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Original Article

Hylonome

Effect of 17β-hydroxysteroid dehydrogenase type 2 inhibitor on bone strength in ovariectomized cynomolgus monkeys

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Abstract

In both sexes, a reduction in sex steroid production with aging impairs the musculoskeletal system. The goal of our study was to test the ability of WH-9062, a novel non-steroidal small molecule inhibitor of the 17β-Hydroxysteroid Dehydrogenase type 2 enzyme, to maintain or improve bone strength without raising serum levels of testosterone or estradiol. Mature, female cynomolgus monkeys with sealed growth plates were allocated into six groups: Sham controls, OVX controls, OVX+Premarin® (15 mg/kg/d), and three groups of OVX monkeys receiving WH-9062 at 1, 5 and 25 mg/kg/day. All treatments were administered by daily oral dosing for 23 weeks. Changes in lipid profile caused by OVX were corrected with WH-9062 and included lowering total of cholesterol and non-HDL cholesterol, and maintenance of initial plasma levels of HDL cholesterol. Only the highest dose of WH-9062 lowered bone resorption relative to OVX controls. Elevated bone specific alkaline phosphatase, osteocalcin, BMC and dynamic bone histomorphometry data resulted in desirable bone balance and bone strength. The obtained results support our theory that inhibition of 17β-HSD type 2 resulted in high local estrogen and/or testosterone levels leading to maintenance of bone formation and bone strength. Collectively, our data demonstrated that the treatment paradigm that utilizes tissue selectivity and receptor bioavailability in conversion of inactive hormones to active forms could be achieved and could result in desirable effects on target tissues such as bone and muscles.

Keywords: Osteoporosis, Cynomologus Monkeys, 17β-Hydroxysteroid Dehydrogenase Type 2 Inhibitor, Bone Histomorphometry, Bone Strength

Introduction

Both men and women experience osteoporosis due to increased bone resorption-driven remodeling[1-4] or impaired capacity of osteoblasts to form bone[5,6]. Although it is well recognized that estrogen deficiency plays a major role in the changes in bone metabolism at menopause[7], there is substantial evidence suggesting that androgens may also be important[8-10]. Androgen as well as estrogen receptors have been found in bone cells from both sexes[11-13]. Positive correlations between bone mass and androgen levels have been reported in pre- and postmenopausal women as well as in elderly men[14-22]. Androgen production was decreased in women with osteoporosis as compared to age-matched controls[23,24]. Not only do androgens increase bone mass in hypogonadal males[25], but anabolic androgenic hormones given alone[26-28] or in combination[29-33] with estrogen are effective in postmenopausal women. Finally, there are studies suggesting that although androgens and estrogens may share the ability to decrease bone resorption, they have different effects on bone formation[34-36]. In contrast to estrogen replacement therapy, which has been associated with a reduction in bone resorption[37-39], the use of androgens is associated with the stimulation of new bone formation[40-42]. The use of estrogen alone in postmenopausal women may actually reduce androgen production by reducing high levels of FSH and LH that can stimulate androgen production by the climacteric ovary[43]. Since stimulation of bone formation is an important goal in established osteoporosis, particularly in older patients, a combined therapy that simultaneously inhibits bone resorption and stimulates bone formation would be optimal.

The super-family of 17β-Hydroxysteroid Dehydrogenases

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Abbreviations	
OVX	Ovariectomy
17β-HSD	17β-Hydroxysteroid Dehydrogenase Type 2
FSH	Follicular stimulating hormone
LH	Luteinizing hormone
E_1	Estrone
E_2	Estradiol
T	Testosterone
A	Androsteneidone
DHT	Dehydrotestosterone
CEE	Conjugated equine estrogens - Premarin®
TAP	Total alkaline phosphatase
BSAP	Bone specific alkaline phosphatase
BMD	Bone mineral density
BMC	Bone mineral content
BMA	Bone mineral area
AST	Aspartate aminotransferaze
ALT	Alanine aminotransferaze

(17β-HSDs) differs in their tissue distribution, substrate and co-factor specificities, and subcellular localization[44]. Each of the 17β-HSD enzymes possesses almost unidirectional activity; types 1 and 3 catalyze reductive reactions of sex steroids; whereas types 2, 4, 5, and 6 are responsible for oxidative pathways. The microsomal 17β-HSD from placenta (designated 17β-HSD type 2) was cloned by expression cloning, and found to be equally active on androgens and estrogens as substrates[45]. The recombinant 17β-HSD type 2 converts the highly active 17β-hydroxysteroids such as estradiol (E_2), testosterone (T), and dihydrotestosterone (DHT) to their less active "keto" forms. In addition, the type 2 enzymes can, to a lesser extent, also convert 20α-hydroxyprogesterone to progesterone[46]. The amino acid sequence of 17β-HSD type 2 indicates an extended hydrophobic amino terminus of approximately 60 amino acids that is characteristic of a trans-membrane signal anchor. The protein contains an amino-terminal type II signal-anchor motif and a carboxyl-terminal endoplasmatic reticulum retention motif that suggests that 17β-HSD type 2 is associated with the membranes of the endoplasmic reticulum[47]. Human and rodent 17β-HSD type 1 predominantly catalyzes reactions opposite to those catalyzed by the type 2 enzyme.

Besides placenta, 17β-HSD type 2 was also found in smaller amounts in the kidney, pancreas, colon, liver, small intestine and prostate[48-50]. Dong and colleagues[51] showed significant activity of 17β-HSD type 2 in cultured human osteoblasts and osteoblast-like osteosarcoma cells MG63 and TE85, but not in SaOS-2. The potential for interconversion of E_1 to E_2, T to A and DHT to A by bone cells could therefore represent an important mechanism for the local regulation of intracellular ligand supply for the estrogen and androgen receptors in the osteoblasts (Figure 1). Since most skeletal changes that occur in both sexes as a result of hypogonadism can be prevented or reversed with conventional hormone replacement therapy[52-56], it is reasonable to assume that maintaining the presence of active forms of sex steroids at the bone tissue level could protect bone mass and structure from deterioration in postmenopausal woman. We hypothesized that there is a natural distribution and bioavailability of estrogen and androgen receptors at various organs. Due to natural atrophy of the uterus and breast following menopause, we postulate that local levels of androstenedione and estrone may be reduced; therefore, these organs may be less likely to show undesirable side effects following treatment with a 17β-HSD type 2 inhibitor. On the other hand muscle, bone and brain that remain active long after menopause should retain relatively high levels of androstenedione and estrone, and therefore should respond to therapy with a 17β-HSD type 2 inhibitor by maintaining relatively high local levels of testosterone and estrogen. In the absence of published pre-clinical or clinical data that can further address this topic we aimed to test the ability of WH-9062, a novel non-steroidal small molecule inhibitor of the 17β-HSD type 2 enzyme to increase bone formation without raising serum levels of testosterone or estrogen and improve bone mechanical properties in ovariectomized cynomolgus monkeys.

