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An Orally Active Selective Androgen Receptor Modulator Is Efficacious on Bone, Muscle, and Sex Function with Reduced Impact on Prostate

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A number of conditions, including osteoporosis, frailty, and sexual dysfunction in both men and women have been improved using androgens. However, androgens are not widely used for these indications because of the side effects associated with these drugs. We describe an androgen receptor (AR) ligand that maintains expected anabolic activities with substantially diminished activity in the prostate. LGD2226 is a nonsteroidal, nonaromatizable, highly selective ligand for the AR, exhibiting virtually no affinity for the other intracellular receptors. We determined that AR bound to LGD2226 exhibits a unique pattern of protein-protein interactions compared with testosterone, fluoxymesterone (an orally available steroidal androgen), and other steroids, suggesting that LGD2226 alters the conformation of the ligand-binding domain. We demonstrated that LGD2226 is fully active in cell-based models of bone and muscle. LGD2226 exhibited anabolic activity on muscle and bone with reduced impact on prostate growth in rodent models. Biomechanical testing of bones from animals treated with LGD2226 showed strong enhancement of bone strength above sham levels. LGD2226 was also efficacious in a sex-behavior model in male rats measuring mounts, intromissions, ejaculations, and copulation efficiency. These results with an orally available, nonaromatizable androgen demonstrate the important role of the AR and androgens in mediating a number of beneficial effects in bone, muscle, and sexual function independent from the conversion of androgens into estrogenic ligands. Taken together, these results suggest that orally active, nonsteroidal selective androgen receptor modulators may be useful therapeutics for enhancing muscle, bone, and sexual function. (*Endocrinology* 148: 363–373, 2007)

SUPPLEMENTAL ANDROGEN THERAPY is used to treat a variety of male disorders characterized by low testosterone (T) levels. In men 75 yr of age and older but otherwise in good health, mean plasma T levels drop to 35% of that of a comparable population of younger men (1). This age-associated T decrease, or andropause, may cause the fatigue, depression, reduced muscle and bone mass, decreased hematopoiesis, and sexual dysfunction sometimes found in elderly men (2). Androgens not only are effective in the elderly, but supplemental androgens have also demonstrated efficacy in the treatment of osteoporosis, including glucocorticoid-induced osteoporosis. They are also efficacious at building muscle and bone in wasting diseases such as cancer and HIV (3). Furthermore, androgens may have a role in the treatment of certain forms of female (4) and male (5) sexual dysfunction.

Despite the mounting evidence that androgens are effective in a growing number of indications for both men and women, they are not routinely prescribed. There are a number of problems with current therapies that limit their usefulness. These include prostate hypertrophy and the potential for induction or acceleration of prostate cancer disease in men, the development of male secondary sex characteristics in women (hirsutism and deepening of voice), and acne in both sexes. The route of administration is also an issue; steroidal androgens cannot be given orally without risking liver toxicity. Injectable T or T esters can cause problems that are associated with abnormally high T immediately after administration. Other topical options such as T patches and gels can cause local skin irritation and spousal transfer and are cumbersome to use.

Although androgens clearly have a valuable effect on bone in the clinic, conversion of T into estrogen by aromatization has clouded the interpretation of the efficacy of androgens directly on bone, in particular because of the well characterized effects of estrogen on bone in females (6). Furthermore, the severe osteoporosis found in an estrogen receptor (ER)-α-negative male has validated the important role the ER plays in the male skeleton (7). That estrogens are important

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Abbreviations: AF2, Activation function 2; AR, androgen receptor; CA, charcoal absorbed; DHT, dihydrotestosterone; DPD, deoxypyridinoline; 17β-E_2, 17β-estradiol; ER, estrogen receptor; FBS, fetal bovine serum; β-Gal, β-galactosidase; GR, glucocorticoid receptor; GRIP1, GR interacting protein 1; IMDM, Iscove's modified Dulbecco's medium; LBD, ligand-binding domain; MMTV, mouse mammary tumor virus; MR, mineralocorticoid receptor; ORDX, orchidectomized; PLSD, protected least significant difference; PPAR, peroxisome proliferator-activated receptor; PR, progesterone receptor; PSA, prostate-specific antigen; RSV, Rous sarcoma virus; SARM, selective AR modulator; T, testosterone.

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does not diminish the need for androgen action in bone. Nonaromatizable androgens are known to be active in cancellous bone without aromatization to estrogens (8). Furthermore, male androgen receptor (AR) knockout mice develop severe osteopenia (8–10).

The development of an orally active, selective AR modulator (SARM) with significant anabolic activity in muscle, bone, and sexual function but with a reduction in the severity of side effects may be useful for the treatment of osteoporosis, frailty, and some forms of sexual dysfunction (11, 12).

Compounds derived from known AR antagonists (bicalutamide and flutamide) have been found to have relatively little effect on prostate while maintaining effects on muscle, but little or nothing is known about their effects on bone or on sexual behavior. These molecules are rapidly cleared in the liver (13, 14) and are thus not suitable for oral administration. Other SARMs have been described, but these exhibit a weak separation between their effects on prostate and bone and are not orally available (14, 15). Moreover, the mechanism by which these SARMs separate anabolic efficacy from prostate effects is unknown (14–20).

The AR is a ligand-regulated transcription factor that modulates gene expression either by binding directly to the promoter of target genes or by affecting gene expression indirectly through the interaction with protein coregulators. It is these interactions that result in the regulation of gene transcription and a response to androgens. The AR contains three domains involved in transcriptional regulatory activity, including a central DNA-binding domain, a C-terminal ligand-binding domain (LBD), and an N-terminal activation domain, all of which are capable of binding coregulatory proteins. Many of these regulatory proteins contain a specific motif of amino acids (LXXLL) that binds directly to the activation function 2 (AF2) region of the LBD of the receptor (21). AR itself has a related sequence, FxxLF, in the N terminus. This sequence interacts directly with the LBD in a hormone-dependent manner (22, 23). The AR N terminus is bound to the C terminus in the presence of hormone in living cells (24). Interaction between the receptor LBD and the LxxLL motif of the coregulatory proteins or the receptor N terminus depends on the nature of the ligand that is bound to the receptor. Some agonist ligands allow this interaction, many antagonists do not, and some ligands alter the affinity and specificity of coactivator interactions (25–27). Ligands that change the coregulator interaction profile may also alter the function of AR when bound to these molecules as has been seen for other steroid receptors (28).

This manuscript describes the discovery and characterization of LGD2226. LGD2226 exhibits anabolic activity in *in vitro* and *in vivo* models of bone and muscle response to androgen, despite reduced interaction of AR with certain coactivators relative to steroids. In contrast to the effects of T and dihydrotestosterone (DHT), prostate growth in the LGD2226-treated animals was markedly reduced. Furthermore, this nonaromatizable compound is fully capable of inhibiting resorption in cancellous bone and enhancing the bone formation rate in cortical bone. The latter is solely a property of androgens, because estrogens under the same conditions inhibit cortical bone formation (29). These effects in bone result in an overall increase in bone strength well beyond the level achieved with endogenous T. LGD2226 is fully efficacious at preventing castration-induced loss of sexual function in male rats. Taken together, these data show that LGD2226 exhibits a beneficial profile that would be useful in a clinical setting characterized by low levels of T, reduced bone mineral density, or reduced muscle mass without some of the side effects of currently marketed steroidal androgens.

Materials and Methods

In vitro binding

Extracts from *Spodoptera frugiperda* 9 (Sf9) moth cells infected with recombinant baculovirus expressing the indicated receptor were used in labeled hormone-binding assays. Growth and purification of recombinant human AR baculovirus was done as described (25–27). Labeled DHT (1–2 nM [^{3}H]DHT) (Sigma Chemical Co., St. Louis, MO) at 130 Ci/mmol was used, and varying concentrations of competing ligand ($0–10^{-5}$ M). Dissociation constant (K_d) values for the analogs were calculated by application of the Cheng-Prussof equation.

