Inhibitory effect of sulphated polysaccharide porphyran (isolated from *Porphyra yezoensis)* on RANKL-induced differentiation of RAW264.7 cells into osteoclasts

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***Abbreviations*:** RANKL, receptor activator of nuclear factor-κB ligand; RANK, receptor activator of nuclear factor-κB; NO, nitric oxide; TNF, tumor necrosis factor; LPS, lipopolysaccharide; TRAP, tartrate-resistant acid phosphatase; NFATc1, nuclear factor of activated T cells 1 protein; MMP-9, matrix metalloproteinase-9; NF-κB, nuclear factor-κB; DMEM, Dulbecco's modified Eagle's minimum essential medium; RT-PCR, reverse transcription-polymerase chain reaction.

ABSTRACT

Safe and efficient therapeutic agents for bone diseases are required in natural sources. We previously found that edible seaweed-derived polysaccharide porphyran exhibited anti-inflammatory effects through the down regulation of NF-κB. The aim of this study was to investigate the availability of porphyran as a therapeutic agent for bone diseases. The effects of porphyran on receptor activator of nuclear factor κB ligand (RANKL)-induced osteoclastogenesis in RAW264.7 cells were examined. Porphyran suppressed RANKL-induced osteoclast formation in a concentration-dependent manner (6.25–50 μg mL^{-1}) without any cytotoxic effects. Furthermore, real-time PCR analyses indicated that porphyran at 50 μg mL^{-1} significantly attenuated the RANKL-induced increase in the mRNA levels of osteoclastogenesis-related marker genes such as nuclear factor of activated T cells (*NFATc1*), *TRAP*, cathepsin K, and matrix metalloproteinase 9 (*MMP-9*) in RAW 264.7 cells. To our knowledge, this is the first report showing that edible-seaweed-derived polysaccharide porphyran can suppress RANKL-induced osteoclastogenesis. Our results suggest that porphyran can be used as a safe therapeutic agent to improve osteoclast-related pathological conditions.

***Keywords*:** *Porphyra yezoensis*; Porphyran; RAW264.7 cells; Osteoclast; RANKL

INTRODUCTION

Bone homeostasis is implemented by continual bone resorption by osteoclasts and bone formation by osteoblasts (Kagiya and Nakamura, 2013). An imbalance of bone metabolism causes various pathological conditions such as osteoporosis and rheumatoid arthritis (Rodan and Martin, 2000). These bone diseases are mostly the consequence of an increase in the number of osteoclasts or osteoclastic activity (Teitelbaum, 2000; Boyle *et al*., 2003; Teitelbaum and Ross, 2003).

Receptor activator of nuclear factor κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) are involved in osteoclast formation from mature monocytes and macrophages (Udagawa *et al*., 1990; Lam *et al*., 2000; Nakashima *et al*., 2011; Kikuta and Ishii, 2013). M-CSF, which is an essential cytokine for macrophage maturation, plays an important role in survival, proliferation, and differentiation of osteoclast precursors (Teitelbaum, 2000). RANKL, on the other hand, binds to its specific receptor called RANK, a member of the tumour necrosis factor receptor superfamily, and activates signalling pathways leading to osteoclastic differentiation of macrophages (Teitelbaum, 2000).

Transcription factors such as nuclear factor κB (NF-κB) and nuclear factor of activated T cells protein 1 (NFATc1) perform crucial functions in osteoclastic differentiation and activation in osteoclast precursor cells stimulated by RANKL (Takayanagi *et al*., 2002). NFATc1 directly regulates the expression of osteoclastogenesis-related marker genes including *TRAP*, matrix metalloproteinase 9 (*MMP-9*), and cathepsin K (Lam *et al*., 2000; Asagiri and Takayanagi, 2007; Sundaram

et al., 2007).

There are studies aimed at finding safe and efficient therapeutic agents for bone diseases in natural sources. It has been reported that certain naturally occurring bioactive substances such as ginger (*Zingiber officinale*) extract and curcumin isolated from *Curcuma longa* have suppressive effects on osteoclastogenesis (Ito *et al*., 2016; Bharti *et al*., 2004).

