

## RESEARCH PAPER

# Differential effects of selective oestrogen receptor modulators (SERMs) tamoxifen, ospemifene and raloxifene on human osteoclasts *in vitro*

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**Background and purpose:** Several selective oestrogen receptor modulators (SERMs) with oestrogen agonist effects in bone cells and without increased risk of breast and endometrial cancer have been developed. Here, we have investigated the effects of different types of SERMs on osteoclast differentiation, bone resorption and apoptosis *in vitro*.

**Experimental approach:** Human peripheral blood-derived CD14<sup>+</sup> monocytes were cultured on bovine bone slices in the presence of RANKL, M-CSF, TNF- $\alpha$  and dexamethasone for seven days. Also, CD14<sup>+</sup> monocytes were co-cultured either with human SaOS-2 or MG-63 osteosarcoma cells, in the presence of parathyroid hormone. Osteoclast cultures were treated with different SERMs. TRACP<sup>+</sup> multinucleated cells and C-terminal telopeptide of type I collagen were used as markers for osteoclast formation and bone resorption, respectively.

**Key Results:** In CD14<sup>+</sup> monocyte cultures, tamoxifen directly inhibited human osteoclast formation and bone resorption, while raloxifene and ospemifene had no inhibitory effect. In the co-cultures either with SaOS-2 or MG-63 cells, ospemifene and raloxifene as well as tamoxifen inhibited osteoclast formation in a concentration-dependent manner. The inhibitory effect was associated with an increased production of osteoprotegerin. The anti-oestrogen ICI 182 780 completely reversed the effects of these SERMs.

**Conclusion and Implications:** Tamoxifen had an oestrogen receptor dependent, direct, inhibitory effect on human osteoclast differentiation and bone resorption, whereas ospemifene and raloxifene required osteoblastic cells to achieve a similar inhibition. The effects of ospemifene and raloxifene were mediated by oestrogen receptors by a mechanism involving paracrine induction of osteoprotegerin in cultures with osteoblast derived osteosarcoma cells.

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**Keywords:** tamoxifen; ospemifene; raloxifene; osteoclast differentiation; bone resorption; osteoprotegerin

**Abbreviations:**  $\alpha$ -MEM,  $\alpha$ -minimum essential medium; CTx, C-terminal telopeptide of type I collagen; FCS, fetal calf serum; IL, interleukin; MACS, magnetic activated cell sorting; M-CSF, macrophage-colony-stimulating factor; MNC, multinucleated cell; OPG, osteoprotegerin; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PTH, parathyroid hormone; RANKL, receptor activator of nuclear factor kappa B ligand; SERM, selective oestrogen receptor modulator; TNF, tumour necrosis factor; TRACP, tartrate-resistant acid phosphatase; WGA, wheat germ agglutinin

## Introduction

Decrease of oestrogen in postmenopausal women results in an increased bone turnover with bone resorption exceeding bone formation. This gradually leads to a general loss of bone mass (Riggs, 1991). In aging women, decreased production of oestrogen has been associated with degenerative changes in

various organ systems including the skeletal, cardiovascular and nervous system (Kanis, 1996; Halbreich, 1997; Grodstein and Stampfer, 1998). In many women, a decreasing level of oestrogen is also associated with menopausal symptoms. Oestrogen replacement therapy effectively protects against degenerative changes as well as menopausal symptoms (Marshburn and Carr, 1992). However, a long-term oestrogen therapy also increases a risk of breast and endometrial cancer (Lobo, 1995; Colditz *et al.*, 1995). Therefore, several attempts have been taken to develop new hormone replacement therapies with beneficial effects on bone (Ward *et al.*, 1993,

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Love *et al.*, 1994), cardiovascular and nervous system (Tonetti and Jordan, 1996) in postmenopausal women, without adverse, stimulatory effects on mammary gland and uterus.

New synthetic, nonsteroidal compounds, the selective oestrogen receptor modulators (SERMs), have been recently developed and shown to possess oestrogen receptor agonistic or antagonistic selectivity in specific target tissues (Draper *et al.*, 1996; Fournier *et al.*, 1996; Yang *et al.*, 1996; Shevde *et al.*, 2000). Raloxifene is a benzothiophene SERM that specifically activates biological responses in bone tissue, without stimulation of mammary gland and uterus (Turner *et al.*, 1988; Black *et al.*, 1994; Fournier *et al.*, 1996; Sato *et al.*, 1996; Yang *et al.*, 1996). Raloxifene binds to oestrogen receptor and it modulates gene transcription as a mixed agonist/antagonist (Compston, 2001). In bone it acts as an oestrogen agonist (Delmas *et al.*, 1997), in mammary gland as an antagonist and in endometrium it has little effect (Delmas *et al.*, 1997). Preclinical and clinical studies have demonstrated that raloxifene prevents bone loss and reduces a risk of fracture (De Launoit *et al.*, 1991; Fuchs-Young *et al.*, 1995; Evans *et al.*, 1994, 1996). More recently, it has been shown that, similar to oestrogen, raloxifene inhibits osteoclast formation in human and mouse bone marrow cultures *in vitro* (Taranta *et al.*, 2002; Ramalho *et al.*, 2002).

Tamoxifen is a triphenylethylene SERM and it has been shown to have effects on bone by inhibiting resorption and increasing bone mineral density in postmenopausal women (Ward *et al.*, 1993; Love *et al.*, 1994; Powles *et al.*, 1996) and affecting osteoclastic bone resorption in ovariectomized rats (Turner *et al.*, 1987, 1988). Organ culture studies indicated that tamoxifen inhibited bone resorption at high concentrations (50–100  $\mu\text{M}$ ) (Stewart and Stern, 1986). In addition, it has been reported that tamoxifen significantly inhibited bone resorption in neonatal rat osteoclast cultures at micromolar concentrations (Arnett *et al.*, 1996).

The recently developed ospemifene is also a triphenylethylene SERM (Qu *et al.*, 2000). Ospemifene prevents bone loss in ovariectomized rats but its uterine effects are minor and it does not stimulate growth of human breast cancer cells either *in vivo* in nude mouse tumors or *in vitro* in cell cultures (Qu *et al.*, 2000). However, in rat osteoclast culture ospemifene did not have any effects on osteoclast survival or bone resorption (Parikka *et al.*, 1998). In addition, ospemifene was found to exert oestrogen-like effects in bone marrow cultures by enhancing osteoblastic differentiation with a mechanism that differs from that of raloxifene (Qu *et al.*, 1999). The effects of ospemifene on osteoblastic differentiation could be inhibited by the pure antioestrogen ICI 182 780, suggesting an oestrogen receptor-mediated mechanism (Qu *et al.*, 1999). In the studies of postmenopausal women, ospemifene has been reported to have bone-restoring activity similar to that of raloxifene (Komi *et al.*, 2006).

