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Marino, S., Bishop, R.T., Logan, J.G. et al. (2 more authors) (2017) Pharmacological evidence for the bone-autonomous contribution of the NFκB/β-catenin axis to breast cancer related osteolysis. Cancer Letters, 410. pp. 180-190. ISSN 0304-3835

https://doi.org/10.1016/j.canlet.2017.09.034

Pharmacological evidence for the bone-autonomous contribution of the NFκB/β□catenin axis to breast cancer related osteolysis

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Running Title: Disruption of NFκB/B-catenin signalling reduces osteolysis.

Abbreviations: PTN, Parthenolide; NFκ□, nuclear factor kappa-B; RANK, receptor activator of NFκ□; RANKL, RANK ligand; TNFα, tumour necrosis factor alpha; IL1β, interleukin 1 beta; CD40L, Cluster of differentiation 40 ligand; TRAF, receptor-associated factor; IKK, IκB kinase; TGFβ, transforming growth factor beta; Runx2, runt related transcription factor 2; M-CSF, mouse macrophage colony stimulating factor; DMSO, Dimethyl sulfoxide; microCT, micro–computed tomography; TRAcP, tartrate-resistant acid phosphatase; Alk Phos, alkaline phosphatase; ALZ, alizarin red; BM, bone marrow; ANOVA, analysis of variance; SD, standard deviation.

Abstract

The NFκB signaling pathway is implicated in breast cancer and bone metastasis. However, the bone-autonomous contribution of NFκB to breast cancer-induced osteolysis is poorly understood. Here, we report that pretreatment of osteoblasts with the sesquiterpene lactone Parthenolide (PTN), a verified NFκB inhibitor, prior to exposure to conditioned medium from human and mouse breast cancer cell lines enhanced osteoblast differentiation and reduced osteoblast ability to stimulate osteoclastogenesis. PTN prevented breast cancer-induced osteoclast formation and reduced the ability of breast cancer cells to prolong osteoclast survival and to inhibit osteoclast apoptosis. In vivo, administration of PTN in immuno-competent mice reduced osteolytic bone loss and skeletal tumour growth following injection of the syngeneic 4T1-BT1 cells and reduced local osteolysis caused by conditioned medium from human and mouse osteotropic breast cancer cell lines. Mechanistic studies revealed that NFκB inhibition by PTN in osteoblasts and osteoclasts was accompanied by a significant increase in β-catenin activation and expression. Collectively, these results raise the possibility that combined targeting of NFκB and β-catenin signalling in the tumour microenvironment may be of value in the treatment of breast cancer related osteolysis.

Keywords: NFκ□; β-catenin; breast cancer; osteolysis; bone.

1. Introduction

Skeletal related events are a common cause of morbidity in patients diagnosed with secondary breast cancer in bone [1-3]. Bone metastases related to advanced breast cancer are osteolytic, and are caused by stimulation of osteoclast formation and bone resorption by breast cancer cells and/or their derived factors in the skeleton [3, 4]. A number of breast cancer- and bone-derived factors including receptor activator of nuclear factor kappa-B (RANK) ligand RANKL, tumour necrosis factor alpha (TNFα), interleukin 1 beta (IL1β□ and Cluster of differentiation 40 ligand (CD40L) influence breast cancer - bone cell interactions[5-8]. Binding of these ligands to their respective receptors activates the canonical NFκB signalling pathway by initiating the recruitment of receptor-associated factors (TRAF) TRAF2 and TRAF6[6-8]. This in turn leads to the binding of IκB kinase (IKK) alpha (IKKα) and beta (I□□β) □□□the regulatory subunit □□□γ[9, 10]. Once activated, the IKKαβγ□complex phosphorylates and triggers the proteasomal degradation of IκB that results in NFκB nuclear translocation[6].

A number of studies have reported that mice deficient in NFκB1/2, TRAF2, TRAF6, IKKα or IKKβ exhibited high bone mass due to osteoclast inhibition[11-15]. Furthermore, pharmacological inhibition of NFκB activation reduced osteoclast number and protected against bone loss in models of inflammation and oestrogen deficiency[16-20]. NFκB activation is also involved in the regulation of osteoblast differentiation and bone formation. For example, □□Fα□□□□□ transforming growth factor beta (TGFβ) induces osteoblast apoptosis[1, 3, 21] and knockdown of IKK in osteoblasts and their precursors enhances bone nodule formation in vitro and stimulates bone formation in mice [22].

