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RESEARCH PAPER

2-Methoxystypandrone represses RANKL-mediated osteoclastogenesis by down-regulating formation of TRAF6–TAK1 signalling complexes

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BACKGROUND AND PURPOSE
2-Methoxystypandrone (2-MS) is a naphthoquinone isolated from *Polygonum cuspidatum*, a Chinese herb used to treat bone diseases. Here we have determined whether 2-MS antagonised osteoclast development and bone resorption.

EXPERIMENTAL APPROACH
RAW264.7 cells were treated with receptor activator of nuclear factor κB (NF-κB) ligand (RANKL) to induce differentiation into osteoclasts. RT-PCR and Western blot were used to analyse osteoclast-associated gene expression and signalling pathways.

KEY RESULTS
The number of multinuclear osteoclasts, actin rings and resorption pit formation were markedly inhibited by 2-MS, targeting osteoclast differentiation at an early stage and without significant cytotoxicity. The anti-resorption effect of 2-MS was accompanied by decreasing dendritic cell-specific transmembrane protein and matrix metalloproteinase-9 (MMP-9) mRNA expression. RANKL-increased MMP-9 gelatinolytic activity was also attenuated by concurrent, but not by subsequent addition of 2-MS. 2-MS markedly inhibited not only the RANKL-triggered nuclear translocations of NF-κB, c-Fos and nuclear factor of activated T cells c1 (NFATc1), but also the subsequent NFATc1 induction. Degradation of IκB and phosphorylation of mitogen-activated protein kinases were also suppressed. RANKL facilitated the formation of singaling complexes of tumour necrosis factor receptor-associated factor 6 and transforming growth factor β-activated kinase 1 (TRAF6–TAK1), important for osteoclastogenesis and formation of such signalling complexes was prevented by 2-MS.

CONCLUSIONS AND IMPLICATIONS
The anti-osteoclastogenic effects of 2-MS could reflect the block of RANKL-induced association of TRAF6–TAK1 complexes with consequent decrease of IκB-mediated NF-κB and mitogen-activated protein kinases-mediated c-Fos activation pathways and suppression of NFATc1 and other gene expression, essential for bone resorption.

Abbreviations
2-MS, 2-methoxystypandrone; DC-STAMP, dendritic cell-specific transmembrane protein; MAPK, mitogen-activated protein kinase; MMP-9, matrix metalloproteinase-9; NFATc1, nuclear factor of activated T cells c1; RANKL, receptor activator of nuclear factor κB (NF-κB) ligand; TAK1, transforming growth factor β-activated kinase-1; TRAF6, tumour necrosis factor receptor-associated factor 6; TRAP, tartrate-resistant acid phosphatase

Introduction

The receptor activator of nuclear factor κB (NF-κB) (RANK) and its ligand RANKL are key molecules in the differentiation and activation of osteoclasts, the professional bone-resorbing polykaryons derived from haematopoietic cells of the monocyte-macrophage lineage (Dougall *et al.*, 1999; Suda *et al.*, 1999). RANK interacts with members of the family of tumour necrosis factor receptor-associated factors (TRAFs) that mediate activation of the inhibitor of κB (IκB) and IκB kinase (IKK) and mitogen-activated protein kinases (MAPKs) (Darnay *et al.*, 1998). Among these TRAF molecules, TRAF6 has been shown to be a pivotal component in the RANK signalling pathway (Kim *et al.*, 1999). Transforming growth factor β-activated kinase (TAK)-1 (TAK1) is a member of the MAPK kinase kinase (MAPKKK) family (Yamaguchi *et al.*, 1995; Ninomiya-Tsuji *et al.*, 1999) that mediates MAPK and IKK activation via interaction with TRAF6 (Mizukami *et al.*, 2002). Recently, Mizukami *et al.* (2002) found that RANKL stimulation not only activated endogenous TAK1 but also formed the TRAF6–TAK1 complex associated with RANK. Certain transcription factors are involved in the expression of genes that characterize osteoclasts (Lee and Kim, 2003). For example, RANKL stimulates activator protein 1 (AP-1) through MAPK activation, and NF-κB through IKK activation. Also, the nuclear factor of activated T cells c1 (NFATc1), a member of the NFAT family of transcription factors, has been characterized as another important regulator of RANKL-induced osteoclast differentiation. In line with the importance of the transcription factors for osteoclast differentiation, genetic studies have shown that deficiency in c-Fos, a component of AP-1, or NFATc1 results in blockade of osteoclastogenesis (Wang *et al.*, 1992; Ishida *et al.*, 2002; Takayanagi *et al.*, 2002). NF-κB double knockout mice, lacking both p50 and p52, exhibit osteopetrotic phenotypes with a prominent decrease in the number of osteoclasts (Iotsova *et al.*, 1997).

Fusion-mediated giant cell formation is critical for osteoclast maturation and bone resorption (Teitelbaum, 2000; Miyamoto and Suda, 2003). Dendritic cell-specific transmembrane protein (DC-STAMP) is a seven transmembrane protein acting as an essential molecule for the cell–cell fusion of osteoclasts (Yagi *et al.*, 2005). DC-STAMP-deficient mice show a complete lack of cell–cell fusion in osteoclasts and the bone-resorbing activity of DC-STAMP-deficient mononuclear osteoclasts is dramatically lower than that of wild-type multinucleated osteoclasts (Yagi *et al.*, 2007). Proteinases also play an important role in bone physiology, including the solubilization of bone matrix, which determines where and when bone resorption is initiated (Delaissé *et al.*, 2000). Cysteine proteinases and matrix metalloproteinases (MMPs) have been identified as the main proteinases active in these processes (Ortega *et al.*, 2003). Among MMPs, MMP-9 is known as the most abundant gelatinolytic MMP in osteoclasts and is the main MMP involved in the invasive activity of osteoclasts (Engsig *et al.*, 2000). The sealing zone, a characteristic feature of mature osteoclasts and serves for the attachment of osteoclasts to the bone surface, is observed as a ringed structure of F-actin dots (actin ring). Disruption of sealing zones results in the suppression of the bone-resorbing activity of osteoclasts (Jurdic *et al.*, 2006). Uncontrollable bone resorption mediated by overactive osteoclasts may result in bone loss and may induce osteopenia/osteoporosis (Allison and Derynck, 2002).

The plant, *Polygonum cuspidatum*, is widely distributed in Asia and is used as a folk medicine in Taiwan to cure bone diseases such as bone fractures or bone loss caused by inflammatory joint diseases. Traditionally, an alcoholic drench of *P. cuspidatum* roots and stems is used for self-medication. However, efforts to evaluate the efficacy of such treatment in experimental settings and to identify responsible principles of its effect on bones have been scarce. After fractionation and purification, we found that 2-methoxystypandrone (2-MS) is a major active component of extracts of *P. cuspidatum*. This compound inhibits RANKL-induced osteoclastogenesis in primary osteoclasts derived from bone marrow macrophages (BMMs) and in a murine preosteoclastic cell line, RAW264.7 respectively. This study further explores the effects of 2-MS on signalling pathways known to be involved in osteoclastogenesis.

Methods

Extraction and isolation of 2-MS

Powdered dried roots of *P. cuspidatum* (10 kg) were refluxed with 95% ethanol (40 L) for 6 h. The ethanolic solution was concentrated in vacuum to obtain a dark-brown ethanolic extract (defined as PC-E) and then partitioned with H_2O : $CHCl_3$ (1:1) to get $CHCl_3$ (PC-E-C) and H_2O (PC-E-W) layers. The $CHCl_3$ layer (PC-E-C) was further repeatedly separated over silica gel (*n*-hexane: ethyl acetate = 9:1–0:1) and Sephadex LH-20 columns (methanol) to yield three known compounds, physcion (76.4 mg), emodin (25.1 mg) and 2-MS (18.3 mg) (Figure 1A). These compounds were identified by comparison of their spectral data with published data (Alemayehu

