

Bone Anabolic Effects of S-40503, a Novel Nonsteroidal Selective Androgen Receptor Modulator (SARM), in Rat Models of Osteoporosis

Keigo HANADA,* Kazuyuki FURUYA, Noriko YAMAMOTO, Hiroaki NEJISHIMA, Kiyonoshin ICHIKAWA, Tsutomu NAKAMURA, Motonori MIYAKAWA, Seiji AMANO, Yuji SUMITA, and Nao OGURO

Central Research Laboratories, Kaken Pharmaceutical Co., Ltd.; 14 Shinomiya, Minamikawara-cho, Yamashina-ku, Kyoto 607–8042, Japan. Received July 29, 2003; accepted August 24, 2003

A novel nonsteroidal androgen receptor (AR) binder, S-40503, was successfully generated in order to develop selective androgen receptor modulators (SARMs). We evaluated the binding specificity for nuclear receptors (NRs) and osteoanabolic activities of S-40503 in comparison with a natural nonaromatizable steroid, 5 α -dihydrotestosterone (DHT). The compound preferentially bound to AR with nanomolar affinity among NRs. When S-40503 was administered into orchietomized (ORX) rats for 4 weeks, bone mineral density (BMD) of femur and muscle weight of levator ani were increased as markedly as DHT, but prostate weight was not elevated over the normal at any doses tested. In contrast, DHT administration caused about 1.5-fold increase in prostate weight. The reduced virilizing activity was clearly evident from the result that 4-week treatment of normal rats with S-40503 showed no enlargement of prostate. To confirm the bone anabolic effect, S-40503 was given to ovariectomized (OVX) rats for 2 months. The compound significantly increased the BMD and biomechanical strength of femoral cortical bone, whereas estrogen, anti-bone resorptive hormone, did not. The increase in periosteal mineral apposition rate (MAR) of the femur revealed direct bone formation activity of S-40503. It was unlikely that the osteoanabolic effect of the compound was attribute to the enhancement of muscle mass, because immobilized ORX rats treated with S-40503 showed a marked increase in BMD of tibial cortical bone without any actions on the surrounding muscle tissue. Collectively, our novel compound served as a prototype for SARMs, which had unique tissue selectivity with high potency for bone formation and lower impact upon sex accessory tissues.

Key words S-40503; selective androgen receptor modulator (SARM); bone anabolic; reduced virilizing activity; rat osteoporosis models

Androgens are well known for their many functions in promoting sexual differentiation and the induction of the male phenotype. In the male, the two endogenous androgens most active in promoting these effects are testosterone and nonaromatizable 5 α -dihydrotestosterone (DHT). They also play important roles in the regulation of bone metabolism. The direct effects of androgen on bone cells is suggested by the presence of androgen receptors (AR) on several human and rat established osteoblast cell lines as well as normal human osteoblast cells (HOB).¹⁾ Androgens increase rates of cellular proliferation and differentiation of osteoblasts, increase TGF- β levels, increase production of matrix proteins and inhibit osteoclast function.^{2,3)} A role of androgens in skeletal regulation is substantiated by numerous studies in human and rodents, demonstrating that chemical or surgical castration, as well as untreated hypogonadism in men and androgen deficiency in women with hypopituitarism, lead to accelerated bone loss.^{2,4–7)} Importantly, the deleterious effects of these conditions on bone can be reversed by treatment with androgens.

One apparently unique effect of androgens is to increase periosteal bone formation in cortical bone, while estrogens depress it.⁸⁾ This reflects a major gender difference in bone size, which is one of important factors determining the bone strength. A number of studies provide the proof of principle that androgens are osteoanabolic in rodents, women and men. Nonaromatizable DHT increased cortical bone volume and periosteal bone formation rates when administered to ovariectomized rats.⁹⁾ The synthetic anabolic steroids, such as nandrolone decanoate or stanozolol, have been shown to increase bone mass in postmenopausal women, possibly *via*

stimulation of bone formation.^{10,11)} Beneficial anabolic effects of androgens on bone in postmenopausal osteoporosis are well-documented in recent studies using combined testosterone and estrogen administration.^{12,13)} On the other hand, bone resorption inhibitors such as estrogens, bisphosphonates, selective estrogen receptor modulators (SERMs) and calcitonin, which are still first line of treatment/prevention of osteoporosis, are not sufficient to restore bone mass for patients who have already lost a significant amount of bone. In addition, bone turnover rate differs from site to site; higher in the cancellous bone of vertebrae than in the cortical bone of the long bones. Therefore, osteoanabolic agents, which increase cortical/periosteal bone formation and bone mass of long bones, would address unmet need in the treatment of osteoporosis especially for patients with high risk of fractures. The osteoanabolic agents also complement the bone resorption inhibitors that target the cancellous bones, leading to a biomechanically favorable bone structure.¹⁴⁾

Despite the beneficial effects of androgens in therapies for osteoporosis, hypogonadism and other androgen deficient diseases, clinical use of them has been limited because of the undesirable virilizing (acne, hirsutism, prostate enlargement and *etc.*) and metabolic actions of androgens.^{15,16)} Current side effect profile of androgen therapies provides a strong rationale for developing tissue-selective androgen agonists for bone. Hence, we developed novel nonsteroidal compound libraries based on the structural information of antiandrogens, bicalutamide and hydroxyflutamide, and steroidal androgens. Among them, we discovered tetrahydroquinolin (THQ) derivatives, which bound to AR as strongly as bicalutamide.¹⁷⁾ Other laboratories have also reported the identifi-

* To whom correspondence should be addressed. e-mail: furuya_kazuyuki@kaken.co.jp

cation of nonsteroidal compounds that possess androgen activity.^{18,19} The discovery of these nonsteroidal androgens offers an opportunity for development of selective androgen receptor modulators (SARMs) for a new treatment of osteoporosis, which can stimulate bone formation with diminished virilizing activities.

