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Comparing the effects of atamestane, toremifene and tamoxifen alone and in combination, on bone, serum lipids and uterus in ovariectomized rats

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ABSTRACT

Complete estrogen blockade remains under investigation as a means to optimize anti-estrogen therapy in breast cancer thus both the efficacy and end-organ toxicities are of interest with combinations. We hypothesized that a steroidal aromatase inhibitor (AI) atamestane (ATA) alone, and in combination with the anti-estrogens tamoxifen (TAM) or toremifene (TOR) would have beneficial effects in ovariectomized (OVX) rats on key end-organ functions including bone and lipid metabolism and on the endometrium. Significant positive effects on bone were noted with ATA, TOR, TAM, ATA + TOR, or ATA + TAM. TOR, TAM, ATA + TOR, or ATA + TAM caused significant decreases in serum cholesterol and low-density lipoprotein cholesterol whereas ATA had no effect. Uterine weight and epithelium lining height were not increased by ATA but were by TOR and TAM. No significant differences were found in the key parameters outlined above between OVX rats given TOR and ATA + TOR, or TAM and ATA + TAM. Our data show that ATA in combination with TOR or TAM is equivalent to TOR or TAM alone in terms of end-organ effects within a range of clinically relevant doses. Further studies of combinations of AIs with anti-estrogens on end-organ function are merited.

1. Introduction

Atamestane (ATA) is a third generation orally bioavailable steroidal aromatase inhibitor (AI) [1–5]. Both selective estrogen receptor modulators (SERMs), tamoxifen (TAM) and toremifene (TOR) are oral, non-steroidal anti-estrogens which compete with estrogen for the estrogen receptors in breast tissue and are used for first-line treatment of hormone dependent advanced breast cancer in patients [6,7]. TOR is a derivative of TAM and has shown anti-tumor activity in patients who have failed prior TAM therapy [8,9]. In postmenopausal women with advanced breast cancer, TOR has been shown to be less tissue-specific than TAM and has partially dose-dependent estrogenic effects [10]. TOR and TAM had tissue-specific and partially dose-dependent estrogenic effects in hypothalamus–pituitary-axis, in the liver and in the vaginal epithelium of postmenopausal women. In some tissues TAM 20 mg/day may be more estrogenic than TOR 60 mg/day.

ATA in combination with TOR was given as a putative "complete estrogen blockade" in a large randomized multicenter Phase 3 clinical trial in which control patients with advanced breast cancer received the non-steroidal AI letrozole [11]. In a prior trial, the ATAC (arimidex, tamoxifen, alone or in combination) adjuvant trial in early stage breast cancer patients, anastrozole plus TAM was inferior to anastrozole given alone [12–14]. In the ATA plus TOR versus letrozole trial time to progression (TTP) of metastatic disease was identical in the two arms. In the ATAC trial the abrogation of the benefit of the AI was presumed to be due to a stimulatory effect of TAM in a low estrogen environment. In vitro and in vivo in a preclinical immature rat uterine model, TOR has been shown to be less agonistic than TAM at low estrogen concentrations [15], possibly borne out by the results of the clinical trial. In the preclinical experiment reported here, we planned to compare the end-organ effects of ATA in combination with TOR and ATA, respectively. These effects cannot be accurately measured in patients with advanced breast cancer and therefore these preclinical results provide important data in the event that a combination of AIs and novel anti-estrogens is considered as adjuvant therapy in women with early stage breast cancer.

For this study, we selected the aged ovariectomized (OVX) Sprague–Dawley rat to mimic the development of estrogen deficiency-induced osteopenia in humans [16–19]. The model is also useful to study the lipid profile resulting from treatment with various endocrine therapies [20]. Our objective was to determine the end-organ effects of ATA, TOR and TAM alone, as well as ATA + TOR and ATA + TAM at all ranges of clinically relevant doses, on bone metabolism, serum lipids and on the uterus.

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2. Materials and methods

2.1. Animals and experimental design

All animals received humane care following study guidelines established by the Massachusetts General Hospital Subcommittee on Research Animal Care. Nine-month-old female Sprague–Dawley rats were obtained from Harlan (Indianapolis, IN, USA). The rats were housed in a pathogen-free environment under controlled conditions of light, humidity and room temperature. They received both food (TD 89222 diet, 0.5% calcium and 0.4% phosphorus; Teklad, Madison, WI, USA) and tap water *ad libitum*. The rats were matched according to body weight and assigned to 13 experimental groups of 12 animals per group: group 1, intact controls; group 2, OVX controls; group 3, OVX + ATA 15 mg/kg; group 4, OVX + TOR 0.01 mg/kg; group 5, OVX + ATA 15 mg/kg + TOR 0.01 mg/kg; group 6, OVX + TOR 0.1 mg/kg; group 7, OVX + ATA 15 mg/kg + TOR 0.1 mg/kg; group 8, OVX + TOR 1 mg/kg; group 9, OVX + ATA 15 mg/kg + TOR 1 mg/kg; group 10, OVX + TAM 0.1 mg/kg; group 11, OVX + ATA 15 mg/kg + TAM 0.1 mg/kg; group 12, OVX + TAM 1 mg/kg; group 13, OVX + ATA 15 mg/kg + TAM 1 mg/kg. Administration of the drugs was initiated 3 days after OVX. ATA (1-methyl-androsta-1,4-diene-3,17-dione) was suspended in a vehicle of 0.9% sodium chloride and 0.085% polyoxyethylene (50) stearate and given twice daily. TOR, and TAM were suspended in 0.5% aqueous methylcellulose 400 and given once daily by oral gavage in a volume of 0.1 ml/100 g of body weight. The animals were weighed weekly and drug doses were adjusted accordingly.