There is also evidence to suggest that NFκB regulates osteoclast and osteoblast activity through its crosstalk with the Wnt/β-catenin pathway[23]. In osteoblasts, activation of β-catenin stimulates

bone formation and enhances the expression of runt related transcription factor 2 (Runx2), a key osteogenic factor essential for osteoblast differentiation [24-27]. Activation of β-catenin inhibits osteoclast formation [28-30] and activation of NFκB by RANKL in osteoclasts leads to β-catenin inhibition [31, 32]. Altogether, these findings implicate the NFκB/β-catenin axis in the regulation of osteoclast and osteoblast activity and bone remodelling.

Over recent years, there has been increasing interest in the role of NFκB in the regulation of breast cancer bone metastasis [33, 34]. NFκB in breast cancer cells enhances osteoclast formation and promotes bone metastasis. Recent work carried out in our laboratories has shown that pharmacologic inhibitors of NFκB reduced osteolysis in a model of breast cancer osteolytic metastasis [33, 34]. However, the role of NFκB – and its interaction with β□catenin - in the regulation of osteoblast and osteoclast changes associated with breast cancer-induced osteolysis has not been investigated. Using a pharmacological approach that utilizes data from experiments using bone cells, organ cultures and immuno-competent mice, we showed that the sesquiterpene lactone Parthenolide (PTN), a verified inhibitor of NFκB[34-37], enhanced β-catenin expression and activity in osteoblasts and osteoclasts in vitro, and protected against breast cancer-induced osteolysis ex vivo and in vivo.

2. Materials and methods

2.1. Reagents and cells

The sesquiterpene lactone PTN was purchased from Tocris Biosciences (Bristol, UK). Parental and osteotropic clones of human MDA-MB-231 and mouse 4T1-BT1 (representative of triple-negative), human MCF7 (hormone sensitive breast cancer cell lines), and the osteoblast-like cells Saos-2 and MC3T3, and the macrophage-like cell (pre-osteoclasts) RAW 264.7 were purchased from ATCC (Manassas, VA). The osteotropic sub clones of the triple negative human MDA-231-BT1 and BT2 and mouse 4T1-BT1 and BT2 were generated by repeated passages in vivo and validated for their

ability to colonize bone and to cause osteolysis[38-40]. Tissue culture medium was obtained from Sigma-Aldrich (Dorset, UK). Primers for quantitative PCR were designed using the Roche website and obtained from Invitrogen (Paisley, UK) and probes were purchased from Roche Diagnostics Ltd. (East Sussex, UK). Antibodies were purchased from Cell Signalling Biotechnology (MA, USA) except rabbit anti-actin was obtained from Sigma-Aldrich (Dorset, UK). Mouse macrophage colony stimulating factor (M-CSF) was obtained from R&D Systems (Abingdon, UK) and RANK ligand (RANKL) was a gift from Patrick Mollat (Galapagos SASU, France)[16].

2.2. Animal experiments

All experimental protocols were approved by the Ethics Committee at the Universities of Edinburgh and Sheffield and were conducted in accordance with the UK Home Office regulations.

2.2.1. Intra-tibial injection in mice

Balb/c female mice (10-week-old) received intra-tibial injection of mouse 4T1-BT1 breast cancer cells (4×10^3 cells) in the left leg and a sham injection of PBS into their right leg. On day 2, animals were divided into two groups (7 mice per group) and received intraperitoneal injection of either vehicle (Dimethyl sulfoxide (DMSO)/water, 1:10) or PTN (10mg/kg/day) for 12 days. Treatment regime used was chosen based on previous in vivo studies, which demonstrated that this agent has anti-resorptive and anti-metastatic effects[34]. Animals were euthanized 14 days post injection of 4T1-BT1 cells and bone architecture was assessed by micro–computed tomography (microCT, Brucker 1172 scanner, Brucker, Belgium)[41]. Skeletal tumor growth was measured on 2D microCT images using Image J (1.34s; NIH, Bethesda, MD, USA) and results were expressed as a percentage of total metaphyseal area.