Material and methods

Testing compound

WH-9062 represents the pyrrolidinone class of compounds that are more than 10 mM potent in steroid receptor (estrogen, progesterone, androgen, and glucocorticoid) binding assays and related hydroxysteroid dehydrogenase (11β-HSD 2, 17β-HSD 1, and 17β-HSD 3) counter screens. The structural formula and physicochemical properties of WH-9062 (4*R*, 5*R*)-5-[(1*S*)-hydroxy (5-(3-pyridyl) (2-thienyl)) methyl]-1-methyl-4-phenylpyrrolidin-2-one) has been described earlier by Wood et al.[57] (Figure 2). WH-9062 demonstrated a favorable biochemical profile *in vitro* showing good potency in bone cells (MG63 cells; IC_{50}=90 nM) and against the isolated 17β-HSD type 2 enzyme (IC_{50}=50 nM). Species specificity of the enzyme was tested using liver microsomes from various species including the rat, guinea pig, mini-pig, monkey and human. The results clearly showed that oxidative 17β-HSD 2-like activity is inhibited similarly in human and primate microsomes, but not in the rat, guinea pig or minipig microsomes, and that WH-9062 inhibited the monkey and human enzyme to a similar degree. When primate 17β-HSD 2 cDNA was PCR-cloned from COS cells, it showed 94% homology to its human counterpart. Therefore, to determine the ability of WH-9062 to influence bone formation we utilized the ovariectomized (OVX) monkey model, an FDA approved large animal model for assessment of safety and efficacy of compounds targeting prevention or treatment of osteoporosis[58,59].

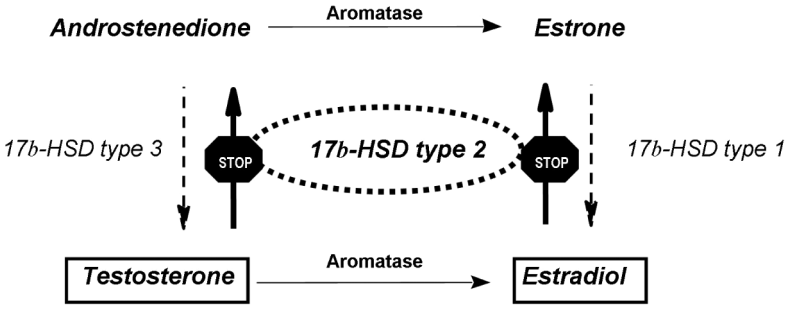


Figure 1. Rationale behind development of 17β-HSD type 2 inhibitor. Inhibition of 17β-HSD type 2 should, according to hypothesis, elevate bone tissue level of estradiol and testosterone, which in turn, will regulate local bone metabolism resulting in increased bone strength.

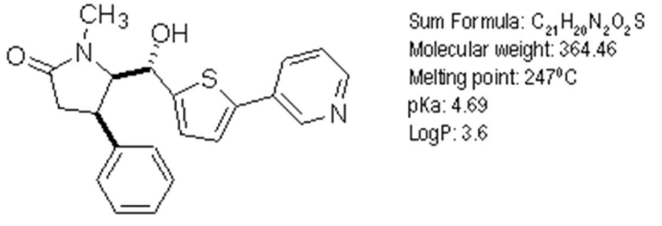


Figure 2. Structural formula and physicochemical properties of WH-9062.

Experimental design and assays

This study was carried out at Wake Forest University School of Medicine (Winston-Salem, NC) and was approved by the school's Institutional Animal Care and Use Committee. Prior to being imported from Indonesia, female cynomolgus monkeys (*Macaca fascicularis*) were screened radiographically to ensure their growth plates were sealed, which indicates skeletal maturity. Their mean age upon arrival was estimated to be 12 years by dentition, but exact age as well as eventual pregnancies could not be determined with certainty. Only monkeys with sealed growth plates and lack of skeletal abnormalities or previous fractures that might interfere with bone densitometry measurements were included in this study. After an initial quarantine of 30 days in a facility approved by the Centers for Disease Control and Prevention, the monkeys were moved into pen housing with each pen accommodating four to six monkeys for an additional two months to stabilize and get trained for oral dosing. Monkeys were either ovariectomized (OVX) (n=90) or sham operated (n=13) and divided into experimental groups that had equivalent body weight, spinal bone mineral density (BMD), and alkaline phosphatase activity. Experimental groups were treated as follows: Group 1: Sham+Vehicle (n=13; Crystal Light); Group 2: OVX+Vehicle (n=16; Crystal Light); Group 3: OVX+CEE (n=15; 15 mg/kg/day, Premarin®, Wyeth Pharmaceuticals); Group 4: OVX+WH-9062 (n=15; 1 mg/kg/day); Group 5: OVX+WH-9062 (n=13; 5 mg/kg/day) and Group 6: OVX+WH-9062 n=15; 25 mg/kg/day). The difficulty in obtaining a larger number of animals as well as monetary constrains prevented us from including baseline or pre-OVX groups that will be highly desirable when conducting studies using prevention protocol. Monkeys were fed once daily with the purified diet containing 0.15% of calcium, 0.15% phosphorus, 30% protein, 55.5% carbohydrate, 9% fiber and 4.5% and 0.20 mg cholesterol/calorie. Based on accumulated experience while working on similar models, the scientists from Wake Forest University suggested to lower Ca and P content in the diet by 50% (standard monkey chow contains 0.3% Ca and 0.3% P) to ensure that the monkeys will develop osteoporosis following ovariectomy and that excess of Ca and P in the diet will not interfere with this process. This diet does not contain phytoestrogens that are usually present in standard "monkey chow" and are believed to act as estrogen analogues. Two monkeys that received 5 mg/kg of WH-9062 were excluded from the data analyses due to the presence of residual ovarian tissue discovered at necropsy.

The treatment with all test materials started two days prior to surgery and lasted for 23 consecutive weeks. All treatments were administered by daily oral dosing, not by gavages but rather by training the monkeys to drink from a syringe. The vehicle in this study was double-strength Crystal Light® a flavored water containing 0.5% methylcellulose and 0.5% Tween 80. The dose volume was 1.5 ml/kg. The Crystal Light formulation was chosen for its taste rather than the solubility of the compound in the vehicle. Pharmacokinetics was assessed in a preliminary single dose and 10-day dose range study utilizing separate cohorts of naive monkeys under identical husbandry conditions. At week 18 of the main study, blood was collected from 12 randomly chosen monkeys per dosing group, shipped to Bayer campus (West Haven, CT) and analyzed for determination of drug plasma concentrations. In addition, organ weights were determined at necropsy for the following tissues: heart, lung, liver, kidney and uterus.