After 16 weeks of treatment, the animals were euthanized by cardiac puncture under ketamine anesthesia. All animals were fasted overnight before blood collection for lipid assays. The uteri were removed for weighing and histological analysis. The whole lumbar spine and femora were excised for bone densitometry, biomechanical testing and bone histomorphometric analyses.

2.2. Bone densitometry

The lumbar spine and left femur of individual rats were scanned by dual energy X-ray absorptiometry using a Lunar PIXImus2 densitometer (GE Medical System Lunar, Madison, WI, USA) with a scan resolution of 0.1 mm × 0.1 mm. Whole left femur and lumbar vertebrae (first through sixth) were placed on a polystyrene tray with water to mimic soft tissue. The bone mineral content (BMC) and area were measured, and bone mineral density (BMD) was calculated automatically as BMC/area (g/cm^2).

2.3. Biomechanical tests

The biomechanical failure properties of the femora and vertebrae were conducted using an Instron 8501 material testing system (Instron Corp., Canton, MA, USA). Force and deformation data were collected at a rate of 25 Hz using a 12-bit data acquisition card (National Instruments, Austin, TX, USA), Labview 5.0 data acquisition software (National Instruments, USA).

The diaphysis of the right femur was tested to failure in three-point bending according to a procedure previously described [21]. Briefly, samples were subjected to a pre-load of 1N and then deformed at a rate of 1 mm/min until failure. The point of failure was defined as a successive drop in load greater than 10%. The body of the fifth lumbar vertebra was tested to failure by unconfined compression using a similar procedure as previously described [22]. Briefly, a pre-load of 2N was applied to the sample and then deformed at a rate of 2 mm/min until failure occurred. The point of failure was defined as a successive drop in load of greater than 5%.

2.4. Bone histomorphometric analysis

Left femora from the rats were cleaned of all soft tissue and fixed in 70% ethanol for 48-h period. Specimens were then dehydrated in graded acetone and embedded in methylmethacrylate. Four-micron sections were cut serially using a microtome (Waltham, MA, USA).

Static histomorphometry was performed on a 4-μm undecalcified Goldner's Trichrome stained section of each proximal femur using a digitizing image analysis system and a morphometric program, OsteoMeasure (OsteoMetrics, Inc., Atlanta, GA, USA), at a magnification of 200×. Measurements of bone were taken from a 15-mm^2 area in the central region beginning 0.2-mm distal to the growth plate. The area selected for measurement covered most of the trabecular bone available for measurement. The following parameters were measured: trabecular bone volume (BV) – percent ratio of trabecular bone volume to total bone volume, mineralized trabecular bone volume (Md.V) – percent ratio of mineralized trabecular bone volume to total bone volume, trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), osteoid volume (OV) – percent ratio of osteoid volume to trabecular bone volume, osteoid surface (OS) – percent ratio of osteoid surface to trabecular bone surface, and osteoid thickness (O.Th). All measurements and calculations were conducted according to the American Society for Bone and Mineral Research (ASBMR) nomenclature and guidelines [23].

2.5. Serum lipid assays

Blood samples were allowed to clot at 4 °C for 2 h, and then centrifuged at 2000 × *g* for 10 min. The serum was transferred to new tubes for lipid assays. Total serum cholesterol (CH), high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol and triglyceride (TG) levels were measured using the Roche Diagnostics' reagents and assayed on a Hitachi 917 Automatic Analyzer (Hitachi, Tokyo, Japan).

2.6. Uterine weight and histology

The uteri were excised, trimmed free of fat, pierced and blotted to remove excess fluid. The body of the uterus was cut just above its junction with the cervix and at the junction of the uterine horns with the ovaries. The uterus was then weighed as wet weight.

10% phosphate buffered formalin-fixed uteri were processed for conventional paraffin embedding. Cross-sections at 4-m thickness were prepared from both horns of each uterus and stained with hematoxylin and eosin. The epithelial lining cells were measured on Eclipse E 800 microscope (Nikon, Japan) equipped with a 40× objective.

2.7. Statistical analysis

Data are expressed as the mean ± standard error of the mean (S.E.M.) of each group. Data were analyzed using a one-way analysis of variance with SAS statistical software (SAS Institute Inc. Cary, NC, USA). Pair-wise comparisons between various groups were performed using a Tukey-Kramer adjustment. Statistical significance was considered at $P < 0.05$.

