

LASOFOXIFENE (CP-336,156), A NOVEL SELECTIVE ESTROGEN RECEPTOR MODULATOR, IN PRECLINICAL STUDIES

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ABSTRACT

Estrogen replacement therapy is reported to reduce the incidence of vertebral fractures in postmenopausal women, however, its compliance is limited because of side effects and safety concerns. Estrogen's side effects on breast and uterine tissues leading to the potential increased risk of uterine and breast cancer limit widespread estrogen usage. Thus, there is a significant medical need for a therapy that protects against postmenopausal bone loss but is free of estrogen's negative effects on reproductive tissues. Selective estrogen receptor modulators (SERMs) have been investigated as an alternative to hormone replacement therapy. One such compound, raloxifene, has been approved for the prevention and treatment of osteoporosis.

Lasofloxifene (LAS), a new, nonsteroidal, and potent SERM, is an estrogen antagonist or agonist depending on the target tissue. LAS selectively binds with high affinity to human estrogen receptors. In ovariectomized (OVX) rat studies, LAS prevented the decrease in femoral bone mineral density, tibial and lumbar vertebral trabecular bone mass at an ED₁₀₀ of about 60 µg/kg/day. LAS inhibited the activation of trabecular and endocortical bone resorption and bone turnover in tibial metaphyses and diaphyses, and lumbar vertebral body in OVX rats. In addition, LAS decreased total serum cholesterol, inhibited body weight gain and increased soleus muscle weight in OVX rats. Similarly, LAS prevented bone loss induced by orchidectomy or aging in male rats by decreasing bone resorption and bone turnover while it had no effect in the prostate. Further, LAS decreased total serum cholesterol in intact aged male rats or in orchidectomized male rats. Synergistic skeletal effects were found with LAS in combination with bone anabolic agents such as prostaglandin E₂ (PGE₂), parathyroid hormone (PTH) or a growth hormone secretagogue (GHS) in OVX rats. In combination with estrogen, LAS inhibited the uterine stimulating effects of estrogen but did not block the bone protective effects of estrogen. In immature and aged female rats, LAS did not affect the uterine weight and uterine histology. In OVX adult female rats, LAS slightly but significantly increased uterine weight. These

results demonstrated that LAS produced effects on the skeleton indistinguishable from estrogen in female and male rats. However, unlike estrogen, LAS had little effect on uterine weight and cellular proliferation of uterus in female rats. In preclinical anti-tumor studies, LAS inhibited human breast cancer growth in mice bearing MCF7 tumors, prevented NMU-induced mammary carcinomas and possessed chemotherapeutic effects in NMU-induced carcinomas in rats.

Therefore, we conclude that LAS possesses the antiestrogenic effects in breast tissue and estrogenic effects in bone and serum cholesterol, but lacks estrogen's side effects on uterine tissue. These data support the therapeutic potential of LAS for the prevention and treatment of postmenopausal bone loss and mammary carcinomas in humans.

INTRODUCTION

Osteoporosis is the loss of bony tissue that results in bones becoming brittle and liable to fracture. Generalized osteoporosis is common in the elderly and in women after menopause. Postmenopausal osteoporosis is a growing health problem throughout the world and the number of women entering the menopausal period will significantly increase over the next 20 years. This increase in the number of postmenopausal women will be accompanied by a significant increase in the incidence of osteoporosis. Today, osteoporosis affects about 25 million Americans. It has been estimated that a 50-year-old woman in the U.S. has about an 11 - 18% lifetime risk of suffering a hip fracture (1). The consequences of hip fracture results in considerable morbidity, loss of independence, and mortality (2).

Many factors, including genetics, environment, nutrition, aging and hormonal factors, contribute to the loss of bone leading to the increased risk of skeletal fractures (3). However, the cause of postmenopausal osteoporosis is, at least in part, due to the decline in levels of circulating estrogen which results in an increased rate of bone loss and osteoporosis (4-8). Prospective clinical studies have shown that the risk of osteoporotic fractures increases as bone mineral density decreases. Kanis et al (9) reported that the risk of fracture increases 1.5 to 3-fold for each standard deviation decrease in bone mineral density. Thus, prevention of bone loss in the postmenopausal period is an effective strategy to reduce the incidence of osteoporotic bone fractures.

Estrogen or hormone replacement therapy (HRT) is effective in preventing the bone loss leading to osteoporosis (10-12) and epidemiological studies have demonstrated that HRT reduces the incidence of bone fractures (11-13). However, HRT's compliance is very low as less than fifteen percent of women prescribed this therapy continue through the second year. Factors that contribute

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to discontinuation of therapy include, objectionable reproductive tissue effects, particularly resumption of menses, breast tenderness, and perceived increased risk of breast cancer (12,14,15). A tissue selective estrogen receptor modulator without estrogenic activity in the breast and uterus would improve the negative side-effect profile of estrogen and overcome these compliance hurdles. A new class of agents, named selective estrogen receptor modulators (SERMs), has been investigated as an alternative to estrogen or hormone replacement therapy in preclinical and clinical studies (16-26). It has been reported that raloxifene, the first agent from this class approved for the prevention and treatment of postmenopausal osteoporosis, prevented bone loss, increased bone mineral density, reduced skeletal fracture, reduced risk of breast cancer, and decreased serum low-density lipoprotein cholesterol without uterine stimulating effects in postmenopausal women (22-24).

Lasofloxifene (LAS), a new, nonsteroidal, potent SERM, is currently in clinical trials for the prevention and treatment of osteoporosis. Prior to the initiation of clinical trials, we performed a series of *in vitro* and *in vivo* preclinical studies to characterize the effects of LAS on bone, serum lipids, breast and uterine tissues (27-30). The purpose of this article is to review and summarize the preclinical pharmacological data demonstrating that LAS has potential in protecting bone mass and averting osteoporotic fractures, lowering serum low-density lipoprotein cholesterol, and reducing the risk of breast cancer in humans.

BINDING OF LAS TO ESTROGEN RECEPTORS (ER α AND ER β)

The ability of LAS to inhibit [3 H]-estradiol binding was measured by a competition binding assay using dextran-coated charcoal as previously described (31). 293T cell extracts expressing either human ER α or human ER β were incubated in the presence of increasing concentrations of competitor and a fixed concentration of [3 H]-estradiol (141 Ci/mmol). IC $_{50}$ values were converted to inhibition constants (K $_i$) according to the Cheng-Prusoff equation, $K_i = IC_{50}/(1 + [L]/K_d)$ (32). The values used in these equations were $K_i = IC_{50}/(1 + [0.5$

nM]/0.6 nM) and $K_i = IC_{50}/(1 + [0.5$ nM]/0.7 nM) for hER α and hER β , respectively.

Competition binding assays measuring displacement of radiolabeled estradiol from expressed estrogen receptors demonstrated high affinity binding of LAS to both ER α (K $_i = 0.27$ nM) and ER β (K $_i = 0.7$ nM). LAS bound to ER α with greater affinity than did estradiol (K $_i = 0.44$ nM), whereas the binding to ER β was approximately equal to that measured for estradiol (K $_i = 0.7$ nM).

TRANSCRIPTIONAL ACTIVATION OF RECOMBINANT ERA IN HEPG2 CELLS

It has been widely proposed that the tissue selective actions of estrogen receptor modulators, such as tamoxifen and raloxifene, can be explained based on the conformational changes induced in the estrogen receptor (33). Our working hypothesis leading to the discovery of LAS, was that we could infer these conformational changes by using cell-based transcriptional reporter assays to assess the functional activity of various ER ligands. The ability of estradiol, raloxifene and LAS to activate transcription of a C3 promoter luciferase reporter construct in HEPG2 cells (a human hepatocellular carcinoma cell line) was measured as described previously (34,35). Briefly, HEPG2 cells were transfected with C3-luciferase plasmid and either wild-type human ER α or a mutated construct called ER-TAF1 (human estrogen receptor containing TAF1 do-main mutated in the TAF2 domain as described in reference 34). The ER-TAF1 construct expressed a receptor that had been mutated by site directed mutagenesis to change amino acids 538, 542 and 545. This triple mutation was designed to disrupt the AF2 functional domain.

