DT56a stimulates gender-specific human cultured bone cells in vitro

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Abstract

DT56a found to have SERM-like properties is used for the treatment of menopausal symptoms and osteoporosis. In vivo experiments demonstrated that DT56a displayed selective estrogenic activity; it stimulated creatine kinase (CK) specific activity in the skeletal tissues but not on the uterus of ovariectomized rats. DT56a, when applied together with estradiol-17β (E2), completely inhibited the E2-stimulated CK, as demonstrated by other SERMs. DT56a stimulated bone formation in a rat model as measured by histological and histomorphometrical parameters. In a clinical study, administration of DT56a (Femarelle) resulted in a considerable elevation of bone mineral density and relief of menopausal symptoms. The aim of the present study was to analyze the effects of DT56a in vitro on human-derived bone cultured osteoblasts (Ob), by measuring its effects, at different concentrations, on DNA synthesis, CK and alkaline phosphatase (ALP) specific activities as well as changes in intracellular [Ca2+] i concentrations. DT56a stimulated CK and DNA synthesis in both pre- and post-menopausal female Ob with maximal effect at 100 ng/ml for both age groups. In addition, DT56a stimulated ALP in Ob from both pre- and post-menopausal women with maximal effect at lower dose of 50 ng/ml, with higher response of pre-menopausal cells. Raloxifene (Ral) inhibited all DT56a-stimulated changes in Ob from both age groups. DT56a, when given together with E2, completely antagonized E2-stimulated effects demonstrating its nature as a phyto-SERM. DT56a also, dose dependency, stimulated the intracellular levels of [Ca2+] i with maximal effect at 10 ng/ml. Male-derived Ob did not respond to DT56a in any parameter. In summary, DT56a stimulated sex-specifically female-derived Ob, indicating its unique nature compared to the compounds currently used for postmenopausal osteoporosis by being bone-forming and not only an anti-resorptive agent.

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1. Introduction

Estrogen is well known for its beneficial effect on osteoporosis [1,2]. Of the 10 million Americans estimated to have osteoporosis, 8 million are women and 2 million are men. Thirty-four million Americans have low bone mass, which puts them at increased risk of developing osteoporosis and related fractures. One in two women and one in four men over age 50 will have an osteoporosis-related fracture in her/his remaining lifetime. Osteoporosis is the cause of some 300,000 hip fractures each year (www.nof.org). Osteoporosis is characterized by reduction in bone mineral density, with the result of fracture after minimal trauma. The effects of estrogen on a tissue is initiated by its binding to estrogen receptor (ER) in the responsive cell. The estrogen-ER complex is then translocated into the nucleus, where it binds to the DNA and modulates the rate of transcription of specific genes. Two ERs have been identified, ERα and ERβ, which differ in their structure and tissue distribution [3] and their biological effect [4]. The two key factors that control tissue selectivity for the estrogen are the structure of its receptor(s) and its interaction with co-regulators [5–6]. However, estrogen can also have non-desired effects that contribute to the development and growth of estrogen-dependent tumors, such as breast cancer [7]. These ominous side effects led to...
extensive research aimed at finding compounds with beneficial estrogenic effects on selected sites, such as the bones [8], and the cardiovascular system [9] without the harmful side effects.

Selective estrogen receptor modulators (SERMs) exhibit a pharmacological profile characterized by estrogen agonist activity in some tissues with estrogen antagonist activity in other tissues [10,11]. Raloxifene functions as an estrogen agonist in skeletal tissues and in the cardiovascular system but not in the uterus. It functions also as an antagonist in the presence of estrogen in skeletal and vascular tissues and in the uterus as well as breast cancer cells in vitro [12,13].

We used the induction of brain type isozyme of creatine kinase specific activity (CK) as a response marker of estrogenic activity [14], which is stimulated by estrogens both in vivo and in vitro in tissues and in cells containing active ER(s) [15–17].

