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RESEARCH PAPER

Thiocolchicoside suppresses osteoclastogenesis induced by RANKL and cancer cells through inhibition of inflammatory pathways: a new use for an old drug

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BACKGROUND AND PURPOSE

Most patients with cancer die not because of the tumour in the primary site, but because it has spread to other sites. Common tumours, such as breast, multiple myeloma, and prostate tumours, frequently metastasize to the bone. To search for an inhibitor of cancer-induced bone loss, we investigated the effect of thiocolchicoside, a semi-synthetic colchicoside derived from the plant *Gloriosa superba* and clinically used as a muscle relaxant, on osteoclastogenesis induced by receptor activator of NF-κB ligand (RANKL) and tumour cells.

EXPERIMENTAL APPROACH

We used RAW 264.7 (murine macrophage) cells, a well-established system for osteoclastogenesis, and evaluated the effect of thiocolchicoside on RANKL-induced NF-κB signalling and osteoclastogenesis as well as on osteoclastogenesis induced by tumour cells.

KEY RESULTS

Thiocolchicoside suppressed osteoclastogenesis induced by RANKL, and by breast cancer and multiple myeloma cells. Inhibition of the NF-κB pathway was responsible for this effect since the colchicoside inhibited RANKL-induced NF-κB activation, activation of IκB kinase (IKK) and suppressed inhibitor of NF-κBα (IκBα) phosphorylation and degradation, an inhibitor of NF-κB. Furthermore, an inhibitor of the IκBα kinase γ or NF-κB essential modulator, the regulatory component of the IKK complex, demonstrated that the NF-κB signalling pathway is mandatory for osteoclastogenesis induced by RANKL.

CONCLUSIONS AND IMPLICATIONS

Together, these data suggest that thiocolchicoside significantly suppressed osteoclastogenesis induced by RANKL and tumour cells via the NF-κB signalling pathway. Thus, thiocolchicoside, a drug that has been used for almost half a century to treat muscle pain, may also be considered as a new treatment for bone loss.

LINKED ARTICLE

This article is commented on by Micheau *et al.*, pp. 2124–2126 of this issue. To view this commentary visit http://dx.doi.org/10.1111/j.1476-5381.2011.01792.x

Abbreviations

ALLN, N-acetyl-leucyl-leucyl-norleucinal; DMEM, Dulbecco's modified Eagle medium; EMSA, electrophoretic mobility shift assay; GST, glutathione transferase; IKK, IκB kinase; IκBα, inhibitor of NF-κBα; M-CSF, macrophage colony-stimulating factor; NBP, NEMO-binding domain peptide; NEMO, NF-κB essential modulator; RANKL, receptor activator of NF-κB ligand; TRAF6, TNF receptor-associated factor 6; TRAP, tartrate-resistant acid phosphatase

Introduction

Enhanced osteoclastogenesis is an important pathological feature in many age-associated bone diseases, such as osteoporosis, osteopetrosis, Paget's disease of bone, malignant bone diseases, rheumatoid and osteoarthritis, periodontal diseases and bacteria-induced osteolysis (Phan *et al.*, 2004). The bone is a dynamic tissue that undergoes continuous adaptation to attain and preserve the skeletal size and shape. This perpetual bone remodelling depends on a fine balance between two dynamic processes, bone formation and bone resorption (Xu *et al.*, 2009). The cell types responsible for these opposing processes are the bone-forming osteoblasts and the bone-resorbing osteoclasts. The osteoclast is a unique bone-resorbing cell derived from the cells of the monocyte–macrophage lineage that removes bone tissue by removing its mineralized matrix and breaking up the organic bone (Boyle *et al.*, 2003). Osteoclast progenitor cells are recruited from haematopoietic compartments, and then differentiate and proliferate towards mature osteoclasts. During this multistep differentiation process, post-mitotic osteoclast precursors progressively express osteoclast-associated markers, like calcitonin receptor and tartrate-resistant acid phosphatase (TRAP). Then, mononuclear pre-osteoclasts fuse together to form multinucleated giant cells. Terminal osteoclast differentiation eventually leads to active bone-resorbing cells (Takahashi *et al.*, 1994).

One of the key factors mediating the differentiation of a monocyte into an osteoclast is the receptor activator of NF-κB ligand (RANKL) (Anderson *et al.*, 1997), a cytokine and a member of the TNF family. RANKL, which is expressed on the surface of osteoblastic/stromal cells, is directly involved in the differentiation of monocytes into osteoclasts (Lacey *et al.*, 1998; Teitelbaum, 2000). Indeed, mice with disruptions in the *rankl* gene show a lack of osteoclasts, severe osteopetrosis and defective tooth eruption, indicating that RANKL is essential for osteoclast differentiation (Kong *et al.*, 1999). This cytokine activates osteoclast formation by stimulating its receptor RANK through its interaction with the adaptor molecule TNF receptor-associated factor 6 (TRAF6) (Yavropoulou and Yovos, 2008). TRAF6 then activates the IκB kinases (IKKs), which phosphorylate and degrade the inhibitor of NF-κB, IκBα (Yavropoulou and Yovos, 2008). Once IκBα is degraded, NF-κB translocates to the nucleus and activates the transcription of specific genes involved in the differentiation process (Xu *et al.*, 2009).

RANKL has also emerged as a major mediator of bone cancer pain (Saad *et al.*, 2008). Bone cancer pain is common in patients who have advanced multiple myeloma or advanced breast, prostate, or lung cancer, which are all tumours with a marked propensity to metastasize to the bone (Coleman, 2001). Multiple myeloma, for example, metastasizes to the bone in approximately 90% of cases (Coleman, 2001; 2006). In addition, about threequarters of patients with advanced breast cancer or hormone-refractory prostate cancer, as well as 40% of patients with advanced non-small cell lung cancer and carcinoma, develop tumour metastases in the bone (Coleman, 2001; 2006). Tumours that metastasize to the bone are a major cause of morbidity and mortality because they induce significant skeletal remodelling, fractures, pain and anaemia (Jimenez-Andrade *et al.*, 2010). In fact, most patients with cancer die not due to the tumour in the primary site, but because it has spread to other sites, such as the bone.

Although bisphosphonates are approved and are frequently used to reduce tumour-induced bone destruction and bone cancer pain, they have unwanted side effects (including induction of arthralgia and osteonecrosis of the jaw) (Drake *et al.*, 2008) and it has yet to be definitively shown that they effectively increase the survival of patients with bone cancer.

Therefore, other treatments are urgently needed. One way to expedite drug development is to discover new uses for older, clinically approved drugs. Thiocolchicoside (Figure 1A), a semi-synthetic drug derived from colchicoside, which is itself a natural glucoside present in the plant *Gloriosa superba*, might be such an agent for inhibiting RANKL and thus perhaps osteoclastogenesis as well. Thiocolchicoside has been used clinically for more than 35 years as an analgesic, a muscle relaxant and to treat a number of orthopaedic, traumatic and rheumatological conditions. Furthermore, since clinical trials have shown that the colchicoside is an efficient and safe treatment for patients with acute low back pain accompanied by muscle spasm (Tuzun *et al.*, 2003; Ketenci *et al.*, 2005; Soonawalla and Joshi, 2008), this drug could rapidly enter phase II studies. Our own group has previously published the potential mechanism for its anti-inflammatory effects (Reuter *et al.*, 2010).