2.2.2. Supracalvarial injection in mice

C57BL/6 female mice (3-weeks-old) were divided into two groups (7 mice per group) and received intraperitoneal injection of either vehicle (DMSO/water, 1:10) or PTN (3mg/kg/day) for 6 days. On day 2, mice were injected subcutaneously over the calvarial bones (supra-calvarial) with 50μL of conditioned medium from human MDA-231-BT1, mouse 4T1-BT1 and human MCF7 breast cancer cells on 5 consecutive days[39]. Osteolytic bone loss in the calvarial bone was assessed using microCT at a resolution of 8μm[41]. All mice injected with conditioned medium from human MDA-231 or MCF7 or mouse 4T1 did not exhibit any obvious physical signs of illness or inflammatory response.

2.3. Micro-computed tomography

Trabecular bone parameters were measured at the left proximal tibia (200 slices distal of the growth plate) using microCT analysis (Skyscan 1172 instrument (Brucker, Belgium) set at 60kV and 150μA [41]. Images were reconstructed by the Skyscan NRecon program and analysed using Skyscan CTAn software (Brucker, Belgium) [41].

2.4. Bone histomorphometry

Bone histomorphometry was performed on the proximal tibial metaphysis from mice as previously described [34, 42]. Briefly bones were fixed, sectioned using a Leica microtome (Solms, Germany) and tissue sections were stained with von Kossa, Paragon, tartrate-resistant acid phosphatase (TRAcP) and aniline blue. Static and dynamic bone histomorphometry were performed on trabecular bone (0.1 and 1mm distal to the growth plate) and three sections per samples were analysed using computer software based on the Aphelion Image Analysis tool kit (Adcis, He0rouville- Saint-Clair, France).

2.5. Cancer cell – mouse calvaria organ co-culture system

The effects of pharmacological inhibition of NFκB on local osteolysis induced by human and mouse cancer cell derived factors was studied ex vivo using an adaptation of the mouse calvarial organ culture as described in[42]. Briefly, neonatal mouse calvarias were isolated from 7-day-old mice, divided into equal halves along the medium sagittal suture and each half was placed into culture on stainless steel rafts in 48-well plates containing standard or conditioned medium. Tissue culture medium containing test agents was changed every 48 hours and the cultures were terminated after 7 days. Bone volume was assessed by using m□□□□CT at a resolution of 5μm.

2.6. Osteoblast cultures

Primary osteoblasts were isolated from the calvarial bones of 2-day-old mice as previously described[43]. Primary calvarial osteoblasts or the osteoblast-like cells Saos-2 and MC3T3 were seeded into 12-well plates at 10 x 10^5 cells per well in standard alpha-MEM or DMEM respectively, supplemented with β-glycerol phosphate (10 μM) and L-ascorbic acid (50 μg/ml) for up to 21 days. Osteoblast cell number, differentiation and bone nodule formation were determined by AlamarBlue assay, alkaline phosphatase (Alk Phos) assay and alizarin red (ALZ) staining as previously described[43].

2.7. Osteoclast cultures

Bone marrow (BM) cells were flushed from the long bones of 3-5 month old mice and primary osteoblasts were isolated from the calvarial bones of 2-day-old mice as previously described[43]. Osteoblasts were plated in 96-well plates (8 x 10^3 cells/well) for 24 hours and BM cells were added (2x10^5 cells/well). M-CSF-dependent osteoclast precursor cells generated as previously described[42] and were plated into tissue culture plates (96 well plates, 15 x 10^3 cells/well; 12 well plates, 150 x 10^3 cells/well) in standard alpha-MEM supplemented with M-CSF (25 ng/ml) and RANKL (100 ng/ml). For studies involving breast cancer cells and their derived factors, M-CSF-dependent osteoclast precursors were plated into 96 well plates (15 x 10^3 cells / well) in alpha-

MEM supplemented with M-CSF (25 ng/ml) and RANKL (100 ng/ml) for 6 hours prior addition of MDA-231-BT1, 4T1-BT1 or MCF7 breast cancer cells (300 cells/well) or their conditioned medium (20% v/v) prepared as previously described [42]. Cultures were terminated by fixation in 4% paraformaldehyde, and stained for Tartrate-Resistant Acid Phosphatase (TRAcP). Resorption pits were visualised in osteoclast cultures plated on Corning® Osteo Assay Surface multiple well plates (Corning, USA) using an Olympus ScanR microscope and resorbed area was quantified by ImageJ.