DT56a (Femarelle, Se-cure Pharmaceuticals, Dalton, Israel) is a unique enzymatic isolate of soybeans. Femarelle has been shown to increase bone mineral density in post-menopausal women [18] and to relieve vasomotor symptoms with no effect on sex hormone levels or endometrial thickness [19]. Its properties as a SERM were shown in previous in vivo and in vitro studies [10,11]. Its properties as a SERM were shown in previous studies: Rats were fed for 4 days with estradiol-17β (E2) or DT56a. Both compounds increased CK activity in the different skeletal and vascular tissues [21]. On the other hand, feeding with DT56a, unlike E2 did not result in CK stimulation in the uterus [20]. Raloxifene (Ral) blocked the stimulation of CK by either DT56a or E2 in all tissues tested, pointing towards a common mechanism of action, utilizing the same type of receptor(s) [20,21].

We also found that DT56a when given simultaneously with E2 inhibited E2’s activity in rat skeletal tissues, vascular tissues and in the uterus, in both immature and ovariectomized female rats (submitted for publication). This phenomenon of mutual annihilation of action between E2 and a SERM such as tamoxifen, tamoxifen methiodide or raloxifene, was previously described in prepubertal female rat tissues and cells in culture [22,23].

In the present study, we assessed the effects of DT56a in vitro on cultured human osteoblasts derived from three different groups: males, pre-menopausal and post-menopausal women. The stimulation of creatine kinase specific activity was measured since it is a convenient estrogen response marker and is induced by estrogens in vivo and in vitro [15–17]. We also measured cell proliferation by [3H] thymidine incorporation (DNA) [15] and also the stimulation of alkaline phosphatase specific activity (ALP), which is a marker of osteoblast activity [24]. We also investigated the early effects of DT56a, which recognized the estrogen receptor, on the interaction with non-genomic ER putative binding sites measuring its effect on cytosolic free calcium concentration ([Ca2+]i) in human female osteoblasts [25]. These actions were compared to E2 which was shown by us to stimulate [Ca2+]i, within 5 s by both influx through voltage-gated channels as well as mobilization from the endoplasmic reticulum [25]. The genomic and non-genomic actions of DT56a were compared to the known membrane and genomic effects of E2.

2. Materials and methods

2.1. Reagents

All reagents were of analytical grade. Chemicals were purchased from Sigma (St. Louis, MO). Raloxifene was extracted from Evista® tablets. DT56a was provided by Secure Pharmaceuticals, as a powder and was dissolved in 1% ethanol in saline.

2.2. Cell cultures

Human bone cells from pre- and post-menopausal women or men, with no known health problems and no medical treatments, were prepared from bone explants, by a non-enzymatic method as described previously [16]. Samples of the trabecular surface of the iliac crest or long bones were cut into 1 mm3 pieces and repeatedly washed with phosphate buffered saline to remove blood component. The explants were incubated in DMEM medium without calcium (to avoid fibroblastic growth) containing 10% fetal calf serum (FCS) and antibiotics. First passage cells were seeded at a density of 3 × 10^5 cells/35 mm tissue culture dish, in phenol red-free DMEM with 10% charcoal stripped FCS, and incubated at 37 °C in 5% CO2. The cells were characterized for osteoblastic features as described before [16].

2.3. Creatine kinase extraction and assay in human osteoblasts

Cells were treated for 24 h with the various agents as specified, scraped off and homogenized by freezing and thawing three times in an extraction buffer, as previously described [15–17]. Supernatant extracts were obtained by centrifugation at 14,000 × g for 5 min at 4 °C in an Eppendorf micro-centrifuge. Creatine kinase activity was assayed by a coupled spectrophotometric assay, as previously described [15–17]. Protein was determined by Coomasie blue dye binding, using bovine serum albumin (BSA) as the standard.

2.4. Alkaline phosphatase extraction and assay in human osteoblasts

After treatment with the different agents, cells were collected and homogenized by freezing and thawing three times in cold PBS and the soluble ALP was obtained by centrifugation at 14,000 × g [24]. Enzyme activity was assayed in an ELISA reader, measuring the hydrolysis of p-nitrophenyl phosphate at 37 °C in a buffer containing 2 mM MgCl2 and 100 mM 2-amino-2-methylisopropanol at pH 10.3. The reaction was stopped with 1 M NaOH and the extracts were
analyzed at 410 nm. Enzyme specific activity was determined as $OD \times 4.3/mg$ protein [24].

