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Protein Kinase C Inhibitor, GF109203X Attenuates Osteoclastogenesis, Bone Resorption and RANKL-Induced NF-κB and NFAT Activity

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Osteolytic bone diseases are characterized by excessive osteoclast formation and activation. Protein kinase C (PKC)-dependent pathways regulate cell growth, differentiation and apoptosis in many cellular systems, and have been implicated in cancer development and osteoclast formation. A number of PKC inhibitors with anti-cancer properties have been developed, but whether they might also influence osteolysis (a common complication of bone invading cancers) is unclear. We studied the effects of the PKC inhibitor compound, GF109203X on osteoclast formation and activity, processes driven by receptor activator of NFκB ligand (RANKL). We found that GF109203X strongly and dose dependently suppresses osteoclastogenesis and osteoclast activity in RANKL-treated primary mouse bone marrow cells. Consistent with this GF109203X reduced expression of key osteoclastic genes, including cathepsin K, calcitonin receptor, tartrate resistant acid phosphatase (TRAP) and the proton pump subunit V-ATPase-d2 in RANKL-treated primary mouse bone marrow cells. Expression of these proteins is dependent upon RANKL-induced NF-κB and NFAT transcription factor actions; both were reduced in osteoclast progenitor populations by GF109203X treatment, notably NFATc1 levels. Furthermore, we showed that GF109203X inhibits RANKL-induced calcium oscillation. Together, this study shows GF109203X may block osteoclast functions, suggesting that pharmacological blockade of PKC-dependent pathways has therapeutic potential in osteolytic diseases.

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Excessive osteoclastic bone resorption is a major hallmark of common pathological bone disorders including osteoporosis, Paget's disease of bone and metastatic breast cancer invasion of bone. RANKL is the key TNF-related factor required for osteoclast differentiation and activity (Lacey et al., 1998; Kong et al., 1999) produced in membrane-bound form by local osteoblasts and soluble form by other cell types such as lymphocytes. Understanding the signaling pathways elicited by RANKL upon binding its cognate receptor (RANK) are critical both for determining the mechanisms underlying the excessive osteoclast-associated bone disorders and for designing anti-osteolytic therapies.

The rapid activation of PKC enzymes forms part of the signal transduction pathways elicited by many hormones, and their phosphorylation of target proteins leads to a variety of cellular responses that include cell proliferation, differentiation and apoptosis (Nakashima, 2002; Diaz-Meco and Moscat, 2012). PKC-dependent intracellular signal transduction pathways significantly influence the progress of inflammation, cancer and metabolic diseases (Nakashima, 2002; Diaz-Meco and Moscat, 2012). PKC enzymes have also been suggested to play a role in bone homeostasis, and specifically in osteoclastic bone resorption, but the involvement of PKC in osteoclastogenesis remains unclear. It is complicated by the existence of over 12 common isozymes of PKC, which are categorized into three classes based on their structure and co-factor requirement for activation. These include the conventional PKCs (α, βI, βII, and γ) which require phosphorylation plus Ca^{+}, diacylglycerol (DAG) and phosphatidylserine as cofactors; the novel PKCs

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(δ, ε, η, μ, and θ) which required DAG but not Ca^{2+}; and the atypical PKCs (λ, ι, and ζ) which are independent of both Ca^{2+} and DAG cofactors (Knopf et al., 1986; Nishizuka, 1986; Dekker and Parker, 1994; Newton, 1995; Keenan and Kelleher, 1998). Bone marrow macrophages and macrophage-like RAW264.7 cells, which form osteoclasts with RANKL treatment, and osteoclasts themselves, all express eight PKC isoforms, namely conventional PKCα, βI, βII, novel PKCδ, ε, μ, and atypical PKCλ and ζ (Lin and Chen, 1998; Larsen et al., 2000; Khor et al., 2013).

An important approach used to study the role of PKC in cellular responses is to block the PKC activity by specific inhibitors, which requires cell permeable, potent and selective compounds. GF109203X, or bisindolylmaleimide I (illustrated in Fig. 1A) is a compound structurally related to the potent PKC inhibitor staurosporine (Toullec et al., 1991). GF109203X inhibits α, βI, βII, and γ subtypes of PKC with similar potency at an IC_{50} of 20 nM. While staurosporine is suggested to be the most potent inhibitor of PKC, it shows strong effects on other protein kinases. In contrast, GF109203X has been shown to display high selectivity for PKC, and is a competitive inhibitor of PKC ATP-binding sites (Toullec et al., 1991). GF109203X selectively inhibits PKC activities in human platelet and Swiss 3T3 fibroblasts (Toullec et al., 1991) but the effect of GF109203X on osteoclast differentiation and function, and in RANKL-mediated signalling, is largely unclear.

In this study, we investigated the effects and the underlying mechanisms of GF109203X in osteoclast formation and function, and RANKL signaling pathways. We observed that GF109203X potently inhibited osteoclast formation and activity, likely resulting from its inhibition of RANKL-dependent NF-κB and NFAT activities, which are critical for osteoclastic functions. This suggests that GF109203X and related compounds may have potential therapeutic use for bone lytic disorders by reducing RANKL-dependent effects on osteoclast formation in addition to its effects on bone resorption.

