Ormeloxifene inhibits osteoclast differentiation in parallel to downregulating RANKL-induced ROS generation and suppressing the activation of ERK and JNK in murine RAW264.7 cells

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Abstract

Ormeloxifene (Orm), a triphenylethylene compound, has been established as a selective estrogen receptor modulator (SERM) that suppresses the ovariectomy-induced bone resorption in rats. However, the precise mechanism underlying the bone-preserving action of Orm remains unclear. In this study, we evaluated the effect of Orm on osteoclast formation induced by receptor activator of nuclear factor κ B ligand (RANKL) in the murine macrophage cell line RAW264.7. We also explored the mechanism of action of Orm by studying the RANKL-induced signaling pathways required for osteoclast differentiation. We found that Orm inhibited osteoclast formation from murine macrophage RAW264.7 cells induced by RANKL in a dose-dependent manner. Orm was able to abolish RANKL-induced reactive oxygen species (ROS) elevation and inhibited the transcriptional activation of two key RANKL-induced transcription factors namely activator protein-1 (AP-1) and NF- κ B through mechanisms involving MAPKs. Activation of two MAPKs, i.e. ERK (MAPK1) and JNK (MAPK8), was alleviated by Orm effectively, which subsequently affected the activation of c-Jun and c-Fos, which are the essential components of the AP-1 transcription complex. Taken together, our results demonstrate that Orm potentially inhibits osteoclastogenesis by inhibiting ROS generation and thereby suppressing the activation of ERK1/2 (MAPK3/MAPK1) and JNK (MAPK8) and transcription factors (NF- κ B and AP-1), which subsequently affect the regulation of osteoclastogenesis. These results provide a possible mechanism of action of Orm in regulating osteoclastogenesis, thereby supporting the beneficial bone-protective effects of this compound.

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Introduction

Estrogen regulates bone metabolism that is controlled by a balance between bone resorption by osteoclasts and bone formation by osteoblasts (Syed & Khosla 2003). The effects of estrogen on osteoblasts include both direct effects resulting in increased bone formation, and an indirect effect, via an osteoblast-mediated interaction with preosteoclasts and osteoclasts, resulting in decreased bone resorption (Galal *et al.* 2007). Estrogen exerts its indirect effect through products secreted by osteoblasts that include receptor activator of nuclear factor κ B ligand (RANKL), colony-stimulating factor-1 (CSF1), and osteoprotegerin (OPG), which are important in differentiation and maturation of osteoclasts. In addition, estrogen treatment has been shown to inhibit osteoclast formation from mononuclear hematopoietic stem cells (Jilka *et al.* 2001) and also affects adhesion and apoptosis of osteoclasts (Saintier *et al.* 2006, Nakamura *et al.* 2007). Decrease in reactive oxygen species (ROS) generation (Lean *et al.* 2003, Maggio *et al.* 2003) and secretion of

pro-inflammatory cytokines interleukin 1 (IL1), IL6, and tumor necrosis factor α (TNF α) by marrow monocytes (Riggs *et al.* 2002) add to the beneficial effects of estrogen. It is well documented that TNF-related activation-induced cytokine (TRANCE; also called RANKL, OPGL, and ODF), originally identified as a member of the TNF family, is expressed by osteoblastic cells and substitutes for stromal cells in osteoclast formation *in vitro* (Asagiri & Takayanagi 2007). Binding of RANKL to its signaling receptor RANK leads to recruitment of TNF receptor-associated factor 6 (TRAF6) to the cytoplasmic domain of RANK (Darnay *et al.* 1999, Kim *et al.* 2009). This results in the stimulation of various intracellular signaling pathways involving MAPKs (ERK (MAPK1), JNK (MAPK8)/SAPK, and p38), PI3K/Akt, and NF- κ B that are activated in osteoclast precursors and mature osteoclasts (Lee & Kim 2003, Kim *et al.* 2005).