As shown in Figure 1A, estradiol activated both wild-type ER α and a mutated ER disrupted in the activation function 2 domain (ER-TAF1). Raloxifene did not activate the wild-type receptor and, in fact, inhibited the reporter activity to below basal levels at doses greater than 1 nM (Figure 1B). Surprisingly, raloxifene was able to stimulate transcription through the ER-TAF1 mutant receptor (35). Since animal models and emerging clinical data indicated that raloxifene exhibited benefi-

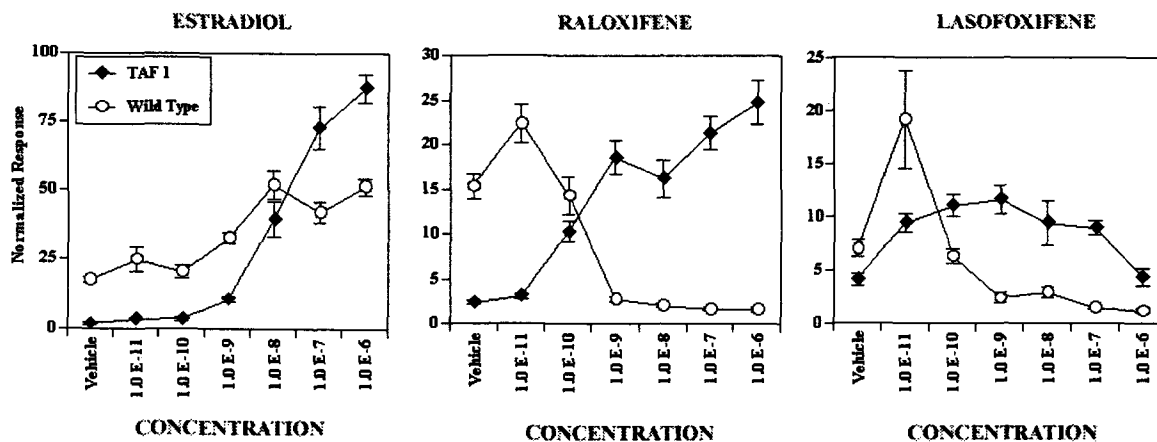


Figure 1: Raloxifene and lasofloxifene exhibit similar activity profiles in transcriptional assays and differ from that of estradiol. The ability of test compounds to activate transcription of C3-luciferase reporter in HEPG2 cells in the presence of either wild-type or mutated (TAF1) estrogen receptor was tested by transient transfection assays. Panel A: estradiol; Panel B: raloxifene and Panel C: lasofloxifene. Error bars represent standard error.

cial bone effects without adverse agonist effects in uterus or breast tissue, we hypothesized that this *in vitro* profile from our transcriptional assays would be predictive of the ER conformational state produced by a tissue selective estrogen agonist. The profile of LAS in these transcriptional assays was qualitatively identical to that of raloxifene: inhibition of basal wild-type ER activity and activation of the ER-TAF1 mutant (Figure 1C). Quantitatively, lasofoxifene was ~10-50 times more potent than raloxifene in its agonist activity at ER-TAF1.

TRANSCRIPTIONAL ACTIVATION OF ENDOGENOUS ERβ IN PRIMARY RAT GRANULOSA CELL

In order to determine the action of LAS on the newly discovered ERβ, we utilized endogenous ERβ present in primary rat granulosa cells as has been reported by O'Brien et al. (36). These cultures were transfected with estrogen responsive luciferase reporter (ERE3-TK-luc) and assayed as has been described previously (31). In these experiments, granulosa cells were isolated from rat ovaries, cultured, and transfected with an estrogen responsive luciferase reporter vector. Estradiol stimulated luciferase activity in these cells in a dose responsive manner with an EC₅₀ of 0.11 nM. LAS did not stimulate the estrogen responsive reporter in this system, as shown in Figure 2. Therefore, we conclude that LAS does not act as an estrogen agonist in ovarian granulosa cells.

EFFECT OF LAS ON BONE IN THE OVARECTOMIZED (OVX) RAT MODEL

As previously described, estrogen deficiency during the menopause results in increased bone turnover leading to bone loss. Ovariectomy (OVX) in rats produces estrogen deficiency and increases bone turnover leading to trabecular bone loss similar to that observed in postmenopausal women (37-40). OVX in female rats causes significant bone loss in the lumbar vertebrae, proximal tibial, and distal femoral metaphyses 4 weeks post-surgery (41,43). The OVX rat is thus an appropriate and recommended model to evaluate compounds for the prevention and treatment of postmenopausal osteoporosis (44,45).

The efficacy of lasofoxifene (LAS) in preventing bone loss was first tested using the OVX rat model (27). Five-month-old OVX rats were treated with lasofoxifene (LAS) at oral doses of 1, 10, 100, or 1000 µg/kg/day, or 17α-ethynyl estradiol (EE) at 30 µg/kg/day (10 rats per group) daily for 4 weeks, beginning 1 day post-OVX. Groups of vehicle-treated sham rats (n = 10) and vehicle-treated OVX rats (n = 10) served as controls. Calcein, a fluorochrome bone marker used to measure bone dynamic histomorphometric parameters, was injected at 10 mg/kg s.c. injection to all rats 12 and 2 days before necropsy. The effects of LAS on cancellous bone mass, bone resorption and bone turnover were compared with those of EE.

As shown in Figure 3, LAS at doses equal to or greater than 1 µg/kg/day completely prevented OVX-induced decreases in bone mineral density (BMD) of whole lumbar vertebrae (LV), determined by dual-energy x-ray absorptiometry (DEXA), and in trabecular bone volume (TBV) of fifth lumbar vertebral body (LV5), determined by cancellous bone histomorphometry. At long bone metaphysis, the minimal effective dose of LAS for preventing the decrease in TBV of proximal tibial metaphysis (PTM) was 10 µg/kg/day, while the minimal effective dose of LAS for preventing the decrease in BMD of distal femoral metaphysis (DFM) was 100 µg/kg/day. The bone protective effects of LAS at the three bone sites, LV, PTM, DFM, were similar to those observed with EE at 30 µg/kg/day. These data illustrated that LAS possessed similar potency and anti-osteopenic effects as estrogen in a rat model of osteoporosis. The anti-resorption or anti-remodeling effects of LAS were also inferred from the effects on serum marker of bone turnover (serum osteocalcin), or by cancellous bone histomorphometric analysis such as osteoclast number, osteoclast surface and bone formation rate/bone surface referent, as shown in Figure 4. Further, LAS induced apoptosis and p53 expression with a concomitant decrease in the number of tartrate-resistant acid phosphatase positive multinuclear cells [TRAP(+)MNC] in rat bone marrow cell cultures *in vitro*, suggesting that