The *Porphyra* genus is classified into red algae and is consumed mainly in East and Southeast Asia, e.g., in Japan, China, and Korea. *P. yezoensis* is important as a raw material for "nori", which is used in the Japanese traditional cuisine sushi. Nori is known to contain some biologically active ingredients such as dietary fibre, taurine, polyunsaturated fatty acids, carotenoids, and mycosporine-like amino acids (porphyra-334), as well as minerals, vitamins, and a relatively high concentration of proteins. Porphyran is one of the main constituents of *P. yezoensis* and is related to agarose, a linear sulphated polysaccharide consisting of D-galactose, 3,6-anhydro-L-galactose, 6-*O*-methyl-D-galactose, and L-galactose-6-sulphate (Morrice *et al*., 1983; Takahashi *et al*., 2000; Yoshimura *et al*., 2006).

Porphyran has diverse biological activities including antitumor, immunomodulatory, antioxidant, antihyperlipidemic, and hypercholesterolemic effects (Inoue *et al*., 2009; Ishihara *et al*., 2005; Kwon and Mam, 2006; Ren *et al*., 1994; Tsuge *et al*., 2004). Our previous study indicates that porphyran inhibits nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW264.7 mouse macrophages through the inhibition of NF-κB activation (Jiang *et al*., 2012). Furthermore, our recent study

showed that discoloured nori with no commercial value contains a much larger amount of porphyran than regular nori does (Isaka *et al*., 2015). Comparative studies revealed that porphyrans obtained from discoloured nori have a relatively smaller molecular size than do those from regular nori, and porphyran derived from discoloured nori has greater antioxidant and anti-inflammatory effects (Isaka *et al*., 2015). More recently, we found that porphyran isolated from discoloured nori has a protective effect against LPS-induced endotoxin shock in mice, and the serum levels of NO and TNF-α are significantly decreased by porphyran treatment (Nishiguchi *et al*., 2016).

To find new bioactivities of porphyran, in this study, we tested whether porphyran isolated from discoloured nori inhibits RANKL-induced differentiation of RAW264.7 cells into osteoclasts.

MATERIALS AND METHODS

Preparation of porphyran

Discoloured nori (*P. yezoensis*) was kindly provided by Japan Fisheries Cooperatives (Saga, Japan), and porphyran was prepared from discoloured nori as reported previously (Isaka *et al*., 2015). The mean molecular size of the porphyran was estimated to be 30 kDa (Isaka *et al*., 2015). Composition analysis revealed that the porphyran used in this study contains 86.4% of galactose, confirming that the porphyran is a highly purified polysaccharide galactan as reported previously (Isaka *et al*., 2015). Porphyran was dissolved in PBS at 50 mg mL^{-1} and passed through a filter with

0.20-μm pore size for sterilisation. PBS (final 0.1% or lower than that in assay system) alone as the vehicle used to dissolve porphyran caused no significant response (data not shown). Other reagents were of the highest grade commercially available.

Cell culture

RAW264.7 cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM containing 4.5 g L^{-1} glucose) supplemented with 10% of foetal bovine serum (FBS) (Moregate Biotech, QLD, Australia), penicillin (100 IU mL^{-1}), and streptomycin (100 μg mL^{-1}) in a humidified atmosphere containing 5% CO_2 and 95% air. Adherent RAW264.7 cells were harvested by mild scraping and collected by centrifugation (270 × *g* for 3 min at 4 °C).

Induction of osteoclast formation

To induce differentiation of RAW264.7 cells into osteoclasts, we used a previously reported method with a slight modification (Hayakawa *et al*., 2015). In brief, RAW264.7 cells resuspended in α-MEM containing 10% of FBS, penicillin (100 IU mL^{-1}), streptomycin (100 μg mL^{-1}), L-alanyl-l-glutamine (2 mM), and L-ascorbic acid 2-phosphate (284 μM) were seeded in 96-well plates (5×10^3 cells $well^{-1}$) with 50 ng mL^{-1} recombinant human soluble RANKL (RANKL, Oriental Yeast Co., Ltd.) and varying concentrations of porphyran and were cultured for 6 days at 37 °C. Negative control cells were incubated in the absence of RANKL and porphyran. After 6 days of

cultivation, TRAP-positive multinuclear cells were detected using the TRAP Stain Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts under a light microscope. Cell viability of RANKL-treated or untreated RAW264.7 cells was measured by an MTT assay as described elsewhere (Isaka *et al*., 2015).