Mineralocorticoid receptor (MR), glucocorticoid receptor (GR), and progesterone receptor (PR) expression in the baculovirus system and binding assays were conducted similarly except that labeled ligands were 1–2 nM [^{3}H]aldosterone (TRK 434; Amersham, Arlington Heights, IL) with a specific activity of 60 Ci/mmol, 1–2 nM [^{3}H]dexamethsone at 84 Ci/mmol, and 2–3 nM [^{3}H]progesterone at 93 Ci/mmol (Sigma), respectively. Each binding assay point was done in duplicate, and each full experiment was repeated three to five times.

Reporter assays

DMEM and Eagle's MEM were obtained from BioWhittaker (Walkersville, MD). All fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). CV-1 cells were obtained from American Type Culture Collection (Rockville, MD).

CV1 or MDA-MB-453 cells derived from a human mammary carcinoma (ATCC HTB 131) were cultured in DMEM supplemented with 10% charcoal resin-stripped FBS. Cells were seeded 48 h before transfection in 96-well microtiter plates. Cells were transiently transfected by the calcium phosphate coprecipitation procedure (30) with a reporter plasmid, MMTV-Luc, containing the mouse mammary tumor virus (MMTV) long terminal repeat linked to luciferase, a β-galactosidase (β-Gal) expression plasmid, pCMV-β-Gal, coding for the constitutive expression of *Escherichia coli* β-Gal, and filler DNA (pGEM) in the presence (CV1) or absence (MDA) of hGR expression in plasmid (RSVhGR).

IL-6 repression assay

The human osteoblast cell line Saos-2 was transfected with a human AR expression plasmid and a luciferase reporter plasmid consisting of the IL-6 promoter controlling luciferase expression. Saos-2 cells (3×10^6) were seeded in a T225 flask and cultured for 24 h in DMEM supplemented with 10% FBS. FuGene 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN), was used to transiently transfect the cells according to the manufacturer's specifications. Transient transfections were performed at a 3:1 (vol/wt) FuGene 6/DNA ratio containing 0.7 μg human AR expression plasmid and 1.4 μg luciferase reporter plasmid per flask of cells for 16 h. Cells were removed from the flask using trypsin and plated into 96-well plates (6×10^3 cells per well) in DMEM-charcoal absorbed (CA) FBS. Compounds were diluted in DMEM-CA FBS containing TNFα (10 ng/ml) and IL-1β (1 ng/ml) and the cell incubated for 16 h in the absence and presence of compound (from 10^{-11} to 10^{-6} M) and with DMEM-CA FBS alone. Medium was removed, and cells were lysed in Triton X-100 buffer.

Prostate-specific antigen (PSA) promoter assay in a prostate cancer cell line (22RV1)

Iscove's modified Dulbecco's medium (IMDM) was obtained from Hyclone. The 22RV1 cells were obtained from American Type Culture Collection. The 22RV1 cells are a human prostate carcinoma epithelial

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cell line derived from a xenograft that was serially propagated in mice after castration-induced regression and relapse of the parental, androgen-dependent CWR22 xenograft. Cells were cultured in IMDM supplemented with 10% FBS. Cells were seeded 24 h before transfection in 24-well plates. Cells were transiently transfected using FuGene 6 (Roche), according to the manufacturer's specifications. Transient transfections were performed at a 6/1 (vol/wt) FuGene 6/DNA ratio containing 25 ng PSA reporter and 25 ng of a β-Gal expression plasmid, pCMV-β-Gal, coding for the constitutive expression of *Escherichia coli* β-Gal, and 50 ng filler DNA (pGEM) per well for 18 h. Compounds were diluted in IMDM-2% charcoal-treated-FBS medium and cells treated for 24 h and lysed and luciferase activity was measured using a luminometer (Torcon).

Two-hybrid assays

Gal4 DNA-binding domain-peptide fusions were constructed by first synthesizing the following oligonucleotides (all shown 5′ to 3′): FxxLF forward, AATTCCCGTCCAGATCCAAGACCTACCGAGGAGCTTTCCAGAATCTGTTCCAGAGCGTGCGCGAAGTGATCT, and FxxLF reverse, CTAGAGATCACTTCGCGCACGCTCTGGAACAGATTCTGGAAAGCTCCTCGGTAGGTCTTGGATCTGGACGGG; and D11/F forward, AATTCCCGTCCAGAGTTGAGAGCGGGAGCAGCAGGTTTATGCAGCTTTTTATGGCGAACGATCTTCTTACCT, and D11/F reverse, CTAGAGGTAAGAAGATCGTTCGCCATAAAAAGCTGCATAAACCTGCTGCTCCCGCTCTCAACTCTGGACGGG.

The forward and reverse pairs were annealed by heating to 95 C and cooling slowly and then cloned into pM (Clontech, Palo Alto, CA) and cut with *Eco*RI and *Xba*I. The resulting plasmids were sequenced to verify the in-frame fusion to the Gal4 DNA-binding domain of the FxxLF (the polypeptide SKTYRGAFQNLFQSVREVI) or the D11/F (VESGSSRFMQLFMANDLLT) (31). For experiments using Gal4-peptides, experiments were conducted as described (25–27). HepG2 liver cells lacking AR were transfected with reporter Gal4D5LUC3, VP16-hAR, Gal4-peptide construct, Rous sarcoma virus (RSV)-β-Gal as a normalization control, and pGEM as carrier DNA. Transfections were carried out using FuGene 6 per the manufacturer's protocol (Roche). After 24 h treatment, cells were lysed and assayed for luciferase and β-Gal activity. All values shown are the mean of three wells and are representative of multiple experiments.

GR interacting protein 1 (GRIP1) coactivation assay

A total of 3×10^6 CV-1 cells (American Type Culture Collection) were transfected with FuGene 6 with 4 μg MMTV-Luc, 0.5 μg RSV-β-Gal, 1 ng RSV-driven hAR expression plasmid, and either 1 μg pGEM or pSG5.GRIP1 expression vector (kindly provided by M. Stallcup, University of Southern California). After 24 h, cells were trypsinized and replated in 96-well plates (30,000 cells per well) with compounds as indicated in Eagle's MEM supplemented with 0.5% charcoal-treated-FBS. Luciferase and β-Gal assays were performed after 24 h of treatment. All values shown are representative of four experiments done in triplicate.

Serum LH determination

Serum (200 μl) was incubated at room temperature for 2–3 d with 100 μl 1:100,000 diluted primary rabbit antibody (NIDDK antirat LH S-11 antibody), and 100 μl of [^{125}I]iodinated LH (Covance Laboratories, Inc., Vienna, VA) diluted to 200,000–300,000 cpm/ml was added and the incubation continued for an additional 24 h. Bound LH was separated from the free hormone by adding 50 μl of 4% normal rabbit antibody and 50 μl of 1:10 diluted goat antirabbit secondary antibody (Antibodies, Inc., Davis, CA) on beads followed by overnight incubation at 4 C and centrifugation. Pellets were counted in a 10-channel γ-counter. The assay has a minimum detectable LH amount of 0.001 ng/tube. The intra- and interassay variability is less than 10%.

Urine deoxypyridinoline (DPD) determination

Urine DPD and creatinine were assayed at SkeleTech, Inc. (Bothell, WA). Both DPD and creatinine were assayed according to the protocol of Metra Biosystems, Inc. (Mountain View, CA).

Data analysis

The data were analyzed for the effects of androgens on orchidectomized (ORDX) rats by ANOVA, followed by Fisher's protected least significant difference (PLSD) test. $P < 0.05$ was the level necessary to achieve statistical significance.