2.5. DNA synthesis in human osteoblasts

Cells were grown until sub confluence and then treated with various hormones or agents as indicated. Twenty-two hours following the exposure to these agents, $[^{3}H]$ thymidine was added for 2h. Cells were then treated with 10% ice-cold trichloroacetic acid (TCA) for 5 min and washed twice with 5% TCA cold with ethanol. The cellular layer was dissolved in 0.3 ml of 0.3N NaOH, samples were aspirated and $[^{3}H]$ thymidine incorporation into DNA was determined [15–17].

2.6. Intracellular calcium concentration

Cells grown on cover slips were washed with Hanks’ Hepes, pH 7.4 buffer, and loaded with $[^{1}H]9262M$ Fura-2/AM for 40 min in the same buffer at room temperature. The glass cover slips carrying the cells were inserted into a cuvette containing Hanks’ Hepes, pH 7.4 buffer. The cuvette was placed in a calorimeter (37 °C) Hitachi F-2000 spectrofluorimeter. DT56a, E$_{2}$ or vehicle alone were added directly to the cuvette under continuous stirring. The Fura-2/AM fluorescence response to intracellular calcium concentration ([Ca$^{2+}$]i) was calibrated from the ratio of 340–380 nm fluorescence values after subtraction of the background levels. The values for $R_{\max}$ and $R_{\min}$ were calculated from measurements using 25$^{[^{1}H]}262M$ digitonin and 4 mM EGTA and enough Tris base to raise the pH to 8.3 or higher [25].

2.7. Statistical analysis

Differences between the mean values of experimental and control group were evaluated by analysis of variance (ANOVA). $p$ values less than 0.05 were considered significant.

3. Results

3.1. The effects of DT56a on CK specific activity in human female bone cells

To determine their responsiveness to DT56a compared to E$_{2}$, human bone cells were incubated with 0.3–300 nM E$_{2}$, or 1–250 ng/ml DT56a. DT56a, similar to E$_{2}$ treated cells, showed an increase in CK specific activity, in both pre- and post-menopausal women (Fig. 1). The increase in the E$_{2}$ treated cells was greater in the pre-menopausal compared to post-menopausal women.

3.2. The effects of DT56a on DNA synthesis in human female bone cells

To determine their responsiveness to DT56a compared to E$_{2}$, human female bone cells were incubated with 0.3–300 nM E$_{2}$, or 1–250 ng/ml DT56a. A similar increase in thymidine incorporation was found in both DT56a and E$_{2}$ groups, in both pre- and post-menopausal cells (Fig. 2).

3.3. The effects of DT56a on ALP specific activity in human female bone cells

To determine their responsiveness to DT56a compared to E$_{2}$, human female bone cell were incubated with 0.3–300 nM E$_{2}$, or 1–200 ng/ml DT56a. A similar increase in thymidine incorporation was found in both DT56a and E$_{2}$ groups, in both pre- and post-menopausal cells (Fig. 2).
Fig. 3. Stimulation by E\textsubscript{2} (0.082–82 ng/ml which is equivalent to 0.3–300 nM) or by DT56a (10–200 ng/ml) of alkaline phosphatase (ALP) specific activity in human female bone cells. Results are mean ± S.E.M. of triplicate culture from 4 pre-menopausal and 4 post-menopausal women expressed as the ratio between ALP specific activity in hormone-treated and control cells (*p < 0.05, **p < 0.01). Basal activity of ALP: 0.20 ± 0.05 u/mg protein (pre-menopausal), 0.26 ± 0.04 u/mg protein (post-menopausal).

incorporation was found in both DT56a and E\textsubscript{2} groups (Fig. 3). In both treatments, the maximal increase was higher in the pre-menopausal cells compared to post-menopausal cells.

3.4. The effects of DT56a on CK specific activity, DNA synthesis and ALP specific activity in human male bone cells

To determine their responsiveness to DT56a compared to E\textsubscript{2} and to dihydrotestosterone (DHT), human male bone cells were incubated with 0.3–300 nM E\textsubscript{2}, with 1–200 ng/ml DT56a or with 30–3000 nM DHT. An increase in all parameters, was found only with DHT at optimum of 300 nM for CK and of 3000 nM for ALP and DNA synthesis (Fig. 4).