3. Results

3.1. Bone mineral density (BMD)

The effects of a 16-week treatment on lumbar vertebral BMD are shown in Fig. 1A. Sixteen weeks after ovariectomy, lumbar vertebral BMD was 11.9% lower in OVX rats than in intact controls ($P < 0.01$). At 16 weeks after treatment, lumbar vertebral BMDs were 7.5%, up to

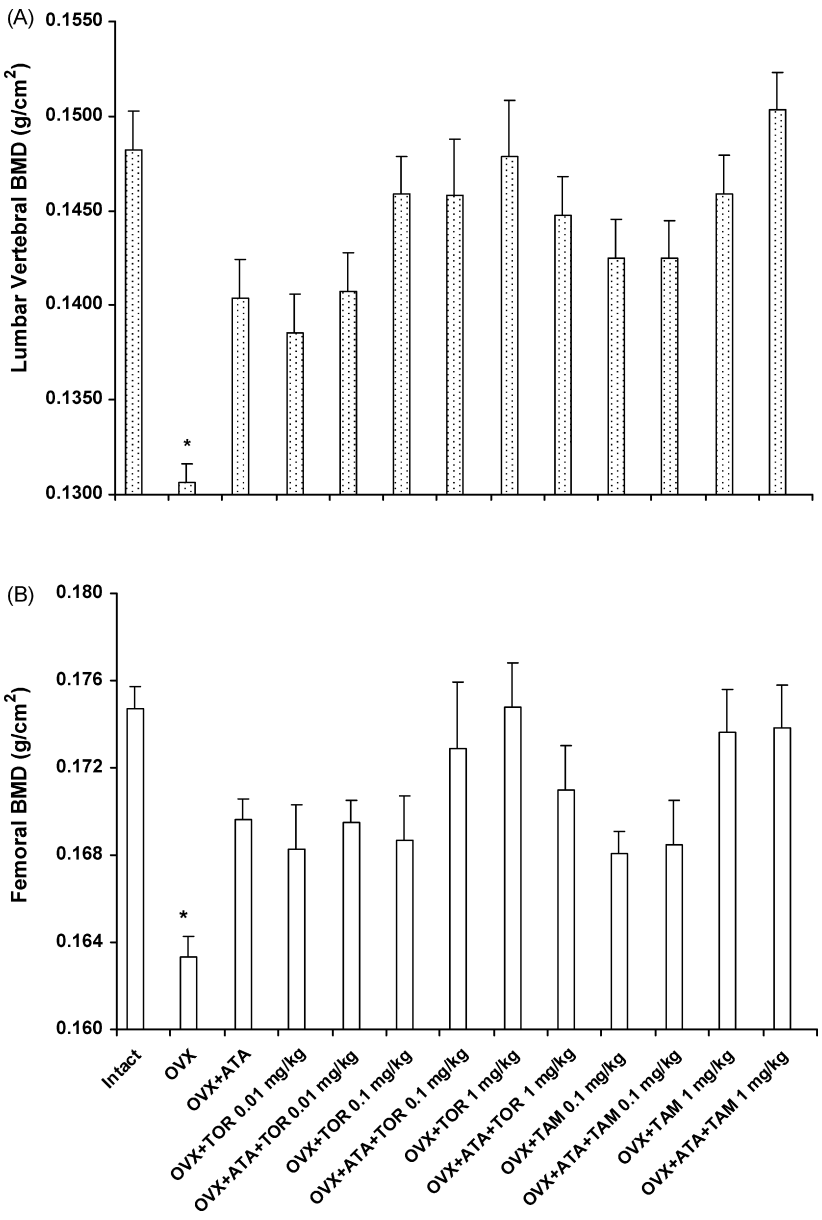

Fig. 1. Bone mineral density of the lumbar vertebrae (A) and left femur (B) after 16 weeks of treatment with ATA, TOR, ATA + TOR, TAM and ATA + TAM. Scale bars represent the mean ± S.E.M., $n = 12$. *Significant difference from all other groups ($P < 0.05$).

13.2%, 14.5%, 14.6% and 15.0%, higher in OVX animals given ATA, TOR, ATA + TOR, TAM, and ATA + TAM, respectively, than in OVX controls (all $P < 0.05$). A similar effect was also observed on femoral BMD measurements (Fig. 1B). Femoral BMD was 6.9% lower in OVX rats than in intact controls ($P < 0.01$). At 16 weeks after OVX, femoral BMDs were 3.9%, up to 7.0%, 5.9%, 6.3% and 6.4%, higher in OVX animals given ATA, TOR, ATA + TOR, TAM, and ATA + TAM, respectively, than in OVX controls (all $P < 0.05$).

No significant differences were seen in lumbar vertebral and femoral BMD between OVX rats treated with all doses of TOR and ATA + TOR, or TAM and ATA + TAM (Fig. 1A and B).

3.2. Biomechanical properties

Ovariectomy or administration with ATA, TOR and TAM alone as well as in combination significantly affected the failure properties of the femur in the three-point bending test (Fig. 2A). Ovariectomy caused a 14.6% decrease in three-point bending strength compared to intact controls ($P < 0.001$). The OVX animals given ATA, TOR, ATA + TOR, TAM, and ATA + TAM had a 7.4%, up to 13.2%, 11.0%, 12.5% and 15.4% increase, respectively, in three-point bending strength compared to OVX controls (all $P < 0.05$). Fig. 2B shows the effects of ATA, TOR, ATA + TOR, TAM, and ATA + TAM on the compressive strength of the fifth lumbar vertebra. Ovariectomy caused a 22.9% decrease in compressive strength ($P < 0.0001$ versus intact controls), which partly recovered with administration of ATA, TOR, ATA + TOR, TAM, and ATA + TAM. Significant increases in compressive strength of the fifth lumbar vertebrae by up to 16.8%, 16.0%, 20.8% and 21.6% (all $P < 0.05$) were seen in the OVX rats given ATA, TOR, ATA + TOR, TAM, and ATA + TAM, respectively.