Compounds and formulations

LGD2226 was synthesized at Ligand Pharmaceuticals, Inc., and fluoxymesterone (9α-fluoro-11α-hydroxy-17α-methyltestosterone) was purchased from Spectrum (New Brunswick, NJ; catalog no. FL-134). Both LGD2226 and fluoxymesterone were formulated as suspensions in a vehicle containing 9.95% polyethylene glycol 400, 0.05% Tween 80, and 0.9% carboxymethyl cellulose in water. The concentrations of test compounds corresponded to doses of 1, 3, 10, 30, and 100 mg/kg per 4-ml vehicle. Calcein and tetracycline hydrochloride (Sigma) were used for bone labeling. Calcein was dissolved in 2% $NaHCO_3$ and tetracycline hydrochloride in sterile water. Both markers, which were used at a concentration of 50 mg/ml, were injected in a volume of 0.1 ml, corresponding to a dose of 5 mg/250 g rat into the perivascular region at the base of the tail.

Animals

All procedures involving animals were approved by Ligand's Institutional Animal Care and Use Committee.

Two-week study

For the 2-wk studies (see Fig. 3), adult (7-wk-old) Sprague Dawley rats were purchased from Harlan (Indianapolis, IN), orchidectomized, and treated using oral gavage immediately after surgery with either vehicle or LGD2226 (1, 3, 10, 30, and 100 mg/kg·d). Twenty-four hours after the last dose, the animals were euthanized by decapitation. The ventral prostate and levator ani muscle were collected, dissected free of adipose tissue, blotted dry, and weighed.

Six-week study

For the 6-wk long-term bone study (see Fig. 4), 7-wk-old male Sprague Dawley rats (~250 g body weight) were used. Animals were housed two to three per cage, fed standard rodent chow (Teklad, 8604) containing 1.46% calcium, 0.99% phosphorus, and 4.96% vitamin D_3 and maintained on a 12-h light, 12-h dark cycle (lights on at 0600 h). Animals were acclimatized for 1 wk before beginning experiments. The first bone-labeling marker, calcein (5 mg/rat·0.1 ml) was administered to the animals by perivascular injection at the base of the tail 3 d before ORDX. At the beginning of the study (d 1), rats were arbitrarily allocated to seven groups, with 10 animals per group. Groups were treated with compound by oral gavage (groups 1–3 were vehicle treated): 1) gonadally intact (baseline control group), 2) sham-operated, 3) ORDX, 4) ORDX treated with fluoxymesterone (100 mg/kg·d), and 5–7) ORDX treated with LGD2226 at 1, 3, and 10 mg/kg·d. On d 2, animals of the baseline control group (group 1) were killed by decapitation, tibiae and femora were isolated and tibiae transferred to 70% ethanol, and femora were stored at −80 C. On d 32 and 40 (10 and 2 d before being killed), rats received tetracycline (5 mg/rat·0.1ml) as a second and third bone-labeling time point. Urine was collected continuously over a 16-h period in metabolic cages and frozen at −80 C. Cleared urine supernatants were sent to SkeleTech for analysis of DPD and creatinine. At the end of the study (6 wk), rats were weighed and then killed by decapitation. Serum was processed and stored at −20 C for further analysis. Long bones, consisting of tibiae and femora of both hind limbs, were isolated and tibiae transferred to 70% ethanol. Femora were stored at −80 C. Tibiae were evaluated using histomorphometric analysis.

Histomorphometry

Histomorphometric analyses of hind limb tibiae were carried out as described (8, 32). Multiple parameters of cortical bone (tibial diaphysis) and cancellous bone (proximal tibia metaphysis) were measured using a digitizing morphometry system. The system consisted of an epifluorescence microscope, a color video camera, and a digitizing pad (Nu-

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monics 2206) coupled to a personal computer and the morphometry program OsteoMetrics (OsteoMetrics, Atlanta, GA).

Cortical bone measurements

Ground transverse sections were used for morphometric analysis of cortical bone. Cross-sections of 150-mm thickness were cut at the tibiofibular junction with a low-speed saw (Isomet) equipped with a diamond wafer blade. The sections were ground to a thickness of 15–20 μm on a roughened glass plate and were mounted in glycerin before microscopic examination under UV illumination to visualize calcein and tetracycline fluorochrome labeling. The following measurements were performed: 1) periosteal bone formation rate ($mm^2/d \times 10^{-3}$), calculated as the area outside of initial calcein label and inside the periosteal surface divided by the labeling period; 2) bone mineral apposition rate ($\mu m/d$), defined as the area outside of the initial calcein label and inside the periosteal surface divided by the product of the labeled surface length (see below) and the labeling period; and 3) periosteal mineralization rate, calculated as the area bounded by each pair of tetracycline labels and divided by the product of the length of the initial label and the labeling period. These measurements are described in greater detail by Baylink *et al.* (33).

Cancellous bone measurements

The tibiae were sectioned transversely at the mid-diaphysis with a low-speed diamond saw and decalcified in 5% formic acid in 10% formalin for several days. The bones were then bisected in the midsagittal plane using a scalpel and dehydrated in ascending grades of alcohol before infiltrating and embedding in glycol-methylmethacrylate, a mixture of methylmethacrylate and 2-hydroxyethylmethacrylate (12.5/1). The resulting blocks were sectioned at an indicated thickness of 5 μm on a Jung-Reichart microtome (Heidelberg, Germany). The sections were mounted on slides and subjected to morphometric analysis. Cancellous bone was examined in the secondary spongiosa of the proximal tibia metaphysis at a standard sampling site no less than 1 mm caudal to the epiphysial growth plate. Measured parameters of cancellous bone included 1) total bone area, defined as the total area of trabecular bone present expressed as a percentage of the total tissue area (bone and marrow area) in the sampling site, which was converted to a volume by multiplication by unit thickness (bone volume/trabecular volume); 2) cancellous bone perimeter, defined as the total trabecular surface length present in the sampling site; 3) single- and double-labeled perimeters; and 4) interlabeled widths.

The measured parameters were then used to calculate 1) percent cancellous bone volume (cancellous bone area/total tissue area $\times$ 100%) and 2) cancellous bone formation rate (double-labeled perimeters + single-labeled perimeters/2) $\times$ interlabeled widths/interval time/cancellous perimeters). All measurements and calculations were carried out according to the standard nomenclature (34).

Sixteen-week study

The 16-wk long-term bone study (see Fig. 4, G–I) was conducted similarly to the 6-wk study with the following exceptions. Nine-month-old male Sprague Dawley rats (~500 g body weight) were housed individually and acclimatized for 2 wk. On d 2, animals of the baseline control group (group 1) were weighed, anesthetized by CO_2, and then euthanized by exsanguination. Ten and 2 d before the termination of the 16-wk study, rats received tetracycline (5 mg/rat·0.1ml) as a second and third bone-labeling time point. At the conclusion of the 16-wk treatment period, bones, serum, and tissues were processed as described above; in addition, lumbar vertebrae (L1–L2 and L3–L5) were excised and processed for further analysis. Tibiae and L1–L2 lumbar vertebrae were transferred into 70% alcohol, whereas L3–L5 lumbar vertebrae and femora were wrapped in saline-soaked gauze and stored in zipped plastic bags at −80 C. Frozen L3–L5 vertebrae and femora were sent on dry ice to SkeleTech for determination of bone mineral density and mechanical properties.

Mechanical testing

Using a material testing system (model 5501R; Instron Corp., Canton, MA), four types of mechanical testing were performed in the femur and the lumbar vertebral body. The load and extension curve was collected by the accompanied software (Merlin II; Instron Corp.). All tests were conducted using a 5-kN load cell at a constant loading rate of 6 mm/min.

