fig. 4), DHT and TFM-4AS-1 significantly increased bone formation rate by 204 and 308%, respectively (Fig. 5A). In the same animals, DHT increased sebaceous gland mean area by 108% and uterus weight nearly 400% (Fig. 5, B and C). In contrast, 10 mpk TFM-4AS-1 increased gland mean area by 33% and did not increase uterus weight. These data indicate that TFM-4AS-1 has reduced effects on the pilosebaceous unit and the uterus at anabolic doses.

Differential Effects of TFM-4AS-1 and Cl-4AS-1 on Prostate and Seminal Vesicle Growth—The above data suggested that TFM-4AS-1 acts as a SARM; however, it remained possible that this property results from limited tissue distribution or 5α-reductase inhibition. Therefore, we compared prostate or seminal vesicle weight in three groups of rats: sham castrated, castrated, or castrated/DHT-supplemented treated with vehicle, intact males. Combined treatment of DHT with TFM-4AS-1 resulted in a dose-dependent inhibition of the DHT-induced increase in seminal vesicle weight (55% reduction by 30 mg/kg/day TFM-4AS-1). Higher doses of TFM-4AS-1 did not achieve greater plasma concentrations and were not tested. In contrast treatment with the antagonist bicalutamide at 10 mg/kg/day resulted in a 97% reduction of the DHT effect. These results confirm that TFM-4AS-1 is a partial agonist in seminal vesicles.

Gene Selectivity in Prostate Tissue—To understand the basis of the reduced effects of TFM-4AS-1 in the male reproductive tract, microarray analyses were performed on the rat ventral prostate 6 and 24 h after a single injection of 3 mg/kg DHT or 10 mg/kg TFM-4AS-1. These time points were chosen to enrich for direct transcriptional events (38). One-dimensional agglomerative clustering of the DHT-regulated RNAs (38) revealed that TFM-4AS-1 generally did not induce or repress
these transcripts to the same extent as DHT. However, closer inspection suggested that some genes are regulated similarly by both (Fig. 6A). To explore this gene selectivity at an individual transcript level, we selected four DHT-responsive transcripts expressed in the prostate, uterocalin (lipocalin 2, Lcn2), FGFR4, IGF-1, and cyclin D1. The activities were normalized to cyclophilin. Nine days after surgery, ORX rats were daily treated with vehicle, 3 mg/kg DHT, or 10 mg/kg TFM-4AS-1 (n = 6/group). Prostate samples were collected at 0.25, 1, 4, and 7 days of dosing. Note that over time TFM-4AS-1 altered uterocalin and FGFR4 RNAs similarly to DHT, whereas it had no effect on cyclin D1 and IGF-1 at any time point. Values are normalized to cyclophilin levels measured within the same reaction and are the mean of six measurements from individual animals (± S.D., * different than vehicle treatment values (p < 0.05 ANOVA).

A

B

FIGURE 6. Microarray analysis of TFM-4AS-1 in rat prostate tissue. A, agglomerative 1-dimensional false-color heatmap (as in Fig. 2) showing the gene expression effects of DHT and TFM-4AS-1 in prostate tissue from castrated males 6 and 24 h after a single injection. Note that TFM-4AS-1 alters the expression of some but not all DHT-regulated genes. Gene expression values are the mean of three measurements each from pooled RNA from three specimens. B, actions of DHT and TFM-4AS-1 on gene expression in prostate was measured by quantitative RT-PCR data for four selected genes, uterocalin (lipocalin 2, Lcn2), FGFR4, IGF-1, and cyclin D1. The values were normalized to cyclophilin. Nine days after surgery, ORX rats were daily treated with vehicle, 3 mg/kg DHT, or 10 mg/kg TFM-4AS-1 (n = 6/group). Prostate samples were collected at 0.25, 1, 4, and 7 days of dosing. Note that over time TFM-4AS-1 altered uterocalin and FGFR4 RNAs similarly to DHT, whereas it had no effect on cyclin D1 and IGF-1 at any time point. Values are normalized to cyclophilin levels measured within the same reaction and are the mean of six measurements from individual animals (± S.D., * different than vehicle treatment values (p < 0.05 ANOVA).