The mechanisms by which these SERMs exert their effects on bone-resorbing human osteoclasts remain largely to be clarified. Both oestrogen receptor isoforms, oestrogen receptor- $\alpha$  and oestrogen receptor- $\beta$  are present in osteoblasts (Eriksen *et al.*, 1988) and osteocytes (Braidman *et al.*, 1995). It has also been reported that oestrogen receptor- $\alpha$  and oestrogen receptor- $\beta$  are present in osteoclasts and these

results have later been confirmed in studies on human (Pensler *et al.*, 1990; Bord *et al.*, 2001; Braidman *et al.*, 2001), chicken (Oursler *et al.*, 1991) and rabbit (Mano *et al.*, 1996). Generally, the levels of oestrogen receptors in osteoclasts are low. In human (Oreffo *et al.*, 1999) and rat (Huang *et al.*, 1998) studies, oestrogen receptor- $\alpha$  was observed in mononuclear preosteoclasts, but not in mature osteoclasts.

We have previously shown that the inhibitory effect of oestrogen on human CD14<sup>+</sup> cell differentiation into osteoclasts is mostly mediated by osteoblastic cells and that oestrogen does not have any direct effect on osteoclast differentiation (Michael *et al.*, 2005a). The aim of this study was to investigate the effects of the SERMs tamoxifen, raloxifene and ospemifene, on human osteoclast differentiation and bone resorption using cultures of a pure population of human peripheral blood-derived osteoclast precursors and their cocultures with human SaOS-2 or MG-63 osteoblast-derived osteosarcoma cells. We found that tamoxifen concentration-dependently inhibited both osteoclast formation and bone resorption, whereas raloxifene and ospemifene had no direct effect on CD14<sup>+</sup> cell differentiation into active osteoclasts or resorption activity of mature osteoclasts. In the cocultures of CD14<sup>+</sup> cells and SaOS-2 or MG-63 cells, all three SERMs concentration-dependently inhibited osteoclast formation. The oestrogen receptor antagonist ICI 182 780 completely reversed the inhibitory effects of tamoxifen in CD14<sup>+</sup> cultures and of all SERMs in cocultures with osteoblastic cells, which suggests that both mechanisms were mediated via oestrogen receptors.

## Methods

### *Bovine bone slices*

Bone slices (7 × 7 mm, 120–140  $\mu\text{m}$  thick) were cut from bovine cortical femur with a low-speed, water-cooled diamond saw (Buehler, IL, USA). Bone slices were cleaned by ultrasonification (MSE, USA) for 2 min in distilled water and then placed in 70% ethanol overnight.

### *Preparation of peripheral blood mononuclear cells (PBMC)*

Buffy coats were obtained from blood of healthy male donors provided by the Finnish Red Cross Blood Bank (Turku, Finland). To isolate PBMC, buffy coats were diluted 1:1 (v/v) in phosphate-buffered saline (PBS), layered over Ficoll Paque Plus solution (Amersham Pharmacia Biotech, Uppsala, Sweden) and centrifuged for 30 min at 400g. The PBMC layer was collected and washed three times with PBS and resuspended in  $\alpha$ -minimum essential medium ( $\alpha$ -MEM) supplemented with 9% fetal calf serum (FCS), 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 IU ml<sup>-1</sup> of penicillin and 100  $\mu\text{g}$  ml<sup>-1</sup> of streptomycin. Cells were counted using a Bürker-Türk chamber.

### *Preparation of purified monocytes*

Immunomagnetic cell separation was performed in accordance with the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). PBMC were washed

with magnetic activated cell sorting (MACS) buffer (PBS, pH 7.2, supplemented with 0.5% BSA and 2 mM EDTA). Clumps were removed by passing the cells through a prefilter and cells were then centrifuged at 400 *g* for 15 min. The cell pellet was resuspended in MACS buffer and 10<sup>7</sup> cells in 80  $\mu$ l of MACS buffer were mixed with 20  $\mu$ l of CD14<sup>+</sup> antibody-coated microbeads and incubated for 15 min at 6–12°C. The cell suspension was applied to a LS-positive selection column that was previously washed with 1 ml of MACS buffer and placed in a magnetic separation unit. The column was rinsed with MACS buffer (3  $\times$  5 ml) and unbound (CD14<sup>-</sup>) cells were discarded. The column was then removed from the magnetic separation unit and the bound cells (CD14<sup>+</sup> monocytes) were flushed with 15 ml of MACS buffer by a syringe plunger. Finally, CD14<sup>+</sup> monocytes were collected following centrifugation at 400 *g* for 10 min.

#### *Osteoclast generation assay*

A total of 1  $\times$  10<sup>5</sup> purified CD14<sup>+</sup> monocytes in 30  $\mu$ l of cell suspension were added to a bovine bone slice placed on Parafilm and incubated for 1 h at 37°C in CO<sub>2</sub> incubator. After the attachment period, bone slices were transferred into 48-well plates (Becton Dickinson, Franklin Lakes, NJ, USA). The cell cultures were maintained in 0.5 ml of  $\alpha$ -MEM containing 9% FCS, 20 mM HEPES, 100 IU ml<sup>-1</sup> penicillin, 100  $\mu$ g ml<sup>-1</sup> streptomycin, (10 ng ml<sup>-1</sup>) macrophage-colony stimulating-factor (M-CSF), (20 ng ml<sup>-1</sup>) receptor activator of nuclear factor kappa B ligand (RANKL), (10 ng ml<sup>-1</sup>) tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and dexamethasone (0.01  $\mu$ M) for 7 days. SERMs at indicated concentrations (0.01–1.0  $\mu$ M) were added to the medium in the beginning of cell culture. Half of the media with treatments were replaced at day 3.

#### *Coculture assay*

SaOS-2 or MG-63 cells were plated in 48-well plates (1  $\times$  10<sup>3</sup> cells/well). Cell cultures were maintained in 1.0 ml of  $\alpha$ -MEM containing 9% FCS, 20 mM HEPES, 100 IU ml<sup>-1</sup> penicillin, 100  $\mu$ g ml<sup>-1</sup> streptomycin and (0.01  $\mu$ M) parathyroid hormone (PTH). On the following day, 1  $\times$  10<sup>5</sup> CD14<sup>+</sup> monocytes/well were added and the cocultures were allowed to grow for 7 days. Half of the media were replaced once a week. SERMs (0.01–1.0  $\mu$ M) were added to the cultures when monocytes were plated and when the media were changed.

#### *Osteoclast resorption assay*

Immunopurified monocytes were allowed to attach on bone slices and osteoclast formation was induced as described above. At day 7, the whole of the culture medium was replaced with fresh medium, and ospemifene, tamoxifen or raloxifene was added. Cultures were maintained for additional 3 days, cell culture media were collected and the C-terminal telopeptide of type I collagen (CTx) released from the resorbed bovine bone slices was measured to investigate osteoclastic bone resorption accord-

ing to the manufacturer's instructions (Osteometer, Herlev, Denmark).

#### *Tartrate-resistant acid phosphatase (TRACP) staining*

At the end of the culture, the cells cultured either on bovine bone slices or culture plates were washed twice with PBS, fixed with 3% *p*-formaldehyde (PFA) (Sigma, St Louis, MO, USA) in PBS for 15 min and rewashed twice with PBS. Cells were stained for TRACP according to the instructions provided by the manufacturer (Sigma). TRACP-positive multinucleated cells (TRACP<sup>+</sup> MNC) containing three or more nuclei were counted under the light microscope.