Real-time RT-PCR analysis

After 6 days of cultivation with RANKL and porphyran, total RNA was isolated from RAW264.7 cells using TRIsure (NIPPON Genetics Co. Ltd., Tokyo Japan) according to the manufacturer's protocol. The extracted RNA (2 μg) was reverse-transcribed into single-stranded cDNA using a PrimeScript® 1st strand cDNA Synthesis Kit (TaKaRa Bio Inc., Shiga, Japan). Quantitative real-time PCR was carried out using the SYBR® Select Master Mix (Applied Biosystems, Foster City, CA, USA) and analysed on a 7300 Real Time PCR System (Applied Biosystems). Quantitative PCR parameters for thermal cycling were as follows: 1 cycle of 2 min at 50 °C, then 95 °C for 2 min, followed by 40 cycles of PCR at 95 °C for 15 s and 60 °C for 30 s. The primers were as follows: TRAP forward primer, 5′-AGGGTGTGATGGTGGGAATG-3′; TRAP reverse primer, 5′-GCTGGGGTGTTGAAGGTCTC-3′; NFATc1 forward primer, 5′-CCAATGAGCCAGGGGATTAG-3′; NFATc1 reverse primer, 5′-GCAGGAGAGGAAAGGTCGTG-3′; cathepsin K forward primer, 5′-GTGGTTCCTGTTGGGCTTTC-3′; cathepsin K reverse primer,

5′-GGTCATATAGCCGCCTCCAC-3′; MMP-9 forward primer, 5′-GCTGAAACCAGACCCCAGAC-3′; MMP-9 reverse primer, 5′-TGACCTGAACCATAACGCACA-3′; β-actin forward primer, 5′-AGGGTGTGATGGTGGGAATG-3′; and β-actin reverse primer, 5′-GCTGGGGTGTTGAAGGTCTC-3′. All the reactions were run in a 20-μL volume in triplicate. The mRNA expression was determined by the $2^{-\Delta\Delta Ct}$ method and was normalised to β-actin as an endogenous control.

Statistical analysis

All the statistical analyses were performed in the GraphPad prism 6 software (GraphPad Software, San Diego, CA, USA) by using one-way ANOVA with Dunnet's or Tukey multiple comparison test. Differences with a p value <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Toxicity of porphyran toward RAW264.7 cells before and after RANKL-induced osteoclastic differentiation

To evaluate the effects of porphyran on the viability of RAW 264.7 cells cultured with or without RANKL for 6 days, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT) assay was performed. In the absence of stimulation with RANKL, porphyran slightly promoted the

growth of RAW 264.7 cells in a concentration-dependent manner, and no cytotoxic effect of porphyran was observed up to a concentration of 50 μg mL^{-1} (Fig. 1A). In our previous studies, the nontoxic nature of porphyran toward RAW264.7 cells after short incubation (24 h) was observed up to the concentration 1,000 μg mL^{-1} (Jiang *et al*., 2012; Isaka *et al*., 2015). Next, we tested the toxicity of porphyran on RAW264.7 cells under conditions of RANKL-induced osteoclastic differentiation. As shown in Fig. 1B, the viability of RAW264.7 cells cultured for 6 days in the presence of RANKL was 72% of the control without RANKL, but in the presence of porphyran at 50 μg mL^{-1}, the viability of RAW264.7 cells recovered to nearly the control level (cells without RANKL). The exact reason for the slight decrease in the viability of RANKL-induced differentiated cells is unclear. MTT assay depends on the mitochondrial dehydrogenase activity, which reflects viability of the cells. Hence, a possible explanation is that the reactivity of differentiated multinucleated giant cells to MTT reagent might be slightly lower than that of undifferentiated RAW264.7 cells, and suppression of RANKL-induced differentiation by porphyran may lead to apparent increase in the viability as a result. After all, it is considered that the possible involvement of cytotoxicity can be ruled out in the effects of porphyran observed in later experiments.