Estradiol concentrations were measured in serum at the Comparative Endocrinology Laboratory of the Yerkes Regional Primate Center (Atlanta, GA). Concentrations of

Parameter	Units	Method	Schedule
Total Alkaline Phosphatase	μL/L	Automated	0, 12, and 22 weeks
Osteocalcin	ng/mL	ELISA	0, 12, and 22 weeks
CTX I	nM	ELISA	0, 12, and 22 weeks
Estradiol	pg/mL	RIA	0, 12, and 22 weeks
Testosterone	pg/mL	RIA	22 weeks
Plasma Lipid Profile	mg/dL	Automated	22 weeks
Ionized Calcium	mg/dL	Electrode	22 weeks
Creatinine, BUN, Glucose	mg/dL	Automated	22 weeks
Liver Enzymes (AST, ALT)	units/liter	Automated	22 weeks
Differential Blood Cell Count	various	Automated	22 weeks

Table 1. Parameters determined in serum at various time points during the course of the study. Abbreviations: CTX I – urinary collagen C-terminal extension peptides; BUN – blood urea nitrogen; AST – aspartate aminotransferaze; ALT – alanine aminotransferaze.

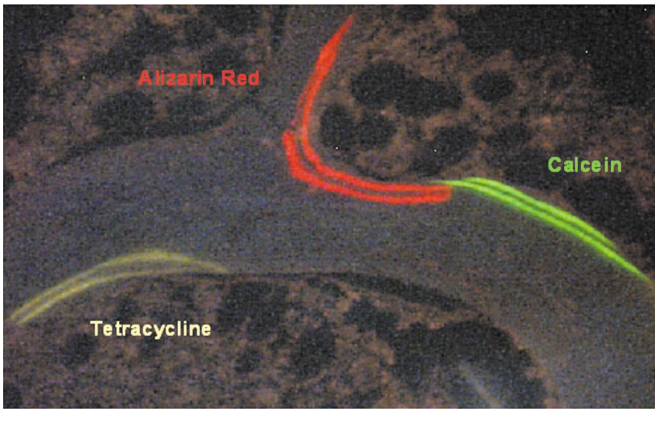


Figure 3. Trabeculae of the cancellous bone from lumbar vertebral body (control cynomolgus monkey). Double labeling (2 weeks apart) of all 3 injected fluorochrome labels are clearly visible under UV light. Magnification x10.

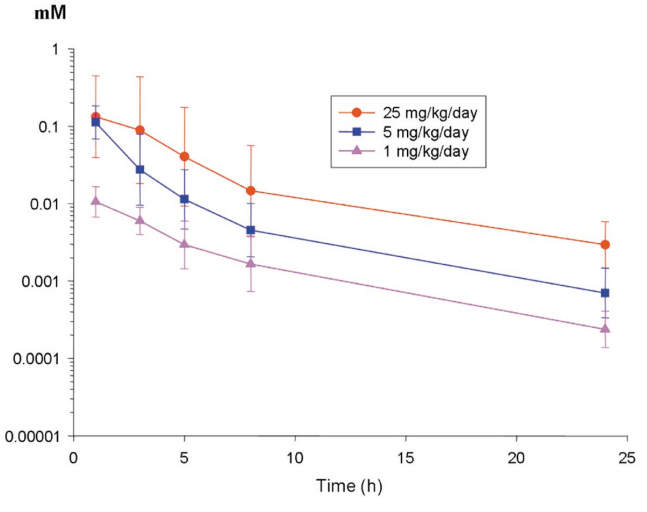


Figure 4. Mean plasma concentration (mM) of WH-9062 following 18 weeks of dosing.

estradiol were determined using a modification of a commercially available RIA kit employing a double antibody technique (Diagnostic Products, Corp., Los Angeles, CA). Levels of serum C-terminal type I collagen cross-linked peptides were determined using the Cross Laps ELISA kit (Osteometer Biotech A/S, Herlev, Denmark). The timetable and methods used to assess serum parameters are depicted in Table 1.

Bone densitometry

In vivo dual energy X-ray absorbitometry (DEXA; Norland, XR-26, Fort Atkinson, WI) measurements of spinal bone mineral density (BMD), bone mineral area (BMA) and bone mineral content (BMC) were done at lumbar vertebrae (2-4) before OVX or sham surgery (baseline), and before necropsy (week 23). Additional *in vivo* measurements of BMD were performed on the fourth lumbar vertebra and midshaft of the femur using a peripheral quantitative computed tomography machine (pQCT; XCT 960A, Stratec, Pforzheim, Germany).

Bone histomorphometry

All monkeys received injections of fluorochrome labels to mark actively forming bone surfaces. Alizarin complexone (60 mg/3ml; 20-25 mg/kg) was given sc during the quarantine period (2 injections/2 weeks apart) to identify sites of active bone mineralization during the acclimatization period. Tetracycline HCl (30 mg/kg) was given IV 14 days prior to and at the time of surgery to identify active bone sites at baseline. Calcein (10 mg/kg) was given sc on a 1-13-1-7 schedule (1 day label, 13 days

Serum Estradiol Concentrations (pg/ml)						
Week	**Sham**	**OVX**	**CEE**		**WH-9062**	
	Vehicle (n=13)	Vehicle (n=16)	15 μg/kg (n=15)	1 mg/kg (n=15)	5 mg/kg (n=13)	25 mg/kg (n=15)
0	60±17	103±28	65±12	51±6	67±15	58±10
12	59±7[a]	5±1	62±9[a]	6±1*	8±1*	5±1*
22	60±11[a]	12±2	65±15[a]	10±1*	11±1*	10±1*
Serum Testosterone Concentrations (ng/dl)						
Week	**Sham**	**OVX**	**CEE**		**WH-9062**	
	Vehicle (n=13)	Vehicle (n=16)	15 μg/kg (n=15)	1 mg/kg (n=15)	5 mg/kg (n=13)	25 mg/kg (n=15)
24	12±2	10±1	11±1	13±1	11±2	18±3
Serum Androstenedione Concentrations (ng/dl)						
Week	**Sham**	**OVX**	**CEE**		**WH-9062**	
	Vehicle (n=13)	Vehicle (n=16)	15 μg/kg (n=15)	1 mg/kg (n=15)	5 mg/kg (n=13)	25 mg/kg (n=15)
24	0.59±0.1	0.49±0.05	0.51±0.1	0.52±0.04	0.51±0.1	0.75±0.1

Significant difference compared OVX vehicle controls at [a]$p<0.001$ and *$p<0.001$ relative to Sham.