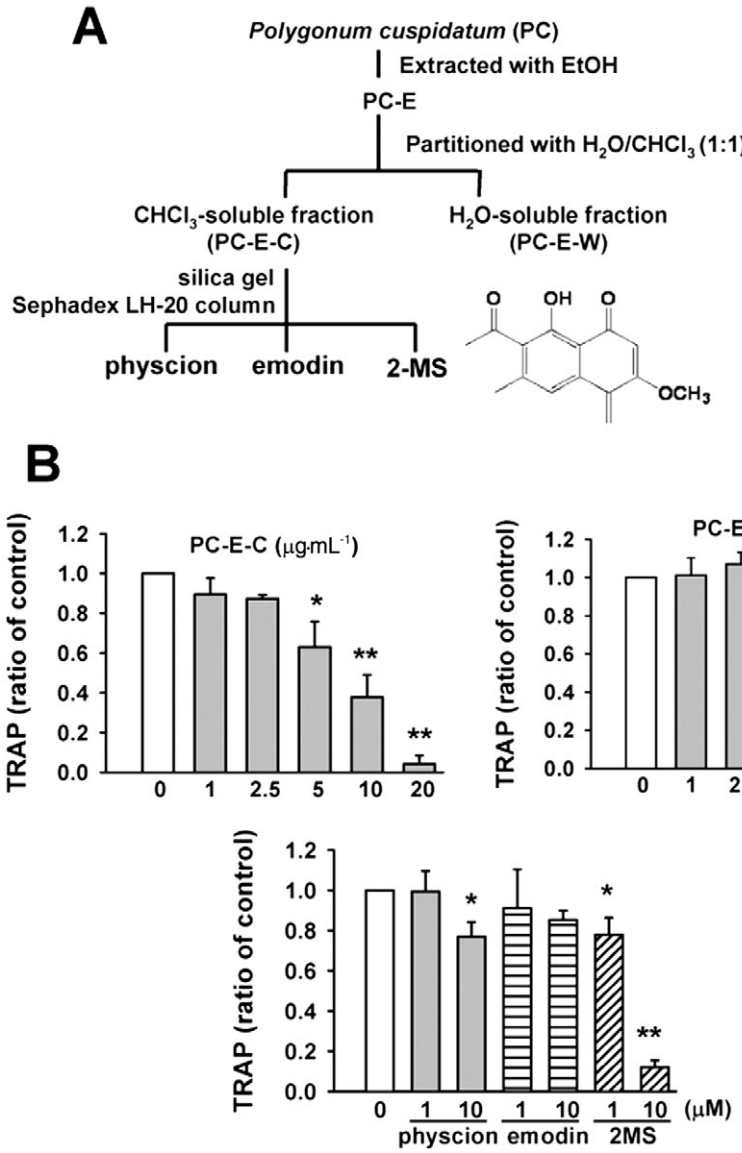

Figure 1

Screening of natural compounds inhibiting RANKL-induced osteoclast differentiation. (A)Scheme of extractions to yield $CHCl_3$-soluble fractions (defined as PC-E-C) and H_2O-soluble fractions (defined as PC-E-W) from the ethanol extract of *Polygonum cuspidatum* (PC-E). The structure of 2-methoxystypandrone (2-MS) is also shown. (B) The effects of two fractions and three pure compounds (physcion, emodin and 2-MS) on RANKL-induced TRAP activity. For differentiation into osteoclasts, RAW264.7 cells were plated in 96-well plates in Dulbecco's modified Eagle's medium containing RANKL (100 ng·mL^{-1}) at 10^3 cells·per well. Cells were further incubated for 72 h. TRAP activity in cell lysates was assayed and expressed relative to those after RANKL alone. Each value is the mean ± SE of four independent experiments, each performed in triplicate. *P < 0.05 and **P < 0.01, compared with the control value.

et al., 1993; Kim *et al.*, 2004). The chemical structure of 2-MS is shown in Figure 1A.

Osteoclast differentiation of RAW264.7 cells

The RAW264.7 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Grand Island, NY, USA) supplemented with antibiotics (100 U·mL^{-1} of penicillin A and 100 U·mL^{-1} of streptomycin), 10% heat inactivated fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel) and maintained at 37°C in 5% CO_2 humidified air. The cells (10^3 cells·per well) were seeded in 96-well plates and incubated to reach ~70% confluence. After that, the media were replaced and the cells were cultured for an additional 3 days in DMEM containing 100 ng·mL^{-1} recombinant murine RANKL (R&D Systems, Minneapolis, MN, USA; defined as differentiation medium) and

different concentrations of 2-MS (1–10 μM). Osteoclast differentiation was analysed by measuring tartrate-resistant acid phosphatase (TRAP) activity as described below.

Osteoclast differentiation of BMMs

Bone marrow cells (BMCs) were freshly isolated from the femur of 5-week-old male ICR mice by flushing the bone marrow cavity with 5 mL of α-minimum essential medium (Gibco BRL) containing antibiotics (Sigma, St Louis, MO, USA). Red blood cells were removed with red blood cell lysis buffer (Sigma). Following centrifugation, cells were suspended in α-minimum essential medium supplemented with 10% FBS (Biological Industries) and antibiotics, and used as BMCs. BMMs were prepared from BMCs cultured for 3 days in the presence of 30 ng·mL^{-1} of macrophage-colony stimulating factor (M-CSF; R&D Systems). The adherent cells (BMMs) were further cultured for 5 days with an osteoclast differentiation medium containing 30 ng·mL^{-1} M-CSF and 100 ng·mL^{-1} RANKL.

TRAP activity measurement and TRAP-positive multinucleated cells (TRAP + MNCs) staining

After culture, RAW264.7 cells or BMMs were lysed and incubated for 1 h with a reaction buffer containing p-nitrophenylphosphate (pNPP). The reaction was stopped with 0.3 N NaOH solution, and optical densities were read and analysed by a microplate spectrophotometer at 410 nm. TRAP activity measured in differentiation medium alone was defined as control. All results were expressed as relative to control. To preclude the possibility that the attenuation in TRAP activity was due to cytotoxicity, cell viability was simultaneously measured by MTT assay. The cells cultivated in differentiation medium alone served as the control. To confirm the generation of multinucleated osteoclasts (MNC), cells were stimulated with RANKL for the whole culture period of 5–6 days. After culture, cells were fixed with 3.7% formalin (Sigma), permeablized with 0.1% Triton X-100 and finally stained for TRAP with the leukocyte acid phosphatase kit (Sigma, Cat. no. 387A-1KT). TRAP-positive MNCs containing three or more nuclei were counted under the light microscope.

Actin ring observation

RAW264.7 cells were cultured in differentiation medium, with or without 2-MS for 6 days. Then, cells were washed three times with ice-cold phosphate-buffered saline (PBS), fixed in 3.7% formalin and permeabilized by incubation for 15 min in 0.1% Triton-PBS. The cells were then blocked with 1% BSA-PBS and incubated with rhodamine-labelled phalloidin for 30 min. The cells were extensively washed with PBS and actin ring formation was visualized using a fluorescent microscope (Carl Zeiss Vision).

Functional bone resorption assay

The resorptive function of mature MNC derived from RANKL-differentiated RAW264.7 cells was analysed on Osteologic Plates (BD BioCoat Osteologic Bone Cell Culture System, BD Biosciences, San Jose, CA, USA). Briefly, cells were cultured in differentiation medium with or without 2-MS for 6 days. Cells were then removed with 1 N NaOH for 20 min, and the slices washed twice with PBS and the resorption pits were stained with Mayer's haematoxylin (Sigma) for 30 s. Finally, bovine bone slices were transferred onto glass slides, mounted with glycerol, covered with glass cover slips and observed under the light microscope. The resorption area was observed under a light microscope and analysed by Image-Pro PlusTM.

Reverse transcription-polymerase chain reaction analysis (RT-PCR)

Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized by using the Superscript II (Invitrogen). PCR was performed with mouse-specific primers: DC-STAMP, 5′-CTTGCAACCTAAGGGCAAAG-3′ (forward) and 5′-TCAACAGCTCTGTCGTGACC-3′ (reverse); MMP-9, 5′-CATTCGCGTGGA- TAAGGA GT-3′ (forward) and 5′-CACTGCAGGAGGTC GTAGGT-3′ (reverse); and GAPDH, 5′-TGAAGG TCGGTGTGAACGGATTTG-2′ (forward) and 5′-CAT GTAGG- CCATGAGGTCCACCAC-3′ (reverse). The sequences of reaction products were confirmed, and the reactions in the absence of reverse transcriptase did not generate any product.

Measurement of matrix metalloproteinase (MMP) activities

For assay of MMP-9 activity, zymography was performed using gelatin gels (Bio-Rad), as described earlier (Milner *et al.*, 2003). After culture with RANKL (100 ng·mL^{-1}) for 4 days in the absence or presence of 2-MS (7.5 μM), respectively, the supernatant was assessed for gelatinolytic activity and the protein concentration was measured by Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA). As the protein levels were not changed by RANKL and 2-MS treatment, the same volumes of culture supernatants were used for the assays. All the samples were separated by electrophoresis on 7.5% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) copolymerized with 0.1% gelatin. Gels were washed

for 1 h in 20 mM Tris-HCl, pH 7.8, and 2.5% Triton X-100 and then incubated in 20 mM Tris-HCl, pH 7.8, 1% Triton X-100, 10 mM $CaCl_2$ and 5 μM $ZnCl_2$ at 37°C overnight. Gels were stained with Coomassie blue R-250; the appearance of light bands indicated latent or active MMP-9 and MMP-2 activity. Further, the same experiment was performed by adding 10 mM EDTA, a chelating agent of bivalent ions, to inhibit MMP activities (data not shown).