Materials and Methods

Reagents

GF109203X was purchased from Sigma (Castle Hill, New South Wales, Australia), and dissolved in dimethyl sulfoxide (DMSO). RAW264.7 cells were obtained from the American Type Culture Collection (Rockville, MD). Alpha modified Eagles Medium (α-MEM) and fetal bovine serum (FBS) were purchased from TRACE (Sydney, Australia). Anti-NFATc1 anti-IκBα, and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (California) and V-ATPase-d2 was generated as previously described (Feng et al., 2009). The luciferase assay

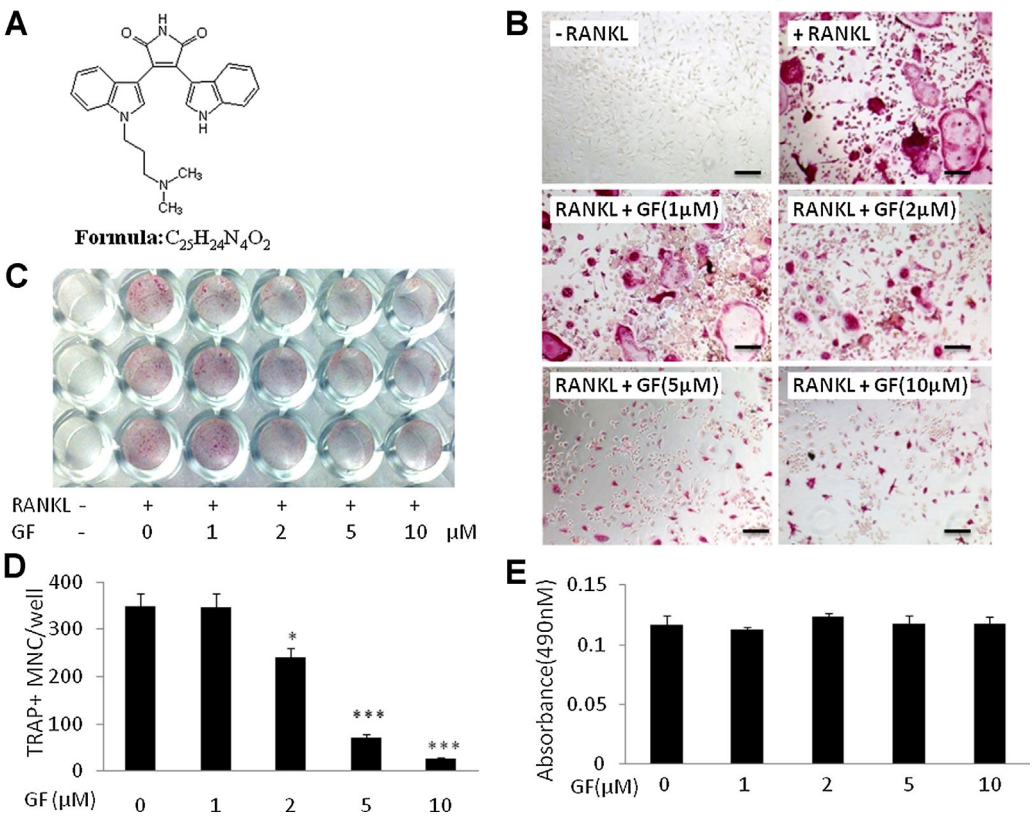

Fig. 1. GF109203X inhibits RANKL-induced osteoclastogenesis. A: Structure of GF 109203X. B: BMM cells were cultured in the presence of RANKL and M-CSF to stimulate osteoclast formation; these cultures were treated with GF109203X as indicated. After 5 days, the cells were fixed and histochemically stained for TRAP activity. Light microscope images depicting the dose-dependent effect of GF109203X on RANKL-induced osteoclast formation at 100× magnification (scale bars, 100 μM). C: Representative image showing the culture plate containing cells stimulated as in B, to indicate the level of TRAP histochemical staining. D: Quantification of the effect of GF109203X on the number of $TRAP^+$ MNCs, counted as osteoclasts. (n = 3). (E) GF109203X did not affect BMM cell viability or proliferation. BMM cells stimulated with M-CSF were incubated with GF109203X for 48 h and then incubated with MTS/PMS mixture for 2 h, then absorbance at 490 nm (proportional to viable cell number) was measured. (n = 3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ relative to vehicle control, RANKL-treated controls.

system and MTS assay system were obtained from Promega (Sydney, Australia). Recombinant GST-rRANKL protein was expressed and purified as previously described (Xu et al., 2000) and recombinant macrophage colony stimulating factor (M-CSF, R&D Systems) was used as previously described (Liu et al., 2013).

In vitro osteoclastogenesis

Freshly isolated bone marrow macrophages (BMM) were cultured in complete α-MEM with M-CSF. BMM cells were seeded on to 96-well plate (6×10^3) and cultured with GF109203X in the presence of 100 ng/ml RANKL and 25 ng/ml M-CSF. Medium was changed every 2 days. After 6–7 days of culture, the cells were fixed with 2.5% glutaraldehyde and histochemically stained to detect TRAP. TRAP positive multinucleated cells with more than three nuclei were counted as osteoclasts.