In the present study, we investigated whether thiocolchicoside can suppress NF-κB activation induced by RANKL and suppress osteoclastogenesis induced by RANKL and cancer cells. We used a homogeneous, clonal, population of RAW 264.7 murine monocytic cells because this system contains no osteoblast/bone marrow stromal cells and no cytokine-like macrophage colony-stimulating factor (M-CSF); both of these features allowed us to focus on RANKL signalling in pre-osteoclast cells. Our results demonstrated that thiocolchicoside suppresses the RANKL-induced NF-κB activation pathway by inhibiting IKK and that this effect correlated with the suppression of osteoclastogenesis induced by RANKL and cancer cells.

Methods

Reagents

A 100 mM solution of thiocolchicoside was kindly provided by Dr Vinod Bansal (Sarv Biolabs, New Delhi, India), prepared in water, stored at 4°C and then diluted as needed in cell culture medium. Dulbecco's modified Eagle medium (DMEM)/F12, RPMI 1640, fetal bovine serum, 0.4% trypan blue vital stain and antibiotic–antimycotic mixture were obtained from Invitrogen (Carlsbad, CA, USA). RANKL protein was kindly provided by Dr Bryant Darnay (Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, TX, USA). Rabbit polyclonal antibodies against IκBα were purchased from Imgenex (San Diego, CA, USA). Antibody against phosphorylated (p)-IκBα (Ser32/36) was purchased from Cell Signaling Technology (Danvers, MA, USA). IKKα and IKKβ antibodies and NF-κB essential modulator (NEMO; also called IKKγ)-binding domain peptide (NBP) were kind gifts from Imgenex.

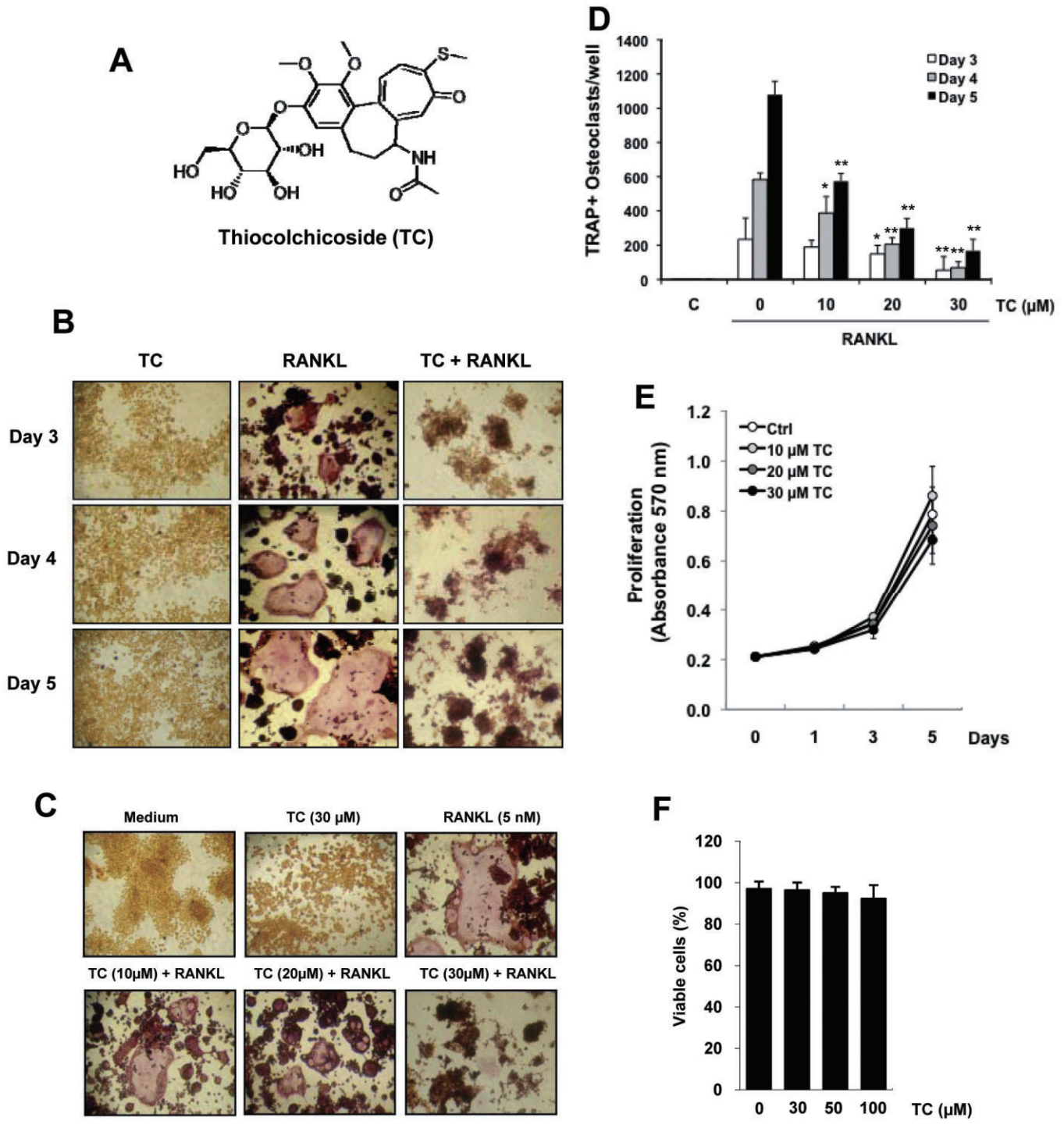

Figure 1

Thiocolchicoside inhibits RANKL-induced osteoclastogenesis. (A) The structure of thiocolchicoside. (B) RAW 264.7 cells (10×10^3 per well) were incubated with 5 nM RANKL, 30 μM thiocolchicoside or both for 3, 4 or 5 days and stained for TRAP expression (red). Magnification, 100× original. (C) RAW 264.7 cells (10×10^3 per well) were incubated with either medium, thiocolchicoside (30 μM) or RANKL (5 nM) alone or with 10, 20 or 30 μM thiocolchicoside plus RANKL for 5 days and then stained for TRAP expression. Magnification, 100× original. (D) Quantification of multinucleated osteoclasts after treatment with medium alone (control; C), with 5 nM RANKL alone or with both RANKL and (10–30 μM) thiocolchicoside for 3, 4 or 5 days. *$P < 0.05$ and **$P < 0.01$ indicate level of significance as compared to cells treated with RANKL alone. (E) RAW 264.7 cells (2×10^3 per 100 μL) were incubated with medium only (Ctrl) or with 10–30 μM thiocolchicoside for 1, 3 or 5 days. Cell proliferation was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. (F) Cells were treated with different concentrations of thiocolchicoside (30–100 μM) and stained with trypan blue. From total number of cells (live + dead), % viable cells were plotted graphically with SD.

p-IKKα/β antibody was purchased from Cell Signaling Technology and phosphorylated ERK 1/2 antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-rabbit and goat anti-mouse horseradish peroxidase conjugates were purchased from Bio-Rad (Hercules, CA, USA). Antibody against β-actin and the leucocyte acid phosphatase kit (387-A) for TRAP staining were purchased from Sigma-Aldrich (St. Louis, MO, USA). Protein A/G agarose beads were obtained from Pierce. [γ-^{32}P]-ATP was purchased from ICN Pharmaceuticals (Mississauga, ON, Canada).

Cell lines

RAW 264.7 (mouse macrophage) cells were kindly provided by Dr Bryant Darnay (Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, TX, USA). For our studies, we used a single clone (#28) that was selected after limited dilution. RAW 264.7 cells were cultured in DMEM/F12 supplemented with 10% fetal bovine serum and antibiotics. This cell line is a well-established osteoclastogenic cell system that has been shown to express RANK and differentiate into functional TRAP-positive osteoclasts when cultured with soluble RANKL (Hsu *et al.*, 1999). Moreover, RANKL has been shown to activate NF-κB in RAW 264.7 cells (Wei *et al.*, 2001). MDA-MB-231 (human breast adenocarcinoma) and U266 (human multiple myeloma) cells were obtained from the American Type Culture Collection. MDA-MB-231 cells were cultured in DMEM, and U266 cells were cultured in RPMI 1640, with 10% fetal bovine serum. All the media were supplemented with penicillin and streptomycin.