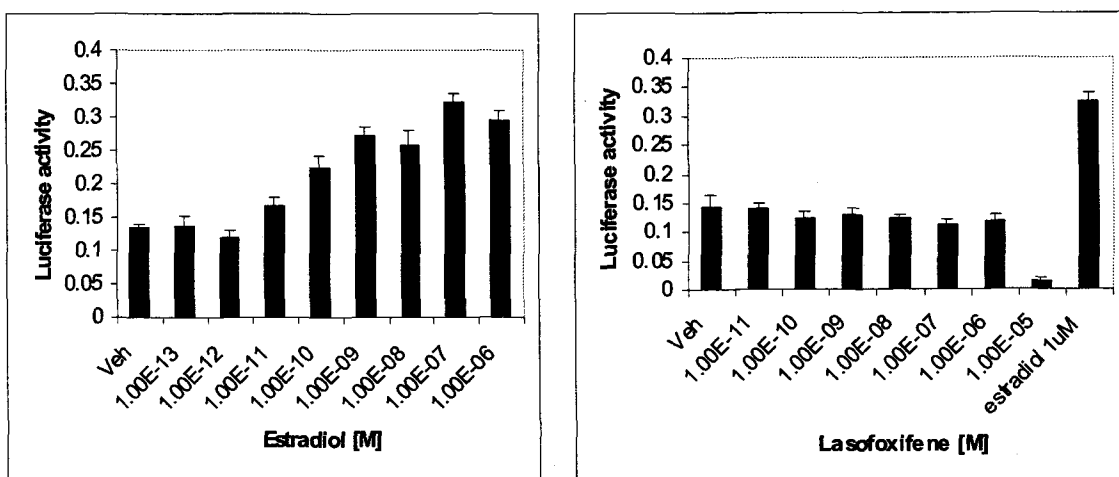


Figure 2: Lasofoxifene does not stimulate granulosa cells. Primary rat granulosa cells were transiently transfected with an estrogen responsive luciferase reporter. Estrogen or lasofoxifene was added at the indicated concentrations and luciferase activity was measured after 36 hours. Each determination was made in replicate (n=6) and error bars represent standard error.

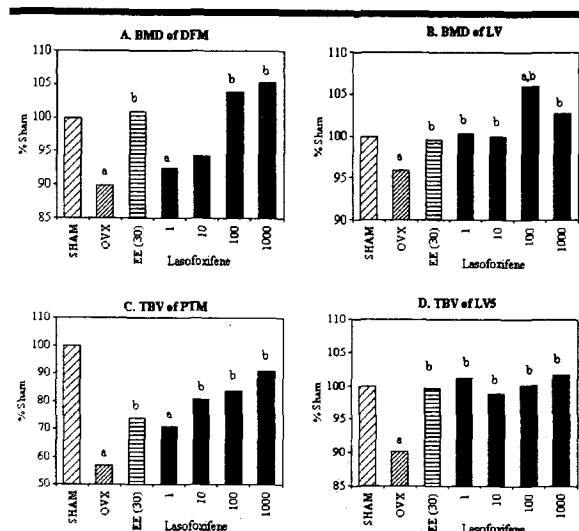


Figure 3: Percent change from sham-operated controls in bone mineral density (BMD) of distal femoral metaphysis (DFM) and whole lumbar vertebral body (LV), and trabecular bone volume (TBV) of proximal tibial metaphysis (PTM) and fifth lumbar vertebral body (LV5) in OVX rats treated with vehicle, 17 α -ethynyl estradiol (EE) at 30 μ g/kg/day, or lasofoxifene (LAS) at oral doses of 1, 10, 100, or 1000 μ g/kg/day for 4 weeks, beginning 1 day post-OVX. Significant prevention of OVX-induced bone loss was observed in OVX rats treated with EE or LAS. Modified from reference 27. a: $p < 0.05$ vs. SHAM; b: $p < 0.05$ vs. OVX.

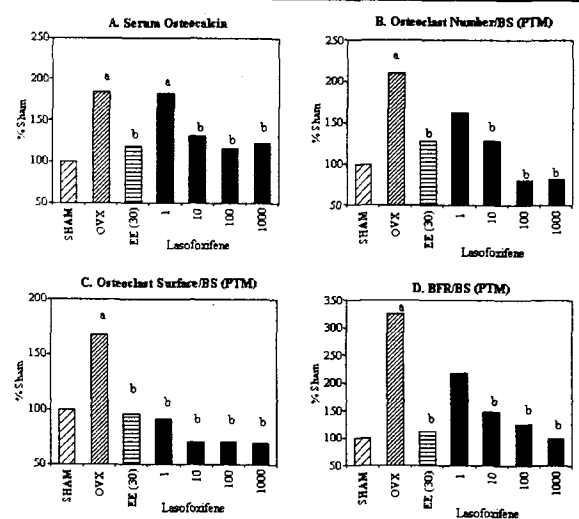


Figure 4: Percent change from sham-operative controls in serum osteocalcin, and osteoclast number/BS, osteoclast surface/BS and bone formation rate/bone surface referent (BFR/BS) of proximal tibial metaphysis (PTM) in OVX rats treated with vehicle, 17 α -ethynyl estradiol (EE) at 30 μ g/kg/day, or lasofoxifene (LAS) at oral doses of 1, 10, 100, or 1000 μ g/kg/day for 4 weeks, beginning 1 day post-OVX. Significant prevention of OVX-induced high bone resorption and turnover was observed in OVX rats treated with EE or LAS. Modified from reference 27. a: $p < 0.05$ vs. SHAM; b: $p < 0.05$ vs. OVX.

the induction of apoptosis may be one of the mechanisms for the estrogen agonistic activities of LAS in bone (27). The anti-resorption or anti-remodeling effects of LAS are very similar to those observed with EE.

Using the same OVX rat model as described above, we sought to more finely characterize the dose-dependent effects of LAS. In the next study, five-month-old OVX rats were treated with LAS at oral doses of 1, 3, 10, 30, 60, 100 or 300 μ g/kg/day (10 rats per group) daily for 4 weeks, beginning 1 day post-OVX. Figure 5 shows the effects of the different doses of LAS on bone mineral content (BMC) and BMD of DFM. Significant protection of loss in BMC and BMD at the DFM was observed at doses equal to or greater than 30 μ g/kg/day with the fully efficacious dose at 60 μ g/kg/day. Similarly, a significant prevention of elevated bone turnover (bone formation rate/bone surface referent, a histomorphometric index of cancellous bone turnover) occurred at doses equal to or greater than 30 μ g/kg/day with the fully efficacy dose at 60 μ g/kg/day, as shown in Figure 5.

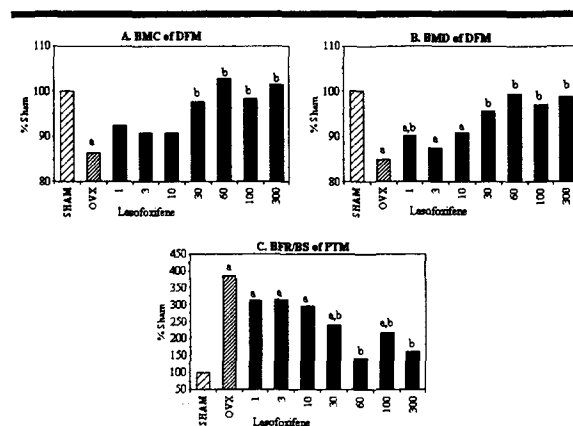


Figure 5: Percent change from sham-operated controls in bone mineral content (BMC) and bone mineral density (BMD) of distal femoral metaphysis (DFM), and bone formation rate-bone surface referent (BFR/BS) of proximal tibial metaphysis (PTM) in OVX rats treated with vehicle or lasofoxifene (LAS) at oral doses of 1, 3, 10, 30, 60, 100, or 300 μ g/kg/day for 4 weeks, beginning 1 day post-OVX. Significant prevention of OVX-induced bone loss and higher bone turnover was observed in OVX rats treated with LAS at doses equal to or greater than 30 μ g/kg/day. a: $p < 0.05$ vs. SHAM; b: $p < 0.05$ vs. OVX.