MTS assay for cell proliferation and viability

The effects of compounds on BMM cell proliferation were examined using MTS assay. The MTS assay was performed according to manufacturer's introduction (Promega, Sydney, Australia). Briefly, 6×10^3 BMM cells per well were seeded onto a 96-well plate, followed by overnight incubation. Cells were incubated with GF109203X for 48 h. MTS/PMS mixture was then added and cells incubated for a further 2 h. MTS stain was measured by spectrophotometric absorbance at 490 nm using an ELISA plate reader (BMG, Germany).

NF-κB and NFAT luciferase reporter gene assays

RAW264.7 cells stably transfected with an NF-κB luciferase reporter construct (Wang et al., 2003) or an NFAT luciferase reporter construct (van der Kraan et al., 2013) were seeded onto a 48-well plate at a density of 1.5×10^5 cells per well. Cells were pre-treated with GF109203X for 1 h prior to RANKL stimulation. Cells were incubated for 6 h (NF-κB luciferase reporter gene assays) or 24 h (NFAT luciferase reporter gene assays), and then lysed for measurement of luciferase activity using Promega luciferase kit and a BMG Polar Star Optima luminescence reader (BMG, Germany).

Analysis of osteoclast activity

BMM-derived osteoclasts were generated on a 30 mm diameter collagen-coated culture plate at the density of 1×10^5 cells/well. Cells were differentiated with 100 ng/ml RANKL and M-CSF until osteoclasts were evident at 5 days incubation. Osteoclasts were then dissociated from the collagen plate using 1 ml/well cell dissociation solution (Sigma) and the same number of cells was cultured in each well of a hydroxyapatite-coated plate. Osteoclasts were cultured in the presence or absence of GF109203X for 48 h with 100 ng/ml RANKL ($n = 6$). Then, half of the wells were fixed with 2.5% glutaraldehyde and histochemically stained for TRAP and osteoclasts counted as above. The rest of the wells were bleached with 10% bleach solution. The areas of hydroxyapatite resorption were observed by light microscopy and analysed using Image J software.

Reverse transcription (RT)-PCR analysis of gene expression

BMM cells were seeded in 30 mm diameter culture wells at the density of 1×10^5/well. Cells were cultured with RANKL in the presence or absence of GF109203X for 5 days. RNA was extracted using Trizol® (Life Technologies, Mulgrave, Australia) in accordance with the manufacturer's protocol. For RT-PCR, single-stranded cDNA was reverse transcribed from 1 μg total RNA using reverse transcriptase with an oligo-dT primer. All PCR reactions were carried out using 1 μl cDNA using cycling parameters as follows: 94°C for 5 min, followed by 30 cycles of 94°C (40 sec), 60°C, (40 sec); 72°C (40 sec), followed by a elongation step of 5 min at 72°C. PCR reactions used specific primers for detecting and quantifying the following genes: Cathepsin K (forward: 5′-GGGAGAAAAACCTGAAGC-3′; reverse: 5′-ATTCTGGGGACTCAGAGC-3′), calcitonin receptor (forward: 5′-TGGTTGAGGTTGTGCCCA-3′; reverse: 5′-CTCGTGGGTTTGCCTCATC-3′), TRAP (forward: 5′-TGTGGCCATCTTTATGCT-3′; reverse:5′-GTCATTTCTTTGGGGCTT-3′), PKC delta (forward: 5′-CAGACCAAGGACCACCTGTT-3′; reverse: 5′-CGTCCCTGTCTAGCATCACA-3′), and 18 sec RNA (forward: 5′-ACCATAGATGCCGACT-3′; reverse: 5′-TGTCAATCCTGTCCGTGTC-3′. PCR reaction products were visualized by electrophoresis in a 1.5% agarose gel in TBS, stained by SybrSafe DNA gel stain (Life Technologies). DNA bands were visualised on an Imagequant LAS 4000 (GE Healthcare, Australia) and quantified using Image J.

Western blot assays

BMM cells were stimulated with RANKL and M-CSF as above, in the presence or absence of GF109203X. Cells were lysed in RIPA buffer for protein extraction. SDS-PAGE electrophoresis was then performed, followed by protein transfer to a nitrocellulose membrane. The membrane was blocked by immersion in a 5% dry skim milk in TBS-Tween solution for 1 h. After washing with TBS-Tween solution, specific antibodies were used. The immunoreactivity was visualized using Enhanced Chemiluminescence (ECL) reagents (Amersham) according to manufacturer's instructions and images acquired on an Imagequant LAS 4000.

Measurement of intracellular Ca^{2+} oscillation

A total of 2.5×10^4 BMM cells were seeded on the bottom of a 48-well plate and cultured with RANKL (100 ng/ml) in the presence of M-CSF for 24 h. Treatment cells were pre-treated with 10 μM GF109203X for 1 h prior to stimulation with RANKL, control cells received M-CSF only. Cells were then incubated in the presence of 5 μM fluo-4 AM, and 0.05% pluronic F-127 (Invitrogen) for 30 min at 37°C in Hank's Balanced Salt Solution (HBSS) supplemented with 1% FCS/1mM probenecid (Assay buffer). Cells were washed twice with Assay buffer and incubated at room temperature for 20 min. The cells were washed again with Assay buffer and then viewed on an inverted fluorescent microscope (Nikon) at an excitation wavelength of 488 nm. Images were captured at 2 sec intervals for 3 min at 400× magnification. Oscillating cells (at least 2 oscillations observed) were analyzed for average peak height (maximum peak intensity minus baseline intensity) per cell, for a minimum of 20 cells per well, and triplicate wells per treatment using Nikon Basic Research Software.