No significant differences were observed in mechanical failure properties between OVX rats treated with all doses of TOR and ATA + TOR, or TAM and ATA + TAM (Fig. 2A and B).

3.3. Bone histomorphometry

A summary of the structural parameters from the static bone histomorphometry is shown in Table 1. The OVX rats had a sig-

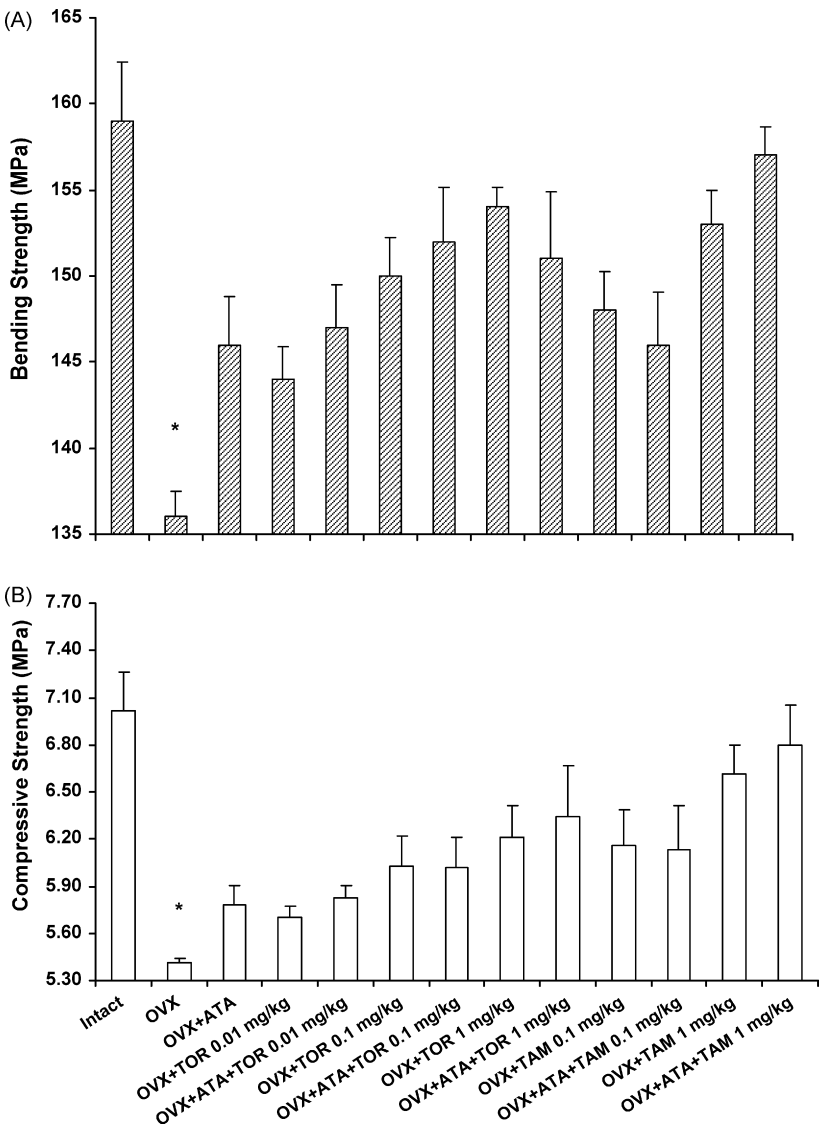

Fig. 2. Mechanical properties of the femora and lumbar vertebrae after 16 weeks of treatment with ATA, TOR, ATA + TOR, TAM and ATA + TAM. Three-point bending strength of the femora (A) and compressive strength of the fifth lumbar vertebrae (B). Scale bars represent the mean ± S.E.M., $n = 12$. *Significant difference from all other groups ($P < 0.05$).

nificantly lower BV, Md.V and Tb.N, as well as significant higher Tb.Sp than the intact controls (all $P < 0.05$). OVX rats treated with ATA, TOR, ATA + TOR, TAM, and ATA + TAM had a significantly higher BV, Md.V and Tb.N, as well as significantly lower Tb.Sp than the OVX controls (all $P < 0.05$). Fig. 3 illustrates the trabecular bone volume (BV) in the proximal femoral metaphysis of intact controls (Fig. 3a), OVX controls (Fig. 3b), OVX rats treated with ATA (Fig. 3c), TOR (Fig. 3d), ATA + TOR (Fig. 3e), TAM (Fig. 3f) and ATA + TAM (Fig. 3g). The reduced amount of trabecular bone in OVX controls comparative with intact control rats, and the significant increase of OVX-induced loss of trabecular bone volume after 16 weeks of treatment with ATA, TOR and TAM alone, as well as in combination were observed.