3.5. The effects of raloxifene and DT56a on CK specific activity, DNA synthesis and on ALP specific activity stimulated by E\textsubscript{2} in human female bone cells

When human female bone cells were incubated with both DT56a and raloxifene or E\textsubscript{2} (Fig. 5a-c), DT56a activity in both age groups was blocked by raloxifene. E\textsubscript{2}‘s activity, was also blocked by DT56a (Fig. 5a-c) as measured by CK specific activity (5a), DNA synthesis (5b) and ALP (5c) in both pre- and post-menopausal age groups.

3.6. The effects of DT56a on intracellular [Ca\textsuperscript{2+}]\textsubscript{i} concentration in human female bone cells

To determine their responsiveness to DT56a compared to E\textsubscript{2}, human female bone cells from post-menopausal origin, were incubated with 0.01–0.10 nM E\textsubscript{2}, or with 0.01–10 ng/ml DT56a (Figs. 6a, b and 7). An increase in intracellular [Ca\textsuperscript{2+}]\textsubscript{i} concentration was found with both agents (Figs. 6a and 7) with higher increase with DT56a and with higher sensitivity to this treatment as determined by the E\textsubscript{2} increased intracellular [Ca\textsuperscript{2+}]\textsubscript{i} concentration at 0.01 nM by about 120% and DT56a at 10 ng/ml by about 240% (Figs. 6 and 7).
Fig. 6. Stimulation by (a) E$_2$ (2.73 ng/ml equivalent to 10 nM) or (b) DT56a (1 ng/ml) of intracellular [Ca$^{2+}$]$_i$ concentration in post-menopausal human bone cells. Results are tracing of the scans described in Section 2.

Fig. 7. Dose dependent stimulation by E$_2$ (0.0273–27.3 ng/ml equivalent to 0.001–0.100 nM) or by DT56a (0.01–10.00 ng/ml) of intracellular [Ca$^{2+}$]$_i$ concentration in post-menopausal human bone cells. Results are quantitations of the tracing of the scans described in Section 2, and are mean ± S.E.M. ($n=4$) (*$p<0.05$, **$p<0.01$).

4. Discussion

Our results demonstrate that DT56a activates osteoblasts in vitro, in all the tested parameters in a sex specific manner, namely it stimulated only female and not male-derived bone cells in both pre and postmenopausal age groups.

DT56a was shown in previous clinical studies to be an effective agent in preventing postmenopausal osteoporosis [18], with no effect on the uterus. Bone loss induced by ovariectomy in female rats, has been widely used as a model for studying postmenopausal osteoporosis [26–32]. Treatment with E$_2$ is known to reverse the osteoporotic changes caused by ovariectomy [30–32].

In the present study, we analyzed the effects of DT56a and E$_2$ on human cultured osteoblasts. DT56a, similarly to E$_2$, stimulated CK specific activity, which is a marker of estrogenic response, and DNA synthesis, which is a measure of cell proliferation, in both pre-menopausal and post-menopausal derived cells. In contrary, ALP activity showed age dependence decline in the response to both treatments. Since ALP is a marker for osteoblastic activity, this decline might reflect the natural decrease in this activity with age.

DT56a like E$_2$ did not stimulated male derived bone cells, which respond only to DHT, demonstrating that like E$_2$, DT56a is sex-specific in its effect on bone cells in culture. DT56a’s activity was blocked by the SERM raloxifene, identical to Ral’s effect on E$_2$, and also displayed a SERM-like activity in blocking E$_2$’s effect.

In the present study, we also showed non-genomic effects of DT56a via putative membranal ER, by increasing intracellular cytosolic free calcium concentration ([Ca$^{2+}$]$_i$). We assume that the increase in [Ca$^{2+}$]$_i$ in both age groups was mediated by two mechanisms: (1) [Ca$^{2+}$]$_i$ influx through voltage-gated
[Ca\(^{2+}\)]\text{\textsc{i}}\) channels and (2) [Ca\(^{2+}\)]\text{\textsc{i}} mobilization from the endomembrane system. The results suggest DT56a to be a bone forming agent. Osteoclast/osteoblast activity is shifted toward bone formation, thus the balance between osteoclastic activation [33,34] at both levels.

The current anti-estrogenic therapies mainly slow down osteoclastic activation [33,34], thus the balance between osteoclast/osteoblast activity is shifted toward bone formation. In the present study DT56a stimulated cultured osteoblasts, suggesting DT56a to be a bone forming agent.

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References