Western blotting

After washing with PBS, total cell lysates were prepared by lysing the cells in cold lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM EGTA, 0.24% sodium deoxycholate, 1% IGEPAL, pH 7.5) containing 25 mM NaF and 2 mM Na_3VO_4. Aprotinin, leupeptin, pepstatin and phenylmethylsulfonyl fluoride (PMSF) were added before each lysis. For isolation of cytoplasmic and nuclear fractions, cells were trypsinized and rinsed in PBS and then lysed for 15 min on ice in cytoplasmic lysis buffer (0.33 M sucrose, 1 mM $MgCl_2$, 0.1% Triton X-100, 10 mM HEPES, pH 7.4) containing phosphatase inhibitors and proteinase inhibitors as above. The cytoplasmic fraction was collected following centrifugation (8,000× *g* for 10 min at 4°C), and then the samples were rinsed in cytoplasmic lysis buffer and centrifuged (15,000× *g* for 10 min at 4°C) to obtain a nuclear pellet. The nuclear pellet was lysed for 15 min on ice in 0.45 M NaCl, 10 mM HEPES, pH 7.4, and the nuclear fraction was collected following centrifugation (Chiou and Chen, 2009). Harvested proteins were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Amersham Bioscience, Piscataway, NJ, USA). After blocking, the membrane was incubated with primary antibodies overnight at 4°C. The primary antibodies used included those to IκB (BioLegend, San Diego, CA, USA), NF-κB (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), c-Fos (Santa Cruz), NFATc1 (Santa Cruz), histone (clone 8E7; Upstate Biotechnology, Inc., Lake Placid, NY, USA), β-actin (Santa Cruz), control and phosphorylated p38, extracellular signal regulated kinase (ERK) and c-Jun N-termainl kinase (JNK) (Cell Signaling Technology, Beverly, MA, USA). Secondary antibody conjugated with horseradish peroxidase was used to allow detection by the enhanced chemiluminescence plus kit (Amersham Biosciences Inc., Piscataway, NJ, USA) and exposed to X-ray film.

Immunoprecipitation assay

RAW264.7 cells plated in 10 cm diameter dishes were stimulated with RANKL before or after 2-MS treatment. Cells were washed once with ice-cold PBS and lysed in lysis buffer [25 mM HEPES (pH 7.7), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM dithiothreitol, with 10 μg aprotinin and 10 μg leupeptin mL^{-1}]. After centrifugation (10,000×*g* for 10 min at 4°C), cell extracts were immunoprecipitated with anti-TAK1 antibody (Santa Cruz). Co-precipitated TRAF6 was detected by immunoblotting with anti-TRAF6 antibody (Santa Cruz). The amounts of TAK1 in each immune complex were determined by immunoblotting using TAK1 antibody. Band signals were visualized by the ECL system (Amersham Pharmacia).

Statistical analysis

Data were expressed as mean ± standard error (SE). All experiments were performed at least three times independently. The data were analysed by one-way analysis of variance (ANOVA) followed by *post hoc* Dunnett's *t*-test for multiple comparisons. A *P*-value < 0.05 was considered significant.

Materials

The MAPK inhibitors (inhibitor of p38,SB203580, inhibitor of ERK, PD98059 and inhibitor of JNK, SP600125) were all purchased from Calbiochem, Darmstadt, Germany.

Results

Effects of different extracts prepared from P. cuspidatum on the RANKL-induced osteoclast differentiation in RAW264.7 cells

Significant elevation of TRAP activity occurred when RAW264.7 cells were cultured with RANKL for at least 3 days. Our preliminary study confirmed that the ethanol extract of *P. cuspidatum* (PC-E, 10–100 $\mu g \cdot mL^{-1}$) was able to suppress RANKL-induced osteoclast differentiation in RAW264.7 cells. After extraction with $H_2O/CHCl_3$ (1:1) (Figure 1A), we found that the chloroform-soluble fraction of PC-E (defined as PC-E-C) retained the activity of suppression of osteoclast differentiation, whereas, the water fraction (defined as PC-E-W) did not (Figure 1B). Three major components – physcion, emodin and 2-MS – were obtained following further analysis of PC-E-C and assays showed that 2-MS was more potent than the others in suppressing osteoclast differentiation, as measured by inhibition of TRAP activity (Figure 1B, bottom).

2-MS inhibited RANKL-induced osteoclastogenesis of RAW264.7 cells and primary BMMs

2-MS reduced the RANKL-induced TRAP activity in RAW264.7 cells, concentration-dependently over a

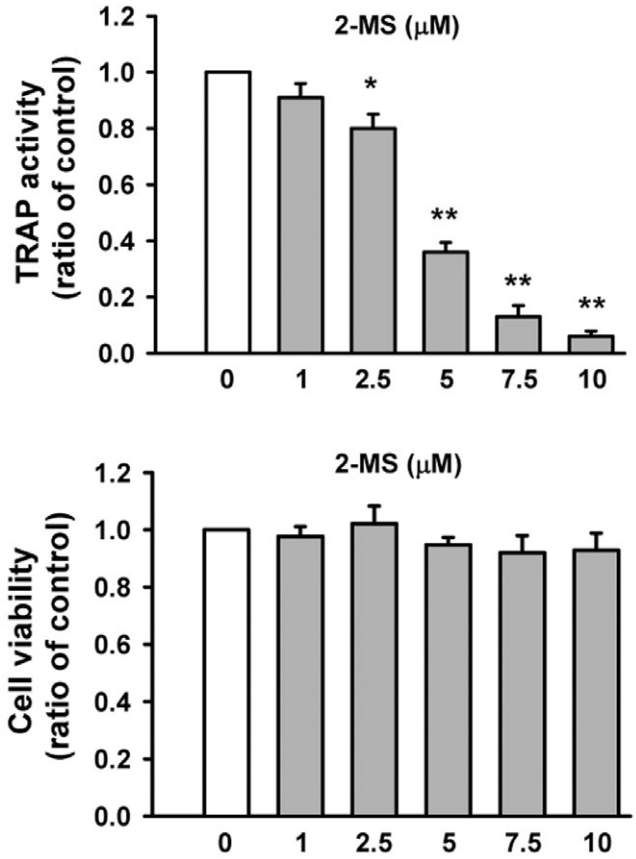

Figure 2

Effects of 2-methoxystypandrone (2-MS) on RANKL-induced TRAP activity and cell viability in RAW264.7 cells. The effect of 2-MS on cell viability was evaluated by MTT assay. For osteoclast differentiation, RAW264.7 cells were treated with RANKL (100 ng·mL^{-1}). Cells were further incubated for 72 h and TRAP activity was assayed in cell lysates. Each value is the mean ± SE of five independent experiments each performed in triplicate. *P < 0.05 and **P < 0.01, different from values after treatment with RANKL alone.

range of concentrations (1–10μM). (Figure 2, upper half). This inhibition of TRAP activity was not due to cytotoxicity as MTT analysis revealed that 2-MS over the same concentration range did not affect cell viability (Figure 2, lower half). Histological assessment showed that many TRAP-positive MNCs were formed in the culture within 6 days in response to RANKL (Figure 3). 2-MS added to the culture for the entire culture period (6 days) concentration-dependently inhibited formation of TRAP-positive cells induced by RANKL (at 2.5 mM, 17 ± 6%;. 5 mM, 42 ± 5%; 7.5 mM, 71 ± 4% and 10 mM, 83 ± 3%; n = 4; P < 0.05) with complete inhibition (~95%) of osteoclast formation at 7.5 μM. Similarly, in BMMs, we found TRAP activity reduced by 2-MS (at 2.5 μM, 17 ± 4%; 5 μM, 42 ± 7%; 7.5 μM, 71 ± 9% and 10 μM, 83 ± 6%; n = 4; P < 0.05), compared with levels in BMMs treated with RANKL alone.