Identification of a Non-steroidal SARM Devoid of 5α-Reductase Activity—Whereas these data support the hypothesis that partial transactivation activity coupled with limited ability to induce the N/C-terminal interaction would be hallmarks of SARMs, TFM-4AS-1 is a steroidal compound that in principle could be affecting any of the number of proteins that recognize the steroidal structure (in addition to 5α-reductase). Thus we wanted to confirm the observations with TFM-4AS-1 using a non-steroidal SARM with no 5α-reductase inhibition. A high-throughput screen identified a class of AR ligands with partial agonist activity; structural optimization yielded FTBU-1 (Fig. 7A). FTBU-1 has an AR binding IC₅₀ of 38 nM, shows no significant binding activity toward GR, PR, MR, or ER, and is devoid of 5α-reductase inhibition. This compound exhibited partial agonist activity in the MMTV transactivation assay (81% of maximal activity) and did not fully stimulate the N/C interaction (5% of maximal activity, Fig. 7B). A separate microarray study in MDA-MB-453 cells was conducted comparing FTBU-1 to DHT. As suggested by the intermediate partial agonist effects in transcription assays, 1 μM FTBU-1 regulated the 294 previously identified DHT-sensitive transcripts in MDA-MB-453 cells to a mean 83.2 ± 29.4% relative to 200 nM DHT, a significant difference (p < 0.0001, Student’s t test) and an intermediate value between those observed with TFM-4AS-1 and Cl-4AS-1. Nearly one-third of all RNAs were >2 S.D. less than the mean for 20 nM DHT (Fig. 7C). Based on these data, FTBU-1 was judged to be a partial agonist like TFM-4AS-1, although with slightly more agonistic activity, and thus was tested in OVX rats. The lowest FTBU-1 dose tested, 10 mg/kg/day sc for 24 days, produced an anabolic response in the periosteum equivalent to that of 3 mg/kg/day sc DHT, but had no significant effect on uterus weight (Fig. 7D). At 10 mg/kg/day, the exposure area under the curve was 77 μm²h (0–24 h). Dosing 9-fold higher, 90 mg/kg/day sc, produced an exposure ~8-fold higher (600 μm²h) and also provided a fully osteoanabolic stimulus, but significantly increased uterus weight by 233% (Fig. 7D), about half the effect of DHT (477%). Therefore, at exposures that were at least 8-fold greater than required for osteoanabolism, FTBU-1 still produced less uterotrophic effects than DHT.
DISCUSSION

For androgen replacement to become widely used it will be necessary to identify androgens with anabolic activities but reduced propensity to induce male secondary sexual traits and stimulate reproductive organs. Here we describe TFM-4AS-1, an anabolic AR ligand with limited effects on reproductive tissues and sebaceous glands. The structurally similar AR ligands, Cl-4AS-1 and TFM-4AS-1, exhibited the profiles of an agonist and a partial agonist, respectively. Cl-4AS-1 fully transactivated the MMTV promoter and repressed the activity of MMP1 promoter. In contrast TFM-4AS-1 only partially (~50%) transactivated the MMTV promoter and antagonized 50% of the activity of a full agonist; thus it can be characterized as a ligand with mixed agonist and antagonist activities. Similar to an AR antagonist, TFM-4AS-1 does not repress the AP-1-sensitive MMP-1 transcriptional cofactors. In these models, activity in one tissue accompanied by lack of activity in another is explained by the selective expression of permissive cofactors. However, TFM-4AS-1 acts as a SARM within a uniform cellular context. Similar data were observed in the prostate, where TFM-4AS-1 regulated uterocalin and FGFR4 similarly to DHT whereas the DHT-responsive genes IGF-1 and cyclin D1 were unaffected. Thus, we propose that TFM-4AS-1 exhibits tissue selectivity because within each cell type it regulates a subset of AR-responsive genes: in some tissues this subset is sufficient to generate a physiological response and in others it is not. In this regard, SARMs should provide valuable insight into molecular requirements for androgenic effects.

Our experiments in castrated rats demonstrated that TFM-4AS-1 is a partial agonist that partially antagonizes both endogenous androgens and co-dosed DHT. However, poor solubility prevented us from testing whether it maintains tissue-selectivity at higher exposures. We identified a non-steroidal SARM, FTBU-1, which has no 5α-reductase activity and improved solubility. This compound closely mimics the in vitro transcriptional profile of TFM-4AS-1, albeit with higher agonistic activity (81% versus 55% MMTV transactivation). Like TFM-4AS-1, FTBU-1 had little effect on the uterus at anabolic doses and at exposures ≥8-fold above those required for anabolism FTBU-1 exhibited 50% less uterotropic activity than DHT. While based on our experience with related compounds, we suspect that this uterotrophic effect is caused by the higher agonism.9 It remains

9 A. Schmidt, S. Harada, D. B. Kimmel, C. Bai, R. L. Vogel, S. Rutledge, A. Scafoneas, F. E. Chen, P. V. Nantermet, M. E. Duggan, G. D. Hartman, T. Prueksaritanont, M. A. Gentile, B. Pennypacker, P. Masarachia, R. Meissner, L. P. Freedman, and W. J. Ray, manuscript in preparation.
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possible that at high doses, tissue-selective SARMs could cause unwanted effects.

The transcriptional profile of our SARMs is distinct from that of the SERMs.Raloxifene, an osteoprotective ER ligand that lacks the agonistic activities of estradiol in breast and uterus (44–47), and is an ER antagonist in transactivation assays but represses ER-controlled AP-1 binding sites (46–48). In contrast, TFM-4AS-1 is an agonist in transactivation assays and does not inhibit AP-1-mediated transcription of the MMP-1 reporter. Unlike the significant body of information describing the clinical properties of SERMs, little clinical information is available regarding the actions of SARMs. However, in a 12-week study in healthy postmenopausal subjects, an AR ligand, MK-0773, which was selected based on similarity to TFM-4AS-1, exhibited SARM-like properties by increasing LBM without affecting markers of skin virilization or endometrial proliferation (49). Thus the properties of SARMs described here might translate into patients and apply broadly to the discovery of new therapeutic androgens.