Ormeloxifene (Orm), a nonsteroidal selective estrogen receptor modulator (SERM), has been developed as a weekly oral contraceptive pill (Singh 2001). Orm binds to both ER α and ER β (Blesson *et al.* 2006). In the

uterus, it modulates estrogen action by classical estrogen antagonism at corepressor recruitment (Daverey *et al.* 2009) and via a nonclassical activator protein-1 (AP-1)-mediated mechanism (Awasthi *et al.* 2007). Orm has also shown anticancer activity in phase II and III clinical trials conducted on terminal cases of breast cancer (Misra *et al.* 1989). Besides antibreast cancer activity, Orm has been demonstrated to cause the inhibition of estrogen-deficient osteoporosis in rats (Arshad *et al.* 2004). It induced the apoptosis of osteoclasts via upregulation of TGF β 3 (TGFB3) expression in rat (Narayana Murthy *et al.* 2006). However, the precise mechanism by which Orm exerts its tissue-specific estrogen agonistic effect and also the antiosteoclastogenic effect remains unknown. This study was carried out to explore the possible mechanism of osteoclastogenesis inhibition caused by Orm using the murine macrophage cell line RAW264.7. RAW264.7 cells serve as an excellent model of osteoclast differentiation as RANKL treatment induces their differentiation into osteoclast-like tartrate-resistant acid phosphatase (TRAP)-positive cells (Hsu *et al.* 1999, Collin-Osdoby *et al.* 2003). Herein, the effects of Orm on RANKL-induced ROS generation, ERK- and JNK-activated c-Jun and c-Fos signaling, during inhibition of osteoclastogenesis and osteoclast differentiation, were studied in RAW264.7 cells (Boyle *et al.* 2003, Forman *et al.* 2004).

Materials and methods

Reagents and antibodies

All cell culture and SDS-PAGE reagents were purchased from Sigma, unless otherwise stated. Antibodies for c-Jun, phospho-c-Jun, c-Fos, JNK, ERK1/2, phospho-ERK1/2, phospho-SAPK, and RANK were purchased from Cell Signaling Technology, Beverly, MA, USA. ER α -specific antibody and secondary antibodies were obtained from Santa Cruz Biotechnologies. Reagents for western blot were obtained from GE Healthcare, Bucks, UK. Amaya kit was procured from Amaya Biosystems, Gaithersburg, MD, USA. Dichlorofluorescein diacetate (DCFH-DA) was purchased from Invitrogen Life Technologies. 17 β -Estradiol (E $_2$) and raloxifene (Ral) were purchased from Sigma. ICI 182 780 was purchased from Tocris, Bristol, UK. Orm was synthesized and kindly provided by the medicinal chemists of the Central Drug Research Institute (Tripathi *et al.* 1997).

Plasmids

Luciferase reporter plasmids *pAPI-luc*, *pNF- κ B-luc*, and *pFR-luc*; expression vectors *pFA-c-Jun*, *pFA-c-Fos*, and *pFA-ATF2*, and negative control plasmid *pFC-dbd* were

purchased from Stratagene, USA. The expression plasmids used were composed of chimeric genes containing the DNA-binding domain of GAL4 fused to the activation domains of c-Jun, c-Fos, or ATF2. *pRL-SV40-luc* was obtained from Promega, and used as an internal control plasmid during transfection.

Cell culture

The murine monocytic RAW264.7 cell line was procured from ECACC (Salisbury, UK). The cells were maintained in low-glucose (1 g/l) DMEM, pH 7.4, supplemented with 10% fetal bovine serum (FBS) and 1 $\times$ antibiotic-antimycotic mix. Once the cells became 70% confluent, they were scraped using the sterile cell scraper and split at a ratio of 1:8. The medium was changed to phenol red-free DMEM containing antibiotics with 10% charcoal-stripped FBS 3 days before starting the experiment. The cell line was maintained at 37 °C with 5% CO $_2$ in humidified incubator.

Characterization and quantitation of osteoclast-like cells

RAW264.7 cells were cultured in a 96-well plate at a density of 5000 cells/well and treated with RANKL at the beginning of the culture and during a medium change on day 3. Osteoclast formation was assessed by counting the total number of multinucleated cells (≥ 3 nuclei), TRAP-positive cells present per well on day 5 (Collin-Osdoby *et al.* 2003). Briefly, after incubation, cells were washed with PBS, fixed in 10% formalin for 10 min, and stained for acid phosphatase in the presence of 0.05 M sodium tartrate (Sigma). Naphthol AS-BI phosphate (Sigma) was used as the substrate. Only TRAP-positive cells, showing a bright pink-colored cytoplasm, were counted by light microscopy.