EFFECTS OF LAS ON TOTAL SERUM CHOLESTEROL IN FEMALE RATS

Since both LDL cholesterol and HDL cholesterol in rats is cleared by the hepatic LDL receptor, cholesterol-lowering effects in the OVX rat can be used to predict the LDL cholesterol lowering efficacy of SERMs. It has been well documented that estrogen and estrogen-like substances such as raloxifene and tamoxifen reduce total serum cholesterol in rats and reduce the LDL cholesterol in human (21,22). Using the OVX rat model, we found that LAS significantly reduced total serum cholesterol as compared with both the sham and OVX controls, as shown in Figure 6A (27). At the highest dose (1000 μ g/kg/d), OVX rats treated with LAS decreased cholesterol by 50% compared with sham controls. Similarly, as shown in Figure 6B, LAS treatment for 4 weeks significantly reduced total serum cholesterol to 46% and 27% of vehicle controls at 10 and 100 μ g/kg/d, respectively, in intact aged (27-month-old) female rats. These data indicate that LAS possesses potentially potent LDL cholesterol lowering capability in humans.

EFFECTS OF LAS ON UTERINE TISSUE OF FEMALE RATS

The effect of estrogen on the uterus is a clinical safety concern as previously noted. OVX in the rat causes significant uterine atrophy and the atrophy can be reversed by treatment with estrogen. Thus, the OVX rat is a sensitive model to evaluate uterine hypertrophy. The uterine weight decreased to a level of about 30% of sham control 4 weeks post-OVX in 4-month-old rats (19,20). As shown in Figure 7A, LAS at doses equal to or greater than 10 µg/kg/day slightly but significantly increased uterine wet weight with no dose-dependent response for up to 1000 µg/kg/day as compared with vehicle-treated OVX rats (27). However, the uterine weight in all LAS treated OVX rats was significantly lower than those of sham controls and OVX rats treated with estrogen. The uterine effects of LAS were further characterized in the immature female rat model and in aged female rat (27). LAS at doses as high as 100 µg/kg/day had no significant effects in uterine weight (Figure 7B-D) and uterine histology such as luminal epithelial thickness in both immature female rats treated for 3 days or in 17-month-old intact female rats treated for 4 weeks.

EFFECTS OF CO-TREATMENT OF LAS AND ESTROGEN ON OVX RATS

As stated above, LAS acts as an estrogen agonist in bone in OVX rats. However, it is not known whether LAS can antagonize estrogen's uterine stimulation effects, and whether LAS can enhance or antagonize estrogen's bone protective effects in OVX rats. To address this question, Sprague-Dawley female rats were sham-operated (n=10) or OVX (n=40) at 5.5 months of age. The OVX rats were treated (oral gavage) with either 17α-ethynyl estradiol (EE) at 30 µg/kg/day or LAS at 100 µg/kg/day, or co-administration with EE and LAS for 28 days (50).

Uterine weight in OVX control rats was significantly decreased (-72%) compared with sham controls as shown in Figure 8A. EE treatment maintained uterine weight in OVX rats to a level that did not differ from sham

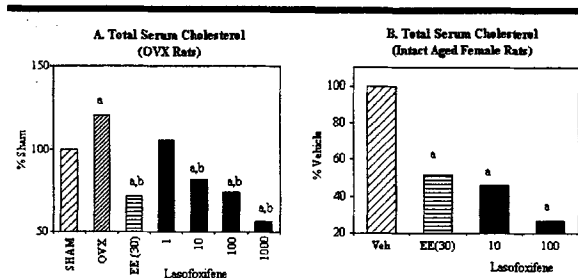


Figure 6: (A), Percent change from sham-operated controls in total serum cholesterol in OVX rats treated with vehicle, 17α-ethynyl estradiol (EE) at 30 µg/kg/day, or lasofoxifene (LAS) at oral doses of 1, 10, 100, or 1000 µg/kg/day for 4 weeks, beginning 1 day post-OVX. LAS significantly reduced total serum cholesterol in OVX rats at doses equal to or greater than 10 µg/kg/day. a: p < 0.05 vs. SHAM; b: p < 0.05 vs. OVX. (B), Percent change from vehicle controls in total serum cholesterol in intact, 27-month-old female rats treated with vehicle, 17α-ethynyl estradiol (EE) at 30 µg/kg/day, or lasofoxifene (LAS) at oral doses of 10 and 100 µg/kg/day for 4 weeks. EE and LAS significantly reduced total serum cholesterol in these rats. Modified from reference 27. a: p < 0.05 vs. vehicle.

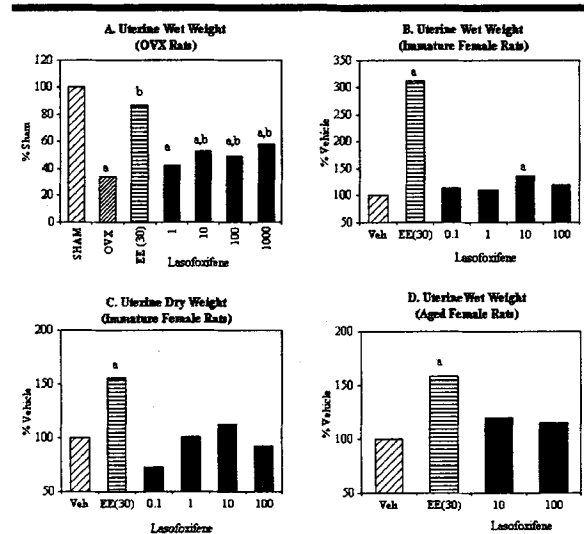


Figure 7: (A), Percent change from sham-operated controls in uterine wet weight in OVX rats treated with vehicle, 17α-ethynyl estradiol (EE) at 30 µg/kg/day, or LAS at oral doses of 1, 10, 100, or 1000 µg/kg/day for 4 weeks, beginning 1 day post-OVX. LAS slightly but significantly increased uterine wet weight in OVX rats at doses equal to or greater than 10 µg/kg/day. a: p < 0.05 vs. SHAM; b: p < 0.05 vs. OVX. (B) and (C), Percent change from vehicle controls in uterine wet and dry weight in intact, 3-week-old immature female rats treated with vehicle, 17α-ethynyl estradiol (EE) at 30 µg/kg/day, or LAS at oral doses of 1, 10 and 100 µg/kg/day for 3 days. LAS had no significant effect in uterine wet and dry weight, except at 10 µg/kg/day, it slightly but significantly increased uterine wet weight in these rats. a: p < 0.05 vs. vehicle. (D), Percent change from vehicle controls in uterine wet weight in intact, 27-month-old aged female rats treated with vehicle, 17α-ethynyl estradiol (EE) at 30 µg/kg/day, or LAS at oral doses of 10 and 100 µg/kg/day for 4 weeks. LAS had no significant effect in uterine wet weight in these rats. Modified from reference 27. a: p < 0.05 vs. vehicle.

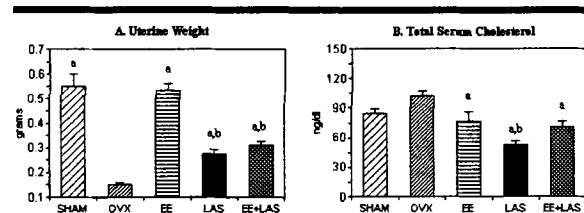


Figure 8: Effects of co-treatment of lasofoxifene (LAS) and 17α-ethynyl estradiol (EE) on uterine weight and total serum cholesterol in OVX rats. The OVX rats were treated (oral gavage) with either EE at 30 µg/kg/day or LAS at 100 µg/kg/day, or co-administration with EE and LAS for 28 days. Error bars represent standard error of mean. a: p < 0.05 vs. OVX; b: p < 0.05 vs. OVX+EE.

controls. LAS-treated OVX rats had slightly but significantly higher uterine weight than OVX controls. Uterine weight in the co-administration group did not differ from LAS alone group and this effect was significantly less than EE alone group, indicating that LAS blocked estrogen's agonistic effects in uteri. Compared with OVX controls, total serum cholesterol decreased significantly by 13%, 22%, and 20% in the EE alone, LAS alone, and co-administration groups, respectively, as illustrated in Figure 8B. These results indicated that

OVX rats co-treated with LAS and EE maintained the cholesterol lowering effects of these agents.