Table 2. Serum levels of estradiol, testosterone and androstenedione at various time points during the course of the study.

no label, 1 day label, 6 days no label, necropsy). This procedure enabled differentiation of bone-forming sites mineralizing at the end of dosing period and prior to necropsy (Figure 3). Structural and dynamic bone histomorphometry analyses were performed on lumbar vertebral bodies (L2; cancellous bone) using standard ASBMR procedures and nomenclature[60]. In brief, bones were fixed in 0.1 M phosphate-buffered 10% formalin, dehydrated in ethanol and embedded undecalcified in methyl metacrylate. Dynamic histomorphometry measurements were performed on unstained, 6 mm thick cross-sections of the lumbar vertebral bodies (L2; 3 sections per monkey) at 20X magnification using a fluorescence microscope (Nikon, Japan) interfaced with a digitizer. The data were collected using a software package (OsteoMetrics, Inc., Atlanta, GA) specifically written for bone histomorphometry. Histomorphometric measurements were done in the central portion of the vertebra, away from the endocortical-cancellous boundary using only calcein labels given 13 days apart. Measurements were made on 6 to 10 fields per section and histomorphometry parameters were calculated according to Parfitt et al.[60].

Mechanical testing

A compression test was used to determine mechanical properties of the lumbar vertebral bodies (L3). Cores of the cancellous bone containing bone marrow were taken along the supero-inferior direction of each vertebra. Cylinders, 3.5 mm in diameter, were cut from the center of the vertebral body and were kept frozen in saline-soaked gauze. Specimens were rehydrated and kept in Ringer's solution for 5 days before mechanical testing was conducted. The cylinders were tested along the vertical axis using a Material Testing System (MTS 810, MTS Corp., Minneapolis, MN). During compression, load-deformation curves and extension curves were collected by the accompanied software and analyzed at SkeleTech, Inc., (Bothell, WA). The measured parameters include: load to fracture (peak of load-deformation curve), energy absorption capacity (area under load-deformation curve), maximum stress (the peak load divided by cross-sectional area of each specimen) and Young's modulus (slope of stress-strain curve).

Statistical analyses

All data were checked to ensure that values were normally distributed and met the assumption for parametric analyses. Values obtained by sequential sampling, such as bone densitometry and clinical chemistry data are analyzed using analysis of covariance (ANCOVA) with repeated measures. For values with no baseline or repeated measures, comparison between groups at single time points was done using analysis of variance or by ANCOVA using pre-treatment values for other variables as co-variates. Differences between groups were examined and significance corrected for multiple comparisons using the Bonferroni correction.

Results

When WH-9062 was administered to monkeys in pilot studies by the oral route, there was significant variability in the level of exposure. After 18 weeks of dosing, plasma samples were collected to determine mean plasma concentrations of WH-9062 (Figure 4). The mean plasma concentrations of

Serum Concentrations of Total Alkaline Phosphatase (*μL/L*)						
Week	**Sham**	**OVX**	**CEE**		**WH-9062**	
	Vehicle (n=13)	Vehicle (n=16)	15 μg/kg (n=15)	1 mg/kg (n=15)	5 mg/kg (n=13)	25 mg/kg (n=15)
0	228±10	226±19	210±14	225±20	224±17	228±13
12	201±9[b]	250±19	157±8[b]	281±21*	258±18*	276±16*
22	185±11[b]	251±17	147±8[b]	294±30*	258±20*	279±18*
% change	***-19%***	***+11%***	***-30%***	***+30%***	***+15%***	***+22%***
Total Plasma Cholesterol Concentrations (mg/dL)						
Week	**Sham**	**OVX**	**CEE**		**WH-9062**	
	Vehicle (n=13)	Vehicle (n=16)	15 μg/kg (n=15)	1 mg/kg (n=15)	5 mg/kg (n=13)	25 mg/kg (n=15)
0	236±15	251±22	270±25	221±13	234±22	240±17
12	267±23	330±33	318±34	285±17	299±22	294±26
22	272±23[a]	347±38	311±31	311±20	290±22	292±23
% change	***+15%***	***+38%***	***+15%***	***+40%***	***+23%***	***+21%***
Plasma HDL Cholesterol Concentrations (mg/dL)						
Week	**Sham**	**OVX**	**CEE**		**WH-9062**	
	Vehicle (n=13)	Vehicle (n=16)	15 μg/kg (n=15)	1 mg/kg (n=15)	5 mg/kg (n=13)	25 mg/kg (n=15)
0	57±4	51±4	47±4	54±4	53±5	57±4
12	54±5	52±5	42±4	58±5	55±5	60±5
22	48±4	49±6	39±4	53±6	51±6	56±5
% change	***-16%***	***-4%***	***-18%***	***-1%***	***-0.2%***	***-0.2%***
Plasma Non-HDL Cholesterol Concentrations (mg/dL)						
Week	**Sham**	**OVX**	**CEE**		**WH-9062**	
	Vehicle (n=13)	Vehicle (n=16)	15 μg/kg (n=15)	1 mg/kg (n=15)	5 mg/kg (n=13)	25 mg/kg (n=15)
0	179±16	200±24	223±27	167±15	181±25	183±17
12	213±26	278±37	276±26	228±20	244±26	234±28
22	224±26[a]	297±43	273±33	258±24	239±26	237±25
% change	***+25%***	***+48%***	***+22%***	***+54%***	***+32%***	***+29%***
Total Cholesterol/HDL Cholesterol Ratio						
Week	**Sham**	**OVX**	**CEE**		**WH-9062**	
	Vehicle (n=13)	Vehicle (n=16)	15 μg/kg (n=15)	1 mg/kg (n=15)	5 mg/kg (n=13)	25 mg/kg (n=15)
0	4.1±0.5	4.9±0.7	5.7±0.8	4.1±0.3	4.4±0.8	4.2±0.4
12	4.9±0.9	4.9±1.1	7.5±1.2	4.9±0.6	5.4±1.1	4.9±0.9
22	5.6±0.9	7.1±1.6	7.9±1.5	5.9±1.0	5.6±1.2	5.2±0.8[a]
% change	***+36%***	***+44%***	***+38%***	***+43%***	***+35%***	***+23%***
Plasma Triglycerides (mg/dL)						
Week	**Sham**	**OVX**	**CEE**		**WH-9062**	
	Vehicle (n=13)	Vehicle (n=16)	15 μg/kg (n=15)	1 mg/kg (n=15)	5 mg/kg (n=13)	25 mg/kg (n=15)
0	41±3	38±4	33±3	46±8	36±4	46±8
12	45±5	51±9	55±7	50±9	38±5	48±8
22	48±5	41±6	48±3	51±11	39±6	52±9
% change	***+17%***	***+7%***	***+45%***	***+10%***	***+8%***	***+13%***
Significant difference compared OVX vehicle controls at: [a]$p<0.05$ and [b]$p<0.001$, and *p<0.001 relative to Sham.						

Table 3. Serum concentrations of Total Alkaline Phosphatase, and plasma concentrations of Total, HDL and Non-HDL Cholesterol and Total Triglycerides at various time points during the course of the study. (% change was calculated between week 0 and week 22).