2.8. Western Blotting

Western blot analysis was used to detect protein expression. Prior to stimulation with test agents or vehicle, cells were incubated in serum free medium for 60 minutes. Test agents or vehicle were added and cells were then gently scraped in standard lysis buffer (0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 1% Triton X-100, 1μM EDTA, 2% (v/v) protease inhibitor cocktail, 10μM of sodium fluoride and 2% (v/v) phosphatase inhibitor cocktail) or cytoplasmic lysis buffer (10 mM Tris (pH 7.5), 100 mM NaCl, 0.05% NP-40, 3 mM MgCl2, 1 mM EGTA supplemented with 2% (v/v) protease and phosphatase inhibitor cocktail). Protein concentration was determined using BCA assay (Pierce, USA). Total protein (50 - 70μg) was resolved by SDS-PAGE (BioRAD, UK) and immunoblotted with antibodies according to manufacturer's instructions. Detection of native and phosphorylated proteins was performed using rabbit monoclonal antibodies (all at 1:1000 dilution, cell Signalling Technology, USA). Immuno-complexes were visualised by an enhanced chemiluminescence detection kit (Pierce, USA) using horseradish peroxidase-conjugated secondary antibody (Jackson labs, UK), and visualised using chemiluminescence (Amersham, UK) on a Syngene GeneGnome imaging system. The intensity of the bands was quantified using GeneSnap software (Syngene, UK) and level of actin (Sigma-Aldrich, UK) was used for normalization.

2.9. Quantitative PCR

Gene expression was detected using quantitative PCR (qPCR). Briefly, samples were lysed using TRIzol reagent and quantified using a nanodrop (Thermo Scientific) according to manufacturer's instructions. Complementary DNA (cDNA) was generated using Invitrogen SuperScript III Reverse Transcriptase kit according to manufacturer's instructions. Primers were designed using the Ensembl genome browser, and Roche website. For amplification of mouse OPG (forward primer: 5'- ATGAACAAGTGGCTGTGCTG-3', reverse primer 5'-CAGTTTCTGGGTCATAATGCAA-3'); mouse RANKL (forward primer: 5'-TGAAGACACACTACCTGACTCCTG-3', reverse primer 5'-CCACAATGTGTTGCAGTTCC -3'); mouse GAPDH (forward primer: 5'-CCTGAATTTTAAGCTACACACAGC -3', reverse primer 5'- CTGGCACTGCACAAGAAGAT -3'), mouse Runx2 (forward primer: 5'-CCACAAGGACAGAGTCAGATTACA-3', reverse primer 5'- TGGCTCAGATAGGAGGGGTA -3'); mouse alkaline phosphatase (ALP, AKP2 (ALPL)) (forward primer: 5'-AAGGCTTCTTCTTGCTGGTG-3', reverse primer 5'GCCTTACCCTCATGATGTCC -3') were used. Levels of gene expression were expressed as copy number per micro-gram of total RNA and GAPDH was used for cDNA normalization.

2.10. Transcription factors assays

Nuclear extracts from cultures of mouse M-CSF dependent osteoclast precursors and osteoblasts were prepared using a nuclear extract kit (Active Motif, Rixensart, Belgium) and DNA binding was measured using TRANSAM ELISA kit for p65 NFκB and c-Fos (Active Motif, Rixensart, Belgium), according to the manufacturer's instructions.