#### *TRACP 5b assay*

TRACP 5b in the culture medium released by osteoclasts was determined as described earlier (Alatalo *et al.*, 2000). Briefly, rabbit anti-human TRACP antiserum was incubated in anti-rabbit immunoglobulin G-coated microtitre plates (EG & G Wallac, Turku, Finland) for 1 h. Culture medium (200  $\mu$ l) was incubated in the wells for 1 h and the bound TRACP activity was detected using 8 mM 4-nitrophenyl phosphate as a substrate in 0.1 M acetate buffer for 2 h at 37°C. Adding 25  $\mu$ l of 0.32 M NaOH to the wells terminated the enzyme reaction and the absorbance at 405 nm was measured with a Victor model 2 instrument (EG & G Wallac). We have already shown that the medium TRACP 5b correlates strongly ( $r=0.94$ ) with the number of TRACP<sup>+</sup> MNC formed in the culture (Alatalo *et al.*, 2000).

#### *Resorption pit staining*

Cells were removed from bovine bone slices by soft brushing and the bone slices were rinsed extensively with PBS. Resorption pits were stained as described earlier (Selander *et al.*, 1994). Briefly, 50  $\mu$ l of peroxidase-wheat germ agglutinin (WGA)-lectin (20  $\mu$ g ml<sup>-1</sup>) in PBS was pipetted onto each bone slice and incubated on Parafilm for 30 min at room temperature. Bone slices were washed twice with PBS and the resorption pits were visualized by incubating them in 3,3' diaminobenzidine tetrahydrochloride solution (0.52 mg ml<sup>-1</sup>) for 15 min at room temperature. Finally, bovine bone slices were transferred onto glass slides, mounted with glycerol, covered with glass cover slips and observed under the light microscope.

#### *Hoechst DNA staining*

Osteoclasts were generated in eight-chamber slides (Nunc A/S, Denmark) as described above. On day 7, the whole media were replaced with fresh culture media and different concentrations of SERMs were added to the cultures and cells were allowed to grow for an additional 3 days. At the end of the culture, cells were fixed with 3% PFA for 15 min and washed twice with PBS. Hoechst stock solution in methanol was diluted in PBS (final concentration 1.25  $\mu$ g ml<sup>-1</sup>) and cells were stained for 5 min in the dark. Ten microscopic fields were observed and the proportions of apoptotic osteoclasts (fragmented or shrunken nuclei) were counted

under the fluorescence microscope (Leitz Aristoplan, Wetzlar, Germany) at 418 nm using  $\times 400$  magnification.

#### Osteoprotegerin (OPG) assay

Cell culture media from cocultures of monocytes and osteoblastic cells were collected and OPG was determined with enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (Biomedica, Austria). Briefly, standards, samples, positive control, assay buffer and biotin-labelled antibody were pipetted into a 96-well plate precoated with monoclonal anti-OPG antibody and mixed. After the incubation for 24 h at  $+4^{\circ}\text{C}$ , the plate was washed five times with the washing buffer, streptavidin-horseradish peroxidase-conjugate was added in the wells and incubated for 1 h at room temperature. The plate was washed five times and tetramethylbenzidine was added as a substrate to develop the colour reaction. After incubation for 20 min at room temperature, stop solution was added and OPG was measured with a Victor model 2 instrument (EG & G Wallac) at 450 nm absorbance.

#### Statistical analysis

The results represent the mean of  $\pm$  s.d. of five replicates and statistical significance was analysed by Student's *t*-test. Statistically significant differences from control, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

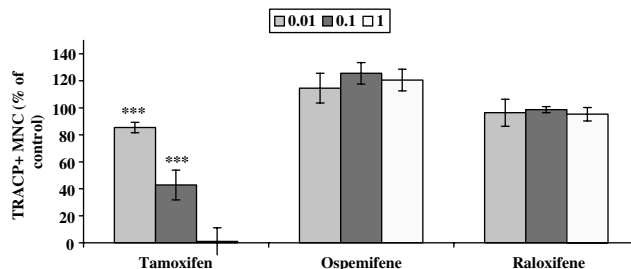
#### Materials

Tissue culture medium, phenol red-free  $\alpha$ -MEM, PBS, HEPES, antibiotics and FCS were purchased from Gibco BRL (Paisley, UK). Soluble human RANKL and TNF- $\alpha$  were from Peprotech (London, UK). Human M-CSF was from R&D Systems (Abingdon, UK). Dexamethasone and PTH were purchased from Sigma (St Louis, MO, USA). Mouse anti-human CD14 antibody coupled to magnetic microbeads and MACS cell separation units were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Ospemifene and tamoxifen were synthesized by Hormos Medical Ltd (Turku, Finland), whereas raloxifene was synthesized by Synthetic Chemistry Laboratory of Hormos Medical Ltd (Oulu, Finland). ICI 182 780 was purchased from Tocris Cookson (Ballwin, MO, USA).

## Results

#### Effect of tamoxifen, ospemifene and raloxifene on human osteoclast differentiation

The effect of the SERMs (0.01–1  $\mu\text{M}$ ) on osteoclastogenesis was studied using *in vitro* differentiation assays. Human peripheral blood osteoclast precursors, CD14<sup>+</sup> monocytes, were cultured on bovine bone slices in the presence of RANKL, M-CSF, TNF- $\alpha$  and dexamethasone for 7 days. These growth factors induced the generation of large MNC that expressed TRACP. TRACP histochemical staining was routinely used to identify osteoclasts in this study. When osteoclast precursors were cultured on bovine bone slices (Figure 1) and treated with tamoxifen, the osteoclast formation was inhibited in a dose-dependent manner as compared to the control culture (100%, nontreated culture),



**Figure 1** Isolated human CD14<sup>+</sup> monocytes were cultured on bovine bone slices in the presence of RANKL, M-CSF, TNF- $\alpha$  and dexamethasone for 7 days and treated with or without 0.01, 0.1, 1  $\mu\text{M}$  SERMs. TRACP histochemical staining was performed at the end of the culture and the number of TRACP positive-TRACP<sup>+</sup> MNC were counted. The number of TRACP<sup>+</sup> MNC in control cultures (= 100%) was  $221 \pm 18$ . \*\*\* $P < 0.001$ .