Cell viability assay

RAW264.7 cells were plated in a 96-well plate at a cell density of 1 $\times 10^3$ cells/well and were incubated for 12 h at 37 °C and 5% CO $_2$. After 12 h, the medium was replaced with fresh medium containing 100 μ M RANKL and the cells were incubated with E $_2$ (10 and 100 nM), Ral (100 nM and 1 μ M), or Orm (100 nM, 1 μ M, and 10 μ M) for 5 days, with a medium change on the third day. One control group with vehicle (ethanol) alone was also included. After the incubation period was over, MTT was added to the wells at a final concentration of 0.5 mg/ml and incubated further for 2 h. The medium was then removed and the formazan crystals thus formed were dissolved by adding DMSO and incubated for a further 15 min at room temperature. Optical density (OD) was measured at 540 nm in a spectrophotometer.

ELISA of ERK1/2 (MAPK3/MAPK1) [pTpY185/187] and JNK [pTpY183/185]

The phosphorylation level of ERK was quantified using ELISA kit (KHO0091; Invitrogen). In brief, RAW264.7 cells were incubated with E₂ (100 nM), Ral (1 μ M), or Orm (10 μ M) for 24 h and then stimulated with 200 ng/ml RANKL for 30 min. At the end of incubation, cell lysate was prepared by lysing the cells in buffer containing protease and phosphatase inhibitors. ERK/JNK activation was measured using 10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na₄P₂O₇, 2 mM Na₃VO₄, 1% TritonX-100, 10% glycerol, 0.1% SDS, and 0.5% deoxycholate following the manufacturer's instructions. OD was measured with MicroQuant ELISA reader (Biotek, Winooski, VT, USA) at 450 nm. The experiments were performed three times with three replicates in each.

Western blot analysis

RAW264.7 cells were incubated with E₂, Ral, or Orm for 24 h and were stimulated with 200 ng/ml RANKL for 30 min. Cell lysate was prepared by lysing the cells in buffer containing 50 mM Tris-HCl, 137 mM NaCl, 1% NP-40, 10% glycerol, 1 mM sodium fluoride supplemented with protease and phosphatase inhibitors. The lysates (30 μ g) were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After blocking with 5% skimmed milk, the membrane was probed with primary antibody (antiphospho-JNK or antiphospho-c-Jun). The same membranes were stripped and reprobed with anti-JNK, c-Jun, c-Fos, and β -actin. Blots were developed using HRP-conjugated secondary antibody and visualized using ECL (Amersham).

The expression level of RANK was also determined by western blotting using anti-RANK and anti-ER α antibodies using the protocol described earlier.

Transient transfection assays

RAW264.7 cells were transfected with a total of 2 μ g DNA using Amaxa kit (Amaxa Biosystems) according to the manufacturer's protocol. The transfection efficiency was almost 40%. Briefly, 2×10^6 cells were pelleted down and suspended in 100 μ l Nucleofector Solution V. Afterward, the cells were transfected with *pAPI-luc* (luciferase reporter plasmid containing three copies of an AP-1 response element) or *pNF- κ B-luc* reporter plasmid (containing NF- κ B binding site). Another set of cotransfection reactions was set in which RAW264.7 cells were cotransfected with pFR-luc and one of the following plasmids: *pFA2-c-Jun* (c-Jun), *pFA-c-Fos* (c-Fos), *pFA-ATF2* (ATF2), or *pFC-dbd* (GAL4). pRL-SV40-luc was also introduced in each case as

an internal control. Nucleofector program D-032 was run and the cells were immediately transferred to the DMEM supplemented with 20% FBS and incubated for 12 h in the incubator at 37 °C with 5% CO₂ in humidified chamber. After nucleofection, the cells were scraped, counted, and seeded into a 96-well plate at a density of 2×10^4 cells/well and were incubated with E₂ (0.1, 1, 10, and 100 nM), Ral, or Orm (10 nM, 100 nM, 1 μ M, and 10 μ M) for 24 h in the presence of RANKL (200 ng/ml). After 24 h, the cells were lysed; firefly luciferase and *Renilla* luciferase activities were measured using Dual Luciferase Assay System (Promega). All values were normalized with internal control, i.e. *Renilla* luciferase. Transcriptional activity was measured as the percentage of normalized luciferase activity, calculated by determining the ratio of normalized luciferase luminescence in the presence of test sample and normalized luciferase luminescence in the unstimulated vehicle-treated group.