Serum Concentrations of Bone Specific Alkaline Phosphatase (μL/L)						
Week	**Sham**	**OVX**	**CEE**		**WH-9062**	
	Vehicle (n=13)	Vehicle (n=16)	15 μg/kg (n=15)	1 mg/kg (n=15)	5 mg/kg (n=13)	25 mg/kg (n=15)
0	86±6	76±6	74±5	82±9	74±7	80±7
12	70±5[b]	84±5	57±3[c]	101±11*	90±9*	100±9*
22	64±4[b]	89±8	49±3[c]	112±15*	94±1*	97±8*
% change	***-26%***	***+17%***	***-34%***	***+36%***	***+27%***	***+21%***
Serum Concentrations of Osteocalcin (nL/mL)						
Week	**Sham**	**OVX**	**CEE**		**WH-9062**	
	Vehicle (n=13)	Vehicle (n=16)	15 μg/kg (n=15)	1 mg/kg (n=15)	5 mg/kg (n=13)	25 mg/kg (n=15)
0	16.3±1.5	17.8±3.0	13.1±1.0	14.5±1.6	13.9±1.8	12.2±1.6
12	26.2±2.5[a]	40.9±2.7	20.0±2.7[b]	45.3±5.4**	39.9±3.9**	36.1±3.8**
22	22.2±1.3[b]	43.2±6.1	17.3±2.0[c]	60.4±7.5[a,*]	45.4±7.0**	35.3±3.9**
% change	***+36%***	***+142%***	***+32%***	***+316%***	***+226%***	***+189%***
Serum Concentrations of CTX I (nM)						
Week	**Sham**	**OVX**	**CEE**		**WH-9062**	
	Vehicle (n=13)	Vehicle (n=16)	15 μg/kg (n=15)	1 mg/kg (n=15)	5 mg/kg (n=13)	25 mg/kg (n=15)
0	6.97±0.9	6.21±0.8	6.04±0.7	6.26±0.6	7.47±0.6	6.69±0.7
12	4.97±0.7[c]	6.81±0.5	4.55±0.6[c]	7.42±0.9*	7.80±0.4*	7.01±0.6*
22	4.57±0.5[c]	7.03±0.7	3.59±0.4[c]	7.94±0.9*	9.15±1.9*	7.26±0.6*
% change	***-35%***	***+13%***	***-41%***	***+26%***	***+22%***	***+8%***

Significant difference compared OVX vehicle controls at: [a]$p<0.05$, [b]$p<0.01$ and [c]$p<0.001$, and *$p<0.01$ and **$p<0.001$ relative to Sham.

Table 4. Serum concentrations of biomarkers of bone metabolism: Bone Specific Alkaline Phosphatase (BSAP), Osteocalcin (OC) and C-telopeptide degradation (CTX I) at various time points during the course of the study.

WH-9062 determined in this efficacy multi-dose study were generally 10-20% lower compared to what was observed in the single dose and 10-day pilot studies (data not shown).

Body weight was not affected by any treatment throughout the study (data not shown). Organ weights (heart, lung, liver, kidneys) recorded at necropsy were normalized relative to total body weight (TBW) and showed no difference between control and treatment groups. As expected, ovariectomy resulted in reduced uterine weight (0.04% of TBW) relative to Sham controls (0.15% of TBW). The dose of Premarin® used in this study was partially effective in preventing uterine atrophy caused by OVX (0.10% of TBW), while treatment with WH-9062 showed no effect on uterine weight (0.04% of TBW). There were no statistically significant changes between treatment groups in any blood chemistry parameters (BUN, Glucose, Creatinine, AST, ALT, Calcium) or differential blood cell counts (WBC, RBC, HGB, MCV, HCT) throughout the study period (data not shown).

There was no significant difference in serum levels of estradiol between groups at the time of OVX or sham surgery (Week 0). Bilateral ovariectomy caused a sharp drop in serum estradiol concentration that was fully prevented by Premarin®, but not by the WH-9062 compound. Six months of dosing with WH-9062 did not significantly increase serum concentrations of estradiol, testosterone or androsteneidone. A trend towards increased serum levels of testosterone was observed in the group of monkeys treated with 25 mg/kg of WH-9062 (Table 2).

There was no significant difference in the serum levels of total alkaline phosphatase between groups at the time of surgery (Week 0). At the end of the study total alkaline phosphatase (TAP) levels in sham and Premarin®-treated OVX monkeys were somewhat lower relative to OVX controls, but these changes were not statistically significant. Dosing with WH-9062 did not significantly influence serum concentrations of TAP (Table 3). During the course of the study, total cholesterol levels rose 38% in OVX control monkeys, but only 15% in sham and OVX monkeys treated with Premarin®. The high and mid dose of WH-9062 showed 21% and 23% percent increases in total plasma cholesterol, while levels in the low dose group rose 40%, similar to OVX controls (Table 3). Similar changes were observed in plasma LDL cholesterol levels but no changes were seen in HDL. A mild increase in total cholesterol/HDL cholesterol ratio was evident in OVX monkeys treated with vehicle and Premarin®, but not in monkeys receiving WH-9062. There was no effect of ovariectomy or treatment on plasma triglycerides (Table 3).

No significant difference in serum bone specific alkaline

Bone Mineral Area (cm^2)						
Week	**Sham**	**OVX**	**CEE**		**WH-9062**	
	Vehicle (n=13)	Vehicle (n=16)	15 μg/kg (n=15)	1 mg/kg (n=15)	5 mg/kg (n=13)	25 mg/kg (n=15)
0	7.02±0.2	7.05±0.2	6.89±0.2	6.95±0.2	6.93±0.1	6.87±0.1
23	7.09±0.2	7.15±0.2	6.95±0.2	6.94±0.2	6.87±0.1	6.97±0.1
% change	***+1.1%***	***+1.2%***	***+1.0%***	***-0.1%***	***-0.9%***	***+1.5%***
Bone Mineral Content (gm)						
Week	**Sham**	**OVX**	**CEE**		**WH-9062**	
	Vehicle (n=13)	Vehicle (n=16)	15 μg/kg (n=15)	1 mg/kg (n=15)	5 mg/kg (n=13)	25 mg/kg (n=15)
0	4.00±0.2	4.05±0.2	3.96±0.2	3.99±0.2	3.95±0.1	3.92±0.1
23	4.14±0.2[b]	3.99±0.2	4.04±0.2[a]	3.93±0.2	3.91±0.1	3.97±0.1[a]
% change	***+3.4%***	***-1.6%***	***+2.2%***	***-1.4%***	***-1.1%***	***+1.4%***
Bone Mineral Density (mg/cm^2)						
Week	**Sham**	**OVX**	**CEE**		**WH-9062**	
	Vehicle (n=13)	Vehicle (n=16)	15 μg/kg (n=15)	1 mg/kg (n=15)	5 mg/kg (n=13)	25 mg/kg (n=15)
0	567±16	570±14	572±12	570±15	569±11	570±11
23	579±16[b]	554±16	578±12[a]	563±15	568±11	569±12
% change	***+2.3%***	***-2.7%***	***+1.1%***	***-1.2%***	***-0.2%***	***-0.1%***