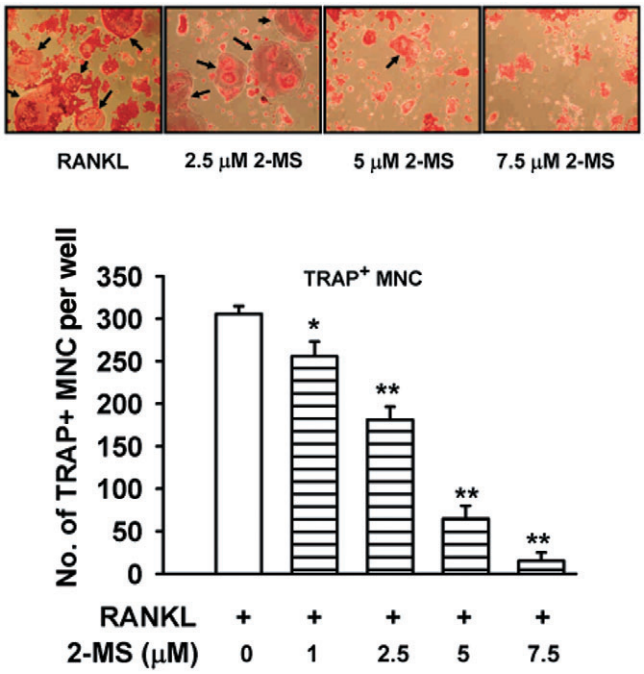

Figure 3

2-Methoxystypandrone (2-MS) inhibited RANKL)-induced formation of TRAP-positive MNC in RAW264.7 cell cultures. Cells were cultured with indicated concentrations of 2-MS in the presence of RANKL (100 ng·mL^{-1}). After 5 days, cells were fixed and stained for TRAP. TRAP-positive multinucleated cells (MNCs ≥ three nuclei) were counted. Arrows indicate TRAP-positive multinucleated cells. Summary values are shown in the bar graph and each value is the mean ± SE of four independent experiments. *P < 0.05 and **P < 0.01, different from values after treatment with RANKL alone.

2-MS at concentrations of 1 and 2.5 μM also dose-dependently reduced the number of TRAP + MNC by 10 ± 3% and 33 ± 7% (P < 0.05), and at 5 and 7.5 μM by 66 ± 5% and 91 ± 9% (n =4; P < 0.01).

2-MS inhibited osteoclastogenesis in the early stage

We wanted to find out which stage of osteoclastogenesis 2-MS would most significantly affect TRAP activity. To do this, RAW264.7 cells were treated with 7.5 μM of 2-MS on different days, from days 0 to 3 after RANKL stimulation according to the design procedure shown in Figure 4 (upper half). After three further days of incubation, we analysed all treated cells for TRAP activity. We found that 2-MS inhibited RANKL-induced TRAP activity by ~80% by concurrent addition (day 0) or by day 1 post RANKL stimulation (P < 0.01) (Figure 4, lower graph). TRAP activity was reduced by 56% on day 2, post RANKL stimulation (P < 0.05). When 2-MS was added to the culture for the final 3 days, it failed to suppress osteoclast differentiation induced by RANKL.

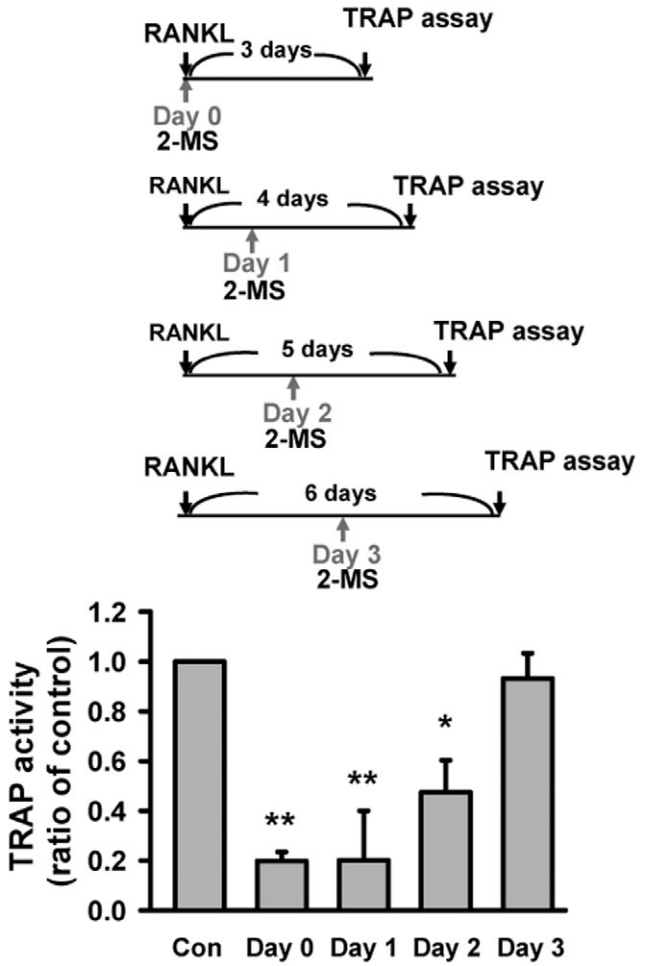

Figure 4

2-Methoxystypandrone (2-MS) inhibited RANKL-induced osteoclast formation at an early stage. RAW264.7 cells were treated with RANKL (100 ng·mL^{-1}), and 2-MS (7.5 μM) was added at the different time (days 0–3 after RANKL stimulation). TRAP activity was assayed after 2-MS addition for further 3 days culture. Data represent the mean ± SE of three experiments performed in triplicate. *P < 0.05 and **P < 0.01, different from values after treatment with RANKL alone.

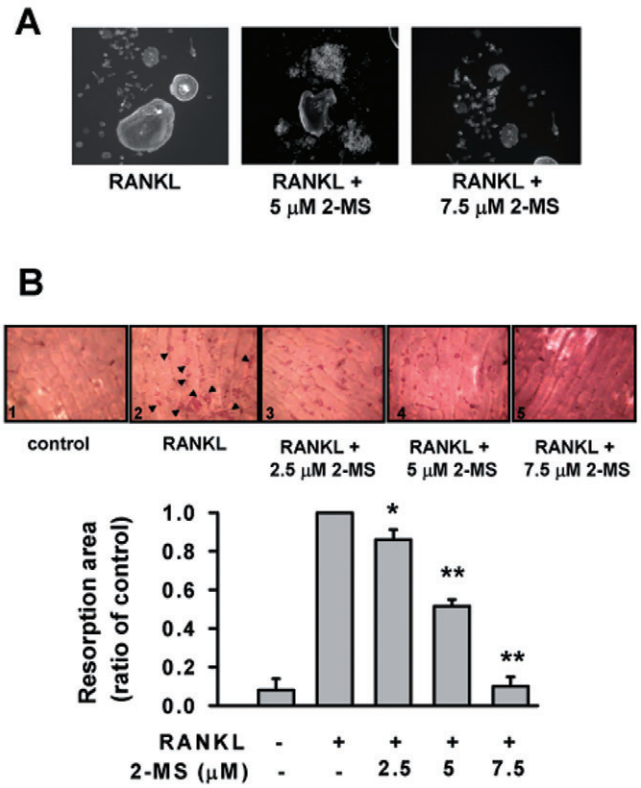

Figure 5

2-Methoxystypandrone (2-MS) inhibited actin ring and pit-forming activity by RANKL in osteoclasts. (A) Effects of 2-MS (5 and 7.5 μM) on actin ring formation in RAW264.7 cells in the presence of RANKL. After culturing for 6 days, cells were fixed with formaldehyde, permeabilized with Triton-X 100 and incubated with rhodamine-conjugated phalloidin. F-actin was detected using a fluorescence microscope. (B) Effect of 2-MS on pit-forming activity in RANKL-activated RAW264.7 cells. The osteoclast preparation was cultured on bone slices with increasing concentrations of 2-MS in the presence of RANKL. After culturing for 6 days, cells were removed from bone slices, and the slices were stained with Mayer's haematoxylin to identify resorption pits. Arrows indicate pit area, and the number of resorption pits was counted. Data represent the mean ± SE of three to five independent experiments. *P < 0.05 and **P < 0.01, different from values after treatment with RANKL alone.