Inhibitory effects of porphyran on RANKL-induced osteoclastic differentiation of RAW264.7 cells

The mouse macrophage cell line RAW264.7 is known as RANK-expressing cells, and has been widely used as an osteoclast precursor model to study osteoclast formation

and function (Cuetara *et al*., 2006). Taking advantage of the simplicity, reproducibility, and reliability of the RAW264.7 cell model, we examined the effects of porphyran in a non-toxic concentration range on RANKL-induced differentiation of RAW264.7 cells into osteoclasts. RAW264.7 cells were cultured in the differentiation medium containing 50 ng mL^{-1} RANKL with varying concentrations of porphyran (0–50 μg mL^{-1}) for 6 days. Under the culture conditions in the absence of porphyran, RAW264.7 cells differentiated into multinucleated giant cells via repeated cell fusion as shown in Fig. 2. To evaluate the degree of osteoclastic differentiation, TRAP staining was conducted. TRAP is a glycosylated monomeric metalloenzyme that is a highly expressed marker enzyme of osteoclasts; thus, a TRAP-staining assay allows us to identify osteoclasts. TRAP-positive multinucleated cells with more than three nuclei were regarded as osteoclasts. As shown in Fig. 2A, porphyran significantly suppressed osteoclastic differentiation of RAW264.7 cells in a concentration-dependent manner. The suppression percentages caused by porphyran toward osteoclast differentiation were 12.9% (at 6.25 μg mL^{-1}), 30.2% (at 12.5 μg mL^{-1}), 47.7% (at 25 μg mL^{-1}), and 76.3% (at 50 μg mL^{-1}). The results suggest that 50 μg mL^{-1} of porphyran is a minimum concentration to attain more than 50% inhibition of osteoclast differentiation. Hence, we evaluated the effects of porphyran at 50 μg mL^{-1} in further experiments. Porphyran at 50 μg mL^{-1} significantly suppressed the formation of RANKL-induced multinucleated giant cells, but the cells were slightly TRAP-positive as seen in Fig. 2B. These results suggested that porphyran may not be able to cause complete suppression of the *TRAP* gene expression induced in RANKL-treated RAW 264.7 cells. As another explanation

for these results, porphyran may have an ability to supress the formation of multinucleated giant cells through the inhibition of cell-cell fusion of osteoclast progenitor. Further studies are needed to clarify these points.

Effects of porphyran on the expression of osteoclastogenesis-related marker genes in RANKL-stimulated RAW264.7 cells

After the binding of RANKL to its specific receptor RANK, a series of intracellular signalling pathways including NF-κB and mitogen-activated protein kinase (MAPK) systems are activated in osteoclast precursor cells with ensuing osteoclastogenesis (Asagiri and Takayanagi, 2007). After the activation of NF-κB and MAPK cascades, expression of downstream transcription factors is induced (Ikeda *et al*., 2004). Because NFATc1 is known as the transcription factor most strongly induced by RANKL stimulation in the early phase of differentiation into osteoclasts, and it regulates the expression of several genes required for osteoclastic differentiation (Asagiri *et al*., 2005), the mRNA expression level of NFATc1 in RANKL-stimulated RAW264.7 cells was analysed by RT-PCR. As shown in Fig. 3A, a significant increase in NFATc1 expression was observed in RANKL-stimulated RAW 264.7 cells; the expression level was nearly three-fold higher than that in the control group (without RANKL stimulation). By contrast, in the presence of porphyran at 50 μg mL^{-1}, NFATc1 expression was evidently suppressed.

In agreement with this result, porphyran also significantly suppressed the mRNA expression of osteoclastogenesis-related genes such as *TRAP*, cathepsin K, and *MMP-9*,