Assay of intracellular ROS

The intracellular generation of ROS was examined using DCFH-DA (Invitrogen) using flow cytometry as described (Lee *et al.* 2005). In brief, RAW264.7 cells were seeded in a 24-well plate at a density so that they

Figure 1 Effect of Orm on osteoclast differentiation in murine RAW264.7 cells. (Panel A) Undifferentiated (a) and differentiated (marked with arrow) (b) RAW264.7 cells. (Panel B) The expression of OC biomarker NFATc1 in differentiated RAW cells. (Panel C) 2×10^5 cells/well were plated in triplicate and multi-nucleated (≥ 3 nuclei), TRAP-positive osteoclasts were quantified, following induction for 5 days with mouse-soluble RANKL in the presence of vehicle, 17 β -estradiol (10 and 100 nM), raloxifene (100 nM and 1 μ M), ormeloxifene (100 nM, 1 μ M, and 10 μ M), or E₂ (100 nM) + ICI 182 780 (100 nM). *** $P < 0.001$ vs control.

become almost 70% confluent after 24 h. The next day, the medium of the wells was changed and the cells were incubated with vehicle, E_2 (10 and 100 nM), Ral (100 nM and 1 μ M), or Orm (100 nM, 1 μ M, and 10 μ M) for 24 h at 37 °C with 5% CO_2 in humidified incubator. Following incubation, the cells were stimulated with RANKL (100 ng/ml) for 15 min at 37 °C. The wells were then washed and incubated in the dark for 30 min in Krebs-Ringer solution (125 mM NaCl, 5 mM KCl, 25 mM HEPES, 6 mM glucose, 5 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 , and 2.4 mM $CaCl_2$, pH 7.4), containing 5 μ M DCFH-DA. After treatment with DCFH-DA, cells were washed with PBS thrice, scraped, and suspended in 400 μ l PBS, and analyzed with a flow cytometer (FACS calibur; BD Biosciences, San Jose, CA, USA). DCF data were recorded at an excitation wavelength of 488 nm and an emission wavelength of 515–540 nm. The 10 000 cells per sample were acquired in histogram using a data analysis program CELL Quest. Dead cells and debris were excluded from the analysis by electronic gating of forward and side scatter measurements.

Statistical analysis

The values were expressed as mean $\pm$ S.E.M. of three independent experiments. The statistical analysis was performed by one-way ANOVA and the significance was determined by Newman–Keuls test.

Results

Inhibitory effects of Orm on osteoclast differentiation in RAW264.7 cells

Monocyte/macrophagic RAW264.7 cells have been described as a model for osteoclast formation (Collin-Osdoby *et al.* 2003). With a view to determine the potential of Orm to affect the osteoclast differentiation, we evaluated whether RANKL-induced osteoclast formation could be inhibited by the molecule. Stimulation of the RAW264.7 cells with soluble mouse RANKL (100 ng/ml) for 5 days led to a profound differentiation of the monocytic–macrophagic cell line into multinucleated (≥ 3 nuclei) TRAP-positive cells. In addition to TRAP-positive staining, the cellular differentiation of RAW264.7 cells was also assessed by observing the expression of *Nfatc1* (Fig. 1A and B).

Quantitation of the number of multinucleated, TRAP-positive cells present on day 5 after treatment of RAW264.7 cells with E_2 (10 and 100 nM), Ral (100 nM and 1 μ M), or Orm (100 nM, 1 μ M, and 10 μ M) revealed that E_2 caused the suppression of RANKL-induced osteoclast formation by almost 70%

at 100 nM concentration while by 50% at 10 nM concentration. Ral mimicked the effect of E_2 in reducing osteoclast formation. Similarly, Orm displayed the antiosteoclastogenic potential in a dose-dependent manner, reducing the TRAP-positive cell number to 50% at 1 μ M concentration (Fig. 1C). Treatment of cells with ICI 182 780 alone or along with E_2 did not caused inhibition in osteoclast formation.

Cell viability assay performed in order to evaluate the cytotoxic potential of Orm, if any, revealed that Orm did not inhibit cell proliferation of RAW264.7 cells at the concentrations used in this study (data not shown).