During the course of screening THQ compounds, we discovered a SARM candidate, designated "S-40503" that bound to AR with nanomolar affinity. To see whether S-40503 selectively binds to AR among the nuclear receptors and whether this compound shows the osteoanabolic effects, we performed competitive receptor binding assays and various animal studies in comparison with a natural AR ligand, DHT. We report here that S-40503 specifically binds to AR and exhibits anabolic activities on bone and muscle in rat models of osteoporosis.

MATERIALS AND METHODS

Animals Sprague-Dawley (SD) rats and Japan White (JW) rabbits were obtained from Japan SLC (Hamamatsu, Japan) and used for the experiments after 1 week of breeding in our animal facility. Animals were housed in a room that was maintained 12 h light–dark cycle under controlled temperature ($23 \pm 2^\circ\text{C}$) and humidity conditions ($55 \pm 15\%$) and allowed free access to commercial standard rodent chow containing 1.15% calcium (Ca) and 0.88% phosphate (P) (MF, Oriental Yeast Co., Tokyo, Japan) and drinking water. Treatment of each animal was conducted in accordance with the guidelines of the Experimental Animal Care and Use Committee of Kaken Pharmaceuticals, Co., Ltd. (Kyoto, Japan).

Chemicals 2-(4-Dimethylamino-6-nitro-1,2,3,4-tetrahydroquinolin-2-yl)-2-methylpropan-1-ol (S-40503) was synthesized by Kaken Pharmaceuticals, Co., Ltd. Bicalutamide was extracted from casodex tablets (AstraZeneca, Osaka, Japan). 5α -Dihydrotestosterone (DHT) was purchased from Wako Pure Chemical (Osaka, Japan). Progesterone (Prog) and 17β -estradiol (E2) were obtained from Nacalai Tesque (Kyoto, Japan). Dexamethasone (Dex) and aldosterone (Aldo) were from Sigma (St. Louis, Mo, U.S.A.) and Acros Organics (Geel, Belgium), respectively. [^3H]testosterone (T), [^3H]E2 and [^3H]Aldo were supplied from PerkinElmer Life Sciences (Boston, MA, U.S.A.). [^3H]Dex and [^3H]Prog were from NEN Life Science Products (Boston, MA, U.S.A.). All steroids and compounds were dissolved in dimethyl sulfoxide (DMSO, Nacalai Tesque) and then diluted with buffer for nuclear receptor binding studies or with olive oil (Nacalai Tesque) for animal studies (5% DMSO).

Competitive Nuclear Receptor (NR) Binding Studies.

AR Assay AR binding analysis was performed as described previously.²⁰ Briefly, crude AR protein fraction (ARF) was prepared from prostates of 11-week-old male SD rats, which were orchietomized (ORX) 3 d before. The tissues were finely cut into ice-cooled ETM buffer (10 mM Tris, 1 mM EDTA, 5 mM DTT, and 10 mM sodium molybdate, pH 7.4), homogenized and ultracentrifuged by Hitachi Micro Ultracentrifuge himac CS 120EX (Tokyo, Japan) at 100000 *g* for 60 min at 4°C . The supernatant was collected and used as the ARF. A 200 μg of ARF (10.0 mg/ml), test compounds, 2.5 nM [^3H]T, and ETM buffer were mixed in a total volume of 100 μl and incubated for 2 h at 4°C . A 300 μl of ETM

buffer solution containing 0.05% dextran T70 (Pharmacia, Uppsala, Sweden) and 1% activated charcoal (Nacalai Tesque) was then added and the mixture was further incubated for 15 min on ice to remove free [^3H]T. After centrifugation at 2500 rpm for 5 min at 4°C , 275 μl of supernatant was harvested into a liquid scintillation vial and 2 ml of Clear-sol I (Nacalai Tesque) was added. The mixture was stirred and allowed to stand. The amount of radioactivity was determined with Beckman Scintillation Counter LS6000IC (Fullerton, CA, U.S.A.).

Progesterone and Estrogen Receptor (PR and ER) Assays

The protocol was based on the method described by McGuire and Dedella.²¹ Crude PR and ER protein fractions (PRF and ERF) were prepared from uteruses of young JW rabbits (1.6 kg of body weight). The tissues were finely cut into ice-cold ETS buffer (10 mM Tris, 1 mM EDTA, and 250 mM sucrose, pH 8.0), homogenized and ultracentrifuged at 12000 *g* for 15 min at 4°C . The supernatant was further ultracentrifuged at 273000 *g* for 60 min at 4°C . Thereafter, the supernatant was collected and used as the PRF and ERF. The binding assays were similarly run using 200 μg of PRF (5.0 mg/ml) and [^3H]Prog (5 nM) or 50 μg of ERF (4.3 mg/ml) and [^3H]E2 (5 nM) as described in AR assay.