which are directly regulated by NFATc1 (Fig. 3B–D) (Teitelbaum and Ross, 2003). Among the genes tested, the inhibitory effect of porphyran on *TRAP* was slightly weaker. This phenomenon may partly explain why the weakly TRAP-stained cells were detected among RANKL-stimulated RAW264.7 cells even in the presence of porphyran (Fig. 2B). On the other hand, cathepsin K and MMP-9 mRNA expression levels were strongly suppressed by porphyran at 50 μg mL^{-1}. Cathepsin K and MMP-9 are proteolytic enzymes highly expressed in osteoclasts and actively participate in the breakdown of the bone matrix and in bone resorption (Sundaram *et al*., 2007; Liu *et al*., 2003). These results clearly indicate that porphyran is capable of inhibiting the RANKL-induced osteoclastic differentiation in RAW264.7 cells through downregulation of multiple osteoclastogenesis-related genes. This effect may be a consequence of the inhibition of NFATc1 expression. Although further studies are necessary to clarify the exact mechanism of porphyran's inhibition of osteoclastic differentiation, porphyran seems to influence an early cellular signalling pathway leading to osteoclastic differentiation of RAW264.7 cells. RANKL activates the NF-κB pathway, which plays a critical role in osteoclast formation (Teitelbaum and Ross, 2003). LPS binds to Toll-like receptor (TLR) 4 on the surface of macrophages and triggers a series of intracellular signalling pathways including the activation of NF-κB, leading to the secretion of cytokines and NO (Takeuchi *et al*., 1999; Akira and Takeda, 2004). RANKL and LPS are quite different stimuli, and their specific receptors on the macrophages are also different, but there seems to be a similarity in the downstream intracellular signalling pathways. Our previous studies indicate that porphyran inhibits

NO and TNF-α production in LPS-stimulated RAW264.7 cells, where translocation of the NF-κB p65 subunit from the cytosol to nucleus and phosphorylation and degradation of the IκB-α subunit are significantly inhibited (Jiang *et al*., 2012). Therefore, these results suggest that porphyran may first inhibit the activation of NF-κB, which in turn leads to the suppression of osteoclastogenesis-related gene expression and eventual inhibition of osteoclastic differentiation of RAW264.7 cells. Further studies are needed to clarify this point.

Regarding the structure–activity relation of porphyran, Isaka *et al*. (2015) have reported that porphyran isolated from a certain lot of nori, which is inactive as is in terms of the inhibition of NO production in LPS-stimulated RAW264.7 cells, became active after fragmentation by acid hydrolysis (Isaka *et al*., 2015). Furthermore, porphyran isolated from discoloured nori was separated into four fractions (F1–F4) by DEAE-anion exchange chromatography, and F1 with the lowest molecular size showed the strongest inhibitory effect on NO production in LPS-stimulated RAW264.7 cells (Isaka *et al*., 2015). These findings suggest that the molecular size of porphyran is a crucial factor for its activity. In addition to the molecular size, sulphate and 3,6-anhydro-galactose levels also seem to be important structural characteristics affecting physicochemical and biological properties of porphyran (Jiang *et al*., 2012; Isaka *et al*., 2015; Nishiguchi *et al*., 2016). On the basis of these findings, we are planning to find the most effective form of porphyran for suppression of osteoclastic differentiation of RAW264.7 cells.

CONCLUSION

To our knowledge, this is the first report that edible seaweed-derived polysaccharide porphyran showed inhibitory effect on RANKL-induced osteoclast in RAW264.7 cells without any cytotoxic effect. RT-PCR analyses also demonstrated that the expression of osteoclastogenesis-related genes such as *NFATc1*, *TRAP*, cathepsin K, and *MMP-9* in RANKL-stimulated RAW264.7 cells are significantly suppressed by porphyran. Our results suggest that porphyran can be a promising therapeutic agent for bone diseases.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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Figure legends

Fig. 1. Toxicity of porphyran toward RANKL-stimulated or unstimulated RAW264.7 cells. The cells were incubated with varying concentrations of porphyran (6.25–50 μg mL^{-1}) without (A) or with (B) RANKL at 50 ng mL^{-1} for 6 days, and then viability of the cells was measured by the MTT assay as described in the text. Each value represents mean ± standard error (n=4). This assay was repeated two times. The asterisks indicate significant differences. (*$p < 0.05$, **$p < 0.01$).