Figure 2 Effect of Orm on RANK expression in RAW cells. (Panel A) RAW264.7 cells incubated with 17 β -estradiol (100 nM), ICI 182 780 (100 nM), raloxifene (100 nM and 1 μ M), or ormeloxifene (1 and 10 μ M), for 24 h, and then stimulated with 200 ng/ml of RANKL for 30 min. (Panel B) Cells were incubated with ICI 182 780 or Orm in the presence of E_2 for 24 h and then stimulated with 200 ng/ml of RANKL for 30 min. Cell lysates were prepared and subjected to western blotting using specific antibody. The β -actin blot is shown as a loading control. Quantification of bands was done by densitometric analysis. The results are representative of three independent experiments. ### P < 0.001, ## P < 0.01 vs vehicle and *** P < 0.001, * P < 0.05 vs vehicle + RANKL group.

Effect on RANK expression

With a view to find out whether Orm caused the suppression of RANK signaling due to altering RANK expression or not, western blot analysis of RANK was performed. The results revealed that Orm

did not cause any significant change ($P>0.05$) at 10 μM concentration (Fig. 2) in RANK expression level. However, at the lower concentration, i.e. 1 μM Orm, there was a significant increase in RANK expression ($P<0.01$). RANKL and ICI 182 780 induced RANK expression significantly ($P<0.001$).

When cells were incubated with Orm in the presence of E_2 , no significant change in RANK expression was observed. However, ICI 182 780, in the presence of E_2 , induced RANK expression significantly ($P<0.01$; Fig. 2B).

Suppression of RANK signaling to ERK and JNK MAPKs

Here, activation of three members of the MAPK family, namely ERK, JNK (also known as SAPK), and p38 was studied. To study the effect of Orm on the MAPK signaling pathway and its comparison with Ral, a known antiresorptive agent, RAW264.7 cells were stimulated with RANKL in the presence of Orm or Ral and the MAPK signaling pathways were examined using antibodies specific for phosphorylated active MAPKs. Both the ERK1/2 and JNK were activated after RANKL stimulation, which was found to be reduced in the presence of E_2 as well as Ral (Fig. 3). Orm also caused a reduction in the activation of these two members of MAPK. Orm seem to reduce RANKL-induced JNK activation at 10 μM concentration. Treatment of cells with ICI 182 780 and E_2 could not suppress the RANKL-induced activation of ERK or JNK. No effect of Orm on p38 activation was observed (data not shown).

In order to see specific effects of Orm on activation of ERK and JNK, we performed ELISA and our results have shown that Orm significantly suppressed the RANKL-induced activation of ERK ($P<0.001$; Fig. 3A) and JNK ($P<0.001$; Fig. 3B).

These results confirmed that RANK signaling was reduced due to the inhibition of the activity of ERK and JNK MAPKs in RAW264.7 cells under the influence of Orm.

Figure 3 Effect of Orm on RANKL-stimulated activation of p-JNK and p-ERK. (Panel A) Quantified degree of p-ERK relative to total ERK expression as determined by ELISA in RAW cells. (Panel B) Quantified degree of p-JNK relative to total JNK expression as determined by ELISA in RAW cells treated with Orm. (Panel C) RAW264.7 cells incubated with 17 β -estradiol (100 nM), ICI 182 780 (100 nM), raloxifene (100 nM and 1 μM), or ormeloxifene (1 and 10 μM), for 24 h, and then stimulated with 200 ng/ml of RANKL for 30 min. (Panel D) Cells were incubated with ICI 182 780 or Orm in the presence of E_2 for 24 h and then stimulated with 200 ng/ml RANKL for 30 min. Cell lysates were prepared and subjected to western blotting using specific antibody. Quantification of bands was done by densitometric analysis. The results are representative of three independent experiments. ### $P<0.001$, # $P<0.01$, * $P<0.05$ vs vehicle and *** $P<0.001$, ** $P<0.01$, * $P<0.05$ vs vehicle + RANKL group.