Glucocorticoid and Mineralocorticoid Receptor (GR and MR) Assays

The binding assays were performed as reported before.^{22,23} Crude GR and MR protein fractions (GRF and MRF) were prepared from livers and kidneys of 8-week-old male SD rats, respectively, which were adrenalectomized 4 d before. The livers and kidneys were finely cut into ice-cooled ETG (20 mM Tris, 1 mM disodium-EDTA, 10 mM DTT, and 10% (w/v) glycerol, pH 7.8) and TEGDM buffer (30 mM Tris, 1 mM EDTA, 1 mM DTT, 10 mM sodium molybdate, and 10% (w/v) glycerol, pH 7.4), respectively and homogenized. The GRF was obtained from the supernatant by twice ultracentrifugation of liver homogenate at 127000 *g* for 70 min followed by at 75600 *g* for 30 min at 4°C . The MRF was done from the supernatant by ultracentrifugation of kidney homogenate at 105000 *g* for 60 min at 4°C . The binding assays were similarly run using 300 μg of GRF (59.5 mg/ml) and [^3H]Dex (10 nM) or 500 μg of MRF (26.2 mg/ml) and [^3H]Aldo (10 nM) as described in AR assay.

Data Analysis Specific binding was defined as the difference between binding of radioligands in the presence (nonspecific binding) and in the absence (total binding) of an excess of unlabelled ligands. Steroid standards were included in each assay. Data were plotted as % inhibition of the change in the radioactivity between specific binding of radioligand and that of test compound at various concentrations. Half-maximal inhibition concentration (IC_{50}) values were determined from a nonlinear regression analysis (probit analysis) of competitive binding curves by the SPSS software program, Regression Models 9.0J (Chicago, IL, U.S.A.). The IC_{50} value is defined as the concentration of competing ligand and required to reduce specific binding by 50%. The inhibition constant (K_i) of test compounds was determined using the Cheng–Prusoff equation,²⁴ $K_i = \text{IC}_{50} / (1 + [\text{L}] / [K_L])$, where L is the concentration of the radioligand, $[K_L]$ is the dissociation constant of the radioligand determined from scatchard plot analysis.

Animal Studies In the following experiments, 11-week-old male (320 to 340 g) and female (200 to 230 g of body

weight) SD rats were used. The animals were randomized by weight into experimental groups. Test compounds and vehicle (95% olive oil and 5% DMSO) were injected subcutaneously (s.c.) once daily in a volume of 0.1 ml/100 g of body weight (BW) in all studies. At the end of the experiment, the rats were euthanized by exsanguinations from the abdominal aorta and served for analysis.

Normal Rat Study The male rats were randomly divided into three groups of eight animals each: vehicle, DHT (10 mg/kg) and S-40503 (30 mg/kg). After 4 weeks of treatments, ventral prostates and levator ani were excised and immediately weighed.

ORX Rat Study The male rats were either bilaterally ORX or sham-operated under ether anesthesia. Upon recovery from the anesthesia, animals were sorted into experimental groups of eight animals each: sham, ORX plus vehicle, ORX plus DHT (0.01 to 10 mg/kg), ORX plus S-40503 (1 to 30 mg/kg). Compound administration began 1 d postsurgery. After 4 weeks of treatments, ventral prostates and levator ani were excised and immediately weighed. Right femurs were dissected free of soft tissue, fixed in 10% neutral formalin and then served for measurement of bone mineral density (BMD).

Immobilized ORX Rat Study The male rats were sciatic neurectomized (NX) or sham-operated under ether anesthesia. Right hindlimbs were denervated by removing a 0.5 mm segment of sciatic nerve at the posterior area of the hip joint.²⁵⁾ The NX rats were further ORX or sham-operated under the anesthesia. The rats were therefore divided into sham (NX- and ORX-sham), NX (ORX-sham) and four NX+ORX groups, which were treated with vehicle, DHT (10 mg/kg), E2 (20 µg/kg) or S-40503 (30 mg/kg). The sham and NX rats were also given the vehicle. Each group consisted of nine animals. Compound administration began 1 d postsurgery. After 4 weeks of treatments, ventral prostates, levator ani and gastrocnemius muscle were excised and immediately weighed. Bilateral tibiae were dissected free of soft tissue, fixed in 10% neutral formalin and then served for measurement of BMD.