2.11. Immunostaining for NFκB

Mature osteoclasts were generated in M-CSF and RANKL-stimulated mouse bone marrow cultures as described above. Cultures were permeabilised in Triton X100 (0.1% v/v), stained with rabbit anti-p65 NFκB (1:200, Santa Cruz Biotechnology, Dallas, USA) and Alexafluor488-conjugated goat–anti-Rabbit antibody (1:200, Fisher Scientific UK, Loughborough, UK) and counterstained

with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride). Cytoplamic and nuclear staining of p65NFκB was visualised using a fluorescence microscope (Zeiss, Cambridge, UK).

2.12. Statistical analysis

Comparison between groups was assessed by analysis of variance (ANOVA) followed by Dunnet's post hoc test (SPSS for Windows, version 11). A p-value value of 0.05 or below was considered statistically significant. The half maximal inhibitory concentration (IC50) values were calculated using GraphPad Prism 4 for windows (V10, Microsoft, USA).

3. Results

3.1. Effects of NFκB inhibition on the growth osteotropic breast cancer cells in vitro

Previous studies have shown that NFκB inhibition reduced the growth of parental breast cancer cell lines [5, 44]. In view of the fact that breast cancer cells in the skeleton (osteotropic) exhibit an altered phenotype compared to their parental counterpart, we assessed the cytotoxic effect of the NFκB inhibitor PTN against a panel of osteotropic, triple-negative (human MDA-231-BT1/2 and mouse 4T1-BT1/2) and estrogen receptor positive (human MCF7) breast cancer cell lines[38-40]. PTN reduced the growth of all cell lines tested in a concentration-dependent manner (Fig. S1). The concentration of PTN that half maximally inhibited cell viability (IC50) was significantly lower in osteotropic cells when compared to their parental control (Table 1). In contrast, PTN had no significant effects on the growth of osteoblasts or bone marrow derived osteoclast precursors at concentrations up to 10μM (Table 1).

3.2. PTN reduces osteoblast support for osteoclastogenesis

Cells of the osteoblast lineage contribute to breast cancer-induced osteolysis through secretion of various osteoclastic factors including RANKL[3, 4, 45]. In this study, we utilized ex vivo organ and in vitro osteoblast culture systems to test the effects of NFκB inhibition on osteoblast support for osteoclastogenesis in the presence of breast cancer cells or their derived factors. In cancer cell -

calvarial organ co-culture, PTN (1μM) inhibited osteoclast formation (Fig. 1A, left panel and Fig. S2A) and osteolysis (Fig. 1A, right panel and Fig. 1B) induced by MDA-231-BT1, 4T1-BT1 and MCF7 breast cancer cells. Addition of conditioned medium from these cells to calvarial osteoblast – bone marrow co-cultures enhanced osteoclast formation and these effects were significantly inhibited in the presence of PTN (1μM) (Fig. 1C). Representative photomicrographs of osteoclasts from the experiment described are shown in Fig. S2B. Pretreatment of calvarial osteoblasts with PTN (5μM) prior to the addition of condition medium from the osteotropic MDA-231-BT1 cells significantly inhibited RANKL/OPG ratio after 24 hours (Fig. 1D). Collectively, these results demonstrate that PTN inhibits osteolysis by disrupting breast cancer – osteoblast – osteoclast crosstalk in the models described.

3.3. PTN enhances osteoblast proliferation, differentiation and bone nodule formation

In view of previous findings that showed that NFκB inhibition in osteoblasts enhances bone formation[24], we examined the effects of PTN on osteoblast proliferation, differentiation and bone nodule formation in the presence and absence of breast cancer derived factors. Treatment of mouse calvarial osteoblasts with PTN (0.01μM) increased alkaline phosphatase activity within 24 hours (Fig. 2A, left panel) and enhanced bone nodule bone formation after 21 days (Fig. 2B, left panel) ($p<0.01$). Representative photomicrographs of bone nodule formation from the experiment described are shown in Fig. 2C. Exposure of mouse calvarial osteoblasts to conditioned medium from human MDA-231-BT1 breast cancer cells inhibited alkaline phosphatase activity after 24 (Fig. 2A, middle panel) and reduced bone nodule bone formation after 14 days (Fig. 2B, middle panel). These effects were completely inhibited by PTN (0.01 – 0.1μM) ($p < 0.01$). Interestingly, conditioned medium from osteotropic 4T1 enhanced alkaline phosphatase activity (Fig. 2A, right panel) in osteoblast cultures. Treatment with PTN (0.01 – 0.1μM) had no effects on alkaline