The osteoid results, a measure of bone formation, were also summarized in Table 1. The osteoid volume (OV) and osteoid surface (OS) in the OVX rats were significantly higher than in the intact

Table 1
Histomorphometric values of the various groups.

Group	BV (%)	Md.V (%)	Tb.N (mm^{-1})	Tb.Sp ((m)	Tb.Th ((m)	OV (%)	OS (%)	O.Th ((m)
Intact	16.35 ± 0.63	16.33 ± 0.72	2.18 ± 0.11	383.4 ± 12.7	75.1 ± 3.1	0.16 ± 0.03	0.58 ± 0.06	10.0 ± 0.29
OVX	10.12 ± 0.31[a]	9.66 ± 0.27[a]	1.39 ± 0.08[a]	644.9 ± 20.6[a]	72.5 ± 2.6	4.28 ± 0.43[a]	13.11 ± 0.73[a]	12.5 ± 0.61
OVX + ATA (15 mg/kg)	15.12 ± 0.37	15.06 ± 0.39	2.21 ± 0.13	385.7 ± 11.2	68.7 ± 4.2	0.35 ± 0.04	1.39 ± 0.05	8.9 ± 0.32
OVX + TOR (0.1 mg/kg)	16.95 ± 0.69	16.91 ± 0.56	2.42 ± 0.07	343.4 ± 14.5	70.1 ± 3.9	0.24 ± 0.05	0.97 ± 0.04	9.1 ± 0.31
OVX + ATA (15 mg/kg) + TOR (0.1 mg/kg)	16.61 ± 0.63	16.57 ± 0.70	2.35 ± 0.08	355.7 ± 12.7	71.9 ± 4.8	0.18 ± 0.02	0.67 ± 0.03	10.5 ± 0.59
OVX + TAM (0.1 mg/kg)	16.64 ± 0.57	16.60 ± 0.61	2.22 ± 0.06	366.6 ± 13.3	72.5 ± 3.7	0.27 ± 0.06	1.05 ± 0.04	9.3 ± 0.28
OVX + ATA (15 mg/kg) + TAM (0.1 mg/kg)	16.26 ± 0.23	16.24 ± 0.34	2.28 ± 0.03	367.4 ± 11.2	71.4 ± 3.9	0.12 ± 0.03	0.38 ± 0.05	12.3 ± 0.39

Data are mean ± S.E.M., $n = 5$.
[a] Significant difference from all other groups ($P < 0.05$).

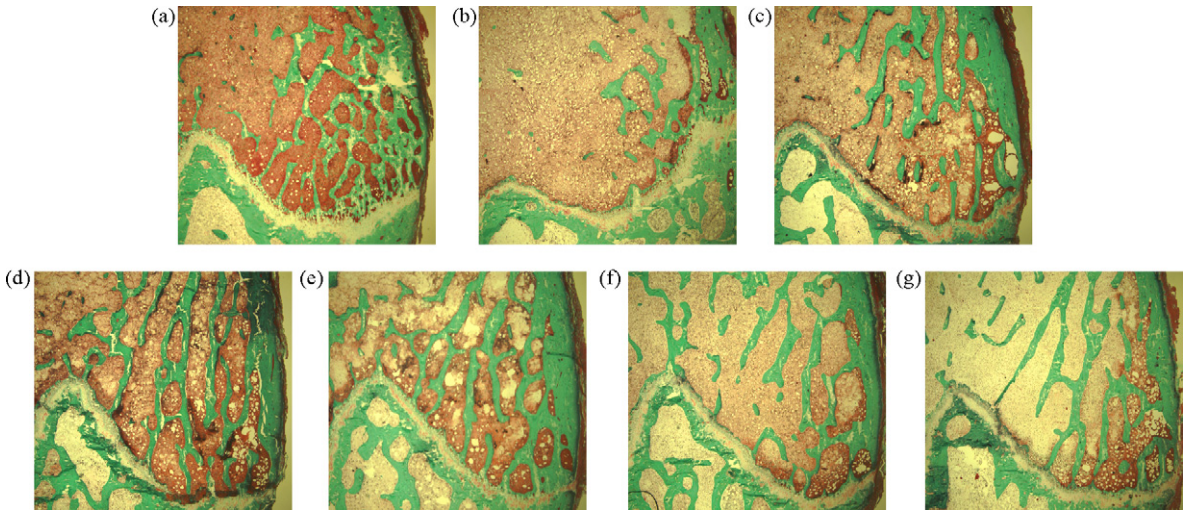

Fig. 3. Goldner's trichrome-stained sections of rat proximal femur metaphysis obtained from intact controls (a), OVX controls (b), OVX rats treated with ATA 15 mg/kg (c), TOR 0.1 mg/kg (d), ATA 15 mg/kg + TOR 0.1 mg/kg (e), TAM 0.1 mg/kg (f) and ATA 15 mg/kg + TAM 0.1 mg/kg (g). Note the reduced amount of trabecular bone in OVX controls comparative with intact control rats, and the significant increase of OVX-induced loss of trabecular bone volume after 16 weeks of treatment with ATA, TOR, TAM along and in combination were observed (magnification of 200×).

controls and in OVX rats administered ATA, TOR, TAM alone and in combination (all $P < 0.05$).