Ovariectomized (OVX) Rat Study The female rats were bilaterally OVX or sham-operated under ether anesthesia. After surgery, all animals were maintained without treatment for 4 weeks to permit bone loss to occur before the beginning of bone anabolic therapy as in established osteoporosis. Thereafter, the OVX animals were regrouped as vehicle, DHT (10 mg/kg), E2 (20 µg/kg) or S-40503 (30 mg/kg) before the start of 8-week treatments. The sham was given the vehicle. During the treatment, the standard chow was replaced with modified AIN diet containing 0.4% calcium and 0.5% phosphate (Oriental Yeast Co.) to proceed a bone loss more progressively. Each group consisted of eight animals. Twenty mg/kg of calcein (Wako Pure Chemical) was injected s.c. on the thirteenth and sixteenth days before euthanasia. After 8 weeks of treatments, bilateral femurs were dissected free of soft tissue, fixed in 10% neutral formalin and then served for measurement of BMD, histomorphometry and biomechanical test. Uteruses were excised and immediately weighed.

Measurement of BMD The tibiae and femurs were divided into 20 equal regions from distal (region 1) to proximal (region 20) of them and the BMD of each region was measured by dual energy X-ray absorptiometry utilizing bone

mineral analyzer (DCS-600, Aloka, Tokyo, Japan).

Biomechanical Analysis To examine the biomechanical strength of the right femurs of OVX rats, three-point bending was performed in the region of femoral midshaft using a load torsion tester (MZ-500S, Maruto, Tokyo, Japan). The maximum load (N), the breaking energy (N/mm) and the stiffness (N/mm) were interpreted and calculated from the load-deflection curve, which was recorded continuously in the computerized monitor linked to the tester as described previously.²⁶⁾

Bone Histomorphometry The femurs of OVX rats with bone labeling were dehydrated with sequential ethanol (70 to 100%) and then embedded without decalcification in methylmethacrylate (Nacalai Tesque) after Villanueva bone staining. A transverse section of femoral midshaft was cut at a thickness 7–10 µm with Jung Rotary Microtome 2065 (Leica Instruments GmbH, Heidelberg, Germany). The measurements of cortical bone were performed with fluorescent microscope (AX-80 systems, Olympus, Tokyo, Japan) and CCD camera (HCC-3800, Olympus) connected to an image analyzing system using SimplePCI software (Compix Inc., Cranberry Township, PA, U.S.A.). Mineral apposition rate (MAR) at the periosteal surface was calculated by dividing the interlabel distance by the time between the calcein labels.

Statistical Analysis Statistical analysis was done using the SPSS Base 11.0J software program (Chicago, IL, U.S.A.). All data are presented as the mean and standard deviation (S.D.). The difference was evaluated by the Student's *t* test when comparing two groups, or by one-way analysis of variance followed by the Dunnett's test for multiple groups. *p* < 0.05 was considered to be statistically significant.

RESULTS

NR Binding Assays Competitive binding assays were performed to determine the affinity of S-40503 for AR as well as the other steroid receptors. S-40503 shares the same receptor binding profile as nonsteroidal antiandrogen, bicalutamide in that they have a high affinity for AR with extremely low affinity for PR, ER GR and MR, although DHT, a nonaromatizable AR ligand used for positive control, showed an approximately 30-fold higher affinity for AR than S-40503 and bicalutamide (Table 1). These data indicated that S-40503 was a strong AR binder with high selectivity.

ORX Rat Study The virilizing and anabolic activities of S-40503 were compared with those of DHT in ORX rats. Prostate was weighed as an index of virilizing activity, while

Table 1. Nuclear Receptor Binding Affinity of S-40503

Compounds	<i>K_i</i> (nM)				
	AR	PR	ER	GR	MR
DHT	0.5±0.2	280	38000	2700	2300
S-40503	14.9±7.2	2100	>390000	6700	13000
Bicalutamide	14.4±6.1	3500	>390000	44000	360000

Each binding assay was conducted in duplicate and *K_i* values were calculated as described under Methods. The values in AR binding assay are mean±S.D. of five experiments. *K_i* value of a natural steroid ligand in each assay was as follows: progesterone, 3.8; 17β-estradiol, 0.5; dexamethasone, 3.0; aldosterone, 44 nM. AR, androgen receptor; PR, progesterone receptor; ER, estrogen receptor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; DHT, 5α-dihydrotestosterone.