Compression test of the fourth lumbar vertebral body

A compression test was used to determine the mechanical properties of the lumbar vertebral body, as described (35). The lumbar vertebral body test specimen was obtained by removing the two epiphyseal ends, posterior pedicle arch, and spinous process from the whole vertebra using a slow-speed saw. An electronic caliper was used to measure the average dorsal to ventral diameter (a), side-to-side diameter (b), and height (h). The maximal load (36), stiffness (S), and energy (W) were obtained from the load and extension curve. The following parameters were calculated from the measured values: cross-sectional area (CSA) = ($\pi \times$ a $\times$ b)/4; ultimate strength (σ) = F_u/CSA; elastic modulus (E) = S/(CSA/h); and toughness (T) = W/(CSA $\times$ h). Breaking strength is the force necessary to compress/crush the bone (newtons). Ultimate breaking strength is the crush force per area (newtons per square millimeter).

Sexual behavior study

LGD2226 and fluoxymesterone were tested for effects on sexual behavior parameters in ORDX hooded Long-Evans rats between 5 and 6 months of age. These animals have previously been shown to respond to androgens (37, 38). Ovariectomized female Sprague Dawley rats (Charles River, MA) were used as stimulus. Rats were housed under a reversed 12-h light, 12-h dark cycle. Before ORDX, male rats were first tested for consistent sex behavior with sexually receptive (stimulus) females. Males that reliably copulated over the course of several weeks of testing were admitted to the treatment phase of the study.

The males were kept sexually active during a 6-wk interval between screening tests and treatment by testing them in 20-min sex behavior tests with stimulus females at 7- to 14-d intervals. The males were castrated and implanted with Silastic capsules (30 mm long; inner diameter, 1.57 mm; outer diameter, 3.18 mm; Dow Corning, Midland, MI) containing 17β-estradiol (17β-E_2) (Sigma; lot 28H0818; 50 μg/ml in olive oil) under isoflurane anesthesia. Before implantation, the Silastic capsules were rinsed twice with 0.1 M PBS (pH 7.4) for removal of 17β-E_2 from the exterior of the capsule and then stirred overnight in PBS to facilitate the release of the 17β-E_2 from the interior of the capsule. The males were subdivided into three groups, and animals in each group were dosed daily by oral gavage (4 ml/kg) with one of the following treatments: vehicle (n = 15), , LGD2226 (100 mg/kg in vehicle) (n = 16), or fluoxymesterone (100 mg/kg in vehicle) (n = 16). Males were then tested weekly at 6- or 7-d intervals starting 1 wk after surgery for 8 wk with stimulus females (treatment phase of sexual behavior testing). The males were killed 4 d after the last test session.

Results

In vitro activity

LGD2226 was synthesized based on structure-activity relationships within a series of bicyclic compounds (6-alkylamino-1*H*-quinolin-2-ones) that exhibited AR agonist and antagonist properties. This compound (6-[bis-(2,2,2-trifluoroethyl)amino]-4-trifluoromethyl-1*H*-quinolin-2-one) binds AR with high affinity (K_d = 1 nM) (Fig. 1, A and B). LGD2226 shows no significant cross-reactivity with the intracellular receptors: GR, PR, MR, ER, retinoic acid receptor, farsenoid X receptor-α, liver X receptor-α, pregnane X receptor-α, peroxisome proliferator-activated receptor (PPAR)-α, PPAR-δ, and PPAR-γ in binding and cotransfection assays (data not shown). In cotransfection assays using CV-1 cells (African green monkey kidney cell line lacking AR) transfected with the androgen-responsive MMTV-Luc reporter and the AR, LGD2226 is a strong, potent agonist, similar to DHT and the synthetic steroid fluoxymesterone (Fig. 1C). DHT, T, fluoxymesterone, and LGD2226 all repressed transcription from the IL-6 promoter in HOS osteoblast bone

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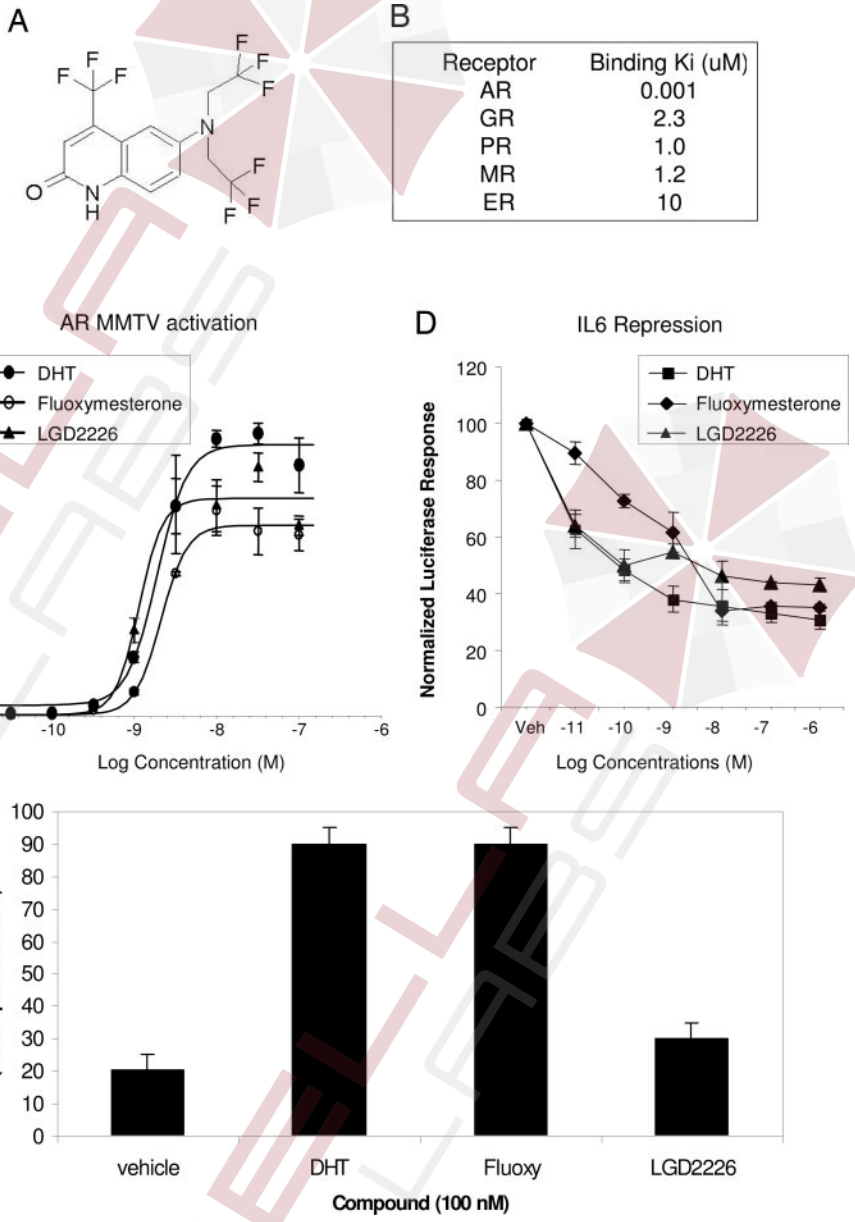


FIG. 1. LGD2226, a selective AR agonist. A and B, The structure (A) of LGD2226 (6-[bis-(2,2,2-trifluoroethyl) amino]-4-trifluoromethyl-1*H*-quinolin-2-one) is shown together with a table (B) showing its high-affinity binding for AR and low-affinity binding for other steroid receptors (GR, PR, and MR). C, Cotransfection experiments demonstrating full activation from the MMTV-Luc reporter in response to increasing concentrations of DHT (●), fluoxymesterone (○), and LGD2226 (▲). The SEM is shown for each dose. D, Strong IL-6 repression by DHT (■), fluoxymesterone (♦), and LGD2226 (▲) is shown. The SEM is also plotted for each dose. E, Cotransfection experiments demonstrating partial agonist activity of LGD2226 in a 22RV1 prostate cancer cell line measuring activation of the PSA promoter by the compound compared with steroidal androgens (DHT and fluoxymesterone) at the same dose. Vehicle is used as a background control, and SEM is shown on *top* of each column with *error bars*.

cells by about 80% (Fig. 1D). Thus, LGD2226 is capable of inducing both transcriptional activation and repression by AR. LGD2226 activity is antagonized by known selective antagonists of AR *in vitro* and *in vivo* (data not shown). Because of the negative effect of androgens on prostate size and prostate cancer, we also tested the activity of LGD2226 in a prostate cancer cell line (22RV1) for activation of the PSA promoter (Fig. 1E). We transfected a PSA/luciferase reporter into 22RV1 cells and measured activity in response to androgens and the SARM LGD2226. The results indicate that in contrast to the MMTV and bone cell assays, LGD2226 exhibits weak partial activity in this prostate cancer cell line.

