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# DT56a (Femarelle): A natural selective estrogen receptor modulator (SERM)[☆]

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## Abstract

A selective estrogen receptor modulator (SERM) is defined as a substance with dissimilar effects on different tissues: agonist in some and antagonists in others. The natural compound DT56a (Femarelle) was shown to activate estrogen receptors in human cultured female derived osteoblasts. It was also shown to relieve menopausal symptoms and to increase bone mineral density with no effect on sex steroid hormone levels and on the endometrial thickness. DT56a, similarly to estradiol-17$\beta$ ($E_2$), stimulated the specific activity of creatine kinase (CK) in skeletal and vascular tissues of female rats, as a marker of estrogen receptor (ER) activation. However, in the uterus, CK was activated only by $E_2$ but not by DT56a. In order to prove that DT56a is a SERM, we examined the mutual interaction between DT56a and $E_2$, at supra physiological doses, in different tissues in both intact and ovariectomized female rats, as well as in human cultured vascular and bone cells. Administration of DT56a or $E_2$ stimulated CK in all tissues tested, but when given simultaneously, in intact immature female rats, DT56a completely abolished $E_2$ stimulation of CK in all organs except in the diaphyseal bone where the inhibition was partial. In ovariectomized female rats, DT56a abolished $E_2$'s stimulation of CK in diaphyseal bone, thymus, uterus and pituitary but caused a partial inhibition in aorta, left ventricle and epiphyseal cartilage. In human bone cells $E_2$ stimulation of CK, of alkaline phosphatase (AP) activity and of DNA synthesis was completely abolished by DT56a in post-menopausal cells and partially inhibited in pre-menopausal cells. In human vascular cells, inhibition of DNA synthesis by $E_2$ was completely abolished by DT56a and $E_2$-induced CK was partially inhibited by DT56a. The results support the finding that DT56a is a SERM; it stimulated different parameters similar to $E_2$, but when given simultaneously, at supra physiological doses, inhibited these $E_2$'s effects. Further investigations regarding intra and extra cellular mechanism of action of DT56a are currently performed.
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## 1. Introduction

Estrogen is well known for its beneficial effect on osteoporosis [1,2] a disease currently affecting more than 25 million women in the U.S. alone, causing some 250,000 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 effect 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 bone and the cardiovascular system [8,9] without the harmful side effects. This group is called SERM's: selective ER modulators [10,11].

SERMs function as estrogen agonists in some tissues and as antagonists in others. For example, the SERM tamoxifen acts as an antagonist of estrogen in breast tissue and is therefore used for the treatment of breast cancer, whereas in bone it acts as an estrogen agonist [12,13]. Raloxifene functions as an estrogen agonist in skeletal tissues and in the cardiovascular system but not in the uterus. Some of the SERMs act also as antagonists in the presence of estrogen in skeletal or vascular tissues and in the uterus as well as breast cancer cells in vitro [14,15]. We used the induction of the brain isozyme creatine kinase specific activity (CK) as a response marker of estrogenic activity since estrogens, both in vivo and in vitro in tissues and in cells containing active ER (S) [16–20], stimulate CK.

DT56a (Tofupill/Femarelle, Se-cure Pharmaceuticals, Dalton, Israel) is a unique enzymatic isolate of soybeans. DT56a has been shown to increase bone mineral density in post-menopausal women [21] and to relieve vasomotor symptoms with no effect on sex hormone levels or endometrial thickness [22]. The selective receptor modulation of DT56a was shown in previous studies: rats were fed for 4 days with estrogen or DT56a. Both compounds induced CK activity in the different skeletal [23] and vascular tissues [24]. On the other hand, DT56a did not activate CK in the uterus while estrogen caused a marked CK activation [23]. The selective estrogen receptor modulator (SERM) raloxifene blocked the stimulation of CK by both DT56a and $E_2$ in all tissues tested, pointing towards a common receptor(s) mechanism of action [23,24]. DT56a also stimulated CK and other parameters in cultured bone cells [25].

Ovariectomized (OVX) female rats were previously described as a model for testing post-menopausal changes [26,27]. The phenomenon of mutual annihilation of action between estradiol-17β ($E_2$) and a selective estrogen receptor modulator such as tamoxifen, tamoxifen methiodide or raloxifene, was previously described in pre pubertal female rat skeletal tissues in culture [28]. This study showed that compounds that activate ER alone, when given together, antagonized each other activity [28,29].