No significant differences were observed in bone histomorphometric parameters between OVX rats treated with all doses of TOR and ATA + TOR, or TAM and ATA + TAM (Table 1).

3.4. Serum lipids

The effects of ATA, TOR, ATA + TOR, TAM, and ATA + TAM on serum lipids are presented in Table 2. Sixteen weeks after ovariectomy, a 34.3% increase in total serum CH, a 28.4% increase in serum LDL and a 12.8% increase in serum TG, respectively, were observed in OVX rats compared to intact controls (all $P < 0.05$). A 16-week administration of TOR, ATA + TOR, TAM, and ATA + TAM to OVX rats caused up to 45.8%, 42.4%, 40.6% and 39.7% reduction, respectively, in serum CH levels compared to the OVX controls (all $P < 0.05$), as well as up to 75.6%, 77.4%, 81.9% and 80.9% reduction, respectively, in serum LDL levels compared to the OVX controls (all $P < 0.05$). After 16-week treatment, OVX rats given TOR, and ATA + TOR had up to a 40.3% and 39.6% decrease, respectively, in serum TG levels compared to OVX controls (all $P < 0.05$).

No significant differences were observed in serum CH and LDL between OVX rats treated with all doses of TOR and ATA + TOR, or TAM and ATA + TAM (Table 2).

3.5. Uterine wet weight and epithelial lining cells

The effects of ATA, TOR, ATA + TOR, TAM, and ATA + TAM on uterine weight in OVX rats are shown in Table 2. Sixteen weeks after ovariectomy, a 72% decrease in uterine wet weight compared to intact controls ($P < 0.01$) was observed. In contrast, OVX rats receiving TOR, ATA + TOR, TAM, and ATA + TAM had an increase in uterine wet weight of up to 54%, 46%, 67% and 58%, respectively, compared to the OVX control animals (all $P < 0.01$). No significant change was observed in uterine wet weight in OVX rats receiving ATA. There were no significant differences between OVX rats treated with all doses of TOR and ATA + TOR, or TAM and ATA + TAM in uterine wet weight.

In Fig. 4 uterine histology illustrates the absence of a stimulatory effect of a 16-week treatment with ATA (Fig. 4c) on the uterine epithelium when compared to OVX controls (Fig. 4b). However, hypertrophy of uterine epithelium was observed after a 16-week treatment with TOR (Fig. 4d and f), ATA + TOR (Fig. 4e and g), TAM

Table 2
Body weight gain, uterine wet weight and serum lipid levels.

Group	Body weight gain (g)	Uterine weight (mg)	CH (mg/dL)	LDL (mg/dL)	TG (mg/dL)	HDL (mg/dL)
Intact	20.9 ± 7.4	525.3 ± 19.5	101.2 ± 5.7[a]	30.6 ± 5.7[a]	55.9 ± 4.6[a]	59.4 ± 3.3[a]
OVX	89.4 ± 5.1[b]	145.9 ± 5.2[b]	135.9 ± 4.1	39.3 ± 4.7	63.1 ± 5.1	84.1 ± 4.2
OVX + ATA (15 mg/kg)	53.7 ± 5.8	141.3 ± 4.4	129.3 ± 3.6	41.1 ± 6.2	66.7 ± 3.2	74.9 ± 4.8
OVX + TOR (0.01 mg/kg)	39.1 ± 4.5	186.3 ± 3.6	99.5 ± 2.8[a]	20.7 ± 5.3[a]	45.6 ± 3.1[a]	69.5 ± 5.7[a]
OVX + ATA (15 mg/kg) + TOR (0.01 mg/kg)	48.3 ± 5.4	178.5 ± 3.8	93.6 ± 2.1[a]	13.3 ± 5.1[a]	51.5 ± 4.2[a]	70.0 ± 4.9[a]
OVX + TOR (0.1 mg/kg)	10.7 ± 4.0	208.3 ± 2.8	87.5 ± 4.5[a]	11.1 ± 3.2[a]	50.3 ± 3.3[a]	66.4 ± 3.6[a]
OVX + ATA (15 mg/kg) + TOR (0.1 mg/kg)	15.3 ± 4.5	201.7 ± 2.1	83.3 ± 3.2[a]	12.2 ± 4.1[a]	52.6 ± 4.1[a]	60.7 ± 3.1[a]
OVX + TOR (1 mg/kg)	−1.9 ± 3.3	224.6 ± 4.5	73.7 ± 2.6[a]	8.9 ± 1.9[a]	37.7 ± 3.7[a]	57.1 ± 2.8[a]
OVX + ATA (15 mg/kg) + TOR (1 mg/kg)	−1.3 ± 1.9	212.8 ± 3.4	78.3 ± 3.2[a]	9.6 ± 2.9[a]	38.1 ± 3.5[a]	60.9 ± 2.9[a]
OVX + TAM (0.1 mg/kg)	−11.2 ± 3.6	241.8 ± 3.9	79.2 ± 2.9[a]	8.3 ± 3.1[a]	69.2 ± 4.7	56.9 ± 3.4[a]
OVX + ATA (15 mg/kg) + TAM (0.1 mg/kg)	0.25 ± 5.6	226.4 ± 6.8	82.0 ± 1.3[a]	8.1 ± 3.7[a]	71.7 ± 4.6	59.5 ± 4.1[a]
OVX + TAM (1 mg/kg)	−8.8 ± 2.0	242.5 ± 7.6	80.7 ± 3.6[a]	7.1 ± 1.8[a]	73.1 ± 4.1	58.9 ± 3.9[a]
OVX + ATA (15 mg/kg) + TAM (1 mg/kg)	−7.4 ± 4.3	229.8 ± 6.9	84.1 ± 3.3[a]	7.5 ± 2.2[a]	75.6 ± 3.9	61.5 ± 4.3[a]

Data are mean ± S.E.M., 12 rats per group.
[a] $P < 0.05$ vs. OVX controls or OVX + ATA.
[b] Significant difference from all other groups ($P < 0.01$).