Acknowledgment—We thank Linda Rhodes, VMD, PhD for help with the prostate studies.

REFERENCES
1. Bachmann, G. A. (1999) Am. J. Obstetrics Gynecol. 180, S308–S311
2. Bain, J. (2007) Clin. Interventions Aging 2, 567–576
3. Vermeulen, A. (2003) J. Steroid Biochem. Mol. Biol. 87, 773–781
4. Marks, L. S., Mostaghel, E. A., and Nelson, P. S. (2008) J. Mol. Endocrinol. 39, 773–781
5. Carmona, R. M., and Perry, P. J. (2004) Drugs Aging 21, 361–376
6. Han, H., Kim, S., Chen, H. Y., Tan, Q., Roher, S. P., Dininno, F., Hammond, M. L., Armour, C. D., Bennett, H. A., Coffey, E., Dai, H., He, Y. D., Kidd, M. J., Hughes, I. A. (2008) J. Biol. Chem. 283, 38948–38961
7. Thompson, J., Tiling, W. D., and Sutherland, R. L. (1994) Eur. J. Cancer 30A, 484–490
8. Isidori, A. M., Giannetta, E., Pozza, C., Bonifacino, V., and Isidori, A. (2003) J. Endocrinol. Invest. 26, 236–241
9. Paech, K., Webb, P., Kuiper, G. G., Nilsson, S., Gustafsson, J., and Pike, J. W. (1997) Science 277, 1508–1510
10. Nantermet, P. V., Xu, J., Yu, Y., Hodor, P., Holder, D., Adamski, S., Gambone, C., Vogel, R., Zhang, H., Kasparcova, V., Bai, C., Harada, S., Schmidt, A., Reszka, A., and Freedman, L. (2004) J. Steroid Biochem. Mol. Biol. 91, 247–257
11. Schmidt, A., Endo, N., Rutledge, S. J., Vilarino, S., Chen, O., Gambone, C., Vogel, R., McElwee-Witmer, S., Bai, C., Freedman, L., and Schmidt, A. (2005) J. Biol. Chem. 280, 38988–38991
12. Hall, E. R., Tiling, W. D., McPhaul, M. J., and Sutherland, R. L. (1992) Int. J. Cancer 52, 778–784
13. Chen, F., Knecht, K., Leu, C., Rutledge, S. J., Scalfano, A., Gambone, C., Vogel, R., Zhang, H., Kasparcova, V., Bai, C., Harada, S., Schmidt, A., Reszka, A., and Freedman, L. (2004) J. Steroid Biochem. Mol. Biol. 91, 247–257
14. Bachmann, G. A. (1999) Am. J. Obstetrics Gynecol. 180, S308–S311
15. Bidder, M., Loewy, A. P., Latifi, T., Newberry, E. P., Ferguson, G., Willis, D. M., and Towler, D. A. (2000) Biochemistry 39, 38917–38928
16. Han, H., Kim, S., Chen, H. Y., Tan, Q., Roher, S. P., Dininno, F., Hammond, M. L., Rodan, G. A., and Balena, R. (1993) Calcified Tissue Int. 53, 278–282
17. Paech, K., Webb, P., Kuiper, G. G., Nilsson, S., Gustafsson, J., and Pike, J. W. (1997) Science 277, 1508–1510
18. Nantermet, P. V., Scalfano, A., Rutledge, S. J., Hodor, P., Holder, D., Adamski, S., Gentile, M. A., Kimmel, D. B., Harada, S., Gerhold, D., Freedman, L. P., and Ray, W. J. (2004) J. Biol. Chem. 279, 1310–1322
19. Yamamoto, M., Fisher, J. E., Gentile, M., Seedor, J. G., Leu, C. T., Rodan, S. B., and Rodan, G. A. (1998) Endocrinology 139, 1411–1419
20. Yamamoto, M., Markatos, A., Seedor, J. G., Marashaghi, P., Gentile, M., Rodan, G. A., and Balena, R. (1993) Calcified Tissue Int. 53, 278–282
21. Paech, K., Webb, P., Kuiper, G. G., Nilsson, S., Gustafsson, J., Kusner, P. J., and Scanlan, T. S. (1997) Science 277, 1508–1510
22. Scalfano, A., Reszka, A. Z., Kimmel, D. B., Hou, X. S., Su, Q., Birzini, E. T., Kim, S., Chen, H. Y., Tan, Q., Roher, S. P., Dininno, F., Hammond, M. L., Rodan, G. A., Towler, D. A., and Schmidt, A. (2008) J. Steroid Biochem. Mol. Biol. 110, 197–206
23. McDonnell, D. P., Clemm, D. L., Hermann, T., Goldman, M. E., and Pike, J. W. (1995) J. Mol. Endocrinol. 9, 659–669
24. Meissner, R. S., Perkins, J. J., Hartman, G. D., Bai, C., Kimmel, D. B., Leu, C. T., Pennickberger, B. L., Prueksaritanont, T., Duggan, M. E., Gentile, M. A., Nantermet, P., Ray, J., and Schmidt, A. (2009) ACS Natl. Meet. 32