In the present study, we examined the interaction between DT56a and $E_2$ when given separately and simultaneously to immature or OVX female rats, and in cultured human vascular and osteoblasts from females either pre- or post-menopausal.

## 2. Materials and methods

### 2.1. Reagents

All reagents were of analytical grade. $E_2$ was purchased from Sigma (St. Louis, MO). DT56a was provided by Se-cure Pharmaceuticals (Dalton, Israel).

### 2.2. Animals

Immature female Wistar rats, weighing about 60 g, were used as intact or were ovariectomized at the age of 25 days. Treatments were administered starting 2 weeks after surgery. The rats were housed in air-conditioned quarters with light from 5:00 to 19:00 h and were exposed to food and water ad libidum. All experiments were carried out according to the regulations of the Committee on Experimental Animals of the Tel-Aviv Sourasky Medical Center.

### 2.3. Cell cultures

#### 2.3.1. Human cultured female osteoblasts (hObs)

Human female bone cells from pre- and post-menopausal women were prepared from bone explants, by a non-enzymatic method as described previously in Ref. [20]. 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 components. 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 $3 \times 10^5$ cells/35 mm tissue culture dish, in phenol red-free DMEM with 10% charcoal stripped FCS, and incubated at 37 °C in 5% $CO_2$.

#### 2.3.2. Human umbilical artery smooth muscle cells (VSMC)

Human umbilical artery vascular smooth muscle cells were prepared as previously described with minor modifications in Ref. [19]. Cells were used only at passages 1–3 when expression of smooth muscle actin was clearly demonstrable.

### 2.4. Creatine kinase extraction and assay

#### 2.4.1. Rat organs

Changes in the specific activity of CK in epiphyseal cartilage (Ep), diaphyseal bone (Di), aorta (Ao), left ventricle of the heart (Lv), pituitary (Pi), thymus (Thy) and uterus (Ut), induced by a single intraperitoneal injection (i.p.) of $E_2$ or DT56a (dissolved in 0.1% ethanol in saline, for both) or both, were assayed in ovariectomized female rats, 2 weeks after surgery, or in 25-day-old immature female rats ($n = 5$ per group for each experiment). Matched control rats ($n = 5$) were injected with 0.05% ethanol in phosphate-buffered saline (PBS). $E_2$ was injected at 5 μg for immature and 10 μg for ovariectomized female rats [17,24,27]. DT56a was

injected at 1600 μg for immature and 3200 μg for ovariectomized female rats. These concentrations were found to be the optimal in dose response experiments [23–24]. Rats were sacrificed 24 h after the injection. The organs examined were removed and stored at −20 °C until processed for assay of creatine kinase activity as described previously in Ref. [17]. Organs were homogenized in buffer. Supernatant extracts were obtained by centrifugation of the homogenates at 14,000 × g for 5 min at 4 °C in an Eppendorf micro-centrifuge. CK was determined by a coupled spectrophotometric assay. Protein was determined by Coomasie brilliant blue dye binding using bovine serum albumin as the standard [17].

### 2.4.2. Human cultured cells

*2.4.2.1. Human cultured female 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 in Ref. [20]. Supernatant extracts were obtained by centrifugation of homogenates at 14,000 × g for 5 min at 4 °C in an Eppendorf micro-centrifuge. CK was assayed by a coupled spectrophotometric assay, as previously described in Ref. [20]. Protein was determined by Coomasie brilliant blue dye binding, using bovine serum albumin as the standard.

*2.4.2.2. Human umbilical artery smooth muscle cells.* Cells were treated for 24 h with the various hormones, protein extraction and assessment of CK activity were carried out according to our previously described protocols in Ref. [19]. Protein was determined by Coomasie brilliant blue dye binding, using bovine serum albumin as the standard.

### 2.5. Alkaline phosphatase extraction and assay

#### 2.5.1. Human cultured female 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 [25]. Enzyme activity was assayed in an ELISA reader, measuring the hydrolysis of *p*-nitro phenyl phosphate at 37 °C in a buffer containing 2 mM MgCl$_2$ and 100 mM 2-amino 2-methyl isopropanol at pH 10.3. The reaction was stopped with 1 M NaOH and the extracts were analyzed at 410 nm. Protein was determined by Coomasie brilliant blue dye binding, using bovine serum albumin as the standard. Enzyme specific activity was determined as OD × 4.3 per mg protein [25].