2-MS inhibited actin ring formation and bone-resorbing function of osteoclasts

As another function of mature osteoclasts, RANKL accelerates actin ring formation, a unique cytoskeletal structure required for bone resorption by osteoclasts (Fuller *et al.*, 1998). Therefore, we addressed the question of whether 2-MS would reduce actin ring formation and bone resorption. RAW264.7 cells were incubated with RANKL for 6 days in the absence or presence of 2-MS. Staining of cells with phalloidin for actin exhibited sealing rings in RAW264.7 cells incubated with RANKL (Figure 5A). Sealing ring formation was either reduced or not observed when cells were co-treated with 5 or 7.5 μM 2-MS. To determine if this suppression of actin ring formation affected the resorptive ability of osteoclast cultures, RAW264.7 cells were plated on Osteologic® slides, and then differentiated to osteoclasts by RANKL for 6 days in the absence or presence of 2-MS. After being cultured for 6 days, many resorption pits were formed on the bone slices (Figure 5B, arrows indicated in second picture). Consistent with its effect on sealing zone formation, RANKL-evoked bone resorption was dose-dependently diminished by concurrent addition of 2-MS (2.5, 5 and 7.5 μM) in RAW264.7 cells (Figure 5B, pictures 3, 4 and 5). These results suggested that 2-MS might inhibit bone resorption through the disruption of actin rings. The

inhibitory effects of 2-MS on pit-forming activity could be caused by toxicity after long-term incubation. We therefore measured the viability of osteoclasts after a 6 day culture on dentine slices by MTT assaym and found no difference in viability with and without treatment with 2-MS, up to 7.5 μM (data not shown), although the number of pits on bone slices was markedly suppressed in the presence of 2-MS. These results suggested that inhibition of the pit-forming activity of osteoclasts by 2-MS was not caused by cytotoxicity.

2-MS inhibited RANKL-induced expressions of DC-STAMP

DC-STAMP is the product of a gene associated with osteoclast differentiation and involved in cell–cell fusion during RANKL-mediated osteoclastogenesis. To confirm the inhibitory effect of 2-MS on the expression of DC-STAMP, RAW264.7 cells were cultured with RANKL in the presence or absence of 2-MS. Compared with untreated cells, DC-STAMP mRNA expression increased twofold in cells treated with RANKL after 4 days of culture (Figure 6A). In the presence of 2-MS, the induction of DC-STAMP mRNA by RANKL was blocked, such that expression of DC-STAMP was reduced by at least 86% with 7.5 μM 2-MS treatment.

2-MS inhibited RANKL-induced expression and activity of MMP-9 in RAW264.7 cells

As MMP-9 is thought to be important in preosteoclast and osteoclast mobility, we next examined whether or not 2-MS could also reduce MMP-9 expression. First, RAW264.7 cells were cultured with or without RANKL, and the level of expression of MMP-9 mRNA was determined by RT-PCR after 4 days of culturing. Compared with untreated cells, MMP-9 mRNA expression increased approximately threefold in cells treated with RANKL. The addition of 2-MS decreased the induction of MMP-9 mRNA by RANKL, with up to 90% reduction after 7.5 μM (Figure 6A, bottom).

We next examined whether or not the inhibitory effect of 2-MS on the induction of MMP-9 mRNA by RANKL resulted in a parallel decrease in the activity of secreted MMP-9 protein. The results showed that the MMP-9 expression by RANKL stimulation for 4 days was paralleled by an enhancement of an unstained band at 92 kDa, revealed by gelatin zymography (Figure 6B, left), indicating the pro-MMP-9 activity was enhanced, compared to that from cells without RANKL stimulation. In the presence of 2-MS, RANKL-induced stimulation of pro-MMP-9 activity was repressed in a concentration-dependent manner. In another set of experiments, after 4 days of culture, the supernatant from a RANKL-differentiated RAW264.7 cell culture was incubated with or without 2-MS (2.5, 5 and 7.5 μM) at 37°C for 24 h. MMP-9 activity was increased by 4 days of RANKL stimulation (Figure 6B, right) and addition of 2-MS from days 4 to 5 did not affect the gelatinolytic activity of MMP-9 secreted by these cultures, suggesting that 2-MS targeted osteoclastic differentiation at an early stage. Conversely, gelatin zymography indicated the latent 72 kDa pro-form of MMP-2 was not affected by RANKL activation, while that of MMP-9 was clearly shown.

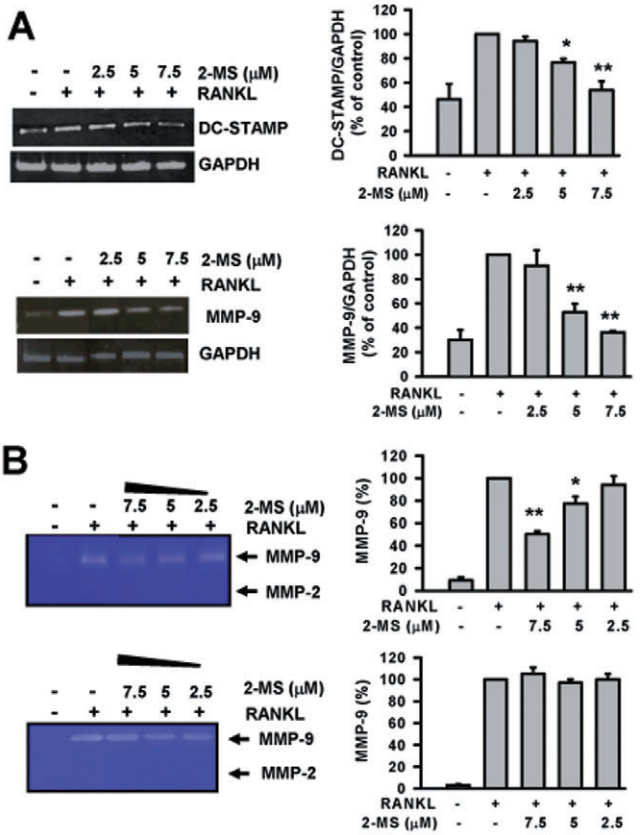

Figure 6

2-MS suppressed RANKL-induced DC-STAMP and MMP-9 mRNA expression and reduced pro-MMP-9 activity. (A) Effect of 2-MS on RANKL-stimulated DC-STAMP and MMP-9 expression in RAW264.7 cells. The relative mRNA levels of DC-STAMP and MMP-9 were measured by RT-PCR, normalized to GAPDH. Data represent the mean ± SE of five independent experiments. *$P < 0.05$ and **$P < 0.01$, different from values after treatment with RANKL alone. (B) 2-MS repressed pro-MMP-9 activity by concurrent (left), but not by subsequent (right) addition of 2-MS. MMP activities were analysed using gelatin zymographic assays. The bands corresponding to pro-MMP-9 activity (92 kDa) and pro-MMP-2 activity (72 kDa) were scanned. *$P < 0.05$ and **$P < 0.01$, different from values after treatment with RANKL alone.

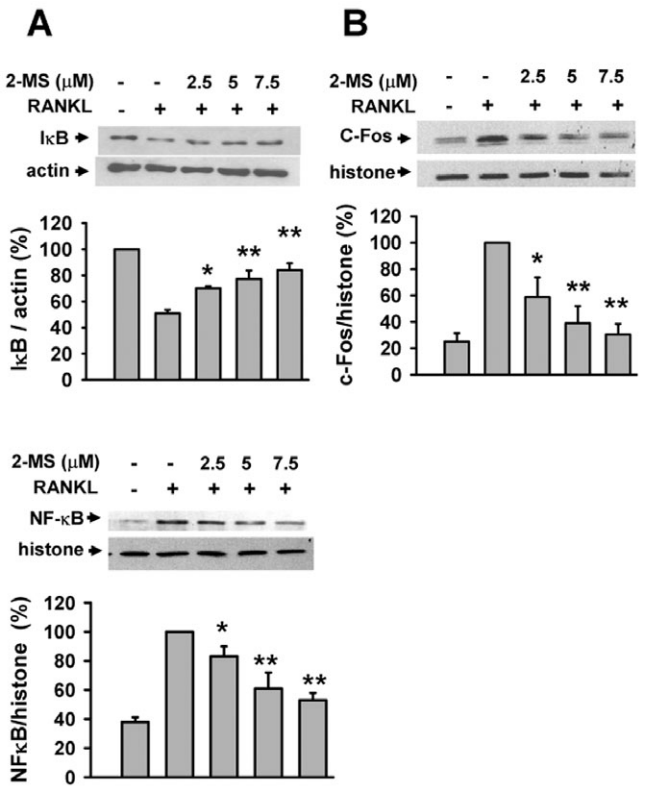

Figure 7

Effects of 2-methoxystypandrone (2-MS) on the activation of IκB/NF-κB (A) and c-Fos (B) in RAW264.7 cells. After 1 day incubation, 2-MS was added to cells 2 h before RANKL and protein samples were prepared after 15 min (for cytosolic IκB degradation assay) or 1 h (for NF-κB and c-Fos nuclear translocation assay) of RANKL stimulation. Activation of signalling molecules were then evaluated by Western blot analysis. Results are expressed as the mean ± SE for each group from four to five separate experiments normalized for histone (for nuclear protein normalization) or β-actin (for cytosolic protein normalization) respectively. *$P < 0.05$ and **$P < 0.01$, *$P < 0.05$ and **$P < 0.01$, different from values after treatment with RANKL alone.