Coactivator interactions

To understand the differential activity of LGD2226, we have used a series of peptide-based two-hybrid assays to analyze the effects of LGD2226 on the structure and function of the AR AF2 pocket on the surface of the LBD. These assays use the Gal4 DNA-binding domain tethered to small interacting peptides that are capable of binding to AR in the presence of ligand (31). The F peptide contains an FxxLF motif derived from the AR N terminus and represents one of the interaction sites between the AR N terminus and the AR LBD (23). The steroidal agonists T and fluoxymesterone efficiently induce the interaction of AR and the peptide; however, LGD2226 is much weaker, exhibiting greatly reduced efficacy and potency (Fig. 2A). In contrast, a peptide with a different sequence (D11/F) (31) demonstrated equal efficacy between the steroids and LGD2226 (Fig. 2B). As expected, 2-hydroxyflutamide, an AR antagonist, was inactive in both assays. As shown in Fig. 2C, LGD2226 was fully capable of antagonizing the fluoxymesterone-induced AR LBD-F-peptide interaction, suggesting that indeed, the peptide was not able to bind to the AR LBD in the presence of LGD2226.

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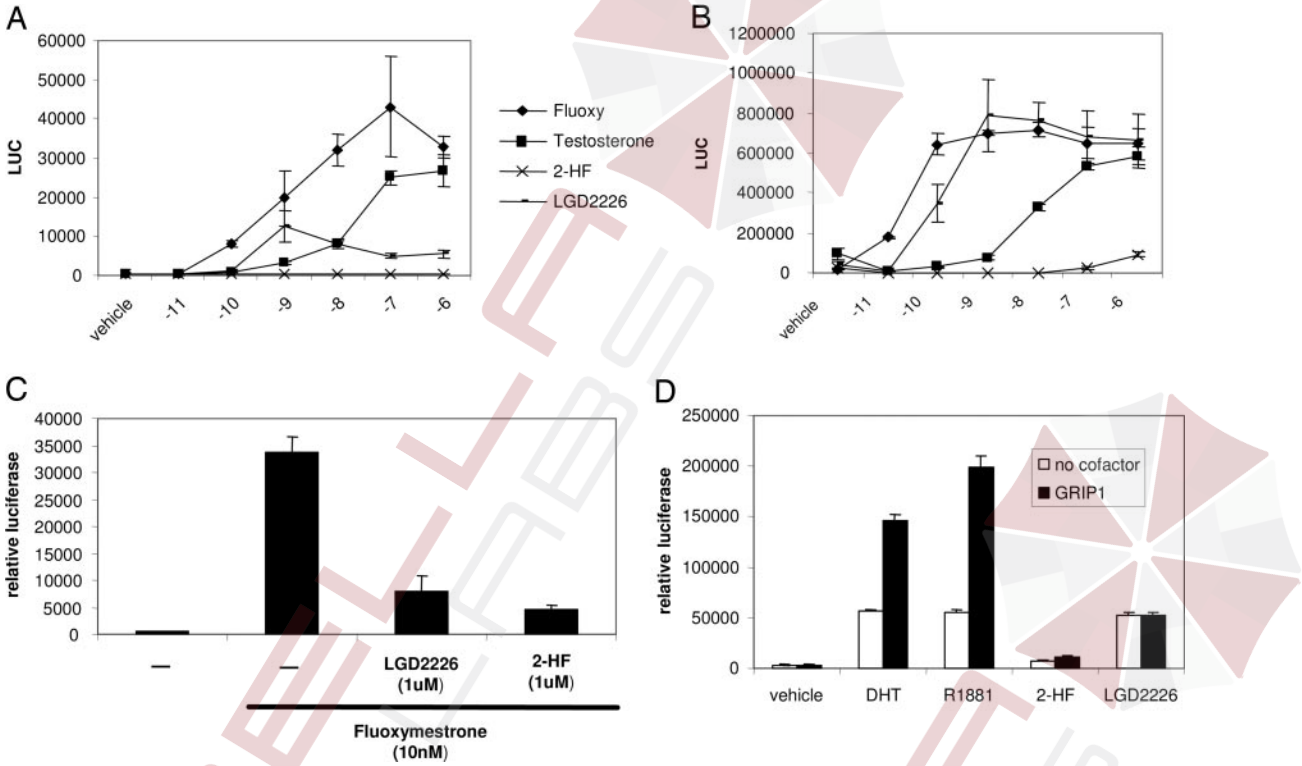


FIG. 2. LGD2226-specific alterations in AR-coactivator interactions. A, HepG2 cells were transfected with plasmids encoding a Gal4-luciferase reporter, Gal4-FxxLF (F-peptide), VP16-hAR, and RSV-β-Gal as a normalization control. After treatment with compounds or reference steroids for 24 h, cells were assayed for luciferase activity. B, Cells were transfected as in A, except that Gal4-D11/F replaced the F-peptide. C, Cells were transfected as in A. Luciferase and β-Gal values were determined after 24 h. Cell treatments were 1) vehicle alone, 2) 10 nM fluoxymesterone, 3) 10 nM fluoxymesterone and 1 μM LGD226, and 4) 10 nM fluoxymesterone and 1 μM 2-hydroxyflutamide. D, CV-1 cells were transfected with AR, MMTV-Luc reporter, RSV-β-Gal, and pSG5-HA.GRIP1. After 24 h, the cells were treated with the different compounds for 24 h at 10 μM. Assays were done as for A.

Similarly, flutamide could also antagonize the F peptide interaction with the AR LBD. The same results were obtained using the full-length N terminus (data not shown). In addition, in another type of coactivation assay, GRIP-1 (also known as steroid receptor coactivator 1) (39, 40) did not enhance LGD2226-mediated transcription but markedly enhanced steroid-mediated transcription (Fig. 2D). This differential interaction was not seen with other coactivators in similar assays (data not shown).

Two-week in vivo study

The strong *in vitro* efficacy profile in bone and muscle cell assays as well as the clearly distinct coactivator binding profile prompted us to test LGD2226 *in vivo*. A 2-wk study was conducted in young adult rats that were ORDX at 8 wk of age and dosed orally from 1–100 mg/kg·d with LGD2226 or T (Fig. 3). T was used for this study because it is routinely used clinically to treat hypogonadism. We have conducted similar experiments with other orally available steroids (fluoxymesterone) with similar results. In Fig. 3, both LGD2226 and T exhibited strong anabolic activity on levator ani muscle weight that was similar to sham at 3 mg/kg and greater than sham at higher doses (Fig. 3A). High levels of androgen typically results in increased growth of the prostate. However, in contrast to the results with the muscle endpoint, LGD2226 produced considerably less prostate growth at all

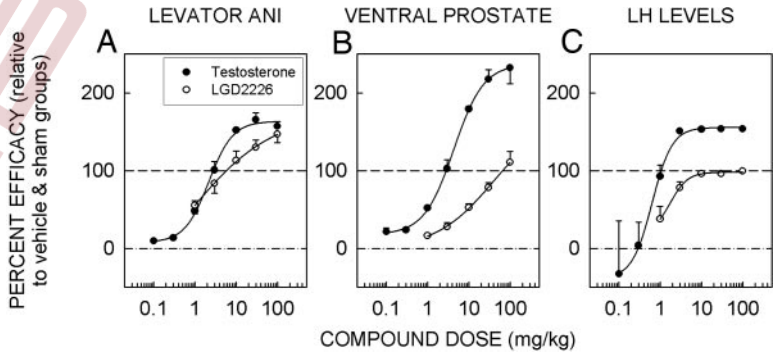


FIG. 3. Tissue-selective agonist activity of LGD2226. A, The muscle weights (levator ani) from eight rats treated for 2 wk with the indicated compound are averaged and plotted as a percentage of sham controls (percent efficacy) together with the SEM. T (●) and LGD2226 (○) were administered in doses up to 100 mg/kg. B, Ventral prostate weights from these same animals are plotted similarly together with the SEM for each dose. C, LH levels in serum are shown relative to sham. In this case, 100% represents sham-like levels of suppression of LH.