Statistical analysis

Quantitative data was analysed using ANOVA and Tukey's *post hoc* test. All data was pooled from at least 3 independent experiments and is shown as mean ± standard error.

Results

GF109203X inhibits RANKL-induced osteoclastogenesis

Cultured BMM cells were used to examine the effect of GF109203X on RANKL-induced osteoclast differentiation. 100 ng/ml RANKL-stimulated BMM cells were treated for 5 days with a range of concentrations (1, 2, 5, and 10 μM) of GF109203X. As shown in Figure 1 (B–D), GF109203X significantly inhibited the RANKL-induced osteoclast

formation at concentrations of 2 μM or greater. To exclude the possibility of cytotoxic effects of GF109203X in this concentration range, MTS assays were employed to test the effect of GF109203X on proliferating BMM cells. This showed that 1, 2, 5, 10 μM GF109203X did not affect BMM cell viability or proliferation (Fig. 1E), and BMM cells treated with RANKL (Supplementary Figure S1).

GF109203X impairs osteoclastic activity

Since GF109203X reduced RANKL-dependent osteoclast formation, we investigated whether osteoclast activity was also affected. For this we assessed the capacity of RANKL-treated osteoclasts to resorb hydroxyapatite substrate in the presence of GF109203X. The same number of osteoclasts was cultured in each hydroxyapatite-coated culture well; after 48 h culture it was evident that treatment with GF109203X at a concentration of 2 μM led to an approximately 50% reduction in osteoclast numbers on hydroxyapatite (Fig. 2A and B), and approximately 80% reduction in resorptive capacity per osteoclast (Fig. 2A and C). These results suggest that GF109203X inhibits osteoclast activity as well as attenuating osteoclast formation. They also indicate that at 2 μM GF109203X is selectively causing the death of mature osteoclasts, whilst having no apparent effect on precursor survival or proliferation as shown in the MTS assay. Considering that PKC delta is a major isoform expressed in osteoclast and plays a key role in bone resorption (Khor et al., 2013), we also assess the effect of GF109203X on PKC delta gene expression. We found that treatment of GF109203X did not alter the PKC delta gene expression (Supplementary Figure S2).

GF109203X inhibits RANKL-induced gene expression

To further characterize the actions of GF109203X in osteoclast differentiation, we examined the effects of GF109203X treatment on osteoclast marker gene expression in cultured BMM cells. The BMM cells were thus treated with M-CSF and RANKL in the absence or presence of GF109203X at 2 μM for 5 days, followed by lysis and RNA extraction. Our results showed that GF109203X reduced the gene expression of RANKL-induced osteoclastic gene markers in these cells; this included expression of cathepsin K, calcitonin receptor, and TRAP (Fig. 3).

GF109203X suppresses RANKL-induced NF-κB activation

To gain insights into the molecular mechanism underlying its inhibitory actions on osteoclast differentiation and activity, the effect of GF109203X on RANKL-induced signalling transduction pathways was examined. To this end, the effect of GF109203X on RANKL-induced NF-κB activity in osteoclast precursors was determined. RAW264.7 cells stably transfected with an NF-κB luciferase reporter construct were pre-treated with various doses of GF109203X ranging from 1 to 10μM for 1 h before stimulating them with 100 ng/ml RANKL for another 6 h. Luciferase activity was then measured; RANKL induced a 10-fold increase in the NF-κB dependent luciferase activity compared to the unstimulated cells. GF109203X had only a modest effect on this signal induction, with 2 μM showing approximately 10% reduction and 10 μM showing approximately 35% reduction in signal (Fig. 4A). Further examination by Western blot showed that, in BMM treated with RANKL, 2 μM GF109203X treatment did not obviously affect IκBα degradation (Fig. 4B).

GF109203X suppresses RANKL-induced NFAT activity, NFATc1 protein levels and calcium oscillations

The effect of GF109203X on RANKL-induced NFAT transcription was also examined by a luciferase reporter assay. RAW264.7 cells stably transfected with an NFAT luciferase