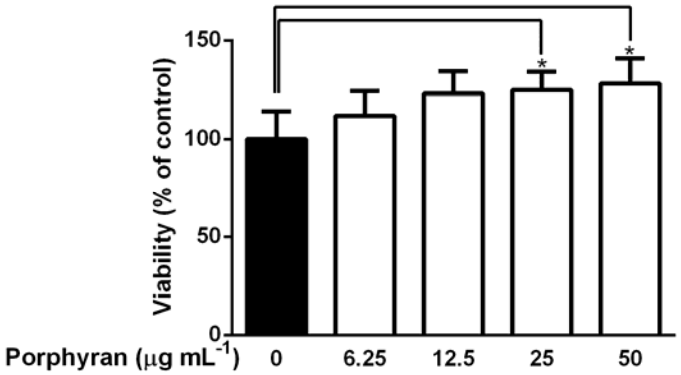
Fig. 2. Inhibitory effects of porphyran on RANKL-induced osteoclastic differentiation of RAW264.7 cells. (A) The cells were cultured with RANKL at 50 ng mL^{-1} in the presence (6.25–50 μg mL^{-1}) or absence of porphyran for 6 days, and then tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells were detected by TRAP staining. The TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts under a light microscope. Each value represents mean ± standard deviation (n=3). This assay was repeated three times. The asterisks indicate significant differences between groups '+ RANKL' and '+ RANKL and porphyran' (*$p < 0.05$, ***$p < 0.001$). (B) RAW264.7 cells in 96-well plates (5×10^3 cells $well^{-1}$) in differentiation medium were cultured with 50 ng mL^{-1} RANKL (RANKL), 50 ng mL^{-1} RANKL and 50 μg mL^{-1} porphyran (RANKL + Porphyran), or without these reagents (Normal) for 6 days at 37°C, and then the cells were subjected to TRAP staining as described in the text. Photographs of the RAW264.7 cells cultured under each condition were taken after 6 days. The scale bar in each picture indicates

200 μm.

Fig. 3. Effects of porphyran on the expression of osteoclastogenesis-related genes in RANKL-stimulated RAW264.7 cells. These cells were cultured with RANKL at 50 ng mL^{-1} in the presence (50 μg mL^{-1}) or absence of porphyran for 6 days. After the extraction of total RNA from the treated RAW264.7 cells, relative mRNA expression levels of NFATc1 (A), TRAP (B), cathepsin K (C), and MMP-9 (D), were analysed by quantitative real time PCR as described in the text. β-Actin served as an endogenous control. Each value represents mean ± standard deviation (n=3). This assay was repeated two times. Asterisks indicate significant differences between groups RANKL and RANKL + porphyran (***$p < 0.001$).

Figure 1

A


Viability (% of control)
150
100
50
0
*
*
Porphyran (μg mL-1)
0
6.25
12.5
25
50

B

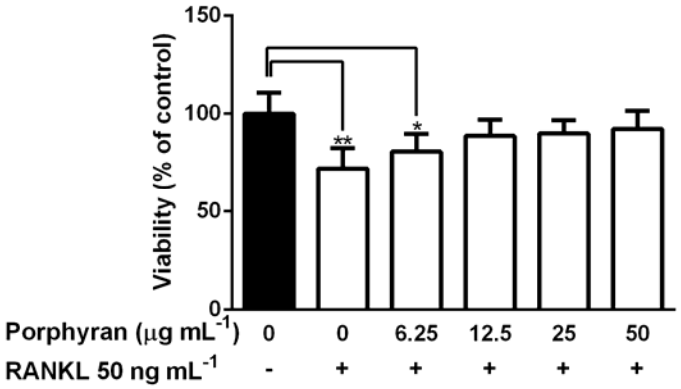
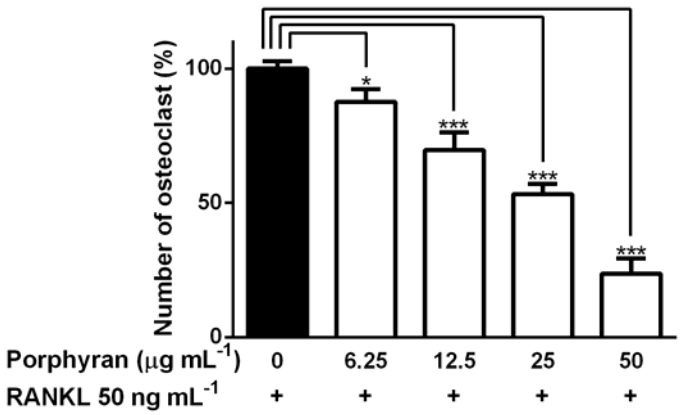

Viability (% of control)
150
100
50
0
**
*
Porphyran (μg mL-1)
0
0
6.25
12.5
25
50
RANKL 50 ng mL-1
-
+
+
+
+
+

Figure 2

A


Number of osteoclast (%)
100
50
0
*
***
***
***
Porphyran (μg mL-1)
0
6.25
12.5
25
50
RANKL 50 ng mL-1
+
+
+
+
+