Osteoclast differentiation assays

RAW 264.7 cells were cultured in 24-well plates at a density of 10×10^3 cells per well and allowed to adhere overnight. The medium was then replaced, and the cells were treated with 5 nM RANKL for 5 days. All cell lines were subjected to TRAP staining using the leucocyte acid phosphatase kit. For co-culture experiments with cancer cells, RAW 264.7 cells were seeded at 5×10^3 cells per well and allowed to adhere overnight. The following day, MDA-MB-231 or U266 cells, at 1×10^3 cells per well, were added to the RAW 264.7 cells, treated with thiocolchicoside, and co-cultured for 5 days before being subjected to TRAP staining.

For conditioned medium experiments, RAW 264.7 cells were seeded at 10×10^3 cells per well and allowed to adhere overnight. The following day, the medium was replaced with 4/5 of RAW 264.7 medium (DMEM/F12) plus 1/5 of conditioned medium from MDA-MB-231 or U266 cells. For that procedure, the supernatant of cultured cancer cells that had been centrifuged was used. Finally, the RAW 264.7 cells were cultured for 5 days and subjected to TRAP staining.

Cell proliferation assay

We employed a method as described previously with minor modification (Pandey *et al.*, 2007). In brief, 2×10^3 of RAW 264.7 cells (in 0.1 mL) per well were incubated with various concentrations of thiocolchicoside, in triplicate, for 1, 3 or 5 days in 96-well plates at 37°C. Thereafter, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution was added to each well. After 2 h of incubation at 37°C, lysis buffer [20% sodium dodecyl sulphate (SDS) and 50% dimethylformamide] was added and the cells were incubated overnight at 37°C. Absorbance was measured at 570 nm using a 96-well multi-scanner (MRX Revelation; Dynex Technologies, Chantilly, VA, USA).

Trypan blue dye exclusion assay for cell viability

Control cells and cells treated with different concentrations of thiocolchicoside were stained with trypan blue. Viable and dead cells were counted using a haemocytometer. We counted around 300 cells for each sample in triplicate. From the total number of cells, % viable cells were plotted graphically.

Phosphatidylserine externalization assay for cell viability

We measured the loss of membrane asymmetry, an indicator of loss in cell viability that occurs when phosphatidylserine moves to the extracellular surface of the membrane using an Annexin V staining kit (Santa Cruz Biotechnology). The assay was performed by following the manufacturer's instructions.

Electrophoretic mobility shift assay

The nuclear extracts from control and treated cells were incubated with ^{32}P-end-labelled 45-mer double-stranded NF-κB oligonucleotide from the human immunodeficiency virus long terminal repeat, 5-TTGTTACAA**GGGACTTTC**CGCTG **GGGACTTTC**CAGGGGGAGGCGTGG-3 (boldface indicates NF-κB-binding sites) for 30 min at 37°C. The resulting DNA–protein complex was separated from free oligonucleotide on 6.6% native polyacrylamide gels. The dried gels were visualized with a Storm 820 optical scanner (GE Healthcare, Piscataway, NJ, USA). Radioactive bands were quantified using a densitometer and Image Quant software. For conditioned medium experiments, RAW 264.7 cells were seeded at 2×10^6 cells per well and allowed to adhere overnight. The following day, the medium was replaced with 4/5 of RAW 264.7 medium (DMEM/F12) and 1/5 of conditioned medium from MDA-MB-231 or U266 cells. For that procedure, we used the supernatant of centrifuged, cultured cancer cells. The RAW 264.7 cells were then cultured for 24 h and assessed for NF-κB activity by using EMSA. Fold values were based on the value for medium without RANKL, which was arbitrarily set at 1.

Western blot analysis

To determine the levels of protein expression, we prepared cytoplasmic and whole-cell extracts (Sung *et al.*, 2007) and fractionated them by using 10% SDS-PAGE. After electrophoresis, the proteins were electro-transferred to nitrocellulose membranes, blotted with each antibody and detected with the chemiluminescence reagent ECL (GE Healthcare).

IKK assay

To determine the effect of thiocolchicoside on RANKL-induced IKK activation, an IKK assay was performed using a method described previously (Wei *et al.*, 2001). Briefly, the

IKK complex from whole-cell extracts (600 μg of protein) of RAW 264.7 cells was precipitated with antibody against IKKα and then treated with protein A/G agarose beads. After 2 h of incubation, the beads were washed with lysis buffer and assayed in a kinase assay mixture containing 50 mM HEPES (pH 7.4), 20 mM $MgCl_2$, 2 mM dithiothreitol, 20 mCi [γ-^{32}P]-ATP, 10 mM unlabelled ATP and 2 μg of substrate glutathione transferase (GST)-IκBα (amino acids 1–54). After incubation at 30°C for 30 min, the reaction was terminated by boiling with SDS sample buffer for 5 min. Finally, the protein was resolved on 10% SDS-PAGE, the gel was dried and the radioactive bands were visualized with a PhosphorImager. To determine the total amounts of IKKα and IKKβ in each sample, the whole-cell protein was resolved on 10% SDS-PAGE, electro-transferred to a nitrocellulose membrane and blotted with anti-IKKα or anti-IKKβ antibody.

Statistical analysis

Results from at least three independent experiments were analysed for statistical significance. The statistical analysis was carried out using a two-tailed unpaired Student's *t*-test. They are expressed as the mean ± SD. $^{**}P < 0.01$ and $^{*}P < 0.05$ when compared to their respective control.

Results

Our study was designed to investigate the effect of thiocolchicoside on osteoclastogenesis induced by RANKL and cancer cells and on RANKL-induced NF-κB activation.

Thiocolchicoside inhibits RANKL-induced osteoclastogenesis

Because RANKL is one of the major cytokines that induces osteoclastogenesis, we used it to induce differentiation of osteoclasts and investigated whether thiocolchicoside can modulate this differentiation. RAW 264.7 cells were incubated with RANKL, thiocolchicoside or both for 3–5 days and allowed to differentiate into osteoclasts (Figure 1B). The morphological observations clearly revealed differentiation of cells into osteoclasts after addition of RANKL (Figure 1B, middle panel) and that the colchicoside suppressed this differentiation (Figure 1B, right panel). This suppression was found to be dose-dependent (Figure 1C). Counting of TRAP+ osteoclasts confirmed that RANKL induced osteoclast differentiation in a time-dependent manner, with the greatest number of TRAP+ osteoclasts at day 5 (Figure 1D), and that thiocolchicoside dose-dependently decreased the number of TRAP+ osteoclasts, with almost complete inhibition at 30 μM at all days examined (Figure 1D).

To exclude the possibility that this observation was due to a reduction in cell proliferation by thiocolchicoside, we analysed the proliferation of RAW 264.7 cells treated with 0–30 μM thiocolchicoside at days 1, 3 or 5. The colchicoside did not significantly affect the proliferation of RAW 264.7 cells (Figure 1E).

To further examine the effect of thiocolchicoside on cell viability, we treated RAW 264.7 cells with 30, 50 and 100 μM thiocolchicoside for 24 h, stained the cells with trypan blue and counted % viable cells of total number of cells. The cell viability was not significantly affected by thiocolchicoside treatment even at 100 μM (Figure 1F). Furthermore, the Annexin V assay indicated 5–8% apoptotic cells at 30–100 μM thiocolchicoside (data not shown).