### 2.6. DNA synthesis

#### 2.6.1. Human cultured female 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, [3H] thymidine was added for 2 h. Cells were then treated with 10% ice-cold

trichloroacetic acid (TCA) for 5 min and washed twice with 5% TCA and then with cold ethanol. The cellular layer was dissolved in 0.3 ml of 0.3N NaOH, samples were aspirated and [3H] thymidine incorporation into DNA was determined [25].

#### 2.6.2. Human umbilical artery smooth muscle cells

Cells were grown until sub confluence and then treated with various hormones or agents as indicated for 24 h. For the last 2 h, [3H] thymidine was added and [3H] thymidine incorporation into DNA was determined as previously described by us in Ref. [19]. DT56a doses were given according to the dose-dependent curves from our previous studies [23].

### 2.7. Statistical analysis

Differences between the mean values obtained from the experimental and the control groups were evaluated by analysis of variance (ANOVA). A *P*-value less than 0.05 is considered significant.

## 3. Results

### 3.1. The effect of DT56a on the stimulation of creatine kinase specific activity by a single injection of estradiol-17β into immature female rats

Immature female rats were injected with E$_2$ (5 μg/rat), DT56a (1600 μg/rat) or both. CK was tested in: epiphyseal cartilage, diaphyseal bone and uterus (Fig. 1), aorta
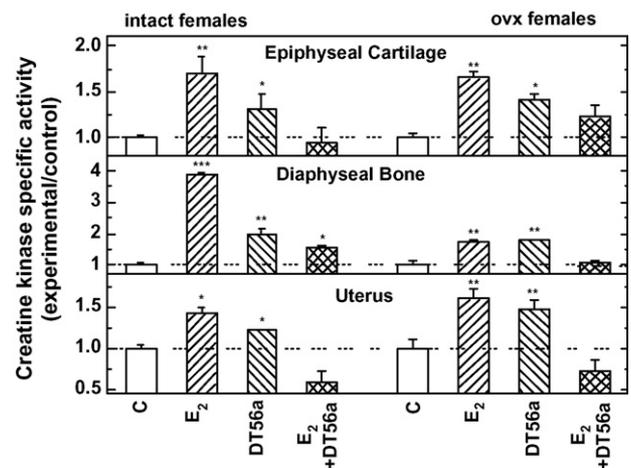


Fig. 1. Creatine kinase activity stimulation by estradiol-17β (E$_2$) (5 or 10 μg) or DT56a (1300 or 3200 μg) or both in epiphyseal cartilage (Ep), diaphyseal bone (Di) and in uterus (Ut) of immature female rats and ovariectomized rats (OVX), respectively. Results are means ± S.E.M. of *n* = 5 (15 assays from 3 independent experiments) and are expressed as the ratios between the specific activities of CK in the treated and control animals. [*]*P* < 0.05; [**]*P* < 0.01; [***]*P* < 0.001. The basal activity of creatine kinase for immature rats was in Ep 0.48 ± 0.10, in Di 0.62 ± 0.03 and in Ut 1.57 ± 0.03 μmol/min/mg protein. In OVX animals: in Ep 0.39 ± 0.02, in Di 0.46 ± 0.09 and in Ut 1.24 ± 0.11 μmol/min/mg protein.
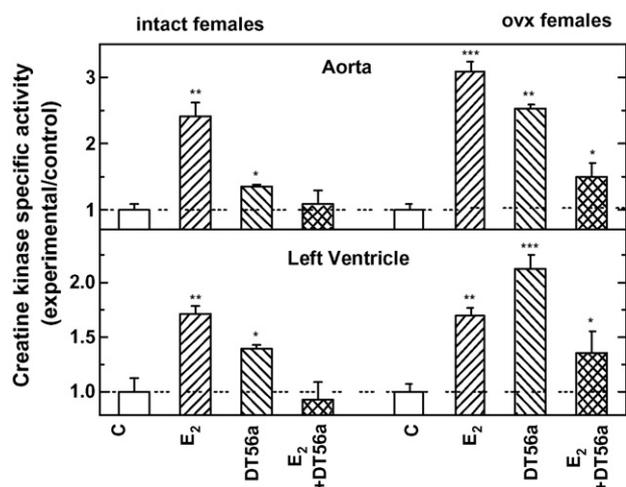
Fig. 2. Creatine kinase activity stimulation by estradiol-17β (E$_2$) (5 or 10 μg) or DT56a (1300 or 3200 μg) in aorta (Ao) and in the left ventricle of the heart (Lv) of immature female rats and ovariectomized rats (OVX), respectively. Results are means ± S.E.M. of $n = 5$ (15 assays from 3 independent experiments) and are expressed as the ratios between the specific activities of CK in the treated and control animals. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. The basal activity of creatine kinase for immature rats was in Ao $0.53 \pm 0.04$ and in Lv $1.92 \pm 0.25$ μmol/min/mg protein. In OVX animals: in Ao $0.49 \pm 0.03$ and in Lv $1.66 \pm 0.15$ μmol/min/mg protein.