Interference with RANKL-induced expression and activation of AP-1 transcription complex partners

As the MAPKs get activated, they translocate into the nucleus and affect the expression and activation of various transcription factors that include c-Fos and c-Jun. c-Fos is considered to be the main target of the ERK pathway while c-Jun is known to get activated in a downstream pathway triggered after JNK activation. As the RANKL-induced ERK and JNK activation was affected by Orm, the expression level of c-Fos and c-Jun was investigated. When RAW264.7 cells were incubated with E₂, Ral, and Orm for 24 h and stimulated with RANKL, it was observed that E₂ and Ral effectively blunted c-Fos expression. Its expression was found to be reduced in a dose-dependent manner after incubation with Orm, similar to that observed in the vehicle-treated group at 10 μM concentration (Fig. 4). Activation and expression analysis of c-Jun revealed no prominent change in the Ral-treated group. The expression of c-Jun was provoked by RANKL and Orm was effective in suppressing the levels of phosphorylated c-Jun at 1 and 10 μM concentrations.

It was interesting to note that the decrease in c-Fos and c-Jun expression and activation was associated with a corresponding decline in RAW264.7 cell transcription, as measured by a significant decrease ($P<0.001$) in luciferase reporter gene activity under AP-1 control at 10 μM concentration when the cells were incubated with Orm (Fig. 5A).

Further, the ability of RANKL to stimulate the transcriptional activity of transfected chimeric genes composed of the DNA-binding domain of GAL4 (pFC-dbd) fused to the activation domains of c-Jun, c-Fos, or ATF2 was examined. Transcriptional activity of pFA-c-Jun was significantly increased by more than threefold after RANKL stimulation (Fig. 5B). E₂ effectively blunted c-Jun transactivation. Orm caused suppression of c-Jun transactivation, which was also shown by Ral at 1 and 10 μM concentration. Mild activation of pFA-c-Fos and pFA-ATF2 was observed on stimulation with RANKL. Ral caused a decrease in transactivation of pFA-c-Fos and pFA-ATF2 at as low as 100 nM (Fig. 5B). Orm did not affect the transactivation of c-Fos at lower concentrations, but it was similar to that of the unstimulated control at 10 μM concentration. By contrast, ATF2 transactivation was not suppressed significantly by Orm, suggesting that Orm may not interfere with the ATF2 activity while forming the transcription complex at AP-1 site. E₂ almost completely blocked induction of each chimera by RANKL and so did Orm and Ral. These findings advocate the role of Orm in affecting transcriptional complex formation as well as the transcriptional activity of transcriptional partners, thereby repressing osteoclast formation.

Figure 4 Effect of Orm on the RANKL-induced expression of p-c-Jun, c-Jun, and c-Fos. RAW264.7 cells were incubated with different concentrations of 17β-estradiol (10 and 100 nM), raloxifene (100 nM and 1 μM), or ormeloxifene (100 nM, 1 μM, and 10 μM) for 24 h and then stimulated with 200 ng/ml RANKL for 30 min. Cell lysates were prepared and subjected to western blotting with the specific antibodies. The β-actin blot is shown as a loading control. Quantification of bands was done by densitometric analysis. The results are representative of three independent experiments. *** $P<0.001$, ** $P<0.01$, * $P<0.05$ vs vehicle + RANKL group.

Effect on transcriptional activation of NF-κB

Activation of the NF-κB transcription factor is an essential step for osteoclast differentiation that takes place via activation of Iκb. Studies suggest that severe osteoporosis develops and mature osteoclasts fail to generate in mice deficient in both the p50 and p52 subunits of NF-κB (Franzoso *et al.* 1997). Therefore,

we next determined whether Orm has any role in affecting NF-κB activation by RANKL. For this, a luciferase reporter construct with a NF-κB binding site was introduced into the RAW264.7 cells, and luciferase activity was determined after incubating the transfected cells with E₂, Ral, or Orm for 24 h. It was observed that RANKL induced transactivation via NF-κB almost twofold, which was reduced to the levels similar to that of unstimulated control in the presence of 100 nM E₂. Orm was able to attenuate the NF-κB activity to almost a similar level at 1 μM concentration (Fig. 5C), suggesting a possible role of Orm by suppressing the activation of NF-κB in mediating its antiosteoclastogenic potential.

Influence on RANKL-stimulated ROS generation

The effect of Orm on ROS generation was evaluated after stimulation of RAW264.7 cells with RANKL. Intracellular ROS was measured using the cell-permeable, oxidation-sensitive dye DCFH-DA by FACS, and it was found that ROS was increased by stimulation with RANKL and also that Orm was able to inhibit its increase after RANKL induction. E₂ and Ral also very effectively caused decrease in ROS generation ($P < 0.001$; Fig. 6, data not shown).