OVX significantly decreased total content (-13%), total density (-14%), trabecular density (-13%), cortical content (-20%), and cortical thickness (-27%) at the distal femoral metaphysis determined by PQCT (see Figure 9 for total density and cortical content). Either EE alone or LAS alone, or co-treatment of LAS and EE completely prevented OVX-induced decreases in above parameters. Figure 10 illustrates that LAS alone, EE alone, or co-administration of LAS and EE completely prevented the OVX-induced increases in osteoclast number (OC.N/BS), osteoclast surface (OC.S/BS), mineral apposition rate (MAR), mineralizing surface (MS/BS) and bone formation rate-surface referent (BFR/BS). OC.N/BS, OC.S/BS, MS/BS, MAR and BFR/BS in the co-administration group did not differ from either EE alone or LAS alone groups.

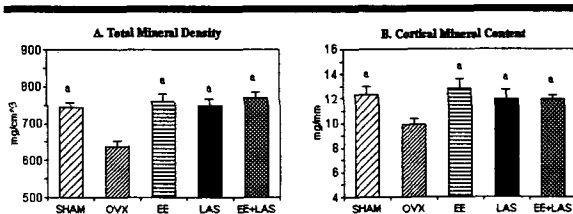


Figure 9: Effects of co-treatment of lasofoxifene (LAS) and 17 α -ethynyl estradiol (EE) on total mineral density and cortical mineral content of distal femoral metaphysis as measured by PQCT in OVX rats. The OVX rats were treated (oral gavage) with either EE at 30 μ g/kg/day or LAS at 100 μ g/kg/day, or co-administration with EE and LAS for 28 days. Error bars represent standard error of mean. a: $p < 0.05$ vs. OVX; b: $p < 0.05$ vs. OVX+EE.

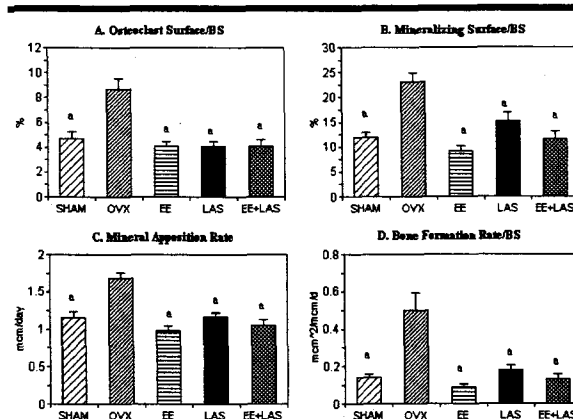


Figure 10: Effects of co-treatment of lasofoxifene (LAS) and 17 α -ethynyl estradiol (EE) on osteoclast surface, mineralizing surface, mineral apposition rate, and bone formation rate-surface referent of proximal tibial metaphysis by histomorphometry in OVX rats. The OVX rats were treated (oral gavage) with either EE at 30 μ g/kg/day or LAS at 100 μ g/kg/day, or co-administration with EE and LAS for 28 days. Error bars represent standard error of mean. a: $p < 0.05$ vs. OVX; b: $p < 0.05$ vs. OVX+EE.

These data support the strategy of using a SERM, such as LAS, for antagonizing the estrogen's uterine effects but maintaining the bone protective effect of estrogen for treatment of postmenopausal osteoporosis.

EFFECTS OF COMBINED TREATMENT OF LAS AND PARATHYROID HORMONE ON OVX RATS

Bone anabolic agents, including prostaglandin E₂ (PGE₂) and parathyroid hormone (PTH), have been shown to restore bone mass and bone strength by increasing activation frequency of bone remodeling units where bone formation exceeds bone resorption in an established osteopenic skeleton (46-48). LAS is a highly potent, orally active SERM, which prevents bone loss by inhibiting bone turnover in OVX rats. We have previously reported that droloxifene, a selective estrogen receptor modulator, did not blunt bone anabolic effects of prostaglandin E₂, but maintained prostaglandin E₂-restored bone in aged, OVX rats (49). In this study, we tested whether combination of an anti-resorptive agent (LAS) with a bone anabolic agent (PTH) would enhance the effects of an anabolic agent in restoring bone mass and strength in an established osteopenic skeleton (OVX rat model) by inhibiting elevated bone turnover associated with anabolic agents.

Sprague-Dawley female rats were sham-operated (n=18) or OVX (n=42) at 6.5 months of age. Sixty days post-surgery, the OVX rats were treated with either bovine PTH-(1-34) at 40 μ g/kg by daily s.c. injection or LAS at 100 μ g/kg by daily oral gavage, or combination of both for 60 days (51).

Compared with sham controls, OVX for 60 days induced significant decreases in trabecular bone volume (TBV, -61%) of proximal tibiae, initial maximal load (-73%) and stiffness (-70%) of distal femora (by indentation test of distal femoral metaphysis), and significant increases in mineralizing surface (+78%), osteoclast perimeter (+100%), osteoclast number (+76%), and bone formation rate/BV (+127%). Treatment with PTH alone restored TBV and initial maximal load and stiffness to the level above the sham controls as shown in Table 1 and Figure 11. Treatment with LAS alone significantly decreased both bone formation and bone resorption and non-significantly increased TBV (+9%) and initial maximal load (+82%) as compared with OVX controls. Combined treatment of PTH and LAS significantly increased TBV (+24%) and initial maximal load (+86%) by decreasing proportionately more bone resorption than bone formation as compared to PTH alone. Both TBV and initial maximal

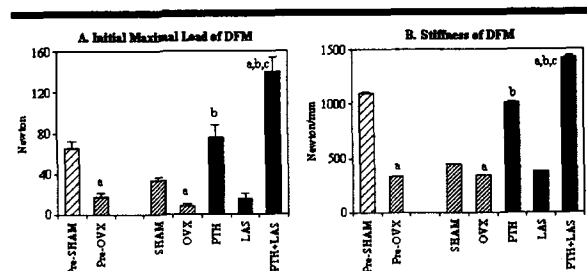


Figure 11: Effects of co-treatment of lasofoxifene (LAS) and parathyroid hormone (PTH) on initial maximal load and stiffness of distal femoral metaphysis by indentation test in OVX rats. Sixty days post-OVX, the rats were treated with either bovine PTH-(1-34) at 40 μ g/kg by daily s.c. injection or LAS at 100 μ g/kg by daily oral gavage, or combination of both for 60 days. Error bars represent standard error of mean. a: $p < 0.05$ vs. SHAM; b: $p < 0.05$ vs. OVX; c: $p < 0.05$ vs. OVX+PTH.

load increased significantly in the combined treatment group when compared to sham controls.

These data showed that the combined treatment of PTH and LAS not only restored bone mass and bone strength to the established osteopenic, OVX rats, but also added extra cancellous bone above that achieved with PTH alone to the proximal tibiae and distal femora of these rats. LAS enhanced the bone restorative effects of PTH by a proportionately greater inhibition of bone resorption than bone formation. These results support the strategy of using anabolic agents such as PTH combined with SERMs such as LAS for treatment of established osteopenia and osteoporosis.