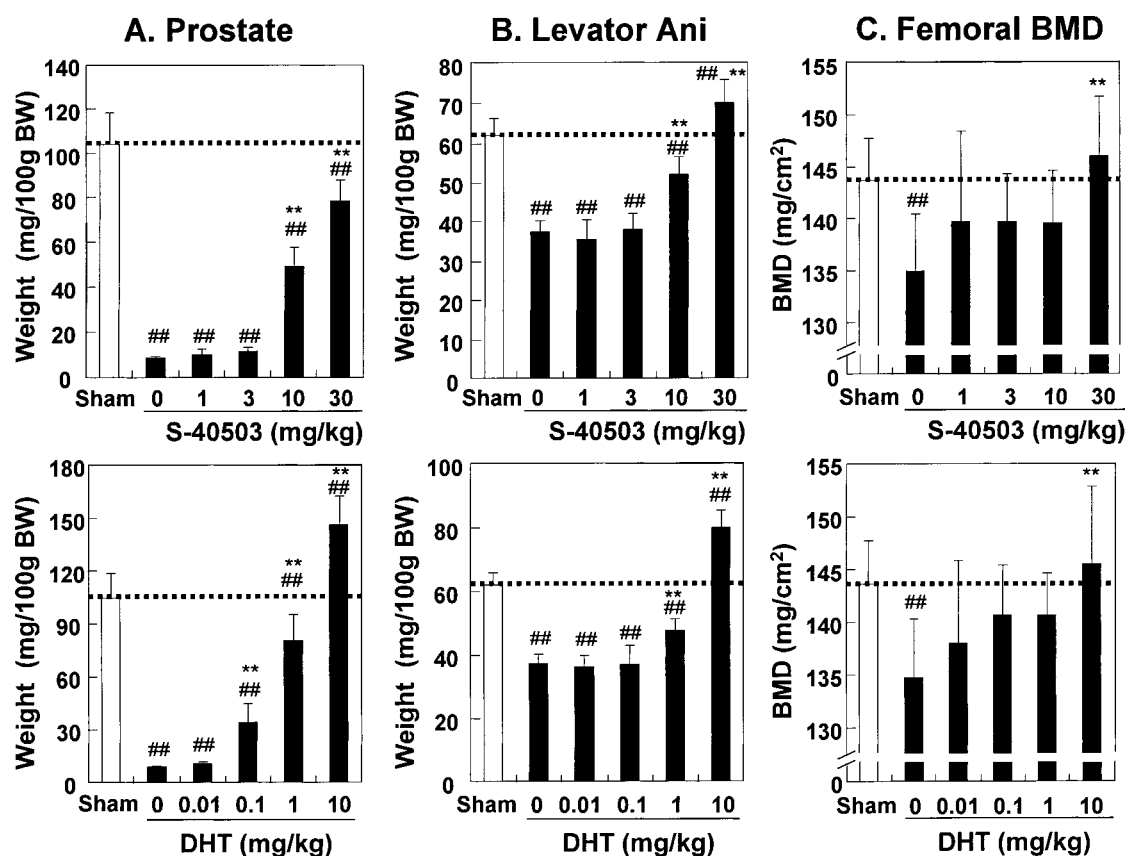


Fig. 1. Effects of S-40503 and 5 α -Dihydrotestosterone (DHT) Treatments on Weights of Prostate (A) and Levator Ani Muscle (B), and Femoral BMD (C) in Orchiectomized (ORX) Rats

The assay procedure was described in experimental methods. Open and closed columns show sham and ORX groups, respectively. Each column and vertical bar represents the mean \pm S.D. of 8 rats. # $p < 0.01$, sham vs. ORX groups; ** $p < 0.01$, vehicle (S-40503 or DHT 0 mg) vs. treatment groups in ORX.

levator ani weight and femoral BMD were measured as that of anabolic activity. Orchiectomy caused a significant decrease in BMD at mid- to proximal regions (regions 12 to 16) and analysis for bone anabolic activity was therefore done at these regions (data not shown). The prostate weight of S-40503-treated rats dose-dependently increased from a dose of 10 mg/kg (A in Fig. 1). At a dose of 30 mg/kg, the prostate weight showed about 75% of that of the sham and reached a plateau, because it was not changed even at 300 mg/kg (data not shown). As presented in B and C in Fig. 1, S-40503 showed anabolic activities on bone and muscle. The compound induced significant increases in femoral BMD and levator ani weight over the sham range at a dose of 30 mg/kg. In contrast, DHT showed no clear dissociation of bone and muscle anabolic activities from the virilizing one (Fig. 1). In the ORX rats treated with 10 mg of the steroid, femoral BMD was significantly increased, and levator ani and prostate were approximately 1.3- to 1.5-fold larger than those of the sham. Thus, S-40503 showed a quite different dose-response profile from that of DHT. Furthermore, the changes in prostate weights correlated well with those in seminal vesicle weights when treated with S-40503 or DHT (data not shown). The data indicated that S-40503 had potent anabolic activities on bone and muscle, but a lesser virilizing activity than a natural AR ligand, DHT.

Normal Rat Study To investigate the virilizing activity of S-40503 in the presence of endogenous androgens, the compound was given into normal rats and prostate stimula-

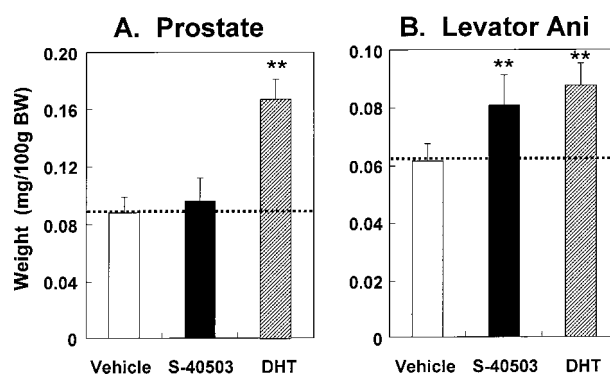


Fig. 2. Effects of S-40503 and 5 α -Dihydrotestosterone (DHT) Treatments on Weights of Prostate (A) and Levator Ani Muscle (B) in Normal Rats

The assay procedure was described in experimental methods. Each column and vertical bar represents the mean \pm S.D. of 8 rats. ** $p < 0.01$, vehicle vs. treatment groups.

tion was compared with DHT. As presented in Fig. 2, administration of DHT (10 mg/kg) significantly increased both prostate and levator ani weights, whereas that of S-40503 (30 mg/kg) did not produce any changes in the prostate in spite of a prominent increase in the levator ani weight. Thus, S-40503 was demonstrated to show a diminished virilizing activity under the physiologically normal as well as under the androgen-deficient conditions.