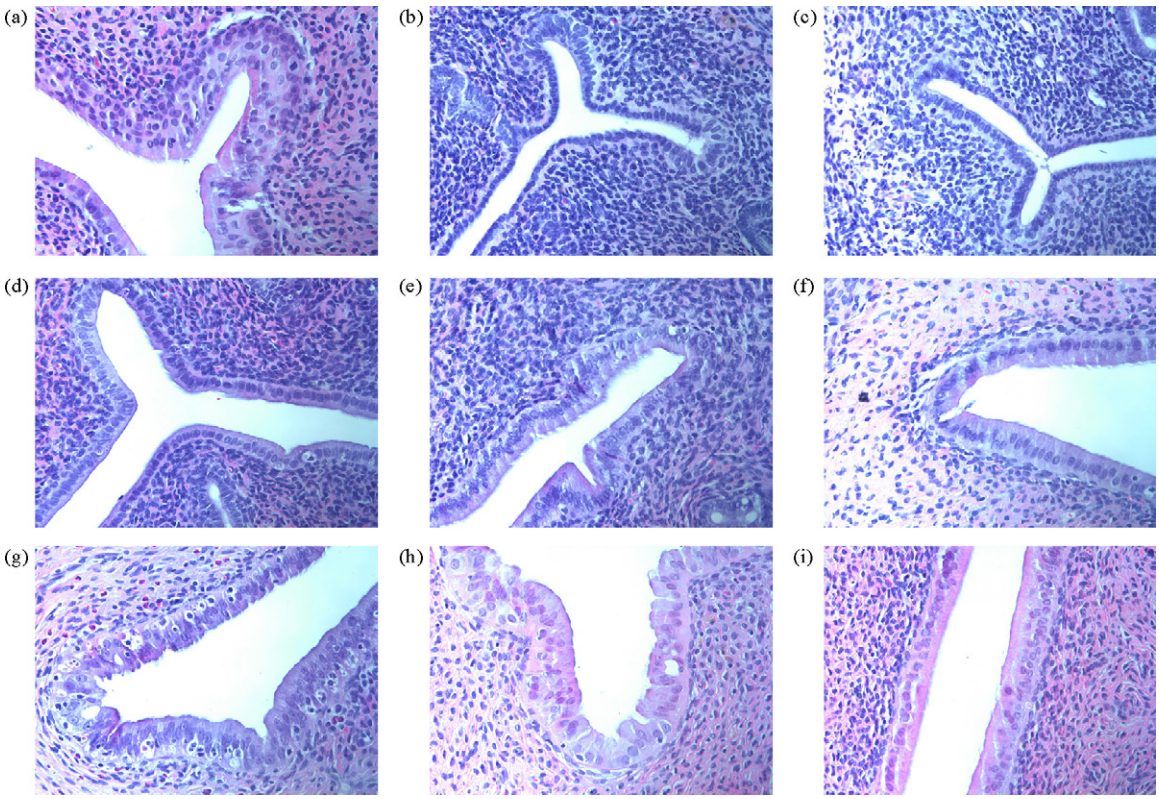

Fig. 4. Hematoxylin- and eosin-stained sections of rat uteri illustrating epithelial lining cells obtained from intact controls (a), OVX controls (b), OVX rats treated for 16 weeks with ATA 15 mg/kg (c), TOR 0.01 mg/kg (d), ATA + TOR 0.01 mg/kg (e), TOR 0.1 mg/kg (f), ATA 15 mg/kg + TOR 0.1 mg/kg (g), TAM 0.1 mg/kg (h) and ATA 15 mg/kg + TAM 0.1 mg/kg (i). Note the absence of stimulatory effect of ATA on the epithelial cells comparative with the OVX controls while hypertrophic effects of TOR and TAM on the uterine epithelial cells was observed in the OVX rat (magnification of 200×).

(Fig. 4h), and ATA + TAM (Fig. 4i). This stimulatory effect correlated with the increase in uterine wet weights observed.

3.6. Gain in body weight

As shown in Table 2, 16 weeks after ovariectomy, gain in body weight in OVX rats was significantly greater than that seen in intact controls (89 g versus 21 g). However, ATA, TOR, ATA + TOR, TAM, and ATA + TAM significantly reduced the gain in body weight seen in OVX controls (all $P < 0.01$). No significant differences in gain in body weight were observed between OVX rats treated with all doses of TOR and ATA + TOR, or TAM and ATA + TAM.