EFFECTS OF COMBINED TREATMENT OF LAS AND PROSTAGLANDIN E₂ ON OVX RATS

Prostaglandin E₂ (PGE₂) restores bone mass by stimulating both bone formation and bone resorption but in favor of bone formation in ovariectomized (OVX) rat skeleton. LAS inhibits bone resorption and prevents bone loss in OVX rats. In this study, we tested whether LAS, a potent anti-resorptive agent, can enhance the bone restoration effects of a sub-optimal dose (1 mg/kg/day) of PGE₂, an anabolic agent, by inhibiting the increased bone resorption associated with PGE₂, therefore, create more positive bone balance in OVX rats.

Sprague-Dawley female rats were sham-operated (n=22) or OVX (n=42) at 3 months of age. Five weeks post-surgery, OVX rats were treated with either vehicle, PGE₂ at 1 mg/kg/d (s.c.), or LAS at 100 µg/kg/d (p.o.), or combined PGE₂ and LAS for 5 weeks (52). Trabecular bone volume (TBV), mineralizing surface (MS/BS), osteoclast number per mm bone surface (Oc.N) and bone formation rate/bone surface referent (BFR/BS) were determined in proximal tibial metaphysis by standard static and dynamic histomorphometric techniques.

As shown in Figure 12, OVX induced a significant decrease in TBV (-33%) and a significant increase in MS/BS (+48%), Oc.N (+39%) and BFR/BS (+114%) at 5 weeks post-surgery as compared to sham controls. A continuous decrease in TBV was seen between 5 weeks and 10 weeks post-surgery in OVX rats (-20%). PGE₂ alone significantly increased TBV as compared with pre-treatment OVX controls (+22%) and OVX controls (+54%). However, TBV in OVX rats treated with PGE₂ was still significantly lower than sham controls (-22%). LAS alone significantly decreased both MS/BS, Oc.N and BFR/BS as compared with OVX controls. TBV in LAS-treated rats was significantly higher than that in OVX controls and did not differ from the pre-treatment OVX controls, indicating that LAS prevented the further trabecular bone loss induced by OVX between 5 to 10 weeks post-surgery. Combination of PGE₂ and LAS increased TBV to the level of sham controls, which was significantly higher than that in PGE₂ or LAS alone treated groups. In the combination treated group, MS/BS significantly decreased by 15% and Oc.N decreased by 69% as compared to PGE₂ alone group, indicating that LAS inhibited more bone resorption than bone formation associated with OVX and PGE₂. Therefore, co-treatment further increased bone mass in the OVX rats when compared to PGE₂ alone. These data showed that LAS inhibited

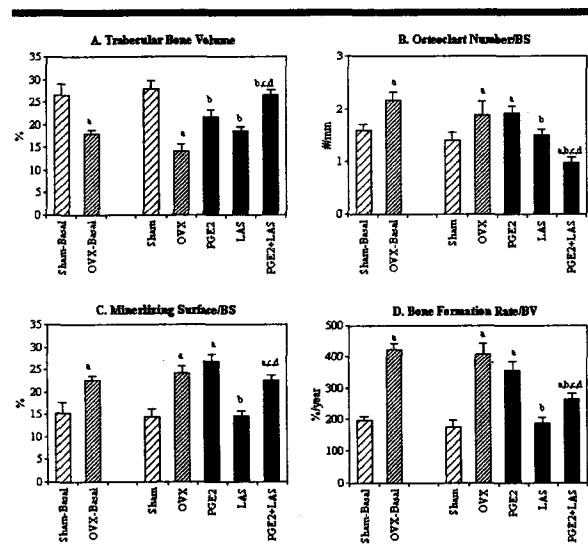


Figure 12: Effects of co-treatment of lasofoxifene (LAS) and prostaglandin E₂ (PGE₂) on trabecular bone volume, osteoclast number, mineralizing surface and bone formation rate/bone volume referent of proximal tibial metaphysis in OVX rats. Five weeks post-OVX, the rats were treated with either PGE₂ at 1 mg/kg by daily s.c. injection or LAS at 100 µg/kg by daily oral gavage, or combination of both for 5 weeks. Error bars represent standard error of mean. a: p < 0.05 vs. SHAM; b: p < 0.05 vs. OVX; c: p < 0.05 vs. OVX+PGE₂; d: p < 0.05 vs. OVX +LAS.

bone resorption and bone turnover, and prevented further bone loss in OVX rats. Furthermore, LAS enhanced the bone restoration effects of PGE₂ in established osteopenic, OVX rats. These results indicated that a combination treatment with an anabolic agent and LAS agent may have more benefits in postmenopausal bone loss than with anabolic agent alone.

EFFECTS OF COMBINED TREATMENT OF LAS AND GROWTH HORMONE SECRETAGOGUE (GHS) ON OVX RATS

Growth hormone increases bone turnover and muscle mass in animals and human as reviewed by Ohlsson et al. (53). An orally active growth hormone secretagogue (GHS), CP-424,391, has been shown to selectively stimulate physiological and secretion of growth hormone *in vivo* (54). However, the effects of GHS on bone mass have not been previously reported. We performed this study to test the effects of GHS (CP-424,391) alone or in combination with LAS on bone mass, muscle mass, total serum cholesterol and uterine weight in established osteopenic, OVX rats. CP-424,391 at 5 mg/kg/day or LAS at 100 µg/kg/day were given alone or in combination by daily oral gavage to OVX rats (surgeries at 3.5 months of age) started at 5.5 months post-surgery for 4 weeks (55). After necropsy, total femoral bone mineral content (TFBMC) was determined *ex vivo* by DEXA. Soleus muscle weight, uterine weight and total serum cholesterol were also determined.

Compared to OVX controls, the GHS and LAS alone significantly increased TFBMC (+8.3% and +7.8%), while the combination of GHS and LAS further increased this parameter (+12.5%), as shown in Figure

13. There was no difference in soleus muscle weight between sham and OVX controls. Treatment with GHS, LAS or combination increased soleus muscle weight significantly by 10.3%, 14.8% and 12.5%, respectively as compared to OVX controls. Interestingly, we found that LAS alone significantly increased soleus muscle weight. The mechanism for this muscle effect of LAS is not known. There was no difference in total serum cholesterol among sham controls, OVX controls and OVX rats treated with GHS. Total serum cholesterol decreased significantly by 49% and 54%, respectively in LAS treated group and combination group compared to OVX controls. No effect on uterine weight was found in GHS or LAS alone groups. However, uterine weight slightly but significantly increased in the combination group compared to OVX controls.

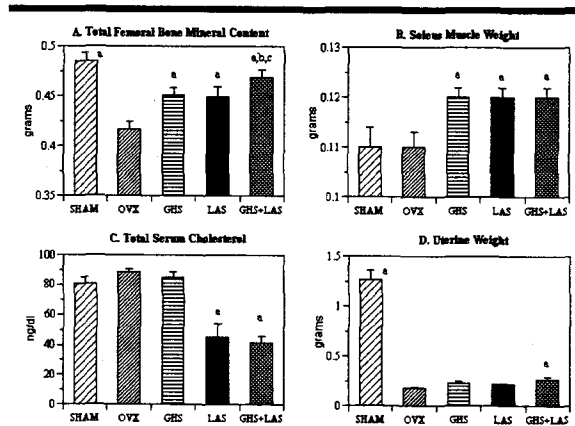


Figure 13: Effects of co-treatment of lasofoxifene (LAS) and growth hormone secretagogue (GHS) on total femoral bone mineral content, soleus muscle weight, total serum cholesterol and uterine weight in OVX rats. Two months post-OVX, the rats were treated by daily oral gavage with either GHS (CP-424,391) at 5 mg/kg or LAS at 100 µg/kg, or combination of both for 4 weeks. Error bars represent standard error of mean. a: $p < 0.05$ vs. OVX, b: $p < 0.05$ vs. GHS, c: $p < 0.05$ vs. LAS.