Discussion

An understanding of the mechanism of the protective effects of estrogen and SERMs in bone has very important implications for the prevention and treatment of osteoporosis. Several bone-protective compounds, such as tamoxifen and Ral (Clemett & Spencer 2000, Goldstein 2010), oppose the differentiating effects of RANKL and reduce the formation of osteoclast-like cells from undifferentiated precursors. Orm is known to prevent ovariectomy-induced bone

Figure 5 Effect of Orm on RANKL-induced activation of AP-1, c-Jun, c-Fos, ATF2, or NF-κB-mediated transcription in RAW264.7 cells. (Panel A) Cells were transfected with a luciferase reporter plasmid *pAP1-luc*. (Panel B) Cells were transfected with a luciferase reporter plasmid *pFR-luc* containing GAL4 binding site along with activator plasmids, pFA2-c-Jun, pFA-c-Fos, or pFA-ATF2. pFC-dbd (GAL4) was also transfected as negative control. (Panel C) Cells were transfected with a luciferase reporter plasmid containing NF-κB-binding sequence. 12 h after nucleofection, the cells were scraped, counted, and seeded into a 96-well plate at a density of 2×10^4 cells/well and were incubated with E₂ (0.1, 1, 10, and 100 nM), Ral, or Orm (10 nM, 100 nM, 1 μM, 10 μM) for 24 h in the presence of RANKL (200 ng/ml). After 24 h, the cells were lysed; firefly luciferase and *Renilla* luciferase activities were measured as relative light units (RLU). Percent transcriptional activity was measured as percentage of normalized luciferase activity of unstimulated vehicle-treated group. The results are representative of three independent experiments. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ vs V + RL; ### $P < 0.001$, ## $P < 0.01$, # $P < 0.05$ vs 100 nM E₂.

Figure 6 Decrease in RANKL-induced ROS production by Orm in RAW264.7 cells. Cells were pretreated with different concentrations of E₂ (10 and 100 nM), Ral (100 nM and 1 μM), or Orm (100 nM, 1 μM, and 10 μM) for 24 h, preloaded with DCFH-DA, and treated with RANKL (100 ng/ml) for 10 min. DCF fluorescence was detected with flow cytometry. Results are expressed as the percentage mean fluorescence intensity of unstimulated vehicle-treated control. The results are representative of three independent experiments. ***P < 0.001 vs stimulated control (V + RL).

loss in rats (Arshad *et al.* 2004, Narayana Murthy *et al.* 2006). This study was carried out to investigate the influence of Orm on the generation of osteoclast and its mechanism of action using an *in vitro* model system, i.e. murine RAW264.7 cells. The RAW264.7 cell line is derived from murine macrophages and has been used as the *in vitro* target of the cloned rat RANKL gene (Xu *et al.* 2000). These cells serve as an excellent model of osteoclast differentiation as RANKL treatment induces their differentiation into osteoclast-like, TRAP-positive cells (David *et al.* 2002, Yamamoto *et al.* 2002, Collin-Osdoby *et al.* 2003). The advantage of this system is that it does not contain any osteoblast/bone marrow stromal cells or cytokine-like macrophage CSF and allows focusing on RANKL signaling in preosteoclast cells.

Our study has demonstrated that Orm can attenuate RANKL-induced osteoclast formation in preosteoclast RAW264.7 cells by suppressing cellular responsiveness to osteoclastogenic factor such as RANKL, an effect that is comparable to that of estrogen. Our results also exclude the possibility of cytotoxicity by Orm, as was evident from the cell viability data.

To gain insight into the molecular mechanisms involved in osteoclast differentiation, the intracellular signaling pathways engaged by RANKL was investigated. JNKs and ERKs are two MAPKs among the family that have been shown to be involved in osteoclastogenesis in *in vitro* cell culture (Shevde *et al.* 2000, Thalhamer *et al.* 2008). RANKL stimulates JNK and subsequently enhances c-Jun activation (Lee *et al.* 2001, 2002, Ikeda *et al.* 2004). Therefore, we assessed the level of phosphorylated JNK in response to RANKL treatment.