Effect of 2-MS on RANKL-activated IκB/NF-κB and AP-1 signal pathways

Activation of NF-κB is important in the activation and survival of mature osteoclasts, as well as osteoclastogenesis (Shiotani *et al.*, 2002). Activity of NF-κB is regulated by its inhibitor, IκB, that forms a complex with NF-κB in the cytoplasm. After degradation of IκB through an ubiquitin/proteasome pathway, NF-κB is subsequently translocated from the cytoplasm into the nucleus. Thus, we examined the effect of 2-MS on changes of IκB and NF-κB after RANKL stimulation. As shown in Figure 7A, RANKL triggered IκB degradation after 15 min incubation and, after 1 h, produced a significant increase in nuclear translocation of NF-κB (65 kD). 2-MS concentration-dependently blocked IκB degradation (figure 7A) In the presence of 2-MS, the subsequent nuclear translocation of NF-κB was also clearly suppressed. We next investigated the effect of 2-MS on c-Fos, a component of AP-1. RANKL stimulation clearly elevated the nuclear level of c-Fos (measured by Western blotting) in RAW264.7 cells at 1 h and this nuclear translocation of c-Fos was decreased by 2-MS (Figure 7B).

RANKL-induced nuclear transport and expression of NFATc1 are down-regulated by 2-MS

Binding of RANKL to RANK activates several transcription factors responsible for promoting osteoclastic gene expression. These are not all activated within the same time frame; early response factors, such as c-Fos, are activated before late-response factors, such as NFATc1 (Yamashita *et al.*, 2007). NFATc1 is one of the key transcription factors involved in osteoclast differentiation by RANKL (Ishida *et al.*, 2002; Takayanagi *et al.*, 2002). In resting T cells, NFATc1 is restricted to the cytoplasm. T-cell activation leads to increased nuclear accumulation of NFATc1. Therefore, we assessed the nuclear translocation of NFATc1 in RANKL-activated RAW264.7 cells, with and without 2-MS treatment. Nuclear translocation of NFATc1 was markedly increased (approximately 1.6-fold) by RANKL stimulation for 24 h over the levels in cells not treated with RANKL (lane 2 in the upper panel of Figure 8). This elevated level of nuclear NFATc1 was reduced by 2-MS in a concentration-dependent manner. The total amount of NFATc1 protein was increased approximately 2.1-fold after stimulation with RANKL for 48 h and this induction was significantly suppressed by co-incubation with 2-MS in a concentration-dependent manner (Figure 8, bottom panel).

Effect of 2-MS on RANKL-induced MAPKs activation in osteoclasts

Several reports have indicated that RANKL induces the activation of three well-known MAPKs (ERK, JNK and p38). These kinases (especially ERK and JNK) also participate in c-Fos and c-Jun activation in osteoclast precursors (Grigoriadis *et al.*, 1994; Li *et al.*, 2002). Based on the findings that 2-MS repressed RANKL-induced nuclear translocation of c-Fos, we next investigated whether or not MAPKs were involved in the inhibition of osteoclastogenesis by 2-MS. We first confirmed that RANKL-elevated TRAP activities were significantly suppressed by SB203580, PD98059 and SP600125, inhibitors of p38, ERK and JNK, respectively (Figure 9A). Our findings indicate that the three MAPKs all participate in the regulation of RANKL-induced osteoclast differentiation in RAW264.7 cells. Furthermore, RANKL strongly activated p38,

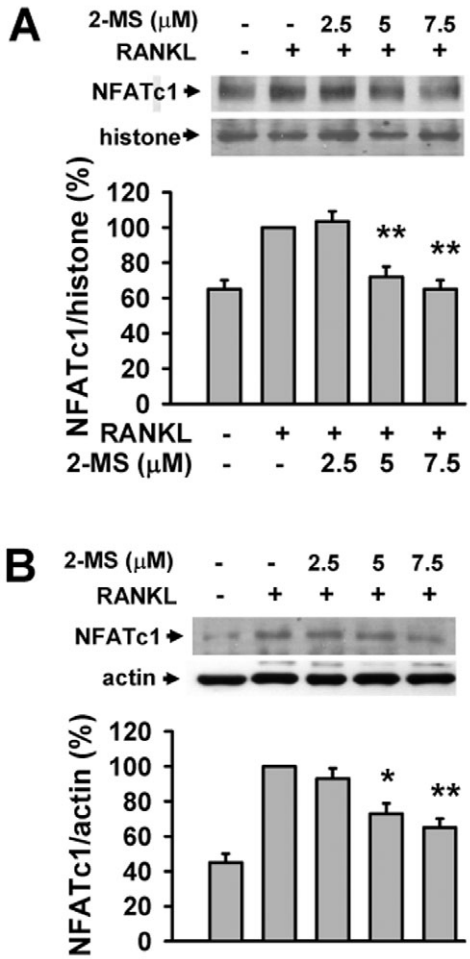

Figure 8

Nuclear translocation and induction of NFATc1 by RANKL were suppressed by 2-MS. (A) RAW264.7 cells were incubated with RANKL (100 ng·mL^{-1}) for 24 h in the absence or presence of 2-MS added 2 h prior to RANKL, then the nuclear proteins were extracted. (B) Cells were stimulated with RANKL (100 ng·mL^{-1}) for 48 h in the absence or presence of 2-MS 2 h prior to RANKL then the total protein were extracted. NFATc1 expression was assessed by Western blotting. Results are expressed as the mean ± SE for each group from four to five separate experiments normalized for histone (for nuclear protein normalization) or β-actin (for total protein normalization) respectively. *$P < 0.05$ and **$P < 0.01$, different from values after treatment with RANKL alone.

ERK and JNK phosphorylation when measured at the early time point of 15 min (Figure 9B). RANKL-induced phosphorylation was inhibited by 2-MS, most clearly for ERK, less for JNK and least effectively for p38 (Figure 9B).

RANKL-induced association of TRAF6–TAK1 complex was repressed by 2-MS

Because TAK1 is able to phosphorylate both MAPK and IKK, this kinase is considered to be at the branching point of the two pathways. Having identified that the inhibition by 2-MS was likely to be at the level of the TAK1/TRAF6 complex, we next analysed whether 2-MS was able to inhibit the formation of the active TAK1/TRAF6 complex. Cell extracts from RAW264.7 cells treated with RANKL were immunoprecipitated with anti-TAK1 antibody and analysed by immunoblotting with anti-TRAF6 antibody. The association of endogenous TAK1 with TRAF6 was observed early at 2 min after RANKL treatment, reaching a peak at 5 min, with subsequent release from TRAF6 at 15 min (Figure 10A). In the presence of 2-MS (2.5–7.5μM), RANKL-induced TRAF6–TAK1 association was decreased (Figure 10B).

Discussion

2-Methoxystypandrone is a naphthoquinone isolated from *P. cuspidatum*, a folk medicine used in Taiwan to cure bone diseases such as bone fractures or bone loss caused by inflammatory joint diseases. In 2001, Singh *et al.* (2001) reported that 2-MS displayed HRV 3C-protease inhibitory activity. However, its effect on osteoclast differentiation has not yet been studied. Our results show that 2-MS dose-dependently inhibited RANKL-induced differentiation and bone resorption of RAW264.7 cells, a homogeneous clonal population of murine monocytic cells. In the absence of osteoblast/bone marrow stromal cells and cytokines like M-CSF or osteoprotegerin, we can rule out the influence of stromal cells and cytokines and focus on RANK signalling in RAW264.7 preosteoclasts. In this study, we found that RANKL-induced differentiation and the function of RAW264.7 preosteoclasts was blocked by concurrent, but not by subsequent addition of 2-MS, suggesting this inhibition occurred at the initial stage of osteoclastogenesis when MNC formation and cell fusion had not occurred yet. However, we did not find obvious cytotoxicity in RAW264.7 preosteoblasts treated with 2-MS at concentrations between 1 and 10 μM.