and the left ventricle of the heart (Fig. 2) or pituitary and thymus (Fig. 3). Significant stimulation of CK by DT56a and E$_2$ was found in all organs tested i.e., in Ep DT56a increased CK by $32 \pm 16\%$ and E$_2$ by $68 \pm 18\%$. In Di, DT56a increased CK by $100 \pm 15\%$ and E$_2$ by $292 \pm 8\%$. In Ut, DT56a increased CK by $24 \pm 2\%$ and E$_2$ by $45 \pm 6\%$. In
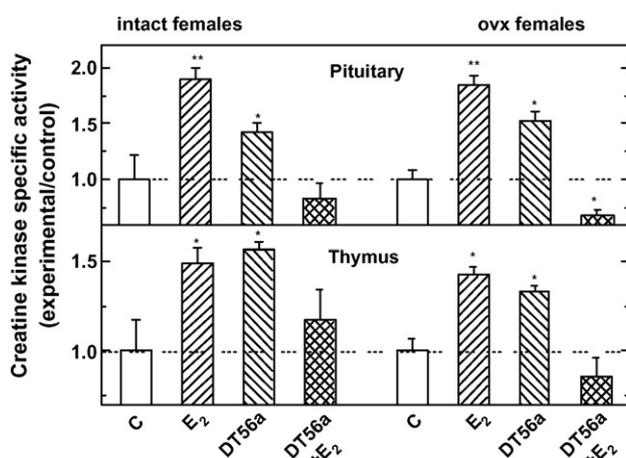


Fig. 3. Creatine kinase activity stimulation by estradiol-17β (E$_2$) (5 or 10 μg) or DT56a (1300 or 3200 μg) in pituitary (Pi) and in thymus (Thy) of immature female rats and ovariectomized rats (OVX), respectively. Results are means ± S.E.M. of $n = 5$ (15 assays from 3 independent experiments) and are expressed as the ratios between the specific activities of CK in the treated and control animals. $^{*}P < 0.05$; $^{**}P < 0.01$. The basal activity of creatine kinase for immature rats was in Pi $2.53 \pm 0.24$ and in Thy $0.92 \pm 0.12$ μmol/min/mg protein. In OVX animals: in Pi $1.49 \pm 0.03$ and in Pi $0.76 \pm 0.15$ μmol/min/mg protein.

Ao, DT56a increased CK by $33 \pm 4\%$ and E$_2$ by $141 \pm 19\%$. In Lv, DT56a increased CK by $40 \pm 4\%$ and E$_2$ by $72 \pm 8\%$. In Pi, DT56a increased CK by $43 \pm 7\%$ and E$_2$ by $90 \pm 10\%$. In Thy, DT56a increased CK by $56 \pm 4\%$ and E$_2$ by $49 \pm 8\%$ (Figs. 1–3). CK stimulation by E$_2$ alone was significantly reduced or completely inhibited when E$_2$ was given together with DT56a i.e., in Ep by $-16 \pm 5\%$, in Di by $-54 \pm 12\%$, in Ut by $-42 \pm 15\%$, in Ao by $22 \pm 7\%$, in Lv by $-7 \pm 7\%$, in Pi by $-16 \pm 13\%$ and in Thy by $17 \pm 17\%$ (Figs. 1–3).