We conclude that a GHS can increase bone mass in the osteopenic OVX rats. Further, these results suggest that combination of a GHS and LAS may have benefits in an aging patient population. These benefits include significant increases in bone and muscle mass and a reduction in total serum cholesterol.

EFFECTS OF LAS ON ORCHIDECTOMIZED RATS

Osteoporosis and associated fractures are a common skeletal disorder in aging men (56), to the extent that one-third of hip fractures occurs in elderly men (57). Results from clinical investigations indicated that bone loss in elderly men is more significantly correlated with declining estrogen levels than with declining androgen levels (56-60). Thus, estrogen deficiency might play an important pathophysiological role in age-related bone loss in elderly men (61). The orchidectomized (ORX) rat model has been used as a model for male osteoporosis in preclinical studies (62-66). In this study, we tested the effects of LAS in the ORX model to determine whether SERMs have potential in prevention of bone loss in the males.

Sprague-Dawley male rats at 10 months of age were divided into six groups with 10 rats per group. The first group was necropsied at day 0 and served as basal controls. The remaining rats were either sham-operated and treated orally with vehicle, or ORX and treated with either vehicle or LAS at 1, 10, or 100 µg/kg/day for 60 days (28). Total serum cholesterol, prostate weight, distal femoral bone mineral density (DFBMD) by dual-energy x-ray absorptiometry, and trabecular bone volume (TBV) and activation frequency (Act.F) of the third lumbar vertebral body (LV3) by bone histomorphometry were determined. Maximal load of the fifth lumbar vertebral body (LV5) was determined by compression tests (28).

Figure 14 shows the age-related decreases in DFBMD (-9%) and TBV (-13%) in sham-operated rats when compared with basal controls. As shown in Figures 14 and 15, ORX induced significant increases in total serum cholesterol (+31%) and activation frequency of bone turnover (+103%), and significant decreases in prostate weight (-89%), DFBMD (-14%), TBV (-23%), maximal load (-17%) when compared with basal controls. Compared with sham controls, ORX induced significant increases in activation frequency. Compared with ORX controls, ORX rats treated with LAS at 10 or 100 µg/kg/day had significantly lower activation frequency, and significantly higher DFBMD, TBV and maximal load. Further, LAS at 10 and 100 µg/kg/day significantly decreased total serum cholesterol by 46% and 68% in ORX rats, while no effect was found in prostate weight when compared with ORX controls. These data showed that LAS prevented bone loss by inhibiting bone turnover associated with aging and orchidectomy in 10-month-old male rats. Further, LAS decreased total serum cholesterol and did not affect the prostate in these rats. These

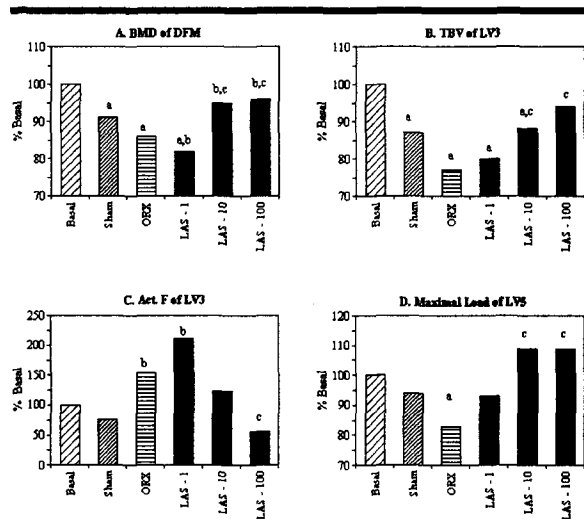


Figure 14: Percent change from basal controls in bone mineral density (BMD) of distal femoral metaphysis (DFM), trabecular bone volume (TBV) and activation of frequency (Act.F) of third lumbar vertebral body (LV3), and maximal load of fifth lumbar vertebral body (LV5) in ORX rats treated with vehicle (sham), or lasofoxifene (LAS) at oral doses of 1, 10, or 100 µg/kg/day for 2 months, beginning 1 day post-ORX. Modified from reference 28. a: $p < 0.05$ vs. Basal controls; b: $p < 0.05$ vs. SHAM; c: $p < 0.05$ vs. ORX.

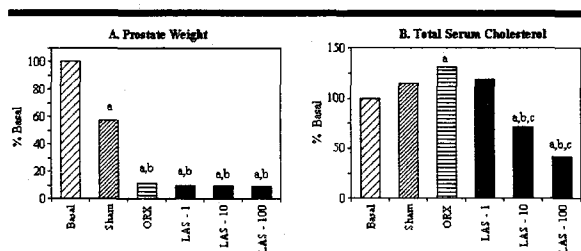


Figure 15: Percent change from basal controls in prostate weight and total serum cholesterol in ORX rats treated with vehicle (sham), or lasofoxifene (LAS) at oral doses of 1, 10, or 100 µg/kg/day for 2 months, beginning 1 day post-ORX. Modified from reference 28. a: p < 0.05 vs. Basal controls; b: p < 0.05 vs. SHAM; c: p < 0.05 vs. ORX.

results suggest that LAS may be a useful therapeutic agent for preventing bone loss in elderly men having some degree of hypogonadism.

EFFECTS OF LAS ON INTACT AGED MALE RATS

In aging males, the loss of gonadal function is more gradual than observed in women in the menopause. Thus, the purpose of this study was to evaluate if long-term (6 months) treatment with LAS can protect against age-related changes in bone mass and bone strength in intact aged male rats. Sprague-Dawley male rats at 15 months of age were treated (daily oral gavage) with either vehicle or LAS at 10 or 100 µg/kg/day for 6 months (29). A group of 15 rats was necropsied at 15 months of age and served as basal controls. LAS at both doses significantly decreased body weight and fat body mass but did not affect lean body mass as compared with vehicle controls (data not shown). No significant difference was found in prostate wet weight among all groups. Total serum cholesterol was significantly decreased in all LAS-treated rats compared with both the basal and vehicle controls. Peripheral quantitative computerized tomographic (pQCT) analysis at the distal femoral metaphysis demonstrated that the age-related decrease in total mineral content was completely prevented by treatment with LAS at 10 or 100 µg/kg/day. Histomorphometric analysis of proximal tibial cancellous bone showed an age-related decrease in trabecular bone volume (-46%) that was completely prevented by treatment with LAS at 10 or 100 µg/kg/day. Moreover, LAS decreased significantly activation frequency of trabecular bone turnover in the proximal tibial metaphysis. Further, LAS at both doses completely prevented the age-related decrease in ultimate strength (-47%) of the fifth lumbar vertebral body. These results demonstrate that treatment with LAS for 6 months in male rats completely prevented the age-related decreases in bone mass and bone strength by inhibiting the increased bone turnover associated with aging. Our data support the potential use of SERMs for protecting against the age-related decrease in bone in elderly men.