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doses when compared with T. Prostate weight reached levels comparable to the intact animals only at 100 mg/kg·d (Fig. 3B). T had almost doubled the prostate weight at 10 mg/kg·d. A large difference in potency and efficacy is also observed when the effect of LGD2226 is compared with the effect of T on LH suppression (Fig. 3C). Note that this graph is expressed as percent efficacy relative to sham; thus, high efficacy equals high (sham-like) LH suppression in this case. LGD2226 also suppressed seminal vesicle growth less efficiently than T (data not shown). These results suggest that LGD2226 is capable of strongly enhancing the development of muscle without excessively stimulating the prostate and the hypothalamus-pituitary-gonadal axis.

Six-week in vivo study

Positive impact on bone is crucial for the development of a SARM; however, long-term studies are necessary to observe androgenic effects on this endpoint. After treating young growing ORDX rats with oral doses of 1, 3, or 10 mg/kg·d LGD2226 for 6 wk, we detected similar activity on LH and similar selectivity of muscle *vs.* prostate size compared with animals treated with fluoxymesterone at 100 mg/kg (Fig. 4, A–C). The animals were also examined for the effects of the treatments on bone structure, strength, and bone biomarkers. DPD is a collagen cross-link, which is predominantly found in the extracellular matrix of bone or cartilage (41). The urinary excretion of DPD is widely recognized as a valuable marker of bone matrix degradation during androgen deficiency in male rats (42–44). DPD levels were decreased in ORDX rats to sham values by LGD2226, suggesting reduced bone resorption (Fig. 4D). To confirm this activity in the bone itself, we examined tibiae from these rats directly using histomorphometry. Mineral apposition rate (number of osteoblasts) and bone formation rate (total osteoblast activity) were calculated based on histomorphometric data from tibiae from these animals. Neither castra-

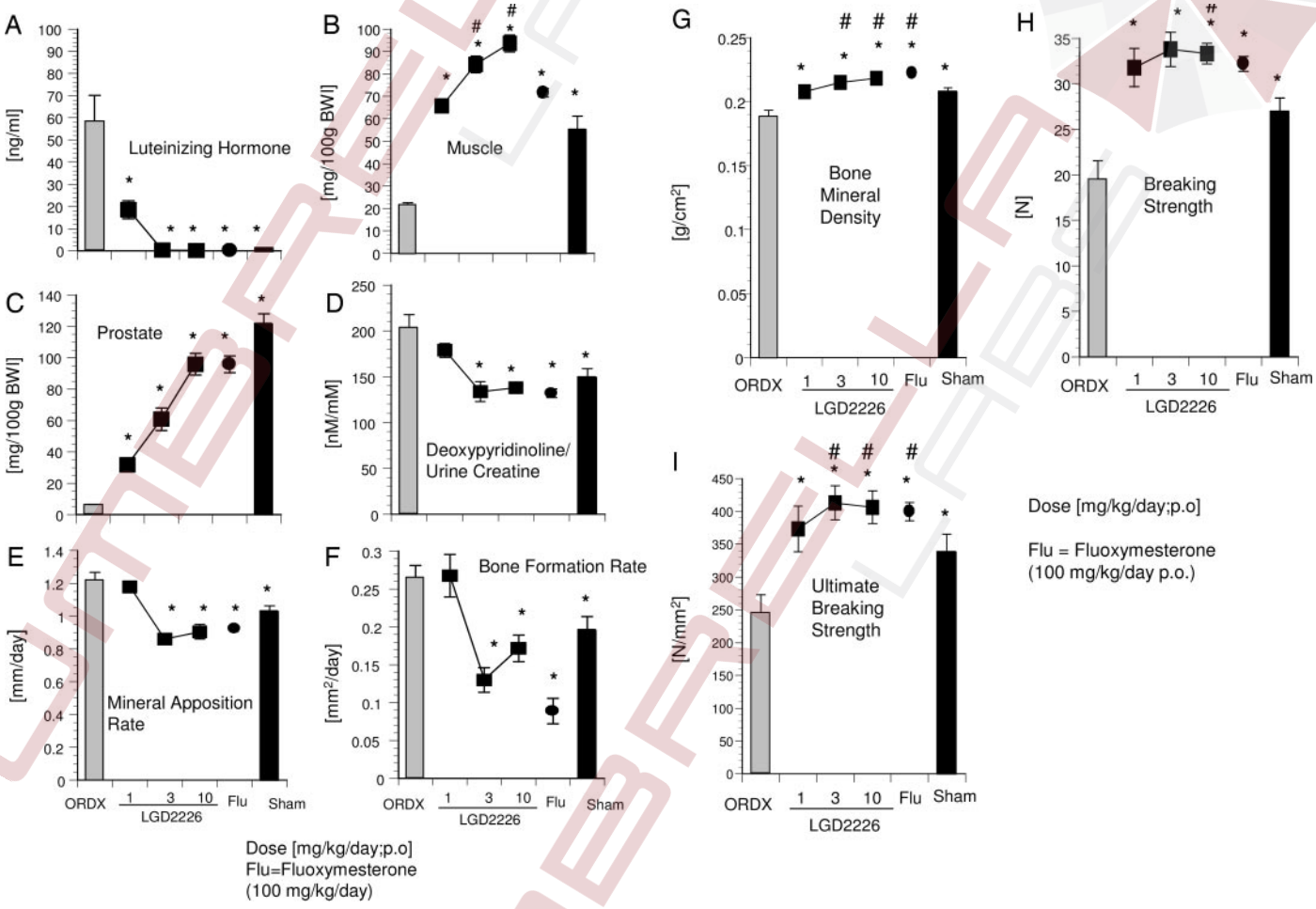


FIG. 4. A–F, LGD2226 is active in bone (6-wk studies). The results of a 6-wk dosing experiment with LGD2226 are plotted for a variety of endpoints including LH (A), muscle (B), prostate (C), DPD/urine creatinine (D), cancellous mineral apposition rate (E), and cancellous bone formation rate/surface area (F). For each panel, various treatment results are shown for castrated rat control (*gray vertical bar*), LGD2226 dose response 1, 3, 10 mg/kg·d (■), fluoxymesterone 100 mg/kg·d (●), and sham animals (*black vertical bar*). The data were analyzed for the effects of androgens on ORDX rats by ANOVA, followed by Fisher's PLSD test. *, $P < 0.05$ was the level necessary to achieve statistical significance from ORDX. G–I, LGD2226 is anabolic in bone (16-wk studies). The results of a 16-wk dosing experiment with LGD2226 are plotted for the bone-related endpoints: G, bone mineral density; H, breaking strength; and I, ultimate breaking strength. The data were analyzed for the effects of androgens on ORDX rats by ANOVA, followed by Fisher's PLSD test. *, $P < 0.05$ was the level necessary to achieve statistical significance from ORDX; #, $P < 0.05$ vs. sham.