4. Discussion

Our long-term intention is to explore the potential of combining aromatase (estrogen synthetase) inhibitors with estrogen receptor antagonists with a view to effecting a "complete estrogen blockade". Our hope is that a combination such as this will be superior to either class of agent given alone in women with hormone dependent breast cancer. We hypothesized that an AI in combination with a less agonistic anti-estrogenic SERM would be more effective than previous studies of an AI in combination with TAM proved to be in patients. A patent protecting specifically the combination of the steroidal AI ATA in combination with SERMS led us to explore this AI in combination. While a number of newer SERMS as alternative to TOR are available, it was necessary for the purpose of our clinical trial to employ a SERM that was an approved treatment for first-line metastatic breast cancer. Thus we chose TOR, as it is a standard first-line treatment and also had been shown to exert a less estrogen agonist signal in the presence of estrogen depletion than TAM. The results of our clinical trial, published recently [11], proved the combination of ATA plus TOR to be equivalent to letrozole as first-line treatment. The rat experiment reported here was designed to understand the end-organ effects of these agents alone (ATA, TAM, TOR) and in combination (ATA plus TAM, as well as ATA plus TOR) as these too will potentially provide additional rationale for pursuing such combination therapies in patients.

Our results reported here confirm that as monotherapy in the OVX rat model, TOR and TAM have positive and beneficial influences on bone and lipid metabolism in as far as they significantly increase BMD and trabecular bone volume, enhance bone mechanical strength, as well as significantly reduce the rise in serum CH, LDL and TG induced by OVX. As monotherapy TOR has a significant and equivalent stimulatory effect on uterine epithelium as TAM. These monotherapy findings are consistent with published studies in this rat model [24–30].

With respect to our findings on lipid metabolism in this OVX rat model it is important to understand the changes in HDL levels in rats compared with those in humans. In both humans and rodents, estrogen lowers cholesterol by upregulating the hepatic LDL receptor, thus resulting in an increased removal of serum cholesterol from the circulation [31]. This effect results in a preferential reduction of LDL cholesterol in humans. However, in the rat, both HDL and LDL cholesterol are reduced, because rat HDL contains apoprotein E (not found in human HDL), which also binds to the hepatic LDL receptor [32]. Thus, in the rat, as opposed to humans, HDL cholesterol is a predominant form of circulating cholesterol, and estrogen therapy lowers both HDL cholesterol and LDL cholesterol. Thus the reduction in both HDL and LDL seen with the administration of TOR or TAM in the OVX rat is in an expected estrogen agonist direction.

ATA given as monotherapy to OVX rats significantly increased BMD, bone mechanical strength and trabecular bone volume. Unlike similar effects in this model with the steroidal AI exemes-

tane, our previous study [30] showed these bone preserving effects are not blocked by the androgen receptor blocker flutamide [33] and thus appear to be non-androgenic [4,30,34,35]. The mechanism of bone preserving effects of ATA remains unexplained. ATA monotherapy also had no stimulatory effect on the uterine epithelium in OVX rats indicating an absence of an intrinsic estrogenic signal on this tissue.

When considering our underlying hypothesis that TOR would be less estrogen agonistic on end-organ function in combination with an AI than TAM, it should be noted that there were no differences on any end-organ effects between ATA + TAM and ATA + TOR. Thus both combinations were equally bone sparing, lowered serum CH and LDL levels equivalently and had a similar stimulatory effect on the endometrial lining.

In summary, ATA plus TOR demonstrated changes in end-organ function that were no different from those seen with ATA in combination with TAM. The effects on end-organs in these experiments confirm the OVX rat as an appropriate preclinical model for evaluation of endocrine changes in postmenopausal women. The changes seen in lipid metabolism, while in a different direction from those in humans, are consistent and explainable as outlined above, and do not negate the usefulness of this OVX rat model. The major unexpected finding in our study is the positive benefits of ATA on bone metabolism that appear not to be due to androgenic properties. As indicated above our overall goal is to determine whether an AI can be combined with an anti-estrogen such as a SERM or a SERD (selective estrogen receptor down-regulator). The hope is that anti-cancer effects will be enhanced by a combination and that end-organ toxicities will be acceptable or beneficial. We showed in our previous manuscript that the steroidal AI exemestane has a positive effect on bone metabolism distinct from letrozole the non-steroidal AI. We are currently testing these differences in a large clinical trial of over 7000 patients comparing exemestane, a steroidal AI to anastrozole, a non-steroidal AI. Our two manuscripts indicate that the steroidal inhibitors have positive effects on bone metabolism in the OVX rat model. While exemestane appears to exert this effect by virtue of an androgenic effect, it remains unclear as to why atamestane does this. Clinical studies will help to clarify these findings and help to determine optimal combinations of endocrine therapies for testing.

Taken together with our previously published clinical results, it is unlikely that ATA plus TOR will be pursued for further clinical development. However, there are two ongoing clinical trials of the AI anastrozole in combination with the "pure anti-estrogen" fulvestrant, an estrogen receptor down-regulator, and the outcome of the clinical efficacy of this combination versus anastrozole alone is eagerly awaited. The end-organ effects of this novel combination will also play a role in ascertaining its overall therapeutic index of efficacy versus toxicity and thereby its potential as a novel therapy in women with hormone dependent early stage breast cancer.

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