EFFECTS OF LAS ON RAT MAMMARY TUMOR MODEL

SERMs such as tamoxifen and raloxifene have been reported to significantly reduce the incidence of breast cancer in women (24,67). A recent report by Cohen and

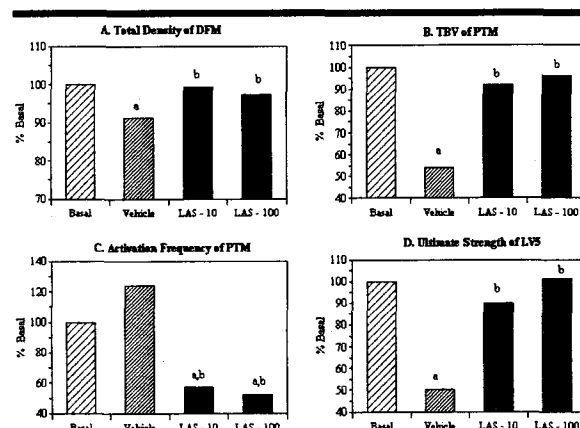


Figure 16: Percent change from basal controls in total density of distal femoral metaphysis (DFM), trabecular bone volume (TBV) and activation frequency of proximal tibial metaphysis (PTM), and ultimate strength of fifth lumbar vertebral body (LV5) in 15-month-old, intact male rats treated with vehicle, or lasofoxifene (LAS) at oral doses of 10 or 100 µg/kg/day for 6 months. Modified from reference 29. a: p < 0.05 vs. Basal controls; b: p < 0.05 vs. vehicle.

co-workers (30) illustrated that LAS had both chemopreventive and chemotherapeutic effects in decreasing tumor growth and was quantitatively comparable with those of tamoxifen in the *N*-nitroso-*N*-methylurea-induced rat mammary tumor model, as shown in Figures 17 and 18. These data reveal that LAS possessed anti-tumor effects similar to tamoxifen in mammary tumor. The potential of LAS in chemoprevention and chemotherapy of breast cancer in human requires further clinical investigation.

SUMMARY AND CONCLUSION

Study results from our *in vivo* and *in vitro* experiments indicated that LAS prevented OVX-induced bone loss in proximal tibial metaphysis and lumbar vertebrae in female rats by inhibiting bone turnover and osteoclastogenesis associated with estrogen deficiency. LAS reduced fat body mass and decreased total serum cholesterol in both young and aged female rats. The bone protective and cholesterol lowering effects of LAS in female rats were identical to those observed with

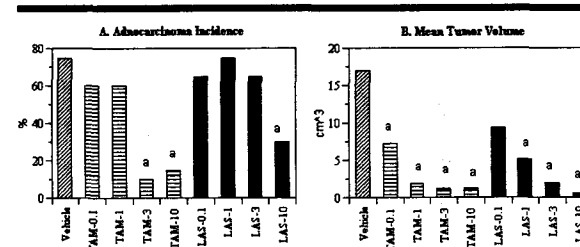


Figure 17: Treatment with tamoxifen (TAM) or lasofoxifene (LAS) produced a marked decrease in NMU-induced tumor appearance. Rats induced to develop mammary tumors with NMU were treated with LAS or TAM at 0.1, 1, 3, or 10 mg/kg at 2 weeks after induction and dosed daily for 8 weeks. TAM or LAS prevented the increases in adenocarcinoma incidence (percentage of rats in the group with at least one adenocarcinoma). Modified from reference 30. a: p < 0.05 vs. Vehicle.

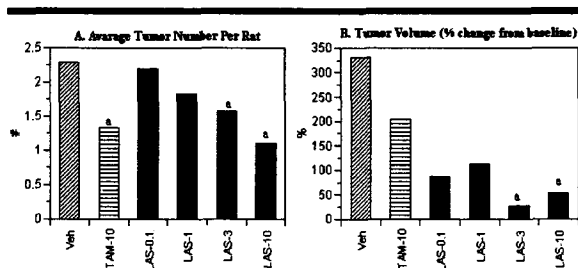


Figure 18: Treatment with tamoxifen (TAM) or lasofoxifene (LAS) decreased average tumor number per rat and tumor volume in NMU-induced tumor rat model. Rats were induced to develop mammary tumors with NMU. During the period of 8 – 15 weeks, treatment with LAS at 0.1, 1, 3, or 10 mg/kg, or TAM at 10 mg/kg was initiated at the appearance of the first tumor equals to or greater than 1cm in diameter. All rats were necropsied at 20 weeks or earlier in case of large necrotic tumors. Modified from reference 30. a: $p < 0.05$ vs. Vehicle.

estrogen. However, LAS diverged from estrogen in its lack of significant estrogenic effects on uterine weight. When co-treatment with estrogen, LAS inhibited the uterine hypertrophy induced by estrogen but maintained the positive bone effects in OVX rats. These data suggest that LAS may be a significant alternative to hormone replacement therapy for prevention and treatment of postmenopausal osteoporosis.

In addition, LAS enhanced the bone restoration effects of anabolic agents such as PTH, PGE₂, GHS in the

established osteopenia, OVX female rat model. These results support the potential strategy for using a combination of an anabolic agent and LAS for the treatment of established osteoporosis in females. In male models of osteoporosis, LAS prevents the bone loss associated with aging and/or orchidectomy in aged male rats without significant increase in prostate weight, indicating that LAS might have therapeutic potential in male osteoporosis. The chemoprevention and therapeutic efficacy of LAS in rat mammary tumor model revealed that LAS may be a useful agent for prevention and treatment of breast cancer in postmenopausal women.

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ABBREVIATIONS

LAS: Lasofoxifene, SERM: Selective Estrogen Receptor Modulator, OVX: Ovariectomy or Ovariectomized, ORX: Orchidectomy or Orchidectomized. ED₅₀: 50% effective dose, ED₁₀₀: fully efficacious dose, IC₅₀: 50% inhibition concentration, EC₅₀: 50% effective concentration.

Table 1. Effects of Combination Treatment of LAS and PTH on Histomorphometric Parameters of Proximal Tibial Trabecular Bone †

Parameters	TBV (%)	Tb.Th (µm)	Tb.N (#/mm)	Oc.N/BS (#/mm)	Oc.S/BS (%)	MS/BS (%)	MAR (µm/day)	BFR/BV (%/yr)
Month 2								
sham controls	27.2±2.3	60±3.1	4.54±0.25	1.2±0.1	5.8±0.8	7.0±0.8	0.93±0.05	82±15
OVX controls	10.7±1.9 a	47±1.3 a	2.22±0.32 a	2.1±0.1 a	11.6±0.8 a	12.4±1.3 a	0.92±0.05	186±30 a
Month 4								
Sham + Vehicle	22.9±1.6	52±1.7	3.83±0.16	1.8±0.1	7.8±0.5	6.1±0.7	0.86±0.04	74±9
OVX + Vehicle	10.2±1.7 a	52±2.7	1.91±0.25 a	2.0±0.1	8.0±0.5	14.4±2.1 a	0.92±0.06	203±45 a
OVX + PTH	29.2±3.3 a,b	109±12 a,b	2.64±0.13 b	1.1±0.1 a,b	4.9±0.7 a,b	38.4±1.7 a,b	1.15±0.06 a,b	302±29 a,b
OVX + LAS	11.2±1.2 a	52±1.7	2.14±0.21 a	1.4±0.1 a,b	6.5±0.7 a,b	9.4±2.1 b	0.63±0.05 a,b	72±16 b
OVX + combination	34.5±2.7 a,b	112±2.9 a,b	3.07±0.2 a,b,c	0.7±0.1 a,b,c	3.5±0.5 a,b,c	35.0±1.1 a,b,c	1.08±0.03 a,b	243±16 a,b

† Mean±SEM; a: $p < 0.05$ vs. sham, b: $p < 0.05$ vs. OVX, c: $p < 0.05$ vs. OVX+PTH, by ANOVA followed by Fisher's PLSD test.

LAS: lasofoxifene at 100 mg/kg/day, oral; PTH: bovine parathoroid hormone-(1-34) at 40 mg/kg/day, s.c. injection.

TBV: trabecular bone volume, Tb.Th: trabecular bone thickness, Tb.N: trabecular bone number, Oc.N/BS: osteoclast number per mm bone surface, Oc.S/BS: % osteoclast surface, MS/BS: % mineralizing surface, MAR: mineral apposition rate, BFR/BV: bone formation rate/bone volume referent.

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