Thiocolchicoside acts at an early step in the RANKL-induced osteoclastogenesis pathway

Complete osteoclast differentiation of RAW 264.7 cells takes up to 5 days after RANKL stimulation. To identify the step at which thiocolchicoside acts in this differentiation pathway, we initially treated the cells with RANKL and, 1, 2, 3 or 4 days later, added thiocolchicoside. As determined by visual observation (Figure 2A, right panel) and counting the number of TRAP+ osteoclasts per well (Figure 2B), we found that thiocolchicoside almost completely inhibited osteoclast formation when the cells were exposed to thiocolchicoside for 1 or 2 days after RANKL stimulation. However, at days 3 and 4 after RANKL addition, osteoclast formation was no longer completely prevented by thiocolchicoside. This result suggests that thiocolchicoside acts at an early step in the osteoclast differentiation pathway.

Thiocolchicoside inhibits osteoclastogenesis induced by cancer cells

Given that osteoclastogenesis is commonly linked with breast cancer and multiple myeloma, we next investigated whether thiocolchicoside inhibits osteoclastogenesis induced by these cancers. RAW 264.7 cells were co-incubated with breast cancer (MDA-MB-231) cells or multiple myeloma (U266) cells and allowed to differentiate for 5 days (Figure 3A and B). MDA-MB-231 cells induced differentiation of RAW 264.7 cells into osteoclasts, and thiocolchicoside inhibited this differentiation (Figure 3A). The same was observed for U266 cells (Figure 3B).

To investigate whether cancer cells act directly or indirectly (by secreting certain cytokines) on osteoclasts, RAW 264.7 cells were co-incubated with only the medium of cultured MDA-MB-231 or U266 cells (i.e. conditioned medium) for 5 days (Figure 3A and B, right panels). The quantified results clearly indicate that conditioned medium alone was sufficient to activate osteoclastogenesis in RAW 264.7 cells, indicating that certain cytokines secreted by tumour cells are responsible for osteoclast differentiation. Furthermore, treatment with thiocolchicoside inhibited osteoclastogenesis induced by the conditioned medium (Figure 3A and B, right panels).

Thiocolchicoside inhibits RANKL-induced NF-κB activation

We next investigated how thiocolchicoside inhibits osteoclast differentiation induced by RANKL and by cancer cells. One major mechanism that has been associated with osteoclastogenesis is activation of the NF-κB pathway. Therefore, we explored whether conditioned medium from cancer cells (MDA-MB-231 and U266) induces NF-κB activation in RAW 264.7 cells and found that they did effectively activate NF-κB (Figure 3C), indicating that this transcription factor is involved in osteoclast differentiation by cancer cells.

We also used EMSA to study the effect of thiocolchicoside on RANKL-induced NF-κB activation in RAW 264.7 cells

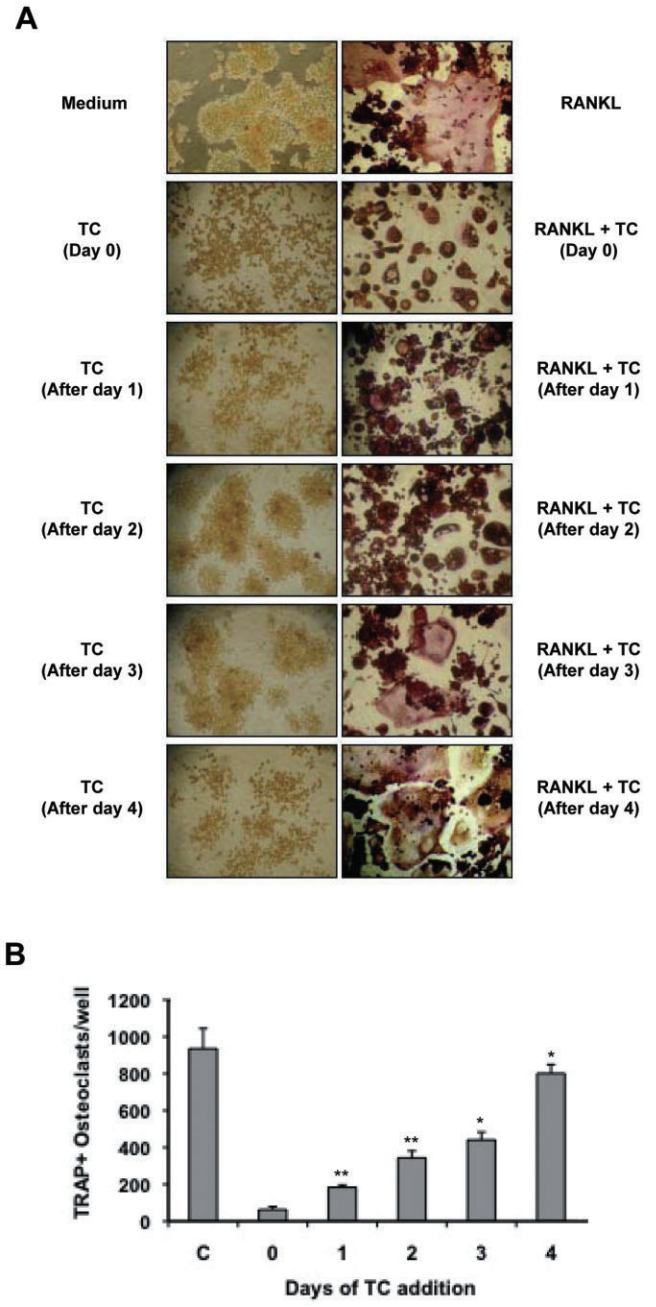

Figure 2

Thiocolchicoside (TC) inhibits RANKL-induced osteoclastogenesis 24 h after stimulation. (A) RAW 264.7 cells (10×10^3 per well) were incubated with RANKL (5 nM) and thiocolchicoside (30 μM) for indicated times and stained for TRAP expression (red). Magnification, 100× original. (B) The number of multinucleated osteoclasts (i.e. those containing three nuclei) was counted. Cells exposed to medium only served as control (C). *$P < 0.05$ and **$P < 0.01$ indicate level of significance as compared to cells treated with RANKL alone.

(Figure 4A). Cells were pretreated with 0, 25, 50, 75 or 100 μM thiocolchicoside for 24 h, and then activated with RANKL for 30 min. Our results show that RANKL activated NF-κB and that the colchicoside inhibited RANKL-induced NF-κB activation at 50 μM. Thiocolchicoside alone did not activate NF-κB.

Thiocolchicoside inhibits RANKL-induced IκBα degradation and phosphorylation

Because the translocation of NF-κB to the nucleus follows the proteolytic degradation of IκBα, we next sought to determine whether the thiocolchicoside-induced NF-κB inhibition was due to inhibition of IκBα degradation. Therefore, we used EMSA to examine the NF-κB expression level after different stimulation times by RANKL in the nucleus (Figure 4B) and for IκBα degradation in the cytoplasm by Western blotting (Figure 5A). RANKL activated NF-κB within 5 min, and thiocolchicoside suppressed this activation (Figure 4B). In accordance with the EMSA results, Western blot analysis showed that RANKL induced IκBα degradation in control cells after 5 min and returned to normal level within 60 min (Figure 5A). In contrast, cells pretreated with thiocolchicoside showed no degradation of IκBα.

IκBα phosphorylation is necessary for IκBα degradation, so we next investigated the effect of thiocolchicoside on IκBα phosphorylation by using the proteasome inhibitor N-acetyl-leucyl-leucyl-norleucinal (ALLN), which prevents RANKL-induced IκBα degradation (Figure 5B). Western blot analysis showed that RANKL and ALLN together induced phosphorylation of IκBα at serines 32 and 36, and that thiocolchicoside pretreatment inhibited this activation in RAW 264.7 cells; thiocolchicoside alone did not induce phosphorylation of IκBα (Figure 5B). These results clearly indicate that thiocolchicoside inhibited RANKL-induced NF-κB activation as well as IκBα degradation and phosphorylation.