OVX Rat Study Bone anabolic effect of S-40503 was further examined in osteoporotic OVX rats. Success of estab-

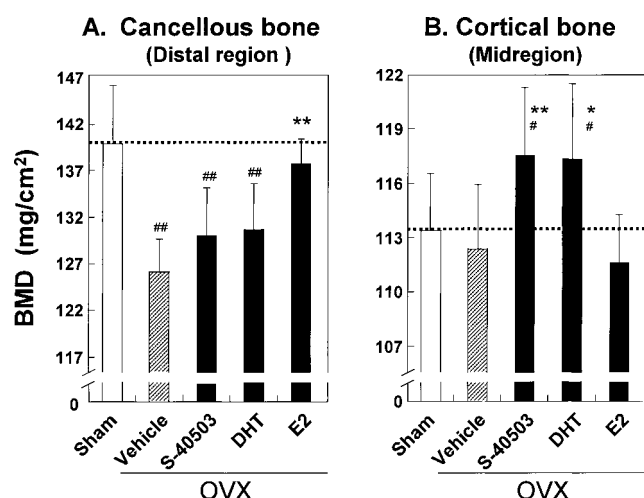


Fig. 3. Effects of S-40503, 5 α -Dihydrotestosterone (DHT) and 17 β -Estradiol (E2) Treatments on Bone Mineral Density (BMD) of Femoral Cancellous (A) and Cortical Bones (B) in Ovariectomized (OVX) Rats

The assay procedure was described in experimental methods. Each column and vertical bar represents the mean \pm S.D. of 8 rats. * p <0.05, ** p <0.01, sham vs. OVX groups; * p <0.05, ** p <0.01, vehicle vs. treatment groups in OVX.

lishment of OVX model was confirmed by the atrophy of uteruses and E2-induced enlargements of them (data not shown). Ovariectomy resulted in a decrease in BMD of femoral cancellous bone, especially at the distal region (regions 1 to 5) (A in Fig. 3), but did not affect the cortical bone at midregion (8 to 12) (B in Fig. 3). Therefore, replacement of E2, well-known anti-resorptive hormone, effectively prevented the cancellous bone loss in OVX rats (A in Fig. 3). On the other hand, the BMD of cortical bone was not also changed after E2 treatment (B in Fig. 3), indicating that metabolic turnover of formation and resorption was extremely low in this bone area. Efficacy of bone anabolic compound was focused on whether it increased the BMD at cortical bone area through a stimulation of bone formation activity. The administration of S-40503 induced a significant elevation in the BMD of femoral cortical bone as well as that of DHT (B in Fig. 3). In contrast, the BMD of cancellous bone was not significantly changed by both treatments (A in Fig. 3). The periosteal MAR at cortical bone area was markedly elevated by S-40503 and DHT treatments, not by E2, indicating that an increase in cortical BMD was ascribed to the enhancement of bone formation (Fig. 4). Table 2 represents the parameters in biomechanical test of femurs treated with S-40503, DHT and E2. As compared with vehicle, S-40503 significantly enhanced maximum load and breaking energy, and tended to increase stiffness. DHT also tended to increase all parameters, but their values were not significant against that of vehicle-treated OVX rats. The OVX study demonstrated that our small compound stimulated bone formation, which led to increase the mass and the biomechanical strength of the cortical bone.

Immobilized ORX Rat Study It was well known that androgens increased muscle mass and stimulated its strength.¹⁶⁾ In our ORX rat experiment, levator ani weight was markedly increased by DHT from a dose of 1 mg/kg (B in Fig. 1). In spite of direct effects of DHT and S-40503 on bone formation in OVX rats, a possibility that enhancement of muscle mass strength indirectly contributed to the increase

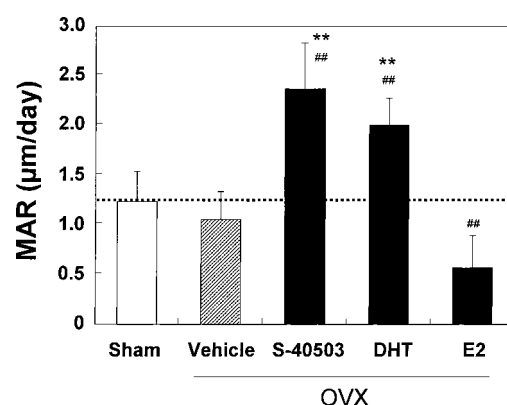


Fig. 4. Effects of S-40503, 5 α -Dihydrotestosterone (DHT) and 17 β -Estradiol (E2) Treatments on Periosteal Mineral Apposition Rate (MAR) of Femoral Cortical Bones in Ovariectomized (OVX) Rats

The assay procedure was described in experimental methods. Each column and vertical bar represents the mean \pm S.D. of 8 rats. ## p <0.01, sham vs. OVX groups; ** p <0.01, vehicle vs. treatment groups in OVX.