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tion nor any dosing treatment of LGD2226 had significant effects on either overall bone density or static or dynamic measurements in cortical bone (data not shown). In cancellous bone, as expected from earlier work in bone (44, 45) and from the DPD data, bone formation and mineral apposition rate were reduced in fluoxymesterone and LGD2226-treated rats (Fig. 4, E and F). The reduced bone formation is due to the direct coupling between bone formation and bone resorption in cancellous bone and indicates a reduction in bone turnover (44, 45); increased bone turnover with a net increase in bone resorption is the mechanism for bone loss after ORDX. For both parameters, LGD2226 was fully efficacious at 3 mg/kg, effectively normalizing these parameters with minimal effect on prostate.

Sixteen-week in vivo study

Because we were unable to see significant effects of LGD2226 on overall bone density and bone formation in cortical bone in this 2-month study in immature rats, we conducted a longer study in skeletally mature ORDX rats dosed orally for 16 wk. Fluoxymesterone treatment at 100 mg/kg for this length of time enlarged the livers of the animals (data not shown). Oral steroidal androgens have been associated with liver toxicity in humans and rodents (46); however, liver enlargement has not been reported in humans. No liver enlargement was seen with LGD2226 at 10 mg/kg (data not shown).

At 16 wk after ORDX, animals exhibited substantial loss of bone density, and this loss was prevented by both the steroidal androgen fluoxymesterone and the nonsteroidal androgen LGD2226 (Fig. 4G). In addition, at the higher doses of 3 and 10 mg/kg, LGD2226 caused frank increases in bone mineral density above sham levels. The breaking strength (newtons) and the ultimate breaking strength (newtons per square millimeter) of LGD2226-treated tibae were above sham at all doses, and 1 mg/kg of LGD2226 was equivalent to 100 mg/kg of fluoxymesterone (Fig. 4, H and I).

Histomorphometric analysis of cortical bone from the 16-wk study revealed that both the periosteal mineral apposition rate and the periosteal bone formation rate were significantly enhanced by treatment with LGD2226. This anabolic activity of LGD2226 was able to prevent bone loss and maintain bone quality in ORDX rats by stimulating bone formation while simultaneously inhibiting bone resorption in cancellous bone.

Sex behavior model

In primates and in rats, nonaromatizable androgens act both on the brain and in the periphery to stimulate male sexual behavior. In rats, the central effects of nonaromatizable androgens do not occur unless the animals are treated concurrently with a behaviorally suboptimal priming dose of estrogen (37, 38). The central and peripheral contributions of androgens to the activation of male sexual behavior are often evaluated by subdividing the behavior into motivation and performance, respectively. Mounts, intromissions (insertion of the penis into the vagina), and ejaculations were scored. Mounting is considered to be the clearest measure of motivation, whereas intromitting is considered a measure of both motivation and performance. Ejaculation is associated with performance. Copulatory efficiency is an overall performance measure describing the animal's ability to achieve an intromission when mounting.

Reliably copulating, 6- to 9-month-old male rats were ORDX and implanted with silastic capsules containing low-dose estrogen and treated with vehicle, LGD2226, or fluoxymesterone. The animals were tested for sexual activity every 6–7 d for 8 wk. The number of tests on which a given rat 1) mounted, 2) intromitted, and 3) ejaculated were determined and expressed in graphical format. Vehicle-treated animals exhibited declines in both motivational and performance indicators during the course of the study. In contrast, oral dosing of these animals with either fluoxymesterone or LGD2226 significantly enhanced sexual function over vehicle alone. This included LGD2226-mediated increases in mounts, intromissions, and ejaculations (Fig. 5, A–C). The

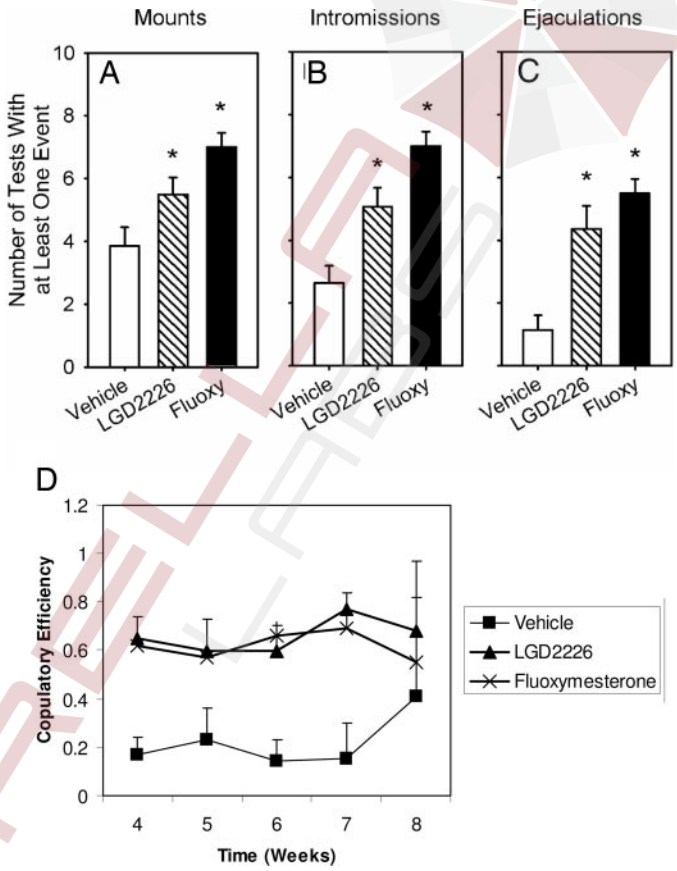


FIG. 5. Sex behavior study. These panels show the number of tests on which ORDX, E_2-treated rats treated with vehicle, LGD2226 100 mg/kg, or fluoxymesterone 100 mg/kg mounted (A), intromitted (B), and ejaculated (C) when tested with sexually receptive female rats. D, Copulatory efficiency for the same animals. Data are expressed as means ± SEM. *, $P < 0.05$ *vs.* vehicle-treated group [one-way ANOVA followed by Fisher's PLSD test (A and B); Kruskal-Wallis test followed by Mann-Whitney *U* test (C)]. At the time of castration, all males were implanted with 30-mm-long Silastic capsules containing 17β-E_2 (50 μg/ml) and began receiving daily oral dosing with fluoxymesterone (100 mg/kg; *black bars*), LGD2226 (100 mg/kg; *hatched bars*), or vehicle (*white bars*) for 8 wk. *, $P < 0.05$ *vs.* vehicle-treated group (two-way ANOVA with repeated measures followed by Fisher's PLSD test).

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SARM also exhibited full efficacy on copulatory efficiency (Fig. 5D) beginning at wk 4. LGD2226 and fluoxymesterone were not significantly different from each other. Thus, both androgens were capable of preventing the loss of motivation and performance that occurs after castration in this rodent model.

Discussion

Steroidal androgens are used in both men and woman for a large variety of diseases including osteoporosis, frailty, hypogonadism, sexual dysfunction, and others (47). Androgens are quite effective because they are anabolic on bone and muscle and act both centrally and peripherally on sexual function.

The search for novel androgens that have a desirable efficacy profile coupled with reductions in the common side effects associated with currently prescribed steroidal androgens is being actively pursued (12–15).

Several groups, starting from the known AR antagonists bicalutamide and flutamide, have developed a series of injectable compounds that exhibit less effect on prostate while maintaining effects on muscle or bone (12–20).