Thiocolchicoside inhibits RANKL-induced IKK activation

Because IKK is required for RANKL-induced phosphorylation of IκBα and thiocolchicoside inhibited the phosphorylation of IκBα, we determined the effect of thiocolchicoside on RANKL-induced IκBα activation by IKK. Results of the immune complex kinase assay showed that RANKL strongly induced the phosphorylation of GST-IκBα by IKK within 2 min, and that thiocolchicoside completely inhibited this phosphorylation (Figure 5C). Neither RANKL nor thiocolchicoside affected the expression of IKKα or IKKβ proteins.

Given that phosphorylation of IκBα by the IKK complex required phosphorylation of the subunits IKKα/β, we next investigated whether thiocolchicoside also inhibited phosphorylation of IKKα/β. RAW 264.7 cells were pretreated with thiocolchicoside and stimulated with RANKL (Figure 5D). Western blot results with p-IKKα/β antibody showed that thiocolchicoside completely inhibited phosphorylation of IKKα/β, suggesting that this mechanism is important for the inhibitory effect of thiocolchicoside on RANKL-induced NF-κB activation.

Inhibition of osteoclastogenesis by thiocolchicoside is NF-κB specific

Given that RANKL-induced osteoclastogenesis is triggered by two main signalling pathways, namely the NF-κB and MAPK pathways, we investigated whether thiocolchicoside pretreatment also affects the MAPK pathway. Cells were pretreated

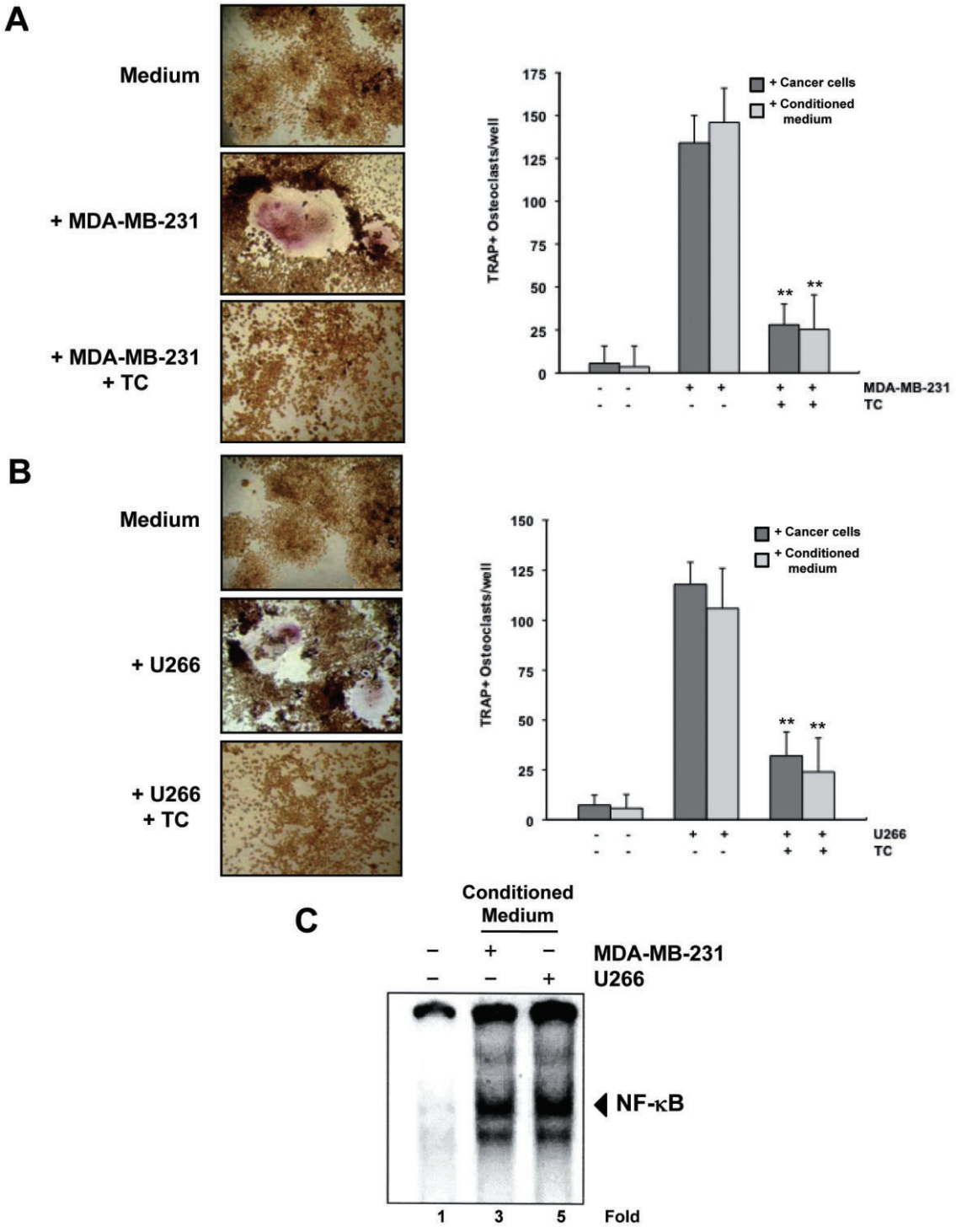

Figure 3

Thiocolchicoside (TC) inhibits osteoclastogenesis induced by cancer cells. (A, left panel) RAW 264.7 cells (10×10^3 per well) were incubated in the presence of MDA-MB-231 cells (1×10^3 per well), exposed to thiocolchicoside (30 μM) for 5 days, and then stained for TRAP expression. Magnification, 100× original. (A, right panel) RAW 264.7 cells (10×10^3 per well) were incubated either in the presence of MDA-MB-231 cells (1×10^3 per well) or conditioned medium from MDA-MB-231 cells, exposed to thiocolchicoside (30 μM) for 5 days, and then multinucleated osteoclasts (i.e. those containing three nuclei) were counted. $^{**}P < 0.01$ indicates level of significance as compared to untreated samples. (B, left panel) RAW 264.7 cells (10×10^3 per well) were incubated in the presence of U266 cells (1×10^3 per well), exposed to thiocolchicoside (30 μM) for 5 days, and then stained for TRAP expression. Magnification, 100× original. (B, right panel) RAW 264.7 cells (10×10^3 per well) were incubated either in the presence of U266 cells (1×10^3 per well) or conditioned medium from U266 cells, exposed to thiocolchicoside (30 μM) for 5 days, and then multinucleated osteoclasts (i.e. those containing three nuclei) were counted. $^{**}P < 0.01$ indicates level of significance as compared to untreated samples. (C) RAW 264.7 cells (2×10^6 per well) were incubated in the presence of conditioned medium from MDA-MB-231 and U266 cells for 24 h and then assessed for NF-κB activity by EMSA.