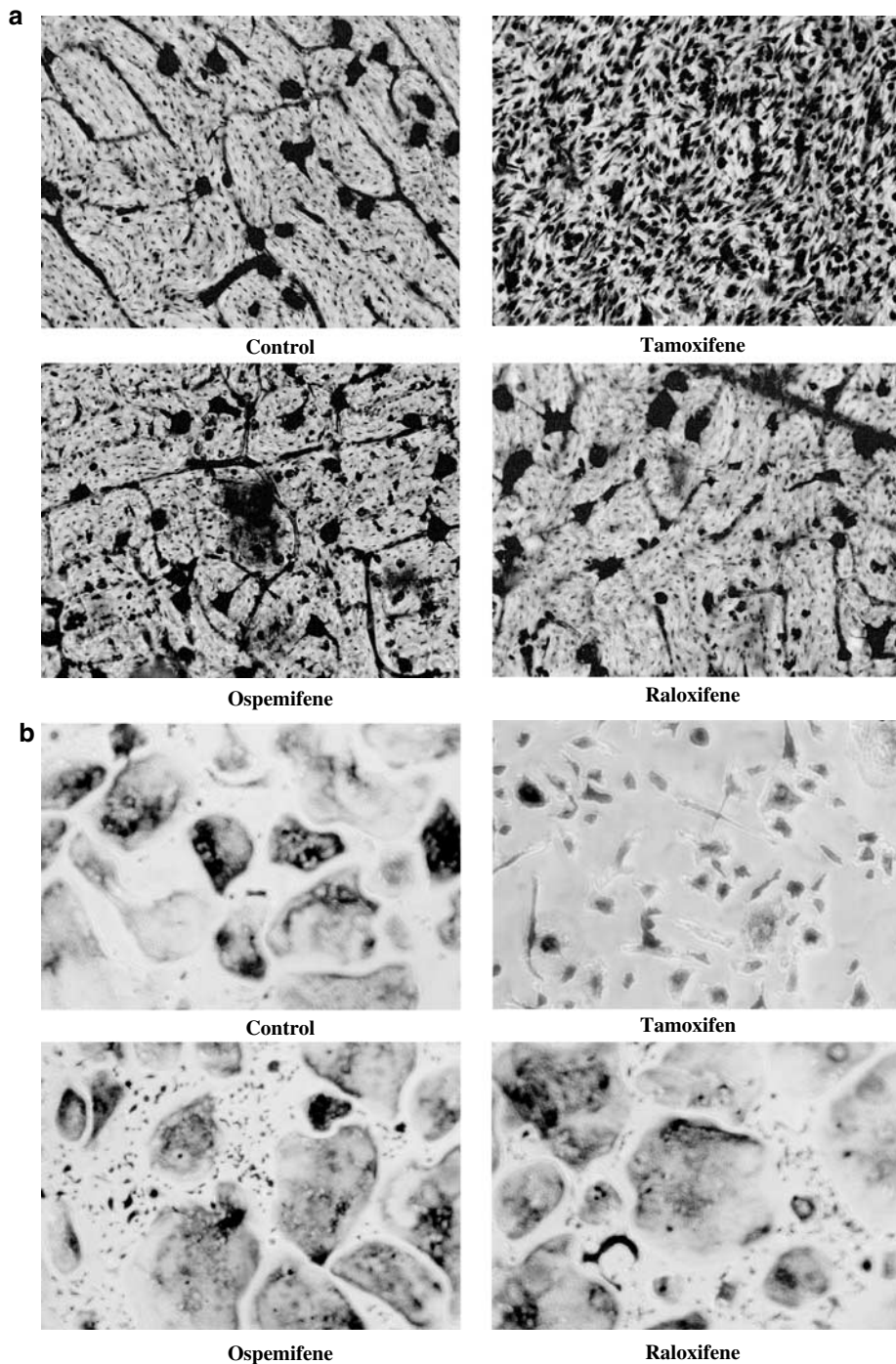
whereas ospemifene and raloxifene did not inhibit osteoclast formation at any given concentration. Figure 2 shows that ospemifene and raloxifene at 1  $\mu\text{M}$  concentration did not inhibit TRACP<sup>+</sup> MNC formation, whereas tamoxifen at the same concentration inhibited the formation of TRACP<sup>+</sup> MNC without affecting the number of TRACP<sup>+</sup> mononuclear cells.

#### Effect of tamoxifen, ospemifene and raloxifene on human osteoclastic bone resorption

Similarly, CD14<sup>+</sup> monocytes were cultured for 7 days on bovine bone slices to induce the differentiation of osteoclasts. The cells were then cultured for another 3 days in the presence of SERMs. CTx analysis (Figure 3a) revealed that tamoxifen (0.01–1  $\mu\text{M}$ ) inhibited osteoclastic bone resorption dose-dependently, compared to the control culture (100%, nontreated culture). CTx values of the cultures, where either ospemifene or raloxifene was present, were similar to the control culture. Figure 3b shows wheat WGA-lectin staining of the resorption pits on the bone slices after the resorption assay was completed. Few resorption pits were formed when osteoclast cultures were treated with tamoxifen. On the other hand, there was a significant number of resorption pits and resorption tracks when osteoclast cultures were treated with either 0.1  $\mu\text{M}$  ospemifene or raloxifene showing that osteoclastic bone resorption was not inhibited by these SERMs.

#### Effect of tamoxifen, ospemifene and raloxifene on osteoclast apoptosis

The extent of osteoclast apoptosis after treatment with different SERMs was estimated by Hoechst DNA staining (Figure 4a and b). The cultures were exposed to SERMs (0.01–1  $\mu\text{M}$ ) for 3 days. When human osteoclasts were treated with 1  $\mu\text{M}$  tamoxifen, all the cells were apoptotic, that is, no viable osteoclasts were observed. In the cultures that were incubated with 0.01 or 0.1  $\mu\text{M}$  tamoxifen, the proportion of apoptotic cells was also clearly increased, relative to the control cultures (0% apoptotic cells). In osteoclast cultures treated with ospemifene or raloxifene, the percentage of apoptotic osteoclasts was 1.6–5.4% over the whole concentration range (0.01–1  $\mu\text{M}$ ). However, none of these values was significantly different from those in control cultures.