Table 2. Enhancement of Biomechanical Strength of Cortical Bones by S-40503

Treatments	Maximum load (N)	Stiffness (N/mm)	Breaking energy (N/mm)
Sham	29.4 \pm 3.8	78.6 \pm 9.4	9.0 \pm 1.5
OVX-Vehicle	31.2 \pm 5.4	79.0 \pm 8.5	10.1 \pm 2.5
S-40503	38.9 \pm 5.9*	92.3 \pm 12.2#	14.9 \pm 4.7*
DHT	33.7 \pm 5.5	84.4 \pm 17.7	11.8 \pm 2.9
E2	30.7 \pm 4.2	78.6 \pm 10.1	9.7 \pm 2.8

The biomechanical parameters of femoral midshaft (midregion) were evaluated as described under Methods. The data are expressed as mean \pm S.D. of eight animals. * p <0.05, # p <0.056 vs. OVX-Vehicle. OVX, ovariectomized; DHT, 5 α -dihydrotestosterone; E2, 17 β -estradiol.

of bone mass could not be excluded. Then, we investigated bone anabolic effects of the compound using immobilized ORX rats, which showed a severe atrophy of in hindlimb muscles such as a gastrocnemius surrounding tibia (sham vs. NX of A in Fig. 5). Indeed, neither S-40503 nor DHT treatments gave significant effects on the gastrocnemius weight in ORX+NX rats (A in Fig. 5). The sciatic neurectomy produced a decrease in the BMD of tibia both at cancellous and cortical bone areas and additional orchietomy promoted their bone loss as shown in Fig. 6. The ORX+NX rats showed a severe bone loss of whole tibia as compared with NX rats (data not shown). Unlike OVX model, E2 treatments restored the BMD of cancellous and cortical bones, although they still had a more stimulatory action on the former. S-40503 treatment resulted in a significant increase in BMD of the cortical bone as well as DHT, whereas they did not have any actions on cancellous bone (Fig. 6). These data strongly supported that the compound and DHT increased cortical bone mass through the bone formation activity and E2 prevented bone loss through anti-resorptive action. Again in this experiment, the rats treated with S-40503 showed an almost similar size of prostate to the sham and NX, and much larger size of levator ani than them (B and C in Fig. 5). In contrast, DHT treatment resulted in approximately 1.8-fold enlargement of prostate in comparison with the sham and NX rats, which was closely proportional to strong muscle anabolic activity. Thus, data suggested that our compound directly stim-

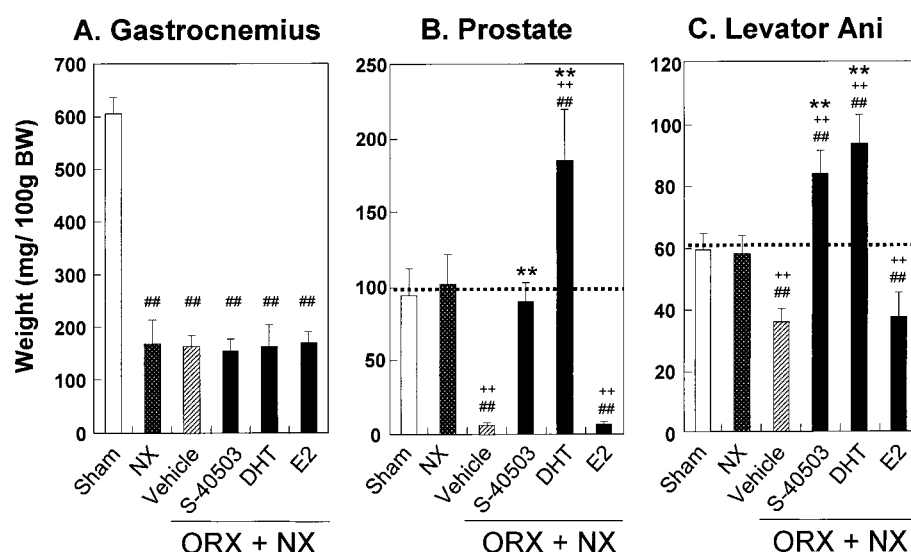


Fig. 5. Effects of S-40503, 5 α -Dihydrotestosterone (DHT) and 17 β -Estradiol (E2) Treatments on Weights of Gastrocnemius Muscle (A), Prostate (B) and Levator Ani Muscle (C) in Orchiectomized Plus Neurectomized (ORX+NX) Rats

The assay procedure was described in experimental methods. Each column and vertical bar represents the mean \pm S.D. of 9 rats. ## p <0.01, sham vs. NX (NX and ORX+NX) groups; ++ p <0.01, NX vs. ORX+NX groups; ** p <0.01, vehicle vs. treatment groups in ORX+NX.