The present molecular pathways underlying osteoclast formation are illustrated in Fig 11 which includes the sequential molecular events induced by RANK (Asagiri and Takayanagi, 2007). The binding of RANKL to RANK results in the recruitment of TRAF6–TAK1, which activates NF-κB and MAPKs. NF-κB is important for the initial induction of NFATc1. NFATc1 binds to its own promoter, thus switching on an autoregulatory loop. The induction of NFATc1 is also dependent on the MAPKs-mediated activation of AP-1. An AP-1 complex containing c-Fos is required for the auto-amplification of NFATc1, enabling the robust induction of

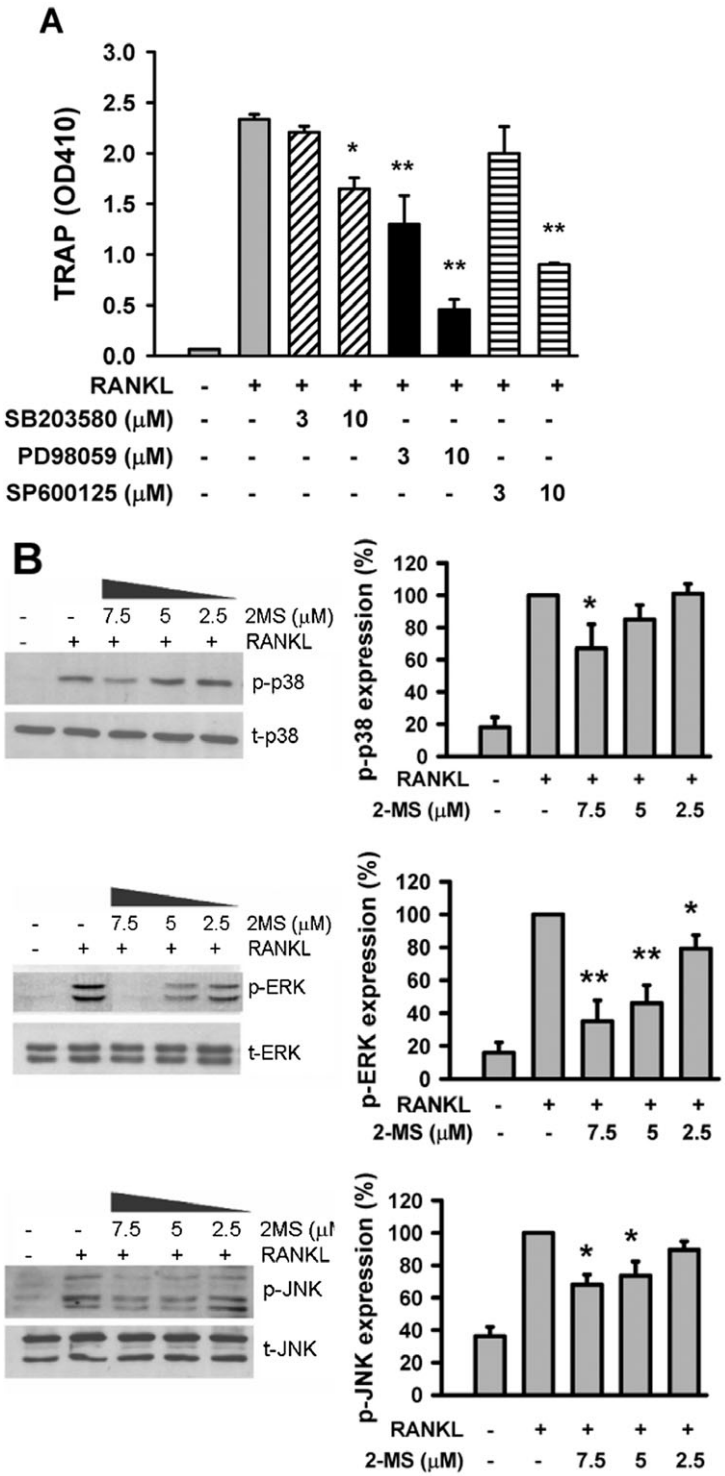

Figure 9

MAPKs regulated RANKL-induced osteoclast differentiation and 2-MS inhibited MAPKs phosphorylation. (A) Effects of SB203580, PD98059 and SP600125 on RANKL-induced TRAP activity. For osteoclast differentiation, RAW264.7 cells were treated with RANKL (100 ng·mL^{-1}) with or without individual MAPKs inhibitors. Cells were further incubated for 72 h and TRAP activity was assayed. Each value is the mean ± SE of five independent experiments each performed in triplicate. *P < 0.05 and **P < 0.01, different from values after treatment with RANKL alone. (B) Effects of 2-MS on RANKL-induced phosphorylation of p38, ERK and JNK in RAW264.7 cells. After 1 day incubation, 2-MS was added to cells 2 h before RANKL and protein samples were prepared 15 min after RANKL stimulation. Activation of signalling molecules were then evaluated by Western blot analysis using control or phosphorylated antibodies against p38, ERK and JNK. Each value is the mean ± SE of five to six independent experiments. *P < 0.05 and **P < 0.01, different from values after treatment with RANKL alone.

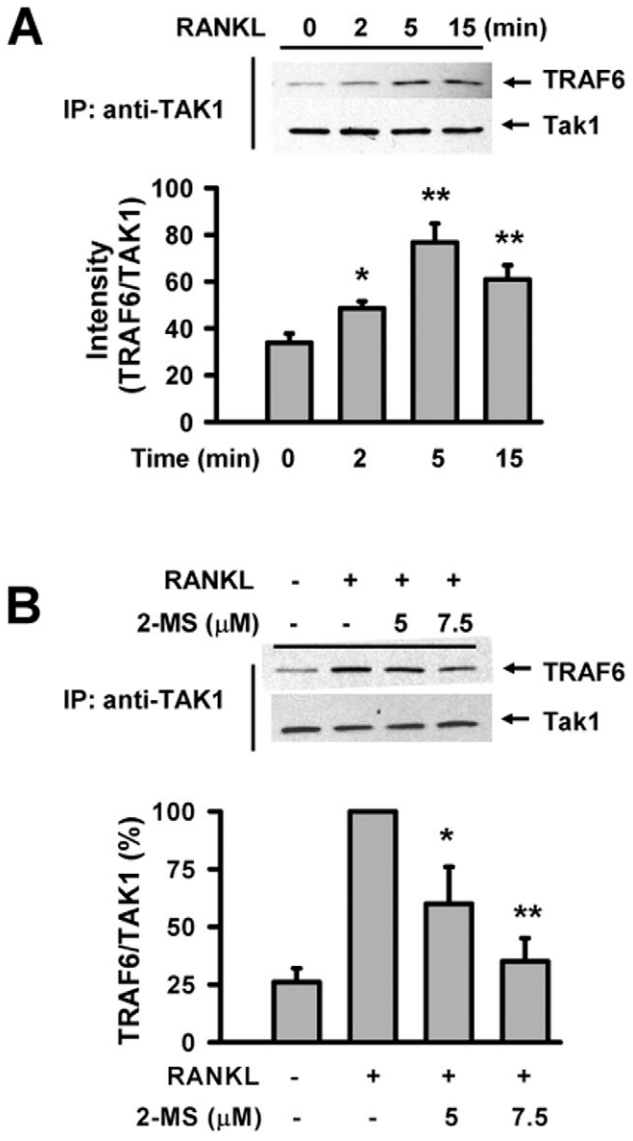

Figure 10

2-MS suppressed RANKL-induced association of endogenous TAK1 with TRAF6. (A) RAW264.7 cells were treated with 100 ng·mL^{-1} of RANKL for indicated times. *P < 0.05 and **P < 0.01, different from values without treatment with RANKL (n = 3 – 4). (B) Effects of 2-MS on the RANKL-induced association of TAK1–TRAF6 complex. Cell extracts were immunoprecipitated with anti-TAK1 antibody. Co-precipitated TRAF6 was detected by immunoblotting with anti-TRAF6 antibody (top panel). The amounts of TAK1 in each immune complex were determined by immunoblotting (bottom panel). *P < 0.05 and **P < 0.01, different from values after treatment with RANKL alone (n = 3 – 4).