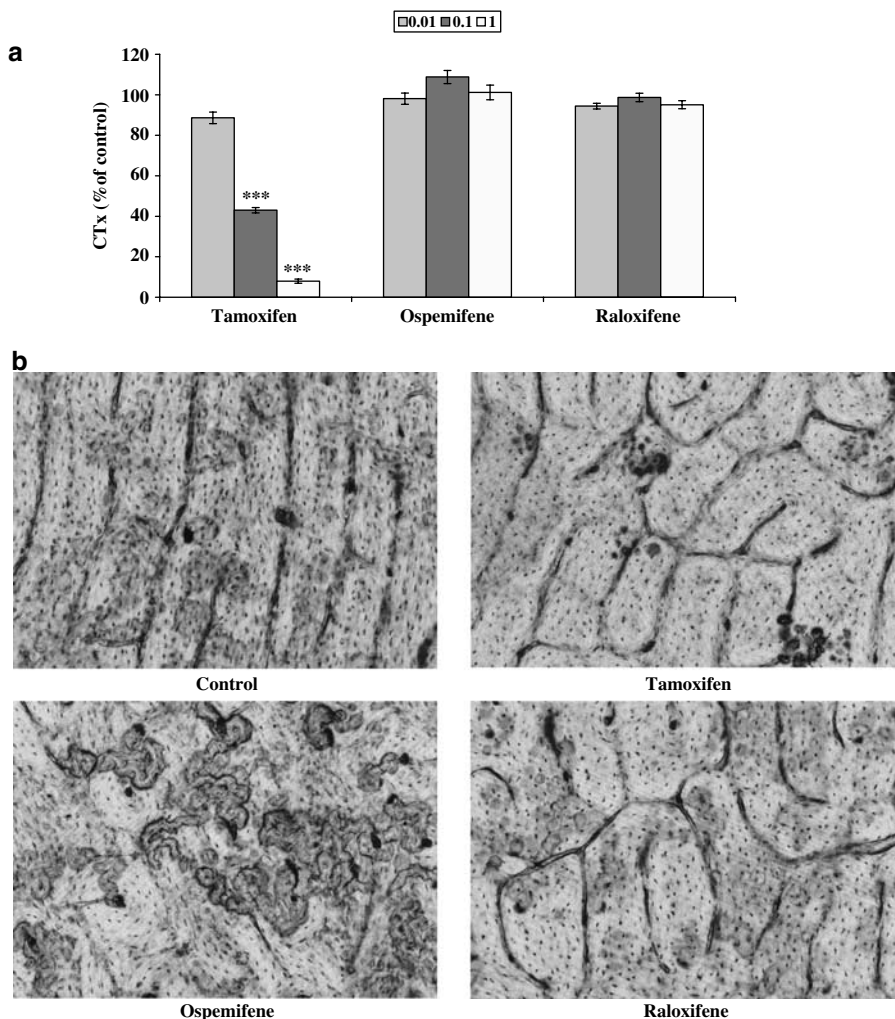


**Figure 2** Effect of  $1 \mu\text{M}$  tamoxifen, ospemifene and raloxifene on TRACP<sup>+</sup> MNC formation during a 7-day culture, either on bovine bone slice (a) or cell culture plate (b). Tamoxifen completely blocked the osteoclast-like cell formation without having a cytotoxic effect on other TRACP<sup>+</sup> mononuclear cells. Microscopic magnification was  $\times 200$ , except  $\times 400$  in tamoxifen-treated cultures.

#### *Time course of the effect of tamoxifen on human osteoclast apoptosis*

As tamoxifen had a potent inhibitory effect on osteoclasts, we decided to study osteoclast apoptosis using shorter time periods (Figure 5). Human CD14<sup>+</sup> monocytes were first induced to differentiate into osteoclasts during a 7-day culture in eight-chamber slides. Tamoxifen ( $0.01\text{--}1.0 \mu\text{M}$ ) was then added and the cultures were continued for 3, 6, 12, 24

and 36 h. At the end of the culture, cells were fixed with 3% PFA and stained with Hoechst and TRACP staining. A significant decrease in osteoclasts was first observed at a 12 h time point in the presence of  $0.1$  or  $1 \mu\text{M}$  tamoxifen and the numbers decreased further at 24 and 36 h. There was a 50 and 80% decrease of osteoclast numbers at a 24 h time point at concentrations of  $0.1$  and  $1 \mu\text{M}$ , respectively. Similarly, at 36 h of culture, there was a significant decrease of osteoclast



**Figure 3** Effects of SERMs (0.01–1  $\mu\text{M}$ ) on the release of CTx by human osteoclasts when cultured on bovine bone slices. Osteoclasts were first induced to differentiate during a 7-day culture. Medium was replaced with new medium containing SERMs and cultures were continued for an additional 3 days. CTx (nM) released from the bone slices was determined as a marker for the resorption activity (a). In the control cultures (= 100%), the CTx value was  $32.6 \pm 2$  nM. In (b), resorption pits were stained with peroxidase-conjugated WGA-lectin, after culturing osteoclasts in the presence of 0.1  $\mu\text{M}$  SERMs. Microscopic magnification was  $\times 200$ . \*\*\* $P < 0.001$ .

numbers, 69 and 84% compared to the control at the respective concentrations.

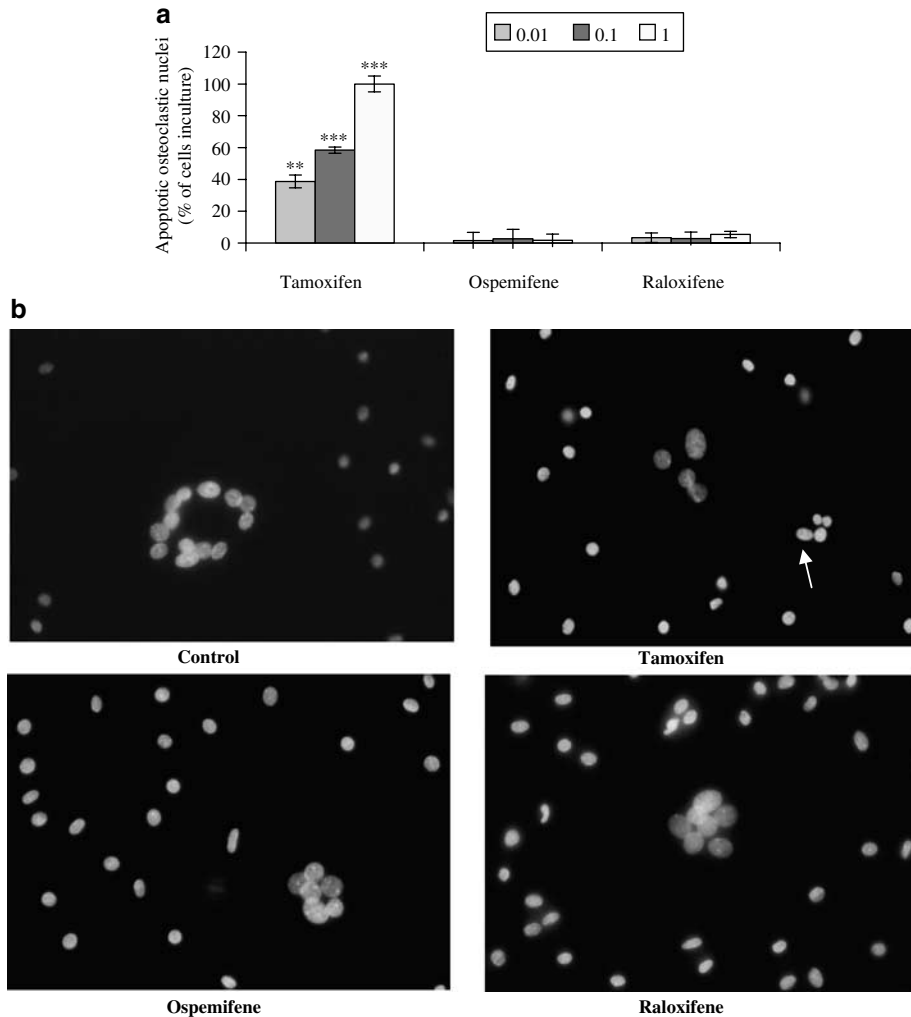
*Effect of tamoxifen, ospemifene and raloxifene in the cocultures of CD14<sup>+</sup> monocytes and SaOS-2 and MG-63 cells*

We also cocultured human peripheral blood-purified CD14<sup>+</sup> monocytes either with human SaOS-2 or MG-63 cells in the presence of PTH for 7 days (Figure 6). Formation of osteoclasts was estimated by measuring TRACP-5b released in the cell culture medium. When CD14<sup>+</sup> monocyte and osteosarcoma cell cocultures were treated either with 0.1 or 1  $\mu\text{M}$  tamoxifen, ospemifene or raloxifene, there was a marked inhibition in the formation of osteoclasts. The inhibition was approximately 50% at 0.1  $\mu\text{M}$  and 63% at 1  $\mu\text{M}$  compared to the control cultures. In addition, raloxifene at its lowest concentration (0.01  $\mu\text{M}$ ) caused a statistically significant inhibition of osteoclast formation (Figure 6a and b).