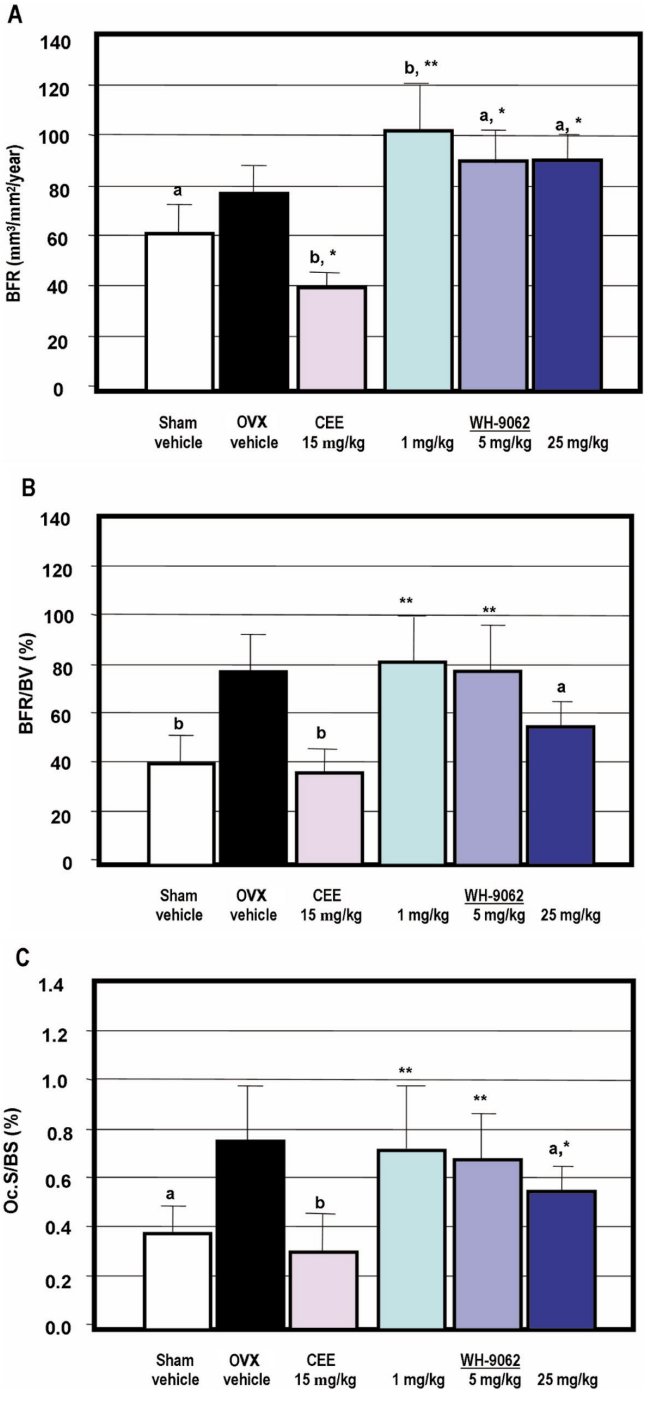
Significant difference compared OVX vehicle controls at [a]$p<0.05$, [b]$p<0.01$.

Table 5. Changes in lumbar vertebral area, mineral content and mineral density obtained *in vivo* by DEXA at the time of OVX surgery and at the end of study. Antero-posterior view was used to obtained scans of lumbar vertebral bodies (L2-L4).

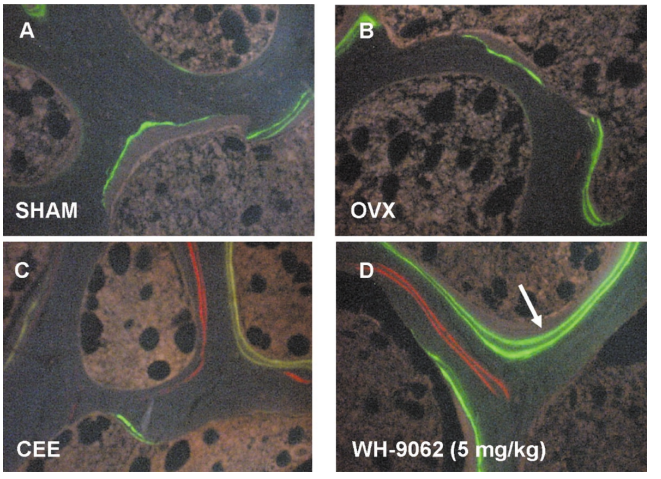
phosphatase (BSAP) between groups was noted at the time of surgery. Ovariectomy induced a slight increase in BSAP, while the sham-operated monkeys treated with vehicle exhibited a slight, but consistent trend toward decreasing serum concentration of BSAP. Treatment with Premarin® resulted in decreased serum levels of BSAP. Compared to OVX controls, all three doses of WH-9062 exhibited a slight, yet consistently higher levels of bone specific alkaline phosphatase with the low dose being the most effective (36%), followed by medium dose (27%) and high dose (21%). During the same period sham monkeys experienced a decrease of 26% and monkeys treated with Premarin® a 34% decrease in BSAP activity (Table 4). Osteocalcin levels were significantly higher in OVX monkeys (+142%) compared to the sham controls (+36%) and Premarin® treated monkeys exhibited a 32% increase. Monkeys treated with WH-9062 showed an elevation in serum osteocalcin that was higher than observed in OVX controls, with the 1mg dose being the most effective with a 316% increase, medium dose showed 226% and high dose 189% increase relative to initial values (Table 4). Sham controls and OVX monkeys treated with Premarin® exhibited a mild, but statistically significant decrease in serum CTX levels. OVX controls and OVX monkeys treated with WH-9062 experienced no change in CTX levels (Table 4).

At the end of the study period there was a significant difference in vertebral BMD between sham and OVX vehicle treated control monkeys. Sham operated monkeys improved BMD by gaining 2.3% relative to baseline values, while OVX controls lost 2.7% of their initial BMD values as a result of OVX surgery. Premarin® completely prevented loss of BMD induced by ovariectomy resulting in 1.1% increase in BMD relative to initial values. DEXA measurements revealed that only monkeys treated with 25 mg/kg of WH-9062 experienced higher BMC relative to OVX controls, with no significant difference between groups in BMD and BMA parameters (Table 5). The volumetric determination appears to detect no change at both spine (LV_4) and femoral midshaft between control and treated groups (data not shown).