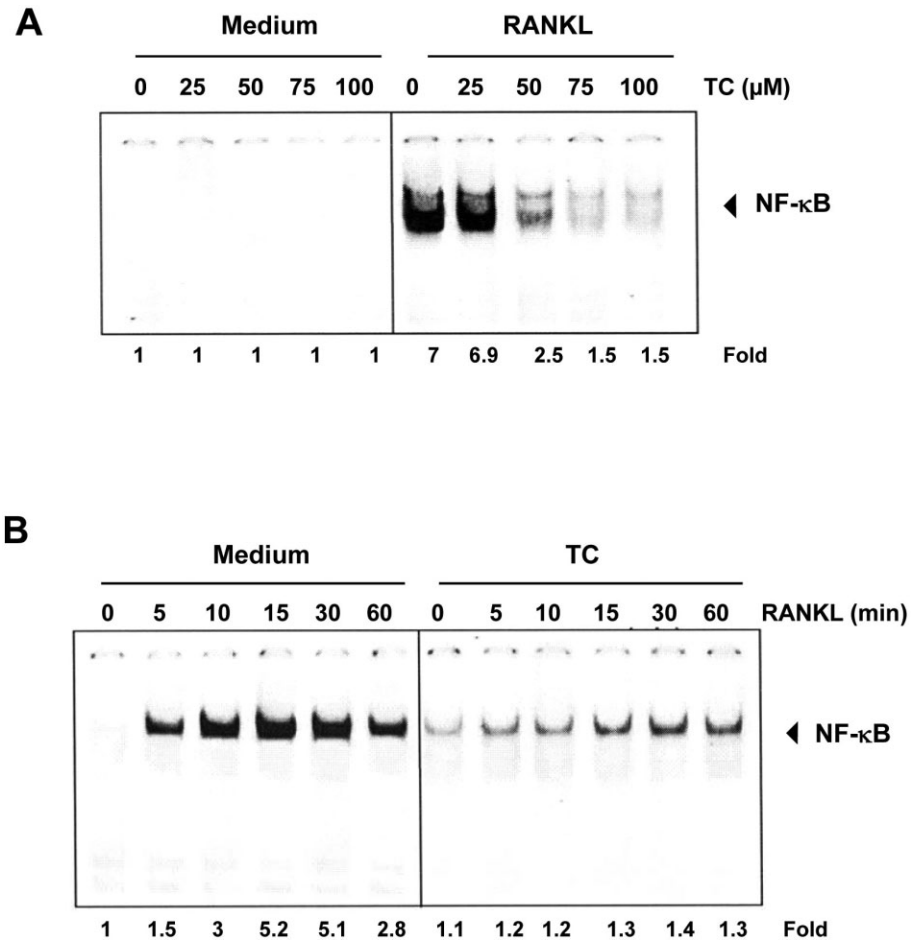

Figure 4

RANKL induces NF-κB activation and thiocolchicoside inhibits it in a dose- and time-dependent manner. (A) RAW 264.7 cells (1.5×10^6 per well) were incubated with different concentrations of thiocolchicoside for 24 h, treated with 10 nM RANKL for 30 min, and examined for NF-κB activation by EMSA. (B) RAW 264.7 cells (1.5×10^6 per well) were incubated with 50 μM of thiocolchicoside for 24 h and treated with 10 nM RANKL for the indicated times and examined for NF-κB activation by EMSA.

with thiocolchicoside for 24 and 48 h before RANKL treatment. We found that RANKL activated ERK in a time-dependent manner and thiocolchicoside pretreatment did not inhibit the activation (Figure 5E). Instead, a slight activation in thiocolchicoside-treated samples was noted at 5 min.

To further ascertain the specificity of NF-κB in osteoclastogenesis, we used a specific inhibitor of the regulatory subunit of the IKK complex, IKKγ, which is also known as NEMO. Whereas the serine kinases IKKα and IKKβ target serines 32 and 36 of the IκBα protein, NEMO regulates the IKK complex activity through its binding to the carboxyl-terminal region of the IKKα and IKKβ subunits, called the NEMO-binding domain. In this regard, a cell-permeable peptide that blocks the NEMO-binding domain would inhibit the association of NEMO with the IKK complex and consequently suppress NF-κB activation and, most likely, osteoclastogenesis. To determine the effect of the NBP on RANKL-induced osteoclastogenesis, we pretreated RAW 264.7 cells with 100 μM NBP for 2 h and then with RANKL for 5 days (Figure 6A and B). Our results showed that the peptide strongly inhibited osteoclast differentiation (Figure 6A and B). Furthermore, when we treated nuclear extracts from RAW 264.7 cells with 100 μM NBP for 2 h and then with RANKL for 30 min, RANKL-induced NF-κB activation was completely inhibited (Figure 6C). These results confirm that NF-κB was responsible for osteoclast differentiation of RAW 264.7 cells, and that inhibition of NF-κB by either thiocolchicoside or NBP prevented osteoclastogenesis.

Discussion

A semi-synthetic derivative of colchicoside, thiocolchicoside, is a natural glucoside present in the plant *G. superba*, which is the national flower of Zimbabwe and the state flower of the Indian state Tamil Nadu. Various preparations of this plant are used in traditional medicines for a variety of complaints in both Africa and India. In western medicine, thiocolchicoside (Muscoril) has been used for more than 35 years as a muscle relaxant, analgesic compound and anti-inflammatory drug to treat a number of orthopaedic, traumatic and rheumatological conditions (Janbroers, 1987). Clinical trials have