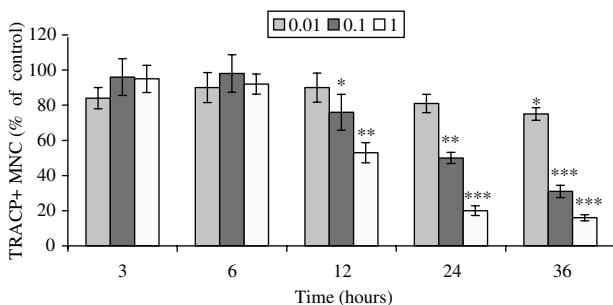
*Effect of oestrogen receptor antagonist ICI 182 780 on osteoclast cultures when treated with tamoxifen, ospemifene and raloxifene*  
We then examined whether the inhibitory effect of SERMs was mediated via oestrogen receptor by using ICI 182 780, an oestrogen receptor antagonist. As shown in Figure 7a, in direct osteoclast culture, ICI 182 780 (in 100-fold higher concentration than tamoxifen) antagonized the inhibitory effect of tamoxifen on osteoclast formation without having any effect by itself. Similarly, when ICI 182 870 was added to CD14<sup>+</sup> and SaOS-2 cell cocultures (Figure 7b) and CD14<sup>+</sup> and MG-63 cell cocultures (Figure 7c), it reversed the inhibitory effect of tamoxifen, ospemifene and raloxifene.

*Effect of tamoxifen, ospemifene and raloxifene on production of OPG*

Using a sensitive sandwich ELISA method, OPG concentrations were measured in the media collected from the cocultures of monocytes and SaOS-2 or MG-63 cells



**Figure 4** Hoechst DNA staining of human osteoclasts treated with or without SERMs (0.01–1  $\mu\text{M}$ ) for 3 days. There were no apoptotic osteoclasts in control cultures (untreated cells, 0%). The proportion of apoptotic nuclei (%) was measured in the cultures with SERMs (a). In (b), a stained culture treated with tamoxifen shows an apoptotic osteoclast (arrow). Microscopic magnification was  $\times 400$ . \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .



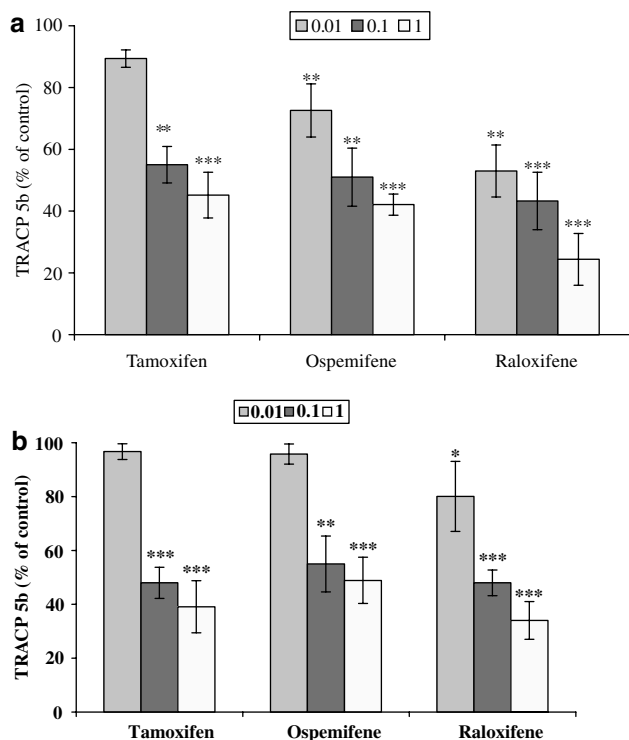
**Figure 5** Time course of the effect of tamoxifen on TRACP<sup>+</sup> MNC formation in CD14<sup>+</sup> monocyte cultures in 8-chamber slides. CD14<sup>+</sup> monocytes were cultured either with or without tamoxifen (0.01–1  $\mu\text{M}$ ) for 36 h. The number of TRACP<sup>+</sup> MNC in the control cultures was  $47 \pm 7.7$ . \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

(Figure 8). In the control cultures (without tamoxifen, ospemifene or raloxifene), OPG concentration was between 0.5–0.8 pM. At 0.1  $\mu\text{M}$  the SERMs caused an up to 6.4-fold stimulation of OPG production on average. At 1  $\mu\text{M}$ , the

stimulatory effect was approximately 12-fold in CD14<sup>+</sup> and SaOS-2 cells cocultures, compared to the control (Figure 8a). Similarly, OPG concentrations in the medium of CD14<sup>+</sup> and MG-63 cells cocultures grown in the presence of SERMs (0.1 and 1  $\mu\text{M}$ ), were increased for up to 4.7- and 7.5-fold, respectively compared to the control (Figure 8b).

## Discussion and conclusions

We have previously reported that high numbers of osteoclasts were produced when CD14<sup>+</sup> monocytic precursors, from peripheral blood, were cultured in the presence of RANKL, M-CSF, TNF- $\alpha$  and dexamethasone with 9% FCS in  $\alpha$ -MEM. In addition, a functional bone resorption assay was developed to estimate the bone resorbing activity of the osteoclasts formed in this culture system (Michael *et al.*, 2005b). We preferred to use blood from male donors as it has been shown that osteoclast formation and resorption capacity is higher in male than in female (Jevon *et al.*, 2002). Osteoclasts were, however, also formed in CD14<sup>+</sup>