phosphatase activity (Fig. 2A, right panel) in these cultures, but PTN further enhanced the increase in bone nodule formation caused by exposure to conditioned medium from 4T1-BT1 (Fig. 2B, right panel). A similar increase in alkaline phosphatase activity (Fig. S3A) and bone nodule formation (Fig. S3B) was observed in cultures of the osteoblast-like cells MC3T3 treated with PTN for 7 days. In addition, exposure of osteoblasts to PTN (0.1μM) increased the mRNA expression of alkaline phosphatase and Runx2 after 24 and 72 hours (Fig. 2D), consistent with enhanced alkaline phosphatase activity and osteoblast differentiation.

3.4. NFκB inhibition by PTN enhances β□catenin activation and expression in osteoblasts

Mechanistic studies in calvarial osteoblasts showed that pretreatment with PTN (5μM) for 1 hour prior to addition of conditioned medium from human MDA-231-BT1 or mouse 4T1-BT1 prevented IκB phosphorylation after 15 minutes (Fig. 3A-B), inhibited NFκB DNA binding after 45 minutes (Fig. 3C) and reduced β-catenin cytoplasmic sequestration and increased its nuclear translocation (Fig. 3D-E) after 3 hours. Exposure of osteoblasts to PTN (1μM) for 24 hours enhanced β-catenin protein level and reduced the expression of IκB (Fig. 3F). These results suggest that the NFκB inhibitor PTN enhances β-catenin activation in osteoblast in the presence of breast cancer derived factors.

3.5. Disruption of the NFκB/β□catenin pathway by PTN in osteoclasts reduces breast cancer-induced osteoclastogenesis

Next, we tested the effects of PTN on breast cancer-induced osteoclastogenesis in the absence of osteoblasts. As shown in Fig. 4 (panels A and B), exposure of mouse M-CSF generated osteoclast precursors to the breast cancer cells MDA-231-BT1, MCF7 and 4T1-BT1 or their derived factors enhanced RANKL-induced osteoclast formation ($p < 0.001$), and these effects were inhibited by PTN in a concentration dependent manner (IC50; 0.13 ± 0.1 for MDA-231-BT1, 0.53 ± 0.2 for 4T1-BT1 and 4.17 ± 1.2 for MCF7 cells). Representative photomicrographs of osteoclasts from the

experiment described are shown in Fig. S2C. Of note, PTN had no effects on the proliferation of M-CSF generated osteoclast precursors (Fig. S2D-E). This excludes the possibility that the inhibitory effect on osteoclast formation was mediated by a reduction in pre-osteoclast cell number. Mechanistic studies in osteoclast precursors showed that exposure to PTN (5μM) for 1 hour inhibited IκB phosphorylation (Fig. 4C) and p65NFκB DNA binding (Fig. 4D) induced by RANKL (100ng/ml) or conditioned medium. Representative microphotographs of p65NFκB nuclear translocation in osteoclasts in the presence and absence of PTN (10μM) are shown in Fig. S4. Additionally, exposure to RANKL (100ng/ml) reduced β-catenin and IκB expression in osteoclast precursor-like cells RAW 264.7 after 48 hours (Fig. 4F), and these effects were reversed by pretreatment with PTN (1μM) (Fig. 4E-F). Collectively, these results demonstrate that pharmacological disruption of NFκB - and its interaction with β-catenin - in osteoclasts by PTN inhibits RANKL and breast cancer cell support for osteoclastogenesis.