Here, Orm and Ral acted similar to E₂ in blunting the phosphorylation of JNK and also of c-Jun. Further, ERK, another MAPK, is known to play a functional role not only in osteoclast differentiation but also in its survival (Lee *et al.* 2001, 2002, Wagner 2002). Orm suppressed the activation of ERK in response to RANKL (Fig. 3). Collectively, the ERK and JNK MAPKs appeared to be the major signaling pathways downregulated by Orm.

The AP-1 transcription factor is a heteromeric protein complex composed of members of Fos, Jun, and ATF families. RANKL is known to activate the AP-1 transcription factor complex, partly through the induction of its critical component c-Fos (Wagner & Eferl 2005). AP-1-mediated transcription is known to play an important role in RANK-regulated osteoclastogenesis (Shevde *et al.* 2000). Orm caused effective suppression in AP-1 transactivation. The suppression of c-Jun has been reported by other groups earlier in the presence of SERMs, e.g. Ral and tamoxifen (Clemett & Spencer 2000, Lee *et al.* 2001). The suppressive effect of Orm on these multiple transcription factors are likely to contribute to the inhibition of osteoclast differentiation and c-Jun-mediated transactivation in the RAW264.7 cells.

Several transcription factors are known to mediate induction of genes implicated in osteoclastic differentiation in response to RANKL. The NF-κB transcription factor is the first one characterized to be activated downstream of RANK. NF-κB is activated by RANKL both in monocytes and RAW264.7 cells (Srivastava *et al.* 2001, Collin-Osdoby *et al.* 2003) and is required *in vivo* for osteoclast formation (Franzoso *et al.* 1997). It is also reported that ER directly inhibits IL6 gene expression by disruption of NF-κB transactivation (Stein & Yang 1995). In this study, Orm was observed to decrease the transcriptional activation of NF-κB induced by RANKL. Similar effects were observed in Ral-treated groups as reported earlier (Bellosta *et al.* 2007, Lee *et al.* 2008). The attenuation of NF-κB by Orm might lead to suppression of cytokines, e.g. IL6. A recent study suggested that NF-κB is also an upstream transcription factor modulating the expression of *Nfatc1* (Takatsuna *et al.* 2005). Therefore, another possibility may be that Orm affects the expression of *Nfatc1*, thereby displaying its property to decrease osteoclast differentiation, although this needs to be examined further.

It is documented that in bone marrow-derived macrophages stimulated with RANKL, intracellular levels of ROS were increased through the RANKL-TRAF6-Rac1-NADPH oxidase (Nox) 1 pathway (Lee *et al.* 2005). The generated ROS is reported to act as an intracellular signal mediator for the activation of MAPKs by RANKL in osteoclast differentiation (Lee *et al.* 2005). Estrogen helps in preventing bone loss by a mechanism involving blunting the production of ROS (Chen *et al.* 2008). Recently, a lignan, sauchinone, having antiosteoclastogenic activity has been reported

to decrease ROS levels in BMM cells, which was described to mediate its osteoclastogenic effect (Han *et al.* 2007). There are reports that N-acetylcysteine inhibits the response of BMM cells to RANKL including ROS production (Lee *et al.* 2005). Our results also demonstrated that RANKL-stimulated ROS generation was decreased in the cells that were treated with Orm. In addition, RANKL-activated ROS is known to induce mitochondrial biogenesis. The signaling pathways controlling osteoclast differentiation and mitochondrial biogenesis cross talk and synergistically promote osteoclastogenesis (Wan 2010). Therefore, in light of our findings, the two mechanisms controlling osteoclastogenesis by Orm may be postulated: the first possibility may be that due to the ROS inhibitory action of Orm, the activation of MAPKs and subsequent signaling events are suppressed. In addition to this, the second mechanism may involve the suppression of cross talk mechanisms between signaling pathways of OC differentiation and the mitochondrial biogenesis.

Taken together, the study demonstrated that Orm inhibits RANKL-induced osteoclastogenesis by reducing ROS generation and attenuates MAPK, AP-1, and NF- κ B activation, ultimately leading to the suppression of downstream signaling molecules. These results provide a possible mechanism of action of Orm in regulating osteoclastogenesis, thereby supporting the potential of this compound toward the beneficial and protective effects on the bone.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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