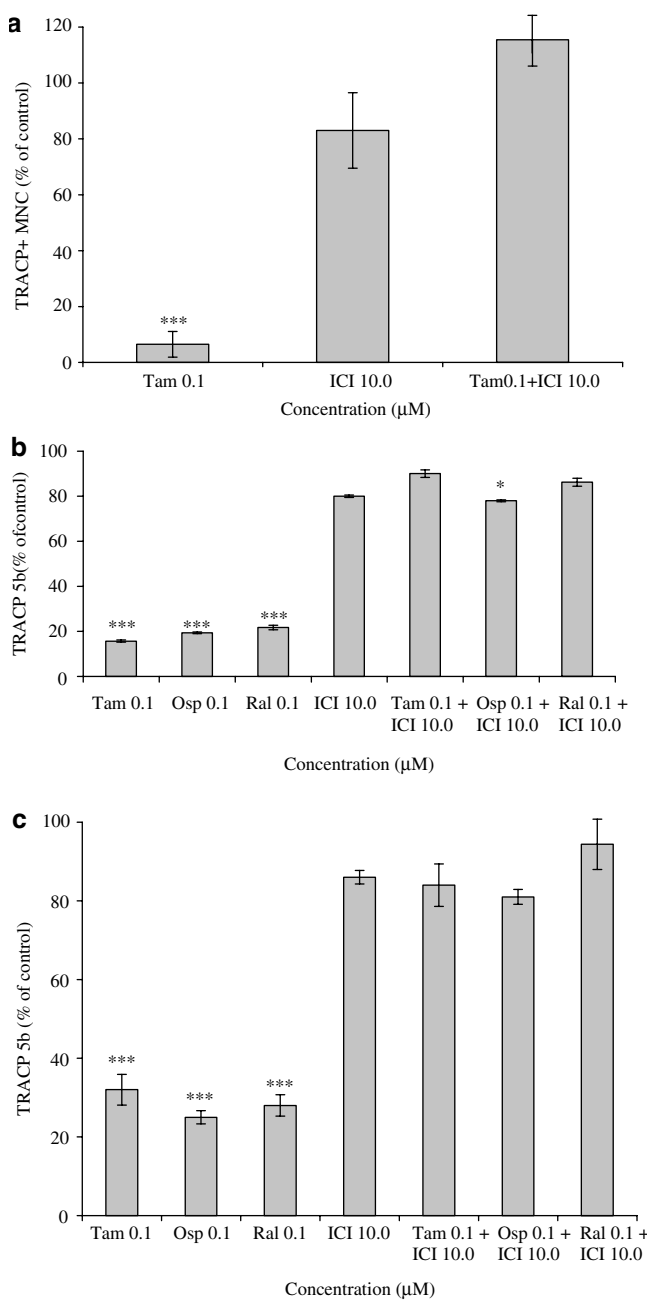


**Figure 6** Human SaOS-2 (a) or MG-63 (b) cells were cocultured for 7 days with human peripheral blood osteoclast precursors, CD14<sup>+</sup> monocytes, in the presence of 10 nM PTH and treated with SERMs (0.01–1 μM). TRACP 5b released by osteoclasts into culture media was measured by ELISA assay (shown as units phosphatase activity l<sup>-1</sup>). The concentration of TRACP 5b in the control culture (100%) was 4.37 ± 0.6 U l<sup>-1</sup>. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001.

monocyte cultures from female donors and the results obtained were comparable to those from the monocyte fraction from male donors.

In this study, we have examined the effects of three different SERMs, tamoxifen, ospemifene and raloxifene all of which have characteristic profiles including bone-sparing effects on differentiation of human peripheral blood-derived CD14<sup>+</sup> monocytes into osteoclasts when cultured either on bovine bone slices or in cell culture plates. To study possible indirect effects of SERMs on osteoclast formation and function, we cocultured CD14<sup>+</sup> cells with human SaOS-2 or MG-63 cells. Our results show that in the CD14<sup>+</sup> monocyte differentiation assay, only tamoxifen was able to inhibit osteoclast formation whereas in cocultures all three SERMs inhibited osteoclast differentiation by a mechanism, that was associated with an increased secretion of OPG and seemed to be mediated by oestrogen receptors.

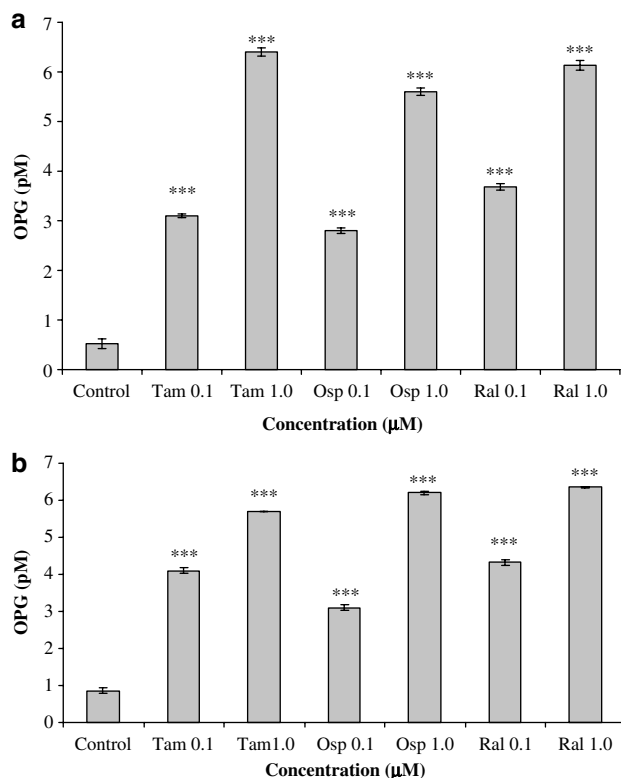
Our results are consistent with earlier studies that showed that tamoxifen inhibits osteoclastic bone resorption in ovariectomized rats (Turner *et al.*, 1987, 1988). Tamoxifen has been reported to block bone resorption at 2–10 μM concentrations in osteoclast cultures of neonatal rats (Arnett *et al.*, 1996) and in organ culture studies, at 50–100 μM concentrations (Stewart and Stern, 1986). In our experiments, the inhibitory effect of tamoxifen on human osteoclasts was observed at much lower concentrations (0.01–1 μM). The explanation of these differences in effective



**Figure 7** Effect of 10 μM ICI 182780 on osteoclast formation when added to CD14<sup>+</sup> monocyte cultures with or without tamoxifen, ospemifene or raloxifene (0.1 μM). Results from CD14<sup>+</sup> monocyte culture are shown in (a); result from CD14<sup>+</sup> and SaOS-2 cell cocultures in (b) and results from CD14<sup>+</sup> and MG-63 cell coculture in (c). ICI 182780 (100-fold excess over SERM) completely antagonized the effect of tamoxifen, ospemifene or raloxifene. \*P < 0.05; \*\*\*P < 0.001.

concentrations may be that we used human monocyte-derived osteoclast cultures, which may be more sensitive to tamoxifen. This was suggested by an observation that tamoxifen markedly decreased osteoclast viability without affecting mononuclear cells. The osteoclast cultures reported earlier (Arnett *et al.*, 1996) were established from bone marrow cells, which also included osteoblasts/stromal cells, in addition to osteoclast precursors.





**Figure 8** OPG concentrations determined in the culture media of co-cultures of SaOS-2 (a) or MG-63 (b) cells and CD14<sup>+</sup> monocytes that were treated with ospemifene, tamoxifen or raloxifene. Note that SERMs significantly and dose-dependently stimulated OPG production in these cultures. \*\*\**p* < 0.001.

Tamoxifen-treated osteoclasts detached from the culture plates, their nuclei were condensed and the cells showed morphological changes associated with apoptosis. Induction of cell death may have been associated with the ionophore-like effect exerted by tamoxifen on the plasma membrane of osteoclasts (Lehenkari *et al.*, 2003). In breast cancer cells, tamoxifen cytotoxicity has been related to nonoestrogen receptor-mediated reduction in cell membrane fluidity (Clarke *et al.*, 1990) and to acute effects on mitochondrial function which were, however, at least partly oestrogen receptor dependent (Kallio *et al.*, 2005). In our experiments, the ability of the anti-oestrogen ICI 182780 to prevent inhibition of osteoclast differentiation by tamoxifen also suggests an oestrogen receptor-mediated mechanism.