### 3.2. The effect of DT56a on the stimulation of creatine kinase specific activity by a single injection of estradiol-17β into ovariectomized female rats

Ovariectomized female rats, weighing about 120 g, were injected with E$_2$ (10 μg/rat), DT56a (3200 μg/rat) or both. CK was tested in; epiphyseal cartilage, diaphyseal bone and uterus (Fig. 1), aorta and the left ventricle of the heart (Fig. 2) or pituitary and thymus (Fig. 3). Stimulation of CK by DT56a and E$_2$ was found in all organs tested i.e., in Ep DT56a increased CK by $42 \pm 5\%$ and E$_2$ by $63 \pm 8\%$. In Di, DT56a increased CK by $85 \pm 4\%$ and E$_2$ by $77 \pm 8\%$. In Ut, DT56a increased CK by $48 \pm 12\%$ and E$_2$ by $64 \pm 18\%$. In Ao, DT56a increased CK by $152 \pm 4\%$ and E$_2$ by $204 \pm 15\%$. In Lv, DT56a increased CK by $113 \pm 12\%$ and E$_2$ by $70 \pm 8\%$. In Pi, DT56a increased CK by $52 \pm 9\%$ and E$_2$ by $85 \pm 8\%$. In Thy, DT56a increased CK by $33 \pm 3\%$ and E$_2$ by $42 \pm 5\%$ (Figs. 1–3). CK activity stimulation by E$_2$ alone was significantly reduced or inhibited when E$_2$ was given together with DT56a i.e., in Ep by $21 \pm 13\%$, in Di by $-15 \pm 8\%$, in Ut by $-30 \pm 14\%$, in Ao by $-44 \pm 22\%$, in Lv by $-37 \pm 19\%$, in Pi by $-32 \pm 5\%$ and in Thy by $-14 \pm 10\%$ (Figs. 1–3).

### 3.3. The effect of DT56a or estradiol-17β or both on the modulation of creatine kinase specific activity, alkaline phosphatase specific activity and DNA synthesis in human female osteoblasts in culture

Human cultured bone cells from pre- or post-menopausal women were incubated with E$_2$ (30 nM), DT56a (200 ng/ml) or both, and CK was tested (Fig. 4). Stimulation of CK activity by DT56a and E$_2$ was found in pre-menopausal cells, DT56a increased CK by $81 \pm 9\%$ and E$_2$ by $79 \pm 8\%$ and in post-menopausal cells, DT56a increased CK by $66 \pm 10\%$ and E$_2$ by $44 \pm 8\%$ (Fig. 4). CK stimulation by E$_2$ alone was significantly reduced or inhibited when E$_2$ was given together with DT56a i.e., in pre-menopausal cells by $29 \pm 5\%$ and in post-menopausal cells by $4 \pm 11\%$ (Fig. 4). Stimulation of alkaline (AP) phosphatase activity by DT56a and E$_2$ was found in pre-menopausal cells, DT56a increased AP by $87 \pm 9\%$ and E$_2$ by $107 \pm 18\%$ and in post-menopausal cells, DT56a increased AP by $156 \pm 22\%$ and E$_2$ by $96 \pm 12\%$ (Fig. 5). AP activity stimulation by E$_2$ alone was significantly reduced or inhibited when E$_2$ was given together with DT56a i.e., in pre-menopausal by $29 \pm 5\%$ and in post-menopausal hObs by $-4 \pm 11\%$ (Fig. 5). Stimulation of DNA synthesis
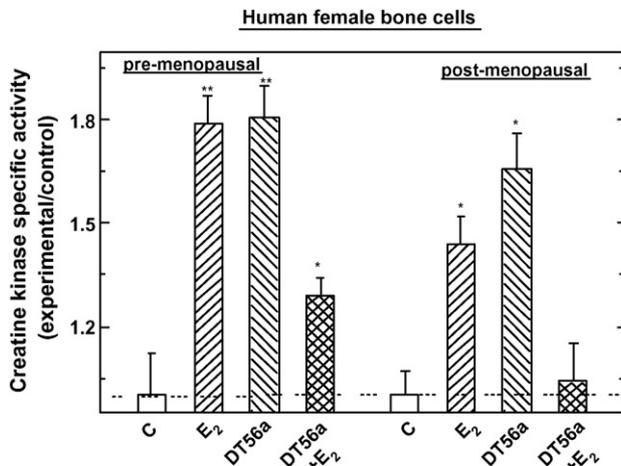
**Human female bone cells**



Fig. 4. Creatine kinase activity stimulation by estradiol-17β (E$_2$) (30 nM) or DT56a (200 ng/ml) in human cultured bone cells from pre- or post-menopausal women. Results are means ± S.E.M. of $n = 5$ (15 assays from 3 independent experiments) and are expressed as the ratios between the specific activities of CK in the treated and control cells. $^*P < 0.05$; $^{**}P < 0.01$. The basal activity of creatine kinase was in pre-menopausal cells $0.033 \pm 0.004$ and in post-menopausal cells $0.025 \pm 0.003$ μmol/min/mg protein.