We have discovered a structurally distinct class of orally active nonsteroidal compounds capable of functioning as SARMs. We describe here a member of this class, LGD2226, that binds competitively to the AR LBD with an affinity in the nanomolar range. This compound is highly selective for the AR, with greater than 1000-fold less affinity for any other nuclear receptor (Fig. 1). In contrast to other described injectable molecules with less selectivity, LGD2226 is a full agonist in a number of *in vitro* efficacy assays and a partial agonist in a prostate cancer cell PSA assay (Figs. 1 and 2). Molecular analysis of the coactivator peptide interaction profile of LGD2226 revealed significant differences between this molecule and reference steroids. This differential profile was generated using a number of peptides capable of binding to the AR in a ligand-dependent fashion. We discovered that one peptide derived from the N terminus of AR itself, despite strong steroid-dependent interactions, was unable to bind efficiently to AR in the presence of LGD2226. This suggested that in much the same way that selective ER modulators alter the conformation of the AF2 coactivator pocket of the ER, LGD2226 alters the structure of this area of the AR in subtle but critical ways such that certain peptides interact with normal affinity, but the binding of others are significantly reduced. The N-terminal interaction has been postulated to play an important role in the regulation of gene expression by the AR in prostate (23). We tested LGD2226 in several versions of N-C interaction assays and found that the compound exhibited reduced activity. Thus, it is likely that when AR binds LGD2226, the N terminus is less tightly bound to the LBD than when the AR is bound to steroids. In addition, we have found that the coactivator GRIP 1 is less able to coactivate a receptor bound to LGD2226 than one bound to steroidal androgens.

This altered coactivator and N-terminal interaction profile likely is the result of alterations in the structure of the receptor LBD helix 12 region. X-ray crystallographic analysis of AR bound to LGD2226 is underway and will be published elsewhere. The functional consequences of this altered coactivator interaction profile were tested *in vivo* in a rat model of androgen deficiency. There is controversy on the relative contribution of AR *vs.* ER to bone structure and function, because of the conversion of T to estrogen. Neither fluoxymesterone nor LGD2226 is subject to aromatization to estrogen; thus our experiments address this issue directly by testing the effects of both nonaromatizable androgens on critical parameters of bone formation, resorption, and strength in ORDX rats as well as other endpoints.

We conducted a 2-wk study in ORDX rats with T or LGD2226 and examined several parameters. We demonstrated significant changes in LH. This critical steroidogenic hormone is under tight negative feedback control by T, and ORDX resulted in the expected rise in serum LH. This increase was entirely and potently prevented by the control steroid and by LGD2226 (Fig. 3A). Muscle weight from these treated animals increased dramatically in response to LGD2226 and the steroid, consistent with the role of androgens in maintaining and enhancing muscle growth. At the same dose that LGD2226 was powerfully anabolic to muscle, prostate growth was limited, not reaching eugonadal levels even at 10 mg/kg (Fig. 3, B and C). This separation in desired efficacy and side effect suggests that this molecule could have beneficial effects in patients in need of the anabolic effects of androgens while avoiding the stimulatory effects on prostate.

The effects on muscle notwithstanding, a critical component of any SARM must be its activity on bone. As expected, after 6 wk of dosing with vehicle, ORDX animals increased bone resorption either because of the decreased activity of the AR in response to endogenous T and DHT or because of the decreased activation by the ER in response to decreased estrogen from aromatized T. After dosing for 6 wk with the nonaromatizable, orally available androgen fluoxymesterone and with LGD2226, organ weights and hormone measurements changed significantly for LH (Fig. 4A), levator ani (Fig. 4B), and prostate (Fig. 4C), but the tissue selectivity noted in the 2-wk study remained. Note that fluoxymesterone is less potent on the organ endpoints than T. In addition, both compounds efficiently blocked the bone resorption marker DPD (Fig. 4D). Examination of the cancellous bone by histomorphometry corroborated the marker data. Castration increased both bone formation rate and mineral apposition rate, and androgen treatment decreased these endpoints (Fig. 4, E and F). This androgen-driven decrease in bone formation rate occurs because of the linkage between resorption and formation of new bone in the cancellous compartment (48). Androgens decrease resorption in cancellous bone on which bone formation depends, and so formation drops as well. Thus, by multiple measures, androgens that act solely through the AR are capable of inhibiting bone resorption in cancellous bone. This result indicates that it is possible for androgens to mediate similar effects as estrogens in the cancellous compartment, suggesting that therapy with androgens would at least mimic the antiresorptive activity of estrogens and bisphosphonates. In contrast, in rat cortical bone, there is very little bone resorption, providing an ideal environment to measure anabolic effects on bone formation di-

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rectly. It is known that estrogen actually inhibits bone formation in cortical bone (32).

To measure bone formation in the cortical compartment, we conducted an extended study of 16 wk of oral dosing with LGD2226 followed by structural and histomorphometric analysis of bones from treated and control animals. Bone mineral density was significantly affected by both castration and treatment with the androgenic compounds. Fluoxymesterone and LGD2226 fully maintained bone density, and LGD2226 at 3 and 10 mg/kg actually increased bone density above eugonadal levels (Fig. 4G). This change in density was accompanied by a change in breaking strength of the bone that was well above eugonadal for all doses of LGD2226 (Fig. 4, H and I). Thus, this SARM not only increased the amount of bone present but also significantly enhanced the strength of that bone. One explanation for this activity is that the compound may be enhancing the rate of bone formation in the periosteum. Using histomorphometry on cortical bone samples, we established that LGD2226 is capable of enhancing both the mineral apposition rate and the bone formation rate in castrated animals. Neither the steroid fluoxymesterone nor LGD2226 was able to fully maintain the rate of bone formation compared with sham, suggesting other testes components may also be important. This beneficial effect of androgens on cortical bone is not a consequence of aromatization to estrogen for two reasons: 1) estrogen in this model is actually inhibitory to cortical bone formation and mineral apposition rate (49) and 2) LGD2226 and fluoxymesterone are both active on building bone and neither one is aromatizable. Thus the benefits generated by these compounds are due to the activation of the AR likely acting directly on osteoblasts. Other well characterized antiresorptive agents such as bisphosphonates (50) and estrogen are incapable of this type of activity. Only true anabolic agents such as steroidal androgens or PTH (51) build bone strength and mass by enhancing the action of osteoblasts. These results demonstrate that LGD2226 exhibits a highly beneficial profile on bone, with both antiresorptive, and, significantly, anabolic bone effects.

There are initial reports of nongenomic effects of androgens on bone (36), which are hypothesized to play a role in mediating the effects of androgens. We know that LGD2226 strongly activates and represses classical androgen-responsive genes through genomic mechanisms, but we have no data on the activity of LGD2226 on potential nongenomic effects.

The role of androgens in libido and sexual function is well established for both males and females (4). Declines in androgen levels can affect multiple aspects of sexual activity in patients. Supplemental topical androgens have provided benefit in a number of clinical trials (52, 53), but their use is confounded by prostate-related side effects in men and androgenic side effects in women as well as skin irritation at the site of application in both sexes.

LGD2226 was tested for activity in a sexual behavior model in rats along with the nonaromatizable androgen fluoxymesterone as a positive control. In this model, the response of male rats to the presence of a sexually receptive female is examined. Castrated males were tested for their receptiveness to females by monitoring behavioral responses. Dosing castrated animals (on a low-priming-dose estrogen) with either fluoxymesterone or the SARM LGD2226 resulted in an improvement in measures of both performance and motivation, including mounts, intromissions, ejaculations, and copulatory efficiency compared with vehicle (Fig. 5).

Thus, LGD2226 is a highly active androgen, capable of anabolic activity on both bone and muscle. In addition, LGD2226 exhibits a safer profile on prostate growth and is able to enhance sexual function and motivation. This beneficial profile is likely the result of alterations in the conformation of the LBD of the AR when it binds to LGD2226. This compound is an orally available SARM, the prototype of a series of molecules that may provide patients with additional bone, muscle, and quality of life enhancement with fewer of the side effects produced by current therapies.

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