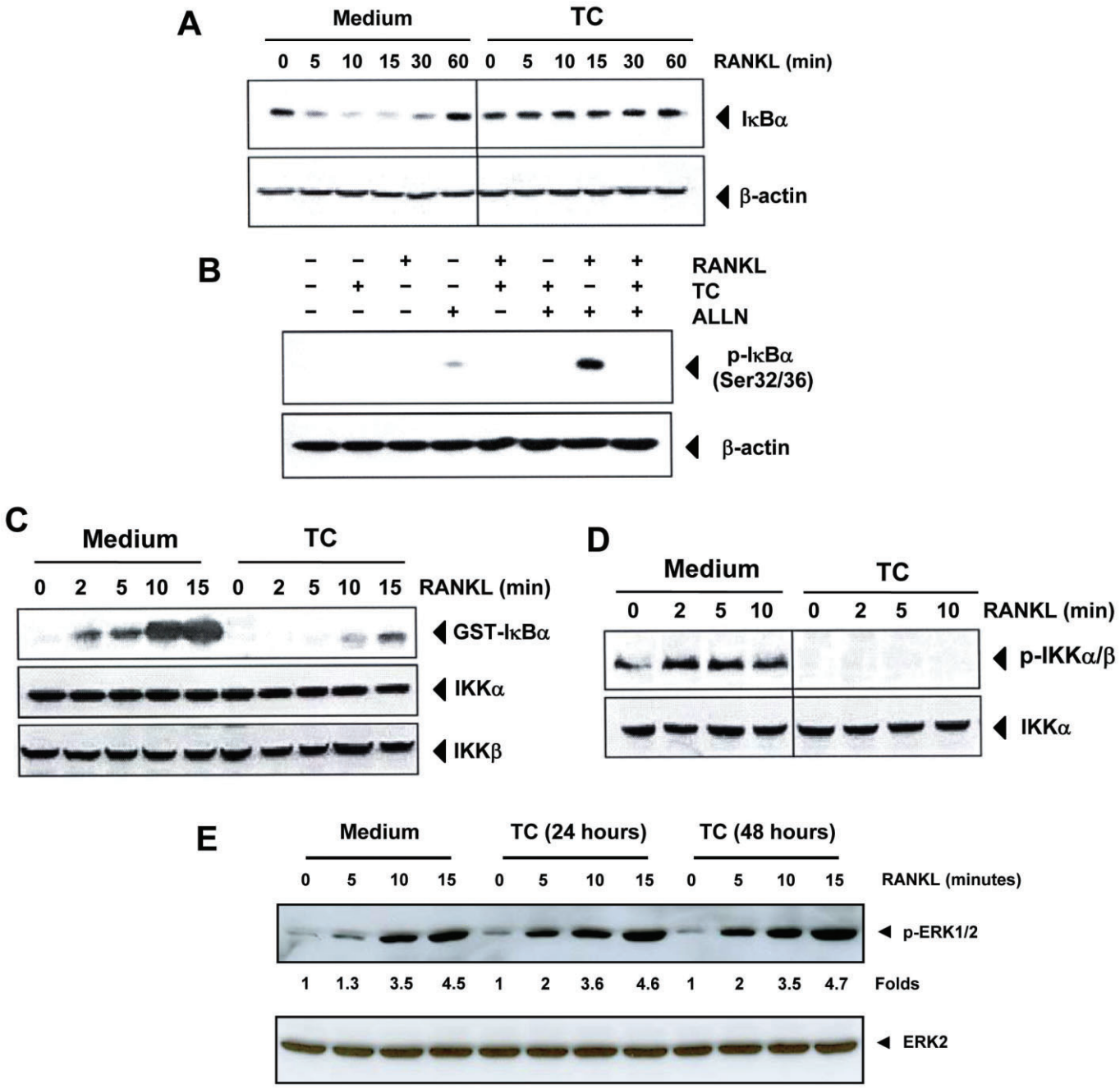

Figure 5

Thiocolchicoside suppresses RANKL-induced IκBα degradation and phosphorylation through inhibition of the IKK activity. (A) RAW 264.7 cells (1.5×10^6 per well) were incubated with 50 μM of thiocolchicoside for 24 h and then treated with 10 nM RANKL for indicated times. Cytoplasmic extracts were examined for IκBα degradation by Western blot using an anti-IκBα antibody. β-Actin was used as a loading control. (B) RAW 264.7 cells (1.5×10^6 per well) were pretreated with thiocolchicoside (50 μM) for 24 h, incubated with ALLN (50 $\mu g \cdot mL^{-1}$) for 30 min and treated with RANKL (10 nM) for 15 min. Cytoplasmic extracts were prepared and analysed by Western blot using an anti-phospho-IκBα antibody. β-actin was used as a loading control. (C) RAW 264.7 cells (3×10^6 per well) were pretreated with thiocolchicoside (50 μM) for 24 h and then incubated with RANKL (10 nM) for up to 15 min. Whole-cell extracts were immunoprecipitated using an antibody against IKKα and analysed with an immune complex kinase assay using recombinant GST-IκBα as described in Methods section. To examine the effect of thiocolchicoside on the level of IKK proteins, whole-cell extracts were analysed by Western blot using anti-IKKα and anti-IKKβ antibodies. (D) RAW 264.7 cells (1.5×10^6 per well) were pretreated with thiocolchicoside (50 μM) for 24 h and incubated with RANKL (10 nM) for up to 10 min. Whole-cell extracts were analysed by Western blot using an anti-p-IKKα/β antibody. IKKα was used as a loading control. (E) RAW 264.7 cells (1.5×10^6 per well) were pretreated with thiocolchicoside (50 μM) for 24 h and 48 h and then incubated with RANKL (10 nM) for up to 15 min. Whole-cell extracts were analysed by Western blot with an anti-p-ERK 1/2 antibody. ERK 1/2 was used as a loading control. The numbers below the blot indicate fold activation in comparison to control cells.

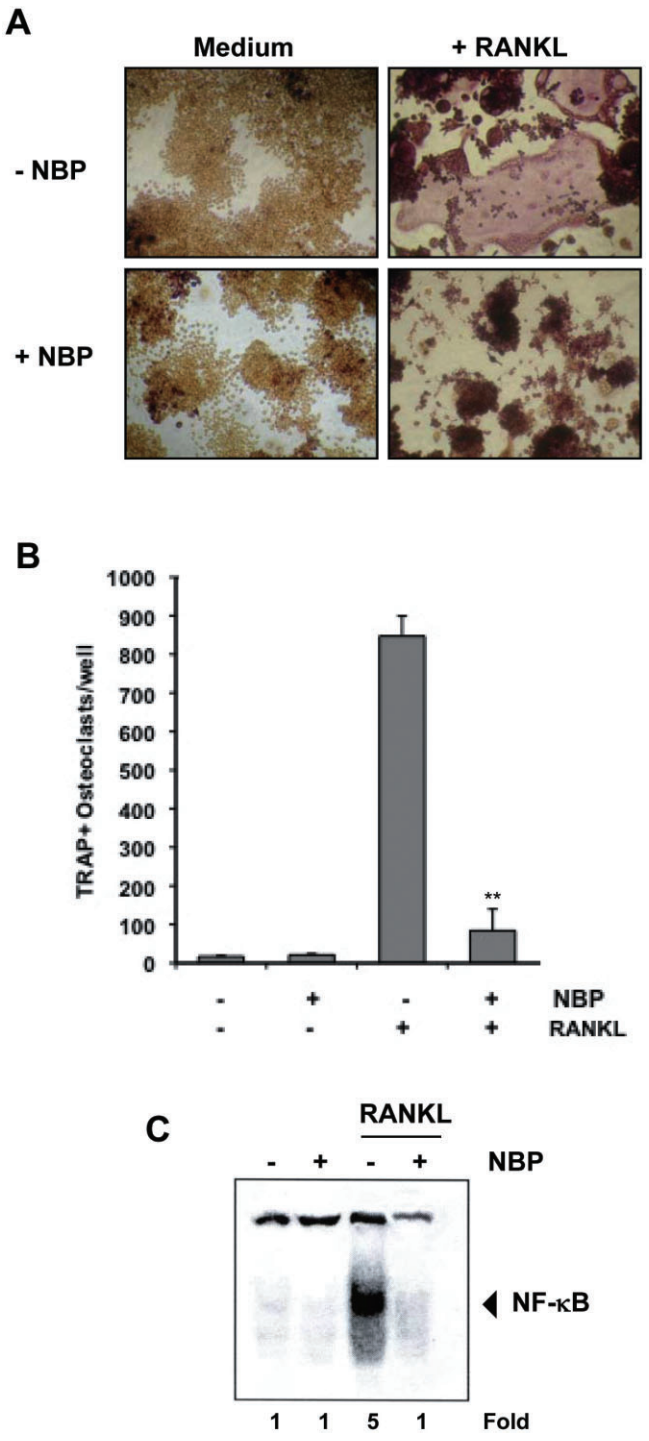

Figure 6

A peptide that targets the NEMO-binding domain inhibits RANKL-induced osteoclastogenesis. (A) RAW 264.7 cells (10×10^3 per well) were pretreated with 100 μM NBP for 2 h, the medium was changed and then RANKL (5 nM) was added for 5 days. Magnification, 100× original. (B) Multinucleated osteoclasts (i.e. those containing three nuclei) were counted. $^{**}P < 0.01$ indicates level of significance as compared to cells treated with RANKL alone. (C) RAW 264.7 cells (1.5×10^6 per well) were incubated with 100 μM of NBP for 2 h, and then incubated with 10 nM of RANKL for 30 min and examined for NF-κB activation by EMSA.

shown that thiocolchicoside is an efficient and safe treatment for patients with acute low back pain accompanied by muscle spasm (Tuzun *et al.*, 2003), and that it is at least as effective as tizanidine, the standard drug used to treat low back pain (Ketenci *et al.*, 2005; Soonawalla and Joshi, 2008). Moreover, biochemical studies have indicated that thiocolchicoside can inhibit the binding of both the [^{3}H]-GABA and [^{3}H]-strychnine receptors to rat cerebrocortical and spinal cord membranes (Biziere *et al.*, 1981; Cimino *et al.*, 1996), suggesting that it induces depression of the CNS and, in turn, myorelaxation, probably via this mechanism (Biziere *et al.*, 1981). Clinical trials have also proven the efficacy and safety of thiocolchicoside in treating acute cervical myofascial pain syndrome (Tuzun *et al.*, 2003; Ketenci *et al.*, 2005; 2009; Soonawalla and Joshi, 2008).