**Human female bone cells**



Fig. 6. [$^3$H] thymidine incorporation into DNA stimulation by estradiol-17β (E$_2$) (30 nM) or DT56a (200 ng/ml) in human cultured bone cells from pre- or post-menopausal women. Results are means ± S.E.M. of $n = 5$ (15 assays from 3 independent experiments) and are expressed as the ratios between the incorporation in the treated and control cells. $^*P < 0.05$; $^{**}P < 0.01$. The basal $^3$H thymidine incorporation: $2550 \pm 100$ dpm/well was in pre-menopausal, and in post-menopausal cells $1850 \pm 75$ dpm/well.

by DT56a and E$_2$ was found in pre-menopausal cells, DT56a increased DNA by $52 \pm 10\%$ and E$_2$ by $134 \pm 18\%$ and in post-menopausal cells, DT56a increased DNA by $76 \pm 11\%$ and E$_2$ by $140 \pm 15\%$ (Fig. 6). DNA synthesis stimulation by E$_2$ alone was significantly reduced or inhibited when E$_2$ was given together with DT56a i.e., in pre-menopausal cells by $-3 \pm 17\%$ and in post-menopausal cells by $5 \pm 11\%$ (Fig. 6).
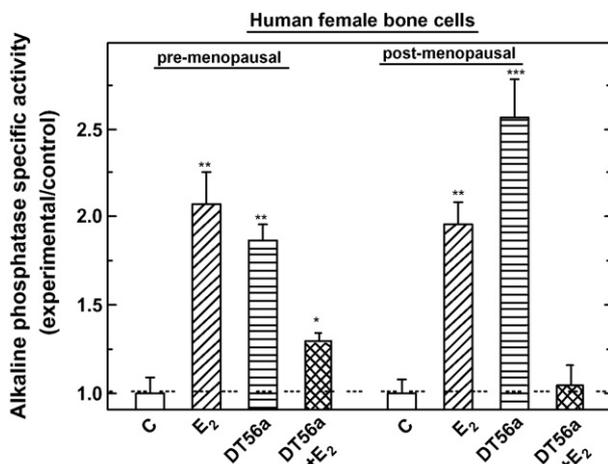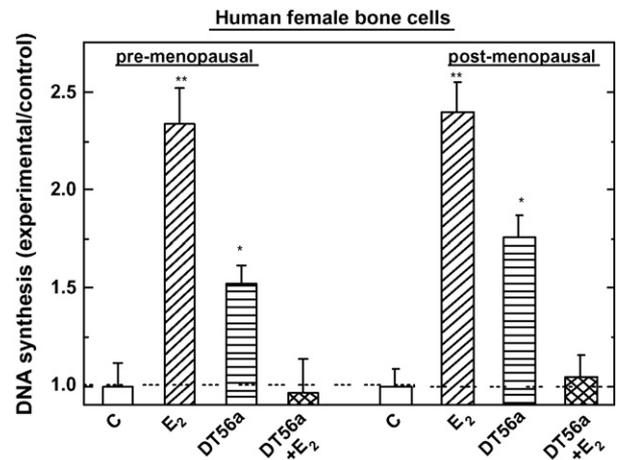
### 3.4. The effect of DT56a or estradiol-17β or both on the stimulation of creatine kinase specific activity and the inhibition DNA synthesis in human vascular smooth muscle cells in culture

Human VSMC were incubated with either E$_2$ (30 nM) or DT56a (200 ng/ml) or both, CK was assayed in those cells (Fig. 7), and stimulation of CK by DT56a and E$_2$ was found. CK was increased by DT56a by $78 \pm 15\%$ and by E$_2$ by
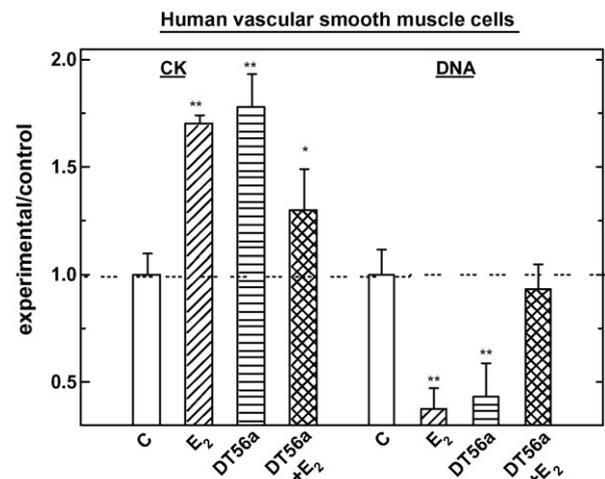
**Human female bone cells**