3.6. PTN induces osteoclast apoptosis

Mature osteoclast survival is a major determinant of osteolytic bone loss [46]. With this in mind, we examined the effects of PTN on osteoclast survival and apoptosis. We generated mature osteoclasts and then exposed these cells to PTN in the presence and absence of M-CSF, RANKL or conditioned medium from human MDA-231-BT1 cells. As shown in Fig. 5 (panel A), exposure of osteoclasts to conditioned medium from MDA-231-BT1 cells (20% v/v) reduced osteoclast death due to withdrawal of M-CSF (25ng/ml) or RANKL (100ng/ml). Treatment with PTN (0.1 - 1μM) exacerbated osteoclast inhibition in these cultures after 24 hours of continuous treatment (Fig. 5A). PTN also induced caspase-3/7 activation in mature osteoclasts after 6 hours (Fig. 5B) and caused osteoclast apoptosis after 24 hours (Fig. 5C), as evident by nuclear condensation and DNA fragmentation as assessed by DAPI and TUNEL assays, respectively. Osteoclast apoptosis in these

cultures was significantly inhibited in the presence of the Caspase inhibitor zVAD-fmk (Fig. 5D), indicative of caspase involvement. We also investigate the effects of PTN on osteoclast activity in the presence and absence of conditioned medium (10% v/v) from MDA-231-BT1. The results of this experiment showed that exposure of mature osteoclasts to MDA-231-BT1 cells significantly increased bone resorption, and this was not affected by PTN treatment (1μM) (Fig. S5).

3.7. PTN reduces breast cancer related osteolysis

We tested the effects of PTN on osteolytic bone damage caused by breast cancer cells or their derived factors in immuno-competent mice (Fig. 6A). Administration of PTN (3mg/kg/3-weekly) in immuno-competent mice 24 hours prior to supra-calvarial injection of conditioned medium from human MDA-231-BT1, MCF7 and mouse 4T1-BT1 breast cancer cells increased bone volume (Fig. 6B). Next, we examined the effects of PTN on skeletal tumour growth and bone loss in the syngeneic 4T1 model of osteolysis. PTN (10mg/kg/3-weekly) was administered in mice 1 day prior to intra-tibial injection of the osteotropic 4T1-BT1 (4×10^3) and continued to day 12 (Fig. 6C). All mice that were treated with vehicle (0.01% DMSO) developed local osteolysis, characterised by a significant loss in trabecular bone volume (% BV/TV), thickness (% Tb.Th) and connectivity (% Conn.D) (Fig. 6D). These changes were significantly inhibited by treatment with PTN (10mg/kg/3-weekly). Detailed microCT analysis of tumor growth in the metaphysis of the proximal tibia showed that PTN reduced the size of lytic lesions (Fig. 6E), indicative of reduced tumour area. Further histomorphometric analysis of bone revealed that PTN reduced osteoclast number (Oc.N/BS, Fig. 6F, left panel), suppressed osteoclast activity (Oc.S/BS, Fig. 6F, right panel) and enhanced osteoblast number (Ob.N/BS, Fig. G, left panel), without affecting active osteoblast surface (Ob.S/BS, Fig. 6G, middle panel) and width (Ob.W, Fig. 6G, right panel).

4. Discussion

Breast cancer cells frequently metastasize to the skeleton in patients with secondary breast cancer [1-3]. Osteoclast and osteoblast activity in the tumour microenvironment plays an essential role in the regulation of breast cancer cell behaviour in the skeleton[1-4]. The NFκB signalling pathway regulates osteoclast and osteoblast differentiation in health and disease[16, 34, 47]. Previous studies have also implicated NFκB in the initiation and progression of bone metastasis [33, 34], and pharmacological inhibitors of NFκB reduced breast cancer-induced osteolysis [33, 34]. However, the bone-autonomous contribution of NFκB to breast cancer-induced osteolysis is poorly understood. This and the fact that most previous studies that have implicated NFκB in bone metastasis were performed in immuno-deficient mice have led us to study the bone cell-autonomous contribution of NFκB to breast cancer related bone cell activity and osteolysis in vitro, ex vivo and in immuno-competent mice.