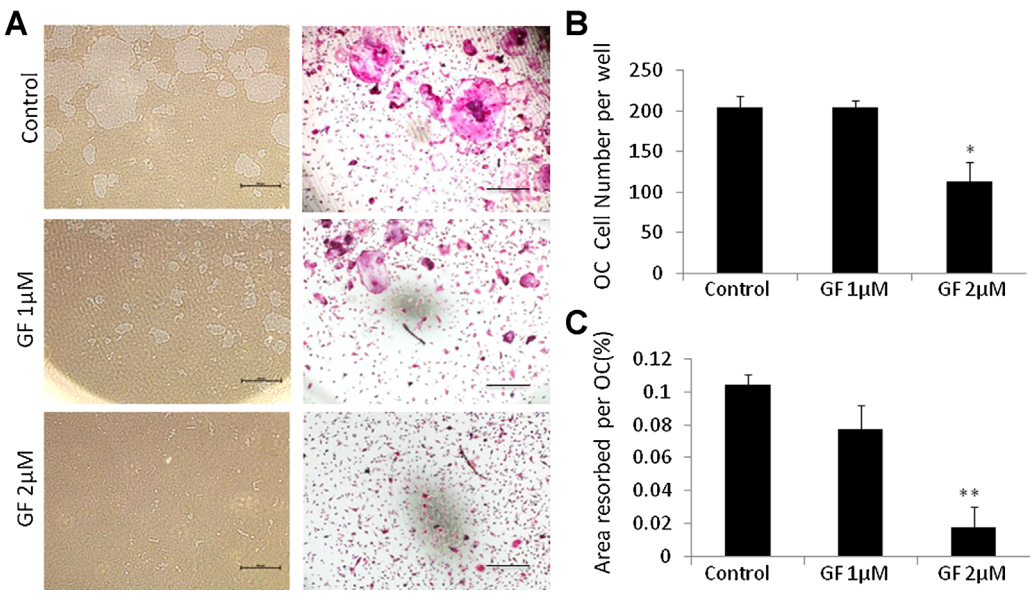

Fig. 2. GF109203X reduces hydroxyapatite resorption by osteoclasts. A: Representative images of osteoclastic resorption and TRAP staining on hydroxyapatite coated surfaces (scale bars, 500 μM). B: Quantification of the effect of GF109203X on the number of TRAP$^+$ MNCs, counted as osteoclasts. (n = 3). C: Percentage of the area of hydroxyapatite surface resorbed per osteoclast. (n = 3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ relative to vehicle control, RANKL-treated cultures.

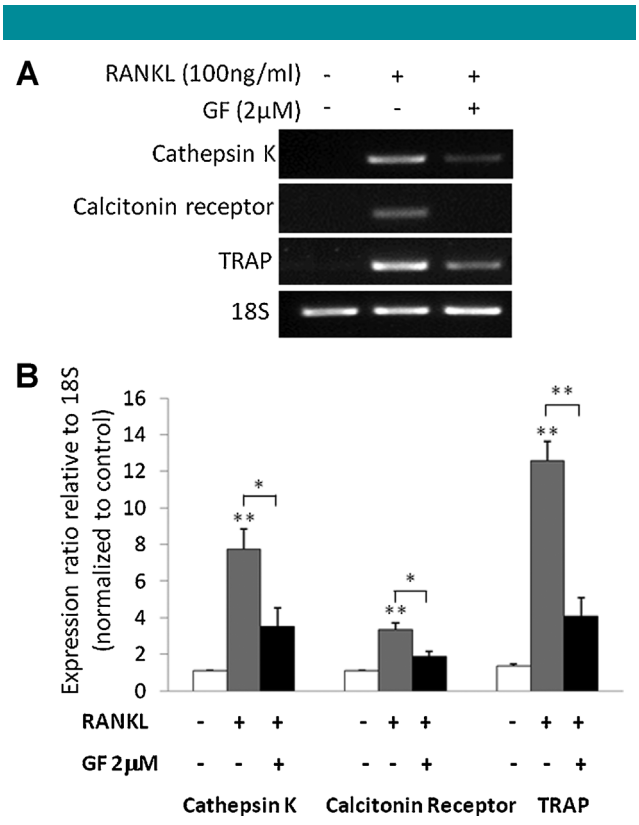

Fig. 3. GF109203X reduces RANKL-induced gene expression in BMM cells treated with RANKL (100 ng/ml) and M-CSF for 5 days. Cellular RNA was extracted and RT-PCR analysis performed using specific primers to analyse gene expression. A: Representative gel showing Cathepsin K, calcitonin receptor, TRAP and 18 sec expression. B: The level of gene expression is shown as the ratio of cathepsin K, calcitonin receptor, and TRAP, relative to 18 S. (n = 3). *$P < 0.05$, **$P < 0.01$ relative to GF109203X untreated control cultures.

reporter construct were pre-treated with various concentrations of GF109203X ranging from 1 to 10 μM for 1 h before stimulating them with 100 ng/ml RANKL for a further 24 h, then luciferase activity measured. RANKL induced a 10-fold increase in the NFAT activity compared to the unstimulated cells and GF109203X significantly decreased the NFAT activity at 2 μM (Fig. 5A).

The transcriptional activity of NFATc1 cannot be distinguished from that of NFATc2, but NFATc1 is the critical factor induced by RANKL and both autoamplifies its own transcription and that of NFATc2 during RANKL-induced osteoclastogenesis (Asagiri et al., 2005). To further examine the effect of GF109203X on NFATc1 protein expression we stimulated the BMM cells for 1, 3, and 5 days with 100 ng/ml RANKL in the presence or absence of GF109203X (2 μM). Western blot analyses showed that GF109203X at 2 μM very strongly reduced RANKL-induced NFATc1 levels at day 5 (Fig. 5B). As NFATc1 activity is critical for the induction of protein expression of V-ATPase-d2 (Feng et al., 2009), and since this protein (in addition to its role in acidification) is required for osteoclast cell fusion that is key to osteoclastogenesis (Lee et al., 2006; Kim et al., 2008) we also investigated the effect of GF109203X treatment on this protein. Consistent with NFATc1 effects, induction of V-ATPase-d2 protein expression was also suppressed by GF109203X (Fig. 5B).

Finally, we examined the effect of GF109203X on calcium oscillation, an NFATc1 upstream pathway as described in materials and methods. The results showed that the intensity of calcium oscillations following RANKL stimulation was significantly reduced in GF109203X treated BMM cells as compared to untreated BMM cells (Fig. 6), consistent with the inhibitory effect of GF109203X on NFATc1 activity.