Dynamic bone histomorphometry performed on cross-sections of LV_2 (3 sections per monkey) revealed changes in bone remodeling between sham and OVX control monkeys. Based on bone formation (calcein fluorescence) recorded during the last 3 weeks of the study, OVX control monkeys showing higher bone formation rate (Figure 5A), higher remodeling index (Figure 5B) and increased bone resorption (Figure 5C) relative to sham controls. Treatment with Premarin® not only prevented an increase in bone formation rate, but also reduced bone resorption and bone remodeling induced by ovariectomy. Monkeys treated with WH-9062 at 1, 5 and 25 mg/kg exhibited more intense bone formation relative to all other groups (Figures 5A and 6). The 25 mg/kg dose of the WH-9062 compound seemed to be the most effective in reducing bone resorption and lowering bone remodeling (Figures 5B and 5C).



Figures 5A-C. (**A**) Bone formation rate (BFR; surface referent); (**B**) Bone formation rate over bone volume (BFR/BV); and (**C**) Osteoclast surface over bone surface (Oc.S/BS) parameter of cancellous bone measured using cross-sections of the 2nd lumbar vertebral body. BFR indicates higher bone formation in OVX vehicle-treated monkeys as compared to sham controls. CEE not only prevented an increase in BFR induced by ovariectomy, but also decreased BFR relative to normally cycling sham control monkeys. Treatment with WH-9062 at 1, 5 and 25 mg/kg/day increased BFR compared to OVX controls. Data are expressed as mean±SE; $^{a}p<0.05$ and $^{b}p<0.01$ relative to OVX vehicle group and $^{*}p<0.05$ and $^{**}p<0.01$ relative to sham controls.



Figures 6A-D. Cancellous bone from L_2 under UV light showing bone mineralization in sham control monkeys (**A**); OVX control monkeys (**B**); OVX monkeys treated with CEE (**C**); OVX monkeys treated with 5mg/kg of WH-9062 (**D**). The fluorochrome bone marker calcein is visible as a green label and was given twice, 19 and 6 days prior to necropsy to label bone surfaces that actively mineralize at the time of calcein injection. Please note intensive calcein labeling (arrow) indicating high bone formation in monkeys treated with WH-9062 (**D**) relative to control groups. Also, note that CEE decreased bone remodeling as visible by conservation of all 3 labels. Magnification x10.

Bone strength evaluation done *ex vivo* on LV_3 using the compression technique indicated that ovariectomy caused a decrease of bone strength that was fully prevented by Premarin®. Despite the relatively small sample size and inherent variations in the test method, there appeared to be a positive protective effect on bone strength; in particular by the mid and high dose of WH-9062, since the results in these two groups were similar to sham controls and significantly higher relative to OVX controls (Figures 7 and 8).

Discussion

Estrogens, via the estrogen receptors (ERα and ERβ) and androgens via the androgen receptor (AR) are involved in bone homeostasis in both men and women regardless of age. Circulating levels of testosterone in women begin to decline in the mid-reproductive years and the levels of the adrenal androgenic steroids, androstenedione and dehydroepiandrosterone, continue to decrease throughout postmenopausal life. This decrease in androgenic hormones, in addition to cessation of ovarian function at the time of menopause, results in an abrupt drop in estrogen synthesis causing osteopenia which, if left untreated, leads to osteoporosis. In both sexes, a reduction in sex steroid production with aging causes increased bone remodeling with bone resorption being the dominant phenomenon due to increased activity and lifespan

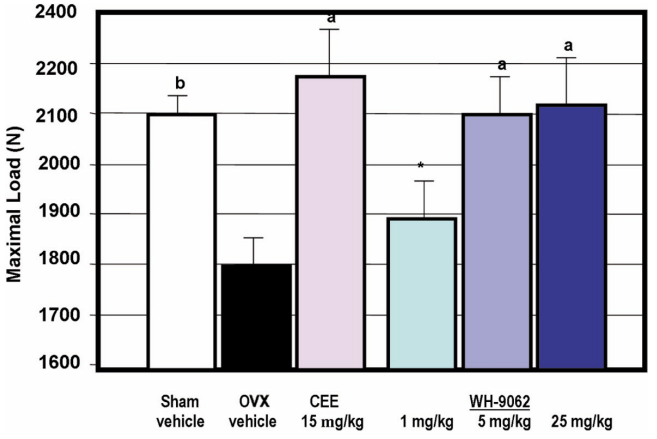


Figure 7. Maximal load, the force required to fracture the vertebral body, measured *ex vivo* on L_3 by using Material Testing System (5501R, Instron Corp.). Bars represent Mean ±SEM of raw data. ANOVA and Bonferroni-adjusted group comparisons were used; $^{a}p<0.05$ and $^{b}p<0.01$ values indicate significant difference relative to OVX controls and $^{*}p<0.01$ relative to sham controls.

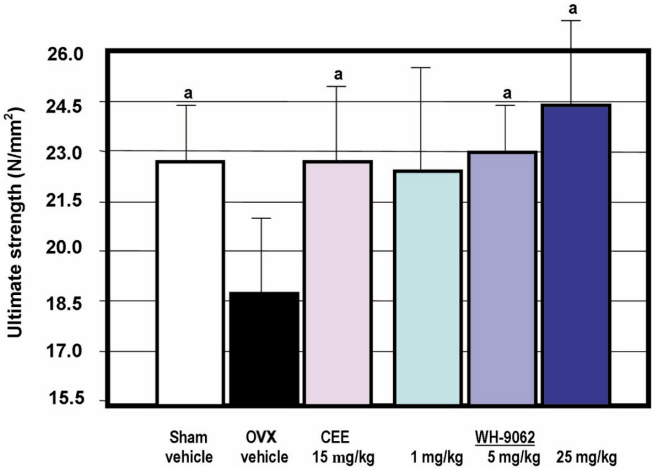


Figure 8. Ultimate strength, the maximum stress bone specimen can sustain, measured *ex vivo* on L_3 by using Material Testing System (5501R, Instron Corp.). Bars represent Mean±SEM of raw data. ANOVA and Bonferroni-adjusted group comparisons were used; $^{a}p<0.05$ and $^{b}p<0.01$ values indicate significant difference relative to OVX controls.

of osteoclasts. While both estrogens and androgens have a biphasic effect on endochondral bone growth during puberty, in adults androgens play the dominant role in regulating bone dimension and geometry, characteristics that, in addition to bone quality, are determinants of overall bone strength[8,11,15,61]. The positive effect of androgens on energy, sense of well-being, sexual function, body composition and muscle and bone mass has been well documented in both sexes, but virilizing side effects following systemic testosterone administration has limited the use of testosterone in postmenopausal women[62,63]. We hypothesized that organ systems in women differ in their sensitivity to testosterone, thus organs that are the targets of potential virilization such as skin, hair, vocal cords, and clitoris may require a higher testosterone concentration for these effects to occur than levels required for induction of beneficial effects in the bone and muscle. Therefore, by appropriately increasing local testosterone levels, clinically beneficial effects on bone and muscle could be dissociated from virilizing side effects. In addition, local increase of androgenic steroids may help to maintain local estrogen synthesis in organs like bone and brain where estrogen has a profound influence on resorption (bone) and cognitive function (brain)[14,15,64].