1

2

3

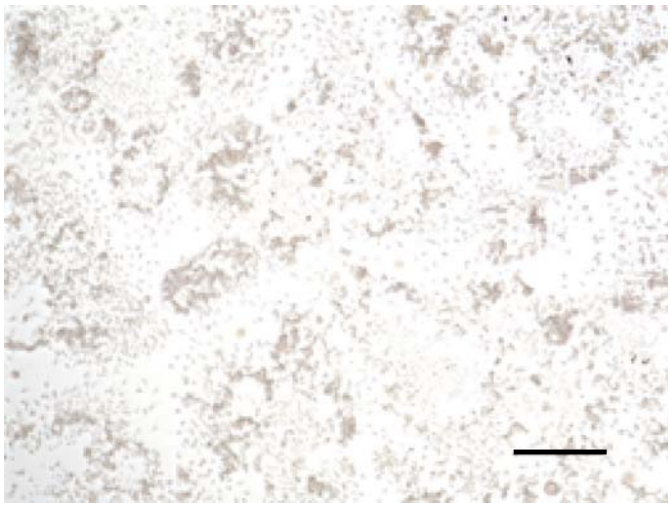
4

5

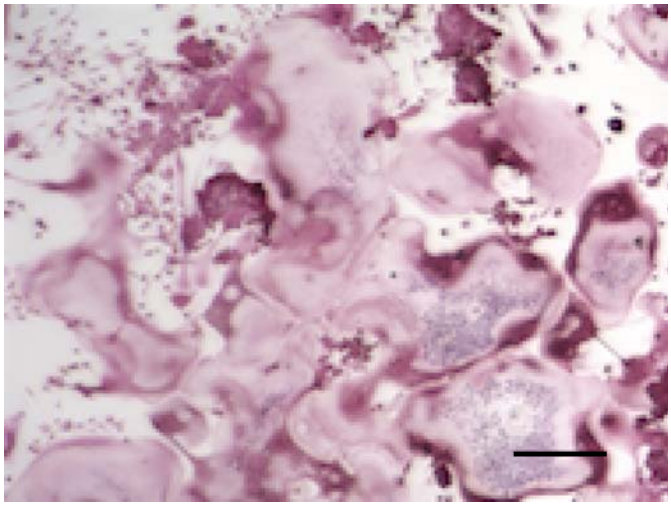
6

7

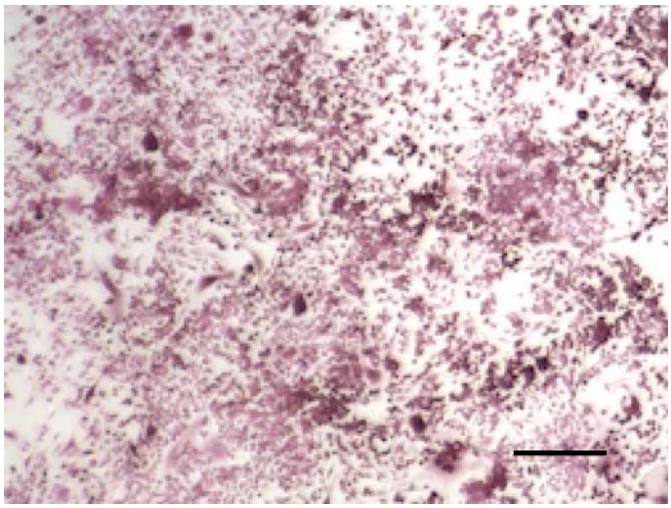
8 **B**



Normal



RANKL

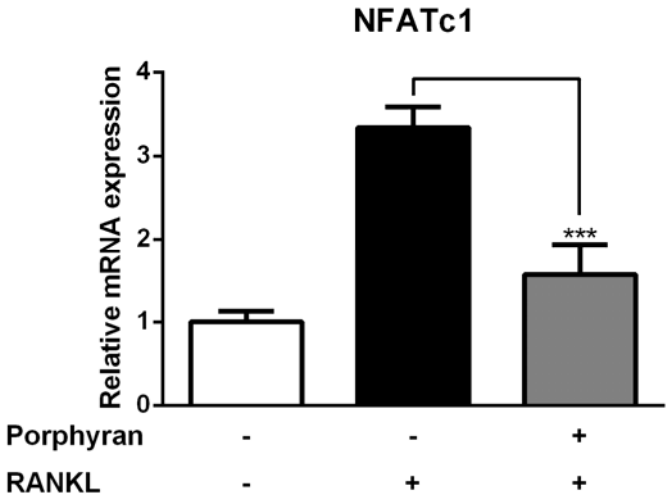


RANKL+Porphyran

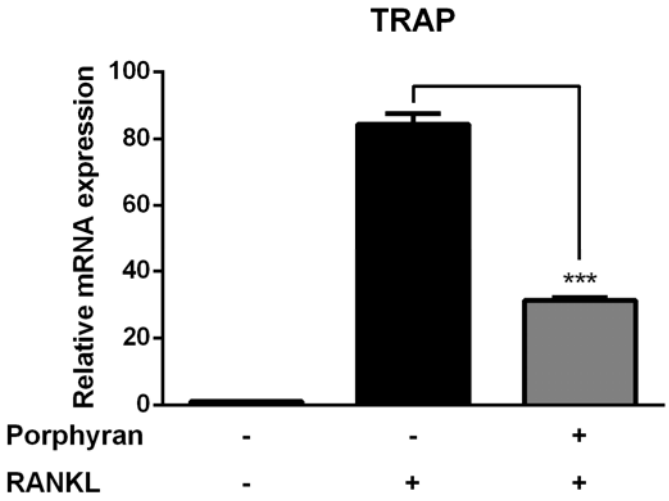
1

Figure 3

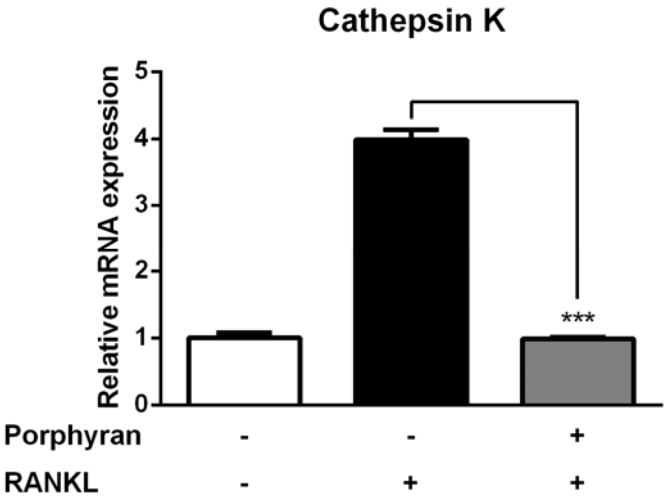
A


NFATc1
Relative mRNA expression