Discussion

In this study, we have found that GF109203X suppresses osteoclastogenesis and bone resorption in vitro and consistent with this reduces the RANKL-induced expression of osteoclast marker genes cathepsin K, calcitonin receptor and TRAP. Furthermore, GF109203X inhibited RANKL-induced NFAT activity and, to a lesser extent, NF-κB activity, indicating a possible mechanism of action and suggesting that GF109203X has potential for use as a treatment for osteolytic bone diseases, particularly those such as metastatic bone disease where GF109203X may also have a clinically useful effect on the underlying tumour.

PKC-dependent pathways are involved in RANKL-induced osteoclast differentiation and the expression of osteoclast-specific genes (Wang et al., 2003), and play a physiological role in osteoclast function (Moonga and Dempster, 1998). More recently, it has been suggested that GF109203X inhibited acid secretion, indicating that acid secretion regulated by PKC might be important for osteoclastic bone resorption. The results presented in this study extend these previous findings and provide further evidence for a significant effect of PKC inhibitor GF109203X in the modulation of osteoclast differentiation and function.

GF109203X is a broad spectrum PKC inhibitor with a highly selective inhibition of PKC isozymes α, β, γ, δ, and ε in vitro (Toullec et al., 1991; Gekeler et al., 1996). The compound also displays only weak anatogonist effects on protein kinase A (PKA), however, PKA has not been found to play a direct role in RANKL-induced osteoclast formation. In the osteoclast lineage, the PKCβI and II gene expression is slightly increased during osteoclastogenesis and may have a functional role in osteoclast differentiation and fusion (Lee et al., 2003). The inhibitory effects of GF109203X on osteoclast differentiation and activity thus might be due to its inhibition of PKCβ, but an influence exerted through other isoforms is also possible. We have previously found that novel-type PKCδ, is a major isoform expressed in osteoclasts and plays an important role in bone resorption (Khor et al., 2013); however, the gene expression of this isoform is not affected by GF109203X. This illustrates the important role that individual PKC enzymes clearly play in aspects of osteoclast biology although the large number of isoforms and their overlapping actions and regulatory interactions make study of individual isoforms difficult in osteoclast lineage cells.

In RANKL-induced signalling pathways, NF-κB activation is critical for osteoclastogenesis (Xu et al., 2009). NF-κB is activated by RANKL both in RAW264.7 cells and monocytes (Wong et al., 1997; Lacey et al., 1998; Hsu et al., 1999; Jimi et al., 1999), and is required for osteoclast formation (Franzoso et al., 1997). Our previous study demonstrated that RANKL activation induced the DNA binding of NF-κB complexes consisting of C-Rel, NF-κB1 (p50), and Rel-A (p65) (Wang et al., 2003). While earlier studies have demonstrated that the inhibitor of PKC, GF109203X, prevents NF-κB dependent transcription, NF-κB DNA binding is unaffected in pulmonary A549 cells (Catley et al., 2004). In this study, GF109203X was found to slightly attenuate the RANKL-induced NF-κB transcription activity, although GF109203X had little effect on IκBα degradation. This suggests that the effect of GF109203X on RANKL-mediated NF-κB activity may be independent from IkBα degradation, or could simply reflect a weak action on NF-κB activity in these cells. This may also be due to the secondary effect of GF109203X on signalling pathways or other unknown regulatory functions that have an impact on

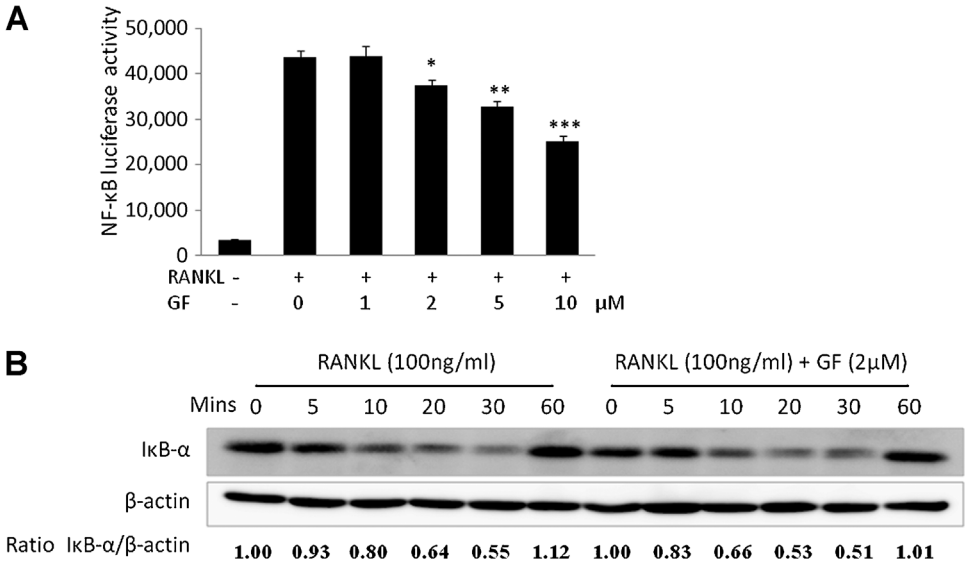

Fig. 4. The effect of GF109203X on RANKL-stimulated NF-κB activity and IκB-α degradation. A: RAW264.7 cells stably transfected with a NF-κB transcriptional luciferase reporter construct, were pretreated with GF109203X (1, 2, 5, and 10 μM) for 1 h and then exposed to RANKL (100 ng/ml) for 6 h and luciferase levels determined. (n = 6). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to untreated control. B: BMM cells pretreated with GF109203X at 2 μM for 1 h were treated with RANKL (100 ng/ml) for 0, 5, 10, 20, 30, and 60 mins as indicated. Representative blots of BMM lysates for IκB-α, and β–actin.