In a previous study, we reported that thiocolchicoside's anti-inflammatory properties are probably due to its ability to inhibit the NF-κB signalling pathway (Reuter *et al.*, 2010), which has also been demonstrated to be a major mediator of bone loss (Xu *et al.*, 2009). For example, mice lacking the NF-κB subunits p50 and p52 and mice deficient in IKKβ show severe osteopetrosis caused by failure of osteoclast formation (Franzoso *et al.*, 1997; Zheng *et al.*, 2006). Furthermore, Abu-Amer and colleagues reported that a IκB super-suppressor blocked osteoclast differentiation and activation (Abu-Amer *et al.*, 2001), and that a dominant-negative IκB protein lacking the NH_2-terminal phosphorylation site lowered NF-κB activation and suppressed recruitment of osteoclasts (Clohisy *et al.*, 2003). Therefore, a compound that inhibits NF-κB is very likely to be able to inhibit osteoclastogenesis as well. Our results indicate that thiocolchicoside inhibits RANKL-induced NF-κB activation by inactivating the IKK complex, through inhibition of the phosphorylation of IKKα/β, and thereby preventing NF-κB activation via inhibition of IκBα phosphorylation and degradation.

In this study, we investigated whether thiocolchicoside, in addition to inhibiting RANKL-induced NF-κB activation, would also inhibit RANKL-induced osteoclastogenesis. For this, we co-treated RAW 264.7 cells with thiocolchicoside and RANKL. RAW 264.7 cells are a homogeneous, clonal population of murine monocytic cells that do not contain any osteoblast/bone marrow stromal cells or cytokine like M-CSF and therefore allow us to focus only on RANK signalling in pre-osteoclast cells. We have previously published that this system parallels with peripheral blood mononuclear cells stimulated with M-CSF and RANKL (Bharti *et al.*, 2004b; Kunnumakkara *et al.*, 2007).

Our results indicated that the colchicoside effectively inhibits RANKL-induced osteoclastogenesis. In addition, a kinetic study indicated that thiocolchicoside acts at an early step in the differentiation process of the osteoclasts. To further confirm that inhibition of the NF-κB signalling pathway is responsible for the arrest of the osteoclastogenesis process in our system, we used a cell-permeable peptide that targets the NEMO-binding domain of the IKKα and IKKβ kinases and so prevents NF-κB activation. This NBP has been shown to inhibit osteoclastogenesis *in vivo* and to delay the onset, lower the incidence and decrease the severity of rheumatoid arthritis (Dai *et al.*, 2004). In addition, previous studies have demonstrated that pharmacological or genetic inactivation of IKKα, IKKβ or both is sufficient for inhibition

of osteoclastogenesis and for prevention of inflammation and osteolytic-induced bone loss (Chaisson *et al.*, 2004; Ruocco *et al.*, 2005). Our results showed that NBP completely blocked RANKL-induced osteoclastogenesis in the same manner as thiocolchicoside. The inhibitory effect of 100 μM NBP was as potent as 30 μM thiocolchicoside, which suggests that the latter is a more potent inhibitor of osteoclastogenesis, at least *in vitro*.

Binding of RANKL to its receptor RANK activates two major signalling pathways leading to osteoclastogenesis, the NF-κB pathway via TRAF6 and the MAPK pathway via activation of ERK and phosphorylation of c-Fos and c-Jun (Bharti and Aggarwal, 2004). While ERK is responsible for osteoclast survival, NF-κB regulates osteoclast activation for bone resorption (Miyazaki *et al.*, 2000). Our studies showed that thiocolchicoside does not affect the MAPK pathway. These studies, together with our results with NBP, suggest that thiocolchicoside's inhibitory effect on osteoclastogenesis is specific to NF-κB inhibition.

To establish thiocolchicoside's effect on cancer cell-induced osteoclastogenesis, we used breast cancer cells and multiple myeloma cells that are known to express RANKL (Lai *et al.*, 2004; Bhatia *et al.*, 2005) and to exhibit constitutive NF-κB activation (Biswas *et al.*, 2004; Bharti *et al.*, 2004a). Breast cancer cells can directly resorb bone, and evidence from clinical studies suggests that the main mechanism responsible for bone destruction in cancer patients is tumour-mediated stimulation of osteoclastic bone resorption (Roodman, 2001). Furthermore, several studies have demonstrated that myeloma cells enhance osteoclast formation and activity through up-regulation of RANKL (Giuliani *et al.*, 2001; 2002; Pearse *et al.*, 2001) or by expressing RANKL themselves (Farrugia *et al.*, 2003; Heider *et al.*, 2003). It is now well defined that osteoclasts, not tumour cells, are principally responsible for the osteolysis observed in bone metastases and directly lead to the bony pathologies observed in these patients (Canon *et al.*, 2008).

In our study, thiocolchicoside inhibited osteoclastogenesis induced by breast cancer and multiple myeloma cells, indicating that this compound is an attractive potential agent for treating patients with metastasis to the bone. Moreover, our studies with RAW 264.7 cells co-incubated with conditioned medium from U266 or MDA-MB-231 cells confirmed that certain factors secreted by tumour cells stimulate osteoclast differentiation. Our laboratory has previously shown that osteoclastogenesis induced by cancer cells effectively correlate with RANKL expression in these tumour cells (Sung *et al.*, 2009; 2011). Furthermore, thiocolchicoside inhibited osteoclastogenesis induced by conditioned medium from U266 or MDA-MB-231 cells, suggesting that the colchicoside acts directly on osteoclasts and not on the proliferation of U266 or MDA-MB-231 cells.

Bisphosphonates are the current standard treatment for patients with bone metastasis or cancer-related bone disease (Terpos and Rahemtulla, 2004; Neville-Webbe and Coleman, 2010). However, not all patients respond to bisphosphonates, and toxicities such as renal impairment or osteonecrosis of the jaw can preclude the use of bisphosphonates (Kyle *et al.*, 2007). The RANKL antibody denosumab (Prolia), a humanized monoclonal antibody against RANKL, was recently approved by the US Food and Drug Administration for the treatment and prevention of post-menopausal osteoporosis and bone loss in patients with hormone-treated prostate or breast cancer (Body *et al.*, 2006). However, denosumab carries black box warnings for serious side effects, such as severe jawbone problems (osteonecrosis), skin problems (dermatitis, rash, eczema and serious skin infections) and hypocalcaemia. In addition, this antibody treatment is expensive.

Therefore, safe treatments for bone loss are still needed. Our results suggest that thiocolchicoside, which is currently approved for treating muscle pain, might be considered as a new drug against bone loss as well. Similarly, the use of aspirin, which is an anti-inflammatory drug and NF-κB inhibitor (Kopp and Ghosh, 1994), has now been associated with an increase in bone mineral density of the hip and lumbar spine (Bauer *et al.*, 1996). Further celecoxib, a non-steroidal anti-inflammatory drug and inhibitor of COX-2, has also been shown to inhibit osteoclastogenesis (Han *et al.*, 2005; Kasukawa *et al.*, 2007).

Overall, our results demonstrate that thiocolchicoside can suppress bone loss induced by cancer cells, and, thus, this drug could be a safe treatment for cancer patients with bone lesions and for patients with osteoporosis, Paget's disease or rheumatoid arthritis. Our future goal is to examine the potential of thiocolchicoside against osteoclastogenesis using clinically relevant animal models before proceeding to cancer patients.

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Conflicts of interest

None.

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