0
1
2
3
4
***
Porphyran - - +
RANKL - + +

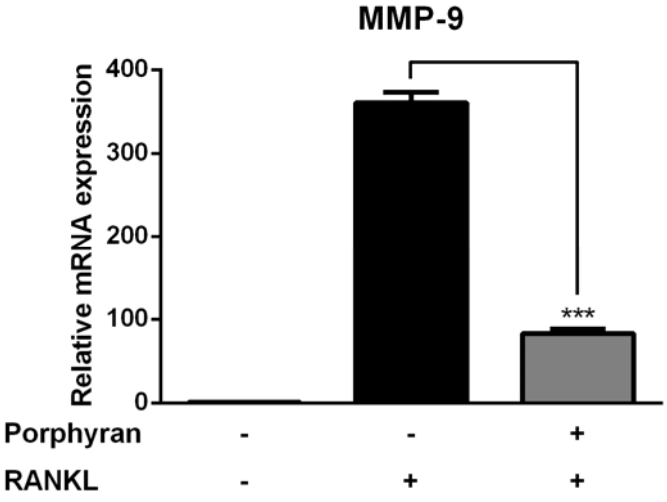
B


TRAP
Relative mRNA expression
0
20
40
60
80
100
***
Porphyran - - +
RANKL - + +

C


Cathepsin K
Relative mRNA expression
0
1
2
3
4
5
***
Porphyran - - +
RANKL - + +

D


MMP-9
Relative mRNA expression
0
100
200
300
400
***
Porphyran - - +
RANKL - + +