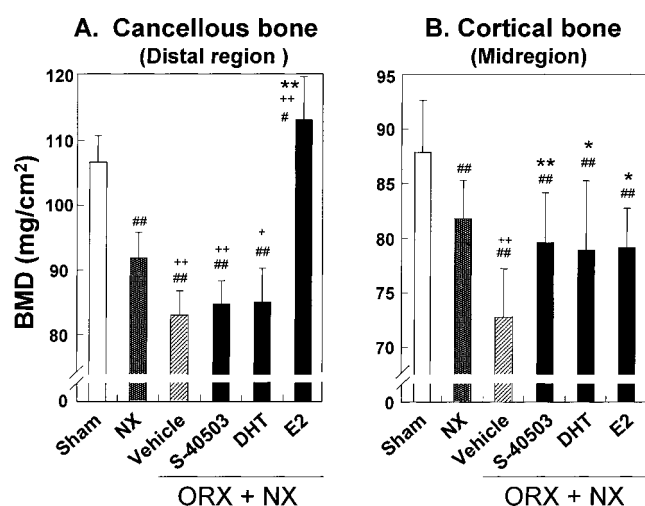


Fig. 6. Effects of S-40503, 5 α -Dihydrotestosterone (DHT) and 17 β -Estradiol (E2) Treatments on Bone Mineral Density (BMD) of Tibial Cancellous (A) and Cortical Bones (B) in Orchiectomized Plus Neurectomized (ORX+NX) Rats

The assay procedure was described in experimental methods. Each column and vertical bar represents the mean \pm S.D. of 9 rats. * p <0.05, ## p <0.01, sham vs. NX (NX and ORX+NX) groups; + p <0.05, ++ p <0.01, NX vs. ORX+NX groups; * p <0.05, ** p <0.01, vehicle vs. treatment groups in ORX+NX.

ulated bone formation and increased cortical bone mass.

DISCUSSION

A novel compound, S-40503, bound to AR as strongly as currently available AR antagonist, bicalutamide. One of the problems with current attempts to develop nonsteroidal AR modulators is cross-reactivity with other steroid receptors. Present study demonstrated that S-40503 had a high affinity for AR, with no measurable or a very feeble affinity for the PR, ER, GR and MR (Table 1).

S-40503 showed bone anabolic activity in a skeletally mature ORX male as an animal model of male hypogonadism

and in OVX female rats as that of postmenopausal osteoporosis. Bone formation activity of the compound was evident from the histomorphometric analysis of periosteal cortical bone in OVX rats. Enhancement of cortical bone strength was also detected by mechanical analysis, demonstrating beneficial effects of S-40503 on bone quality. The immobilized ORX study strongly suggested that S-40503 had a direct bone formation activity on the cortical bone. At present, it is unclear whether androgens have anti-resorptive activity. Recently, a few studies reported that androgens inhibited bone resorption activity *in vitro*.^{3,27,28} However, it is unlikely that S-40503 has anti-resorptive activity *in vivo* as well as DHT, because they did not exert significant effects on cancellous bones of OVX and immobilized ORX rats (Figs. 3, 6). Since metabolic turnover is the most active in a cancellous bone, it is susceptible to the anti-resorptive hormones and drugs, and immobilization treatment. Indeed, bone loss is prominent in the cancellous bone in OVX and NX rats. Supplement them with estrogen, anti-resorptive hormone, dramatically prevented a cancellous bone loss (Figs. 3, 6). Histomorphometric analysis is now on progress to clarify the anti-resorptive and bone formation activities of the compound.

A novel approach to the treatment of osteoporosis in men, and possibly women, is the SARMS that can stimulate formation of new bone with substantially diminished virilizing activity. The virilizing activity of S-40503 demonstrated by an increase in prostate weight was significantly reduced as compared with DHT in ORX and immobilized ORX rats (Figs. 1, 5). Histology of prostates did not show any abnormal changes such as hyperplasia and neoplastic growth of epithelium (data not shown). Moreover, administration of S-40503 into normal rats did not induce prostate enlargement as seen in treatment with DHT (Fig 2). These *in vivo* data suggest that S-40503 has less impact on sex accessory tissues than steroids while maintaining bone anabolic activity. The compound also showed anabolic activities on the muscle. Taken together, these results suggest that S-40503 served as a proto-

type for SARMS, which had unique tissue selectivity and may be useful therapeutics for both bone and muscle in androgen-deficient and osteoporotic patients through their anabolic activities.

Many coregulators normally show tissue-specific distribution, and the levels of different coactivators, for example CBP, can vary dramatically among specific cell types. Such differences in expression levels might indicate cell specificity of NR-mediated transcriptional regulation, and might partially explain how the same gene can be regulated differentially in different cell types or tissues.²⁹ In recent report, cell-type and promotor-specific differences in recruitment of coregulators, SRC-1, plays a critical role in determining the tissue-selective functions of SERMs, tamoxifen and raloxifene.³⁰ It is likely that the similar mechanisms are integrated into SARM function.

The search for tissue-selective SARMS has been driven by both clinical and research needs. Analysis for the expression of SARM activities at a molecular level could give a clearer understanding of the physiology and cellular biology of androgen actions. In addition, compounds with SARM activities may provide better treatment for patients with a variety of androgen-related endocrine disorders as well as a new type of anabolic agents for osteoporosis.

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