In humans, tamoxifen has bone-sparing effects and the therapeutic concentration of circulating tamoxifen in humans is approximately 0.5 μM but, together with active metabolites, it can be as high as 2–3 μM (Trump *et al.*, 1992; Peyrade *et al.*, 1996). Our present data indicated that tamoxifen at 0.01–1 μM can be lethal to human osteoclasts cultured in a medium containing 9% FCS. It is thus possible that this effect could be involved in the bone-sparing effect of tamoxifen. An anti-resorptive skeletal profile has been firmly established for tamoxifen in postmenopausal breast cancer patients (Clemons *et al.*, 2002; Yoneda *et al.*, 2002). An increase in bone mineral density has been observed in them, although this has not been seen in the premenopausal

patients (Powles *et al.*, 1996). Data from breast cancer prevention trials suggest that this skeletal effect may prevent fractures as well in postmenopausal patients, although the findings were not statistically significant (Fisher *et al.*, 1998). Experiments in rats have additionally proven that *in vivo* tamoxifen prevents the increase in osteoclast number and activity that is associated with the bone loss resulting from ovariectomy (Turner *et al.*, 1987, 1988). Another SERM, toremifene, that effectively inhibits breast cancer growth and that is used in breast cancer treatment (Pagani *et al.*, 2004) also shows some bone-sparing activity (Tiitinen *et al.*, 2003) and breast cancer cells (Kallio *et al.*, 2005) are very similar to those of tamoxifen. Although not studied here, one could expect that the effects of toremifene on osteoclastogenesis to be rather similar. The major metabolites of tamoxifen and toremifene (4-hydroxy- and *N*-desmethyl-derivatives) may also contribute to bone effects *in vivo* but this possibility remains to be studied.

Raloxifene treatment has been demonstrated to reduce the risk of both osteoporotic vertebral fractures (Ettinger *et al.*, 1999) and invasive breast cancer (Cummings *et al.*, 1999) in postmenopausal women and it is becoming important in prevention of vertebral fractures in postmenopausal women. It has also been shown to inhibit bone resorption in ovariectomized rats (Evans *et al.*, 1996). In mouse and human bone marrow cultures, raloxifene has been shown to inhibit osteoclast formation (Ramalho *et al.*, 2002; Taranta *et al.*, 2002) by inhibiting TNF-α action on osteoblastic cells (Taranta *et al.*, 2002) or by upregulating transforming growth factor-β3 expression (Narayana Murthy *et al.*, 2006). It has also been shown that raloxifene enhances OPG production and concurrently suppresses interleukin-6 (IL-6) secretion in normal human osteoblastic cells that express predominantly oestrogen receptor-α (Viereck *et al.*, 2003). Raloxifene treatment has also been reported to downregulate the production of bone resorption-enhancing cytokines such as IL-1α, IL-1β, IL-6 and TNF-α by human osteoblastic cells and murine osteoblastic cells (Taranta *et al.*, 2002; Cheung *et al.*, 2003). In a clinical study of postmenopausal women, raloxifene has been shown to decrease significantly the levels of IL-6 and TNF-α in serum, whereas bone densities were increased (Gianni *et al.*, 2004). In our experiments, raloxifene did not have inhibitory effects on the formation of osteoclasts from CD14<sup>+</sup> cells and on the *in vitro* bone resorption by the osteoclasts formed. However, in the cocultures with human osteoblastic cells, raloxifene inhibited osteoclast formation. Furthermore, our results suggested that raloxifene inhibition was associated with increased production of OPG from MG-63 and SaOS-2 cells cocultured with purified monocytes. In our experiments, the level of raloxifene stimulation of OPG production was similar to that induced by estradiol, being approximately 5–10-fold higher than in control cultures (Liao *et al.*, 2002).

Ospemifene also caused a statistically significant inhibition of osteoclast formation when CD14<sup>+</sup> monocytes were cocultured with human osteoblastic cells. This suggests that ospemifene action was also mediated through osteoblastic cells through oestrogen receptors. Ospemifene has been found to stimulate bone formation *in vitro* (Qu *et al.*, 1999)

and to have bone-sparing effect and to reduce bone turnover in ovariectomized rats (Qu *et al.*, 2000) and in postmenopausal women (Komi *et al.*, 2004, 2006).

Our studies showed that the oestrogen receptor antagonist, ICI 182780, completely reversed the inhibitory effect of tamoxifen in CD14<sup>+</sup> monocyte cultures as well as that of tamoxifen, raloxifene and ospemifene in cocultures with osteoblast-derived cells. These findings suggest that the effects of these SERMs on osteoclast formation in human peripheral blood-derived osteoclast cultures are mediated by oestrogen receptors. Both MG-63 (Mahonen and Maenpaa, 1994; Vidal *et al.*, 1999) and SaOS-2 (Sutherland *et al.*, 1996; Vidal *et al.*, 1999) cells express oestrogen receptors. It has also been shown that OPG production in MG-63 cells is stimulated by oestrogen and IL-1 $\alpha$  (Vidal *et al.*, 1998; Su *et al.*, 2003). OPG is also produced by human osteoblastic cell line, HOB, and the human bone marrow stromal cell line, HCCL, when treated with raloxifene (Cheung *et al.*, 2003; Viereck *et al.*, 2003). No data on the effect of either tamoxifen or ospemifene on OPG production by osteoblastic cells have previously been available. Our results show that in cocultures, tamoxifen, raloxifene and ospemifene all have an oestrogen agonist effect on OPG production by osteoblastic cells. This is in accordance with our previous results on oestrogen effects on osteoclast differentiation (Michael *et al.*, 2005a).

OPG has been identified as a soluble member of the TNF-receptor family (Yasuda *et al.*, 1998), which acts as a paracrine factor within the bone microenvironment to decrease bone resorption. OPG is likely to be a major regulatory factor of bone metabolism, as it is produced by osteoblasts and stromal cells and its production is regulated by many of the major calcitropic hormones and cytokines, such as oestrogen, 1,25-dihydroxyvitamin D<sub>3</sub>, PTH, TNF- $\alpha$  and IL-1 $\beta$  (Hofbauer *et al.*, 1998). OPG administered to normal rats increased bone density by inhibiting osteoclastogenesis and prevented bone loss following ovariectomy. Moreover, hepatic overexpression of OPG in transgenic mice induced osteopetrosis (Simonet *et al.*, 1997) and targeted ablation of OPG led to the early onset of osteoporosis (Bucay *et al.*, 1998; Mizuno *et al.*, 1998).

In conclusion, our results suggest that human osteoclast formation and bone resorption is inhibited by tamoxifen both directly and indirectly through osteoblastic cells, whereas ospemifene and raloxifene seem to act indirectly only. Increased osteoblastic production of OPG was associated with the indirect SERM inhibition of osteoclastogenesis and both direct and indirect mechanisms appeared to be mediated via oestrogen receptors.

## Conflict of interest

The authors state no conflict of interest.

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