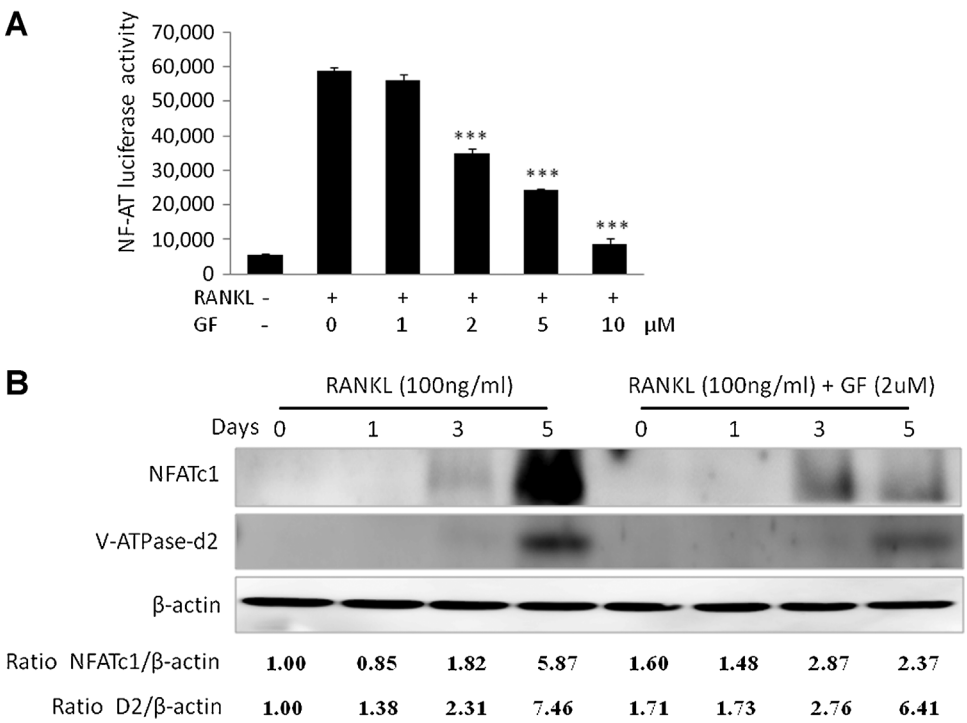

Fig. 5. GF109203X reduces NFAT activity and protein expression of NFATc1 and V-ATPase-d2. A: RAW264.7 cells, stably transfected with a NFAT transcriptional luciferase reporter construct, were pretreated with GF109203X (1, 2, 5, and 10 μM) for 1 h and then exposed to RNAKL (100 ng/ml) for 24 h. (n = 6). ***$P < 0.001$ compared to untreated control. B: BMM cells were pretreated with GF109203X for 1 h prior to RANKL (100ng/ml) stimulation for 0, 1, 3, and 5 days. Representative blots of cell lysates for NFATc1, V-ATPase-d2 and β-actin expression.

