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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