Non-human primates demonstrate many advantages over other animal models for osteoporosis, as their endocrine, gastro-intestinal and musculoskeletal systems closely resemble the same systems in humans[59,65]. Female macaque monkeys cycle monthly and have a hormonal pattern similar to that of humans. Results from several studies show similarities between non-human primates and humans in response to estrogen cessation; however, spontaneous menopause does not occur in macaques[66-68]. In this study, bone remodeling and strength were assessed in OVX monkeys following six months of treatment with a 17β-HSD type-2 inhibitor, a treatment duration that is equivalent to 1.5 human years. Despite suboptimal formulation and oral consumption dosing instead of controlled oral gavages, reasonable pharmacokinetic separation was achieved between the three doses of WH-9062 used. The results of treatment with WH-9062 were compared to OVX controls treated with vehicle or Premarin®.

Ovariectomy caused a sharp drop in serum estradiol concentration that was fully corrected by Premarin®, but not by the WH-9062 compound. At 12 weeks post-ovariectomy the average serum level of estradiol in OVX controls and WH-9062 treated monkeys was 10 times lower than the level of estradiol in sham controls (6 and 60 pg/ml, respectively). Estradiol levels in the OVX control and WH-9062-treated animals rose to 10 pg/ml by week 22, similar to results reported by Hotchkiss et al.[69] using a similar animal model. These results indicate a possible increase in adrenal production of estradiol and/or conversion of testosterone to estradiol in ovariectomized monkeys. It is well established that in humans testosterone serves not only as an androgenic hormone, but also as a pro-hormone that can be converted by many tissues including muscle, bone, breast, skin, brain, ovary and liver into two active metabolites, estradiol and dihydrotestosterone, that are needed in order to exert specific activity at target tissues. For example, in order for testosterone to be effective on skin, its obligatory 5-α-reduction to dihydrotestosterone is needed[70]; while testosterone's effects on bone resorption, gonadotropin suppression, plasma lipids, cognitive and other brain functions require its aromatization to estradiol[71]. In contrast to human data

where ovariectomy caused decreases in serum testosterone and androstenedione levels of about 50% each[72], ovariectomized monkeys in our study did not demonstrate changes in serum concentrations of testosterone and androstenedione. This observation may raise an important question regarding the appropriateness of this model for study of some aspects of osteoporosis in women. Maintenance of serum testosterone levels after surgical ovariectomy in monkeys may be one of the reasons why this species shows bone loss at the lumbar spine after ovariectomy, but no change in cortical BMD[69]. A study by Tok and colleagues[14] revealed that endogenous androgens influence bone mineral density in postmenopausal women, however, androgen effects could be different in different bone types. In our study, ovariectomy and/or treatment modalities did not significantly change serum levels of testosterone and androstenedione, although both hormones were somewhat higher in monkeys treated with the high dose of WH-9062 compound relative to sham and OVX controls.

It is well known that postmenopausal women are at higher risk for developing cardiovascular events and that these events in women with osteoporosis may be proportional to the severity of osteoporosis[73]. OVX monkeys in our study showed 38% higher cholesterol and 48% higher non-HDL cholesterol relative to sham controls at the end of the study period, a change that was somewhat corrected by Premarin® since those monkeys experienced only 13% higher cholesterol and 18% higher non-HDL cholesterol compared to controls. The WH-9062 compound showed efficacy similar to Premarin® in attenuating the rise in serum cholesterol seen in OVX monkeys, in particular the high dose, since monkeys in that dose group experienced only 7% higher cholesterol and non-HDL cholesterol compared to sham controls.

Collectively, serum assays of bone metabolism, spinal BMD data and bone histomorphometry data from this study are consistent with hallmarks of osteoporosis seen in postmenopausal women that are associated with decreased bone strength and increased propensity to fractures. The decline in bone remodeling in Premarin®-treated monkeys is dominated by markedly decreased bone resorption followed by lack of bone formation resulting in maintenance of BMD and preservation of bone strength. The combined results of systemic and local measurements of bone resorption led us to believe that monkeys treated with the high dose of WH-9062 experienced decreased bone resorption, possibly through local increase of estrogen. On the other hand, bone formation activity in all WH-9062-treated monkeys was elevated, in particular bone specific alkaline phosphatase and osteocalcin, findings that were paralleled by bone formation data and BMC. Taken together, data from monkeys treated with a high dose of WH-9062 indicate that decreased bone resorption and increased bone formation lead to desirable bone balance, and support our theory that inhibition of 17β-HSD type 2 resulted in high local estrogen and/or testosterone levels leading to maintenance of bone formation and bone strength. Studies in humans revealed that combined administration of testosterone and estrogen implants increased lean body mass and decreased body fat more than estrogen implants alone[74], suggesting that increased testosterone could be highly beneficial for musculoskeletal health in postmenopausal women, but also for treatment of type II osteoporosis and frailty in both men and women.

In contrast to the dramatic decline in estradiol and progesterone production that occurs at menopause, decline in DHEAS and testosterone becomes apparent in the decade prior to menopause and is gradual and progressive[75-77]. It is currently believed that in young menstruating women, adrenal and ovarian daily production of androgens equals 300 mg of testosterone, and that approximately half of the circulating testosterone is derived from the ovaries[78,79]. It is also believed that the adrenal gland produces testosterone precursors (DHEA, DHEAS, Androstenedione) whose peripheral conversion to testosterone contributes to the remaining 50% of circulating testosterone[80]. In healthy women, approximately 50-60% of testosterone is bound to sex hormone binding globulin, 30-40% to albumin and only 0.5-3% is unbound. The free hormone hypothesis assumes that only the free and loosely bound (albumin bound) hormone exerts biologic effects. We believe that better understanding of systemic and local effects achieved with inhibitors of 17β-HSD may lead to discovery of compounds with better efficacy and selectivity capable of increasing testosterone or estrogen levels at target organs such as bone and muscle. Rather than give the combination therapy of estrogen and testosterone and raise serum levels of these hormones with an increased chance to induce undesirable side effects, we are banking on natural androgen production by the adrenals in postmenopausal women. Our data demonstrate the treatment paradigm that utilizes tissue selectivity and receptor bioavailability in conversion of inactive hormone to active form achieving and resulting in desirable effects on target tissues such as bone, muscles, cardiovascular system and brain.

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