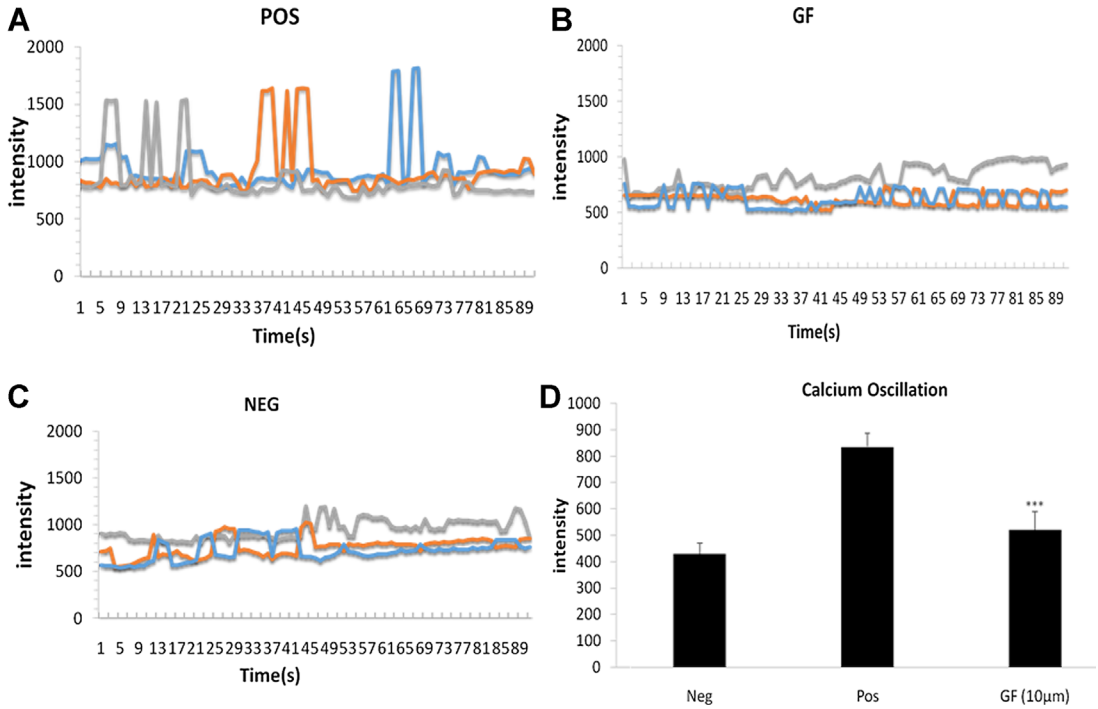

Fig. 6. GF109203X suppresses RANKL-induced calcium oscillation. Representative images showing (A) calcium oscillation pattern in BMM cells stimulated with RANKL (100 ng/ml) (Pos. group), (B) cells pre-treated with GF109203X for 1 h prior to stimulation with RANKL (GF group), and (C) control cells received M-CSF only (Neg. group). Quantitative analysis of oscillating cells (maximum peak intensity minus baseline intensity, n = 20,) is presented (D). ***$P < 0.001$ relative to RANKL-treated cells.

NF-κB. We also demonstrated reduced mRNA expression of osteoclast marker genes following treatment with GF109203X, which are downstream targets of NF-κB activation. The inhibition of RANKL-mediated NF-κB activation by GF109203X might partly explain the inhibitory effect of GF109203X on osteoclastogenesis, however, is unlikely to be the predominant mechanism.

NFATc1 is a critical transcription factor for osteoclastogenesis whose overexpression can drive osteoclast formation independently of RANKL; it is also highly regulated and many osteoclastogenesis inhibitors affect NFATc1 levels (Takayanagi, 2005). Indeed, we observed that GF109203X abrogated RANKL-induced NFATc1 levels and NFAT activity, which is highly suggestive of an inhibitory action upon osteoclast commitment. NFATc1 levels are regulated by transcriptional control and by the actions of the Ca^{2+}-dependent phosphatase calcineurin, which stabilises the protein and allows its nuclear transportation and transcriptional activity. Previous studies have found that NFATc1 can auto-amplify transcription of NFATc1 itself and NFATc2 with which it cooperates to bind NFAT response elements in gene promoters (Asagiri et al., 2005) such as V-ATPase-d2, cathepsin K and β3 integrin. It should be noted that such NFAT activity requires coordinating with other transcription factors such as MITF, PU.1, NFκB, and AP-1 during RANKL-induced osteoclastogenesis (Crotti et al., 2008; Balkan et al., 2009; Feng et al., 2009). We demonstrated that GF109203X could decrease NFATc1 protein expression, as well as that of the NFATc1 downstream target V-ATPase-d2. This indicates that GF109203X inhibits osteoclastogenesis via an NFATc1-mediated pathway. Since NF-κB and NFATc1 coordinate to promote expression of most osteoclast marker genes, GF109203X is able to suppress the transcription of osteoclast-specific genes such as TRAP and cathepsin K, contributing to the inhibitory effect of GF109203X on osteoclast formation and function. Consistent with a report that GF109203X reduces intracellular Ca^{2+} levels in DDT1 MF-2 cells (Sipma et al., 1996), our results further demonstrate that GF109203X suppresses RANKL-induced calcium oscillations in BMM, indicating that GF109203X is preventing upregulation of NFATc1 in response to RANKL stimulation by inhibiting calcium dependent NFAT activation.

PKC inhibitors have been tested for their inhibitory effects on a wide range of cancer cells, such as pancreatic cancer cells, non-small cell lung cancer cells, and breast cancer cells (El et al., 2007; Tekle et al., 2008; Wu et al., 2010). GF109203X also inhibited the differentiation of SH-SY5Y human neuroblastoma cells induced by phorbol ester (Heikkila et al., 1993). Our data indicate that GF109203X could also inhibit osteoclastogenesis and bone resorption by regulation of RANKL-induced signaling pathways. Thus, the dual properties of GF109203X as an anti-cancer and anti-osteoclast agent might be useful for treatment of breast cancer-induced osteoclast activation in bone microenvironments. It should be noted, however, that GF109203X has not been investigated for its influence on other bone cells such as osteoblasts in vivo. It is also likely that any action to reduce osteoclast number in vivo, if confirmed, is likely to cause a concomitant reduction in bone formation due to the coupling between osteoclast and subsequent osteoblast action in bone remodelling. This is less a consideration with rapid pathological osteolysis than with slower progressing conditions such as osteoporosis. Future study will examine the role of GF109203X in vivo using mouse bone metastasis model (Yoneda et al., 2001).

In summary, these results suggest that PKC-dependent pathways play an important role in osteoclast differentiation

and activity which is probably exerted through influences on the RANKL-induced NFAT signalling pathway. PKC inhibitors, namely GF109203X, might be potential therapeutics for bone lytic disorders. Moreover, our study suggests that GF109203X or similar compounds might be an effective agent for inhibiting the osteolysis caused by cancer cells.

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