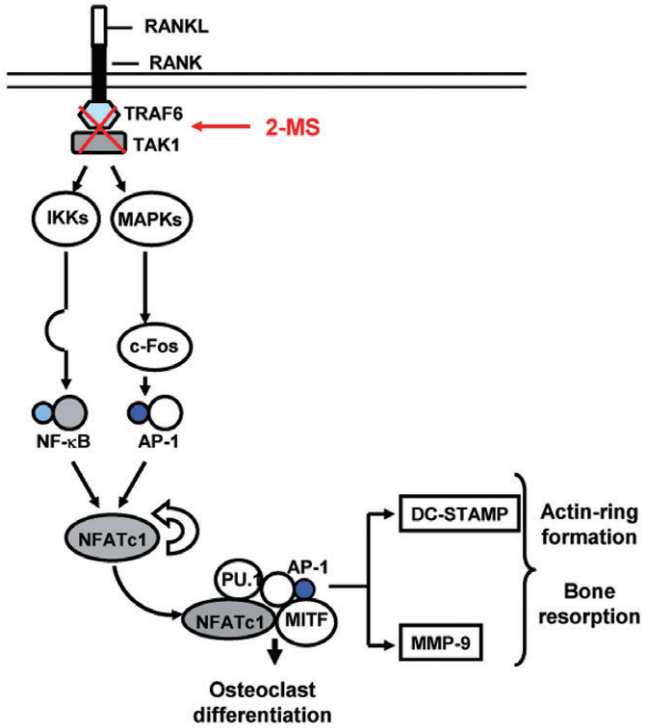

Figure 11

Proposed intracellular actions of 2-MS to suppress RANKL-induced osteoclastogenesis in RAW264.7 cells. 2-MS, 2-methoxystypandrone; AP-1, activator protein 1; DC-STAMP, dendritic cell-specific transmembrane protein; MAPKs, mitogen-activated protein kinases; MITF, microphthalmia-associated transcription factor; MMP-9, matrix metalloproteinase-9; NFATc1, nuclear factor of activated T cells c1; RANKL, receptor activator of nuclear factor κB (NF-κB) ligand; TAK1, transforming growth factor β-activated kinase (TAK)-1; TRAF6, tumour necrosis factor receptor-associated factor 6.

NFATc1. Finally, NFATc1 cooperates with other transcriptional partners to activate osteoclast-specific genes (Feng, 2005; Takayanagi, 2007).

RANKL induces the formation of mature, active osteoclasts by regulating various transcription factors such as NF-κB, MITF, c-Fos and NFATc1 (Teitelbaum and Ross, 2003). RANKL activates AP-1 partly through an induction of its critical component, c-Fos (Wagner and Eferl, 2005). The role of c-Fos in osteoclastogenesis has been revealed by knockout experiments in which c-Fos knockout mice exhibit serious osteopetrotic phenotypes due to the failure of osteoclast formation (Wang *et al.*, 1992). RANKL also induces the expression of NFATc1 through c-Fos and auto-amplification by NFATc1 (Asagiri *et al.*, 2005). It has been reported that NFATc1-deficient embryonic stem cells fail to differentiate into mature osteoclasts in response to RANKL (Takayanagi *et al.*, 2002). In addition, exogenous overexpression of NFATc1 in BMMs efficiently induces differentiation of these cells into osteoclasts even in the absence of RANKL (Takayanagi *et al.*, 2002; Kim *et al.*, 2005). In our study, 2-MS significantly suppressed not only RANKL-induced c-Fos nuclear translocation but also NFATc1 nuclear transport and further induction. It has been shown that c-Fos induces *Nfatc1* expression and that c-Fos and NFATc1 cooperatively regulate osteoclastogenesis in

response to RANKL stimulation (Takayanagi *et al.*, 2002). Thus, we suggest that the inhibition of RANKL-induced c-Fos activation by 2-MS is a relevant factor in the suppression of downstream NFATc1 signal pathways.

Our results showed that RAW264.7 pre-osteoclasts cultured with RANKL for 4 days were able to generate TRAP-positive MNC (Figure 3). After 6 days of culture with RANKL, actin ring formation and resorption pits were observed (Figure 5). Consistent with previous results (Takayanagi *et al.*, 2002; Kim *et al.*, 2005), we found the expression of NFATc1 was markedly up-regulated 2 days after RANKL stimulation (Figure 8B, bottom). In addition, various proteins related to osteoclast function, such as MMP-9, were found to be expressed later (4 days after RANKL stimulation in the present study) than NFATc1, suggesting that NFATc1 might induce the expression of these function-related proteins. Te role of NFATc1 in osteoclast fusion has been revealed by many investigators. Kim *et al.* (2008) reported that NFATc1 binds directly to the promoter regions of the gene for the fusion-mediating molecule DC-STAMP and induces the expression of this gene, thereby regulating osteoclast multinucleation. In addition, NFATc1 inactivation by cyclosporin A attenuates the expression of DC-STAMP as well as osteoclast fusion. Thus, the prevention of RANKL-induced actin ring formation and bone resorption by 2-MS may be at least in part, attributable to down-regulating the key fusion-mediating molecule DC-STAMP via direct targeting of NFATc1. Additionally, MAPKs have been implicated in RANK signalling by regulation of AP-1 activation (Lee *et al.*, 2002). Our results showed that 2-MS significantly inhibited RANKL-induced phosphorylation of MAPKs, especially ERK and JNK. A reasonable explanation for this might be that 2-MS suppressed c-Fos expression via the inhibition of upstream MAPKs activation.

The importance of NF-κB in RANK signalling pathways for osteoclastogenesis has been confirmed by genetic studies (Iotsova *et al.*, 1997; Boyce *et al.*, 2005). In the classical NF-κB signalling pathway, IκB is degraded in an ubiquitin-dependent manner upon phosphorylation. When NF-κB is released from IκB degradation, it translocates to the nucleus to exert transcriptional activity. In our study, 2-MS attenuated not only RANKL-induced degradation of IκB, but also the subsequent nuclear translocation of NF-κB (Figure 7A), suggesting that the effect of 2-MS on osteoclast differentiation is also likely to be dependent on the classical NF-κB activation pathway. As a multifunctional second messenger activated by RANKL, TRAF6 is critical for RANK-induced activation of downstream signal factors such as NF-κB. It has been shown recently, in the context of osteoclastogenesis, that TRAF6 forms a signalling complex containing RANK and TAK1-binding protein (TAB)2, resulting in TAK1 activation (Mizukami *et al.*, 2002), and subsequent activation of NF-κB, AP-1 and p38 pathways, which are crucial for osteoclast differentiation, survival and function (Boyle *et al.*, 2003). These results are observed in TRAF6-deficient mice which develop osteopetrosis due to decreased numbers of osteoclasts (Lomaga *et al.*, 1999). In the interleukin-1 signalling pathway, TAK1 MAPKKK mediates MAPK and IKK activation via interaction with TRAF6, and TAB2 acts as an adapter linking TAK1 and TRAF6. Mizukami *et al.* (2002) also demonstrated that TAK1 and TAB2 participate in the RANK signalling pathway. To clarify the role of TRAF6 and TAK1 in our model, RAW264.7 cells were treated with RANKL and changes in TRAF6–TAK1 complexes was measured. Based on our immunoprecipitation observations, stimulation with RANKL indeed evoked a rapid and significant accumulation of TRAF6–TAK1 protein complexes and the amount of such complexes was less in the presence of 2-MS. The data presented here demonstrate that 2-MS might disrupt intracellular signal propagation by targeting the formation of upstream TRAF6–TAK1 complexes.

The results presented here indicated that 2-MS potently inhibited osteoclastogenesis. This was shown by the suppression of the number of TRAP + MNCs, expression of DC-STAMP and MMP-9, actin ring formation and finally of bone resorptive activity. Furthermore, 2-MS significantly suppressed not only RANKL-induced osteoclast differentiation and pit formation in RAW264.7 cells (IC_{50} ~ 3 μM), but also inhibited the same phenomenon in mouse BMMS treated with both RANKL and M-CSF (IC_{50} ~ 6 μM) (data not shown). These results suggest that 2-MS might be beneficial for bone resorption-associated diseases. Nevertheless, further studies should be carried out in order to determine the biological efficacy of 2-MS in *ex vivo* or *in vivo* studies.

In conclusion, the present study demonstrated that 2-MS significantly suppressed osteoclastogenesis, and suggests that this inhibitory activity could result from its potential to down-regulate the RANKL-induced formation of the signalling complex TRAF6–TAK1 that regulates the expression of osteoclast-associated genes (such as MMP-9 and DC-STAMP) by modulating the activation and expression of transcriptional factors such as NF-κB, c-Fos and NFATc1 (Figure 11). Further studies can explore whether 2-MS acts on targets beyond the formation of TRAF6–TAK1 complexes, by interfering

with the recruitment of TRAF6–TAK1 to RANK or the binding of RANKL to RANK to block osteoclastogenesis.

Acknowledgements

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Conflicts of interest

None.

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