Fig. 5. Alkaline phosphatase activity stimulation stimulation by estradiol-17β (E$_2$) (30 nM) or DT56a (200 ng/ml) in human cultured bone cells from pre- or post-menopausal women. Results are means ± S.E.M. of $n = 5$ (15 assays from 3 independent experiments) and are expressed as the ratios between the specific activities of AP in the treated and control cells. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. The basal activity of alkaline phosphatase was in pre-menopausal cells $0.20 \pm 0.05$ U/mg and in post-menopausal cells $0.26 \pm 0.04$ U/mg protein.

**Human vascular smooth muscle cells**



Fig. 7. Inhibition of [$^3$H] thymidine incorporation into DNA and stimulation of creatine kinase activity (CK), by estradiol-17β (E$_2$) (30 nM) or DT56a (200 ng/ml) in human cultured vascular smooth muscle cells. Results are means ± S.E.M. of $n = 5$ (15 assays from 3 independent experiments) and are expressed as the ratios between the specific activities of CK or $^3$H thymidine incorporation in the treated and control cells. $^*P < 0.05$; $^{**}P < 0.01$. The basal $^3$H thymidine incorporation was $1275 \pm 50$ dpm/well was and in post-menopausal cells $1850 \pm 75$ dpm/well, the basal activity of creatine kinase was $0.016 \pm 0.002$ μmol/min/mg protein.

$70 \pm 4\%$ (Fig. 7). CK stimulation by $E_2$ alone was significantly reduced or inhibited when $E_2$ was given together with DT56a by $30 \pm 19\%$ (Fig. 7). DNA synthesis in VSMC, was significantly inhibited by either DT56a or $E_2$; DT56a inhibited DNA by $56 \pm 15\%$ and $E_2$ by $62 \pm 10\%$ (Fig. 7). DNA synthesis inhibition by $E_2$ was abolished when $E_2$ was given together with DT56a by $-6 \pm 11\%$ (Fig. 7).

## 4. Discussion

In the present study, we showed that CK specific activity induced by $E_2$, was completely or partially inhibited when DT56a was administered at supra physiological doses together with $E_2$. The same pattern of CK stimulation by the compounds alone and inhibition when given together, was also seen in human cultured osteoblasts from women at different ages and in cultured human vascular smooth muscle cells. In addition, this phenomenon was not restricted to the induction of CK activity, but was shown also in the stimulation of alkaline phosphatase activity and DNA synthesis in osteoblasts, as well as the inhibition of DNA synthesis in VSMC.

The phenomenon of mutual annihilation of activity between $E_2$ and selective estrogen receptor modulators were previously described. SERMs can be full agonists when provided alone, but complete antagonists at supra physiological doses in the presence of estrogen [28,29]. When a SERM like tamoxifen, tamoxifen methiodide or raloxifene was given together with $E_2$ to pre pubertal immature female rats, the effect of those compounds on the induction of CK specific activity in diaphysis, epiphysis and the uterus was antagonized [28]. In the present study, we found that DT56a although active by itself in the different organs, antagonized $E_2$ activity when applied together, similar to the other SERMs. The results obtained by these studies suggest that DT56a can be classified as a SERM. The question whether this phenomenon is caused by desensitization of estrogen receptor(s) or their rearrangement or by changes in co-activators and/or co-repressors or by other undiscovered mechanisms, remains unsolved and is the subject of future investigations in these and other experimental systems. In preliminary experiments (data not shown) we found that DT56a modulates mRNA expression of ER$\alpha$ and ER$\beta$ in different patterns. In human osteoblasts ER$\alpha$ was not changed and ER$\beta$ was increased. If we speculate that in these cells $E_2$ is stimulating via ER$\alpha$ and DT56a via ER$\beta$ it might explain our results. On the other hand, in VSMC both ER$\alpha$ and ER$\beta$ are increased to different extents. The change in ERs ratios might explain our results. But it has still to be further analysed in order to understand the mechanism of the findings.

## Acknowledgments

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