Previous studies showed that NFκB inhibition reduced osteolysis and this effect was attributed – at least in part - to the reduction in number of breast cancer cells metastasizing to bone [33, 34]. Here, we provide pharmacological evidence to show that bone cell-specific inhibition of NFκB by PTN reduced osteolysis induced by conditioned medium from human and mouse breast cancer cells. Data from in vitro bone cell and organ cultures and histomorphometric analysis of bone confirmed that this agent inhibited breast cancer- and RANKL-induced osteoclast formation and reduced the ability of breast cancer cells to prolong osteoclast survival and to inhibit osteoclast apoptosis. It is important to note however that this agent had no direct effect on bone resorption by mature osteoclasts at concentrations that do not affect osteoclast survival (Fig. S5A-C). Breast cancer- and bone-derived mediators other than RANKL are implicated in the regulation of breast cancer – bone cell interactions [3, 4]. In this study, we show that PTN also inhibited NFκB activation by TNFα and TGFβ in cultures of osteoclasts and their precursors that have been primed with MDA-

231-BT1 conditioned medium (Fig. S6). These findings are entirely consistent with the pro-apoptotic and anti-resorptive effects of this agent, and of great interest in demonstrating that NFκB inhibition suppresses excessive osteoclast formation caused by RANKL, breast cancer cells and other osteolytic mediators such as TNFα and TGFβ that are often present in the bone tumour microenvironment.

Osteolysis supports the growth of metastatic breast cancer cells in the skeleton [1-3]. Previous studies have shown that breast cancer cells in the skeleton exhibit an altered phenotype compared to their parental counterpart [1-4]. Here, we show that osteotropic sub-clones of the human MDA-231 and mouse 4T1 breast cancer cells were sensitive to treatment with PTN when compared to parental cells. Furthermore, PTN inhibited tumour area in mice injected with the osteotropic 4T1-BT cells, suggesting an anti-tumour effect. Whilst we cannot exclude the possibility that PTN exerts a direct inhibitory effect on tumor growth in vivo, it is likely that the anti-tumour effect observed in our in vivo model was mainly due to osteoclast inhibition. Evidence to support this hypothesis comes from the anti-osteoclast effects observed in vitro, ex vivo and in vivo, and from in vitro studies that showed that the concentrations of PTN that needed to inhibit growth of breast cancer cells were at least 10 times higher than those that inhibited osteoclast formation.

NFκB inhibition is associated with increased osteoblast differentiation and maturation[22]. With this in mind, we examined the effects of PTN on osteoblast differentiation and activity in the presence and absence of conditioned medium from human and mouse breast cancer cells [21, 22, 27]. PTN reduced osteoblast inhibition by breast cancer cells, as evidenced by marked increase in osteoblast differentiation and bone nodule formation, and also by the fact that PTN enhanced ALP expression and activity, increased Runx-2 expression and stimulated β-catenin activation in osteoblasts. Moreover, PTN had no effects on osteoblast apoptosis in the presence or absence of

conditioned medium from breast cancer cells (data not shown). It is important to note however that administration of PTN in mice stimulated osteoblast number but had no significant effects on bone formation rate in the 4T1 model described. Future in vivo studies in models of osteolysis are needed to evaluate the consequences and implications of osteoblast stimulation by PTN in the treatment of breast cancer bone metastasis.

NFκB cooperates with multiple signaling pathways in bone and breast cancer cells [32]. One of the transcription factors that is essential for osteoclast formation is c-Fos [48]. In this study, we showed that exposure of osteoclasts and their precursors to PTN reduced the phosphorylation and DNA binding of c-Fos by TNFα-, RANKL- or MDA-231-BT1 conditioned medium (Fig. S7). Previous studies have implicated β-catenin in the stabilization and cytoplasmic sequestration of p65NFκB[32]. Wnt/β-catenin signalling plays a role in the regulation of osteoclast and osteoblast differentiation, and Sclerostin, a secreted inhibitor of Wnt/β-catenin signalling reduces bone formation[25-27, 32]. In broad agreement with these findings, we showed that NFκB inhibition by PTN in osteoblasts and osteoclasts was accompanied by a significant increase in β-catenin activation and expression. Notwithstanding this, we cannot exclude the involvement of other mechanisms since PTN has been reported to affect a wide array of cellular processes including DNA methylation and generation of reactive oxygen species[37].

In conclusion, our present data together with previous results obtained from immuno-deficient mice [33, 34] suggest that NFκB plays a key role in the regulation of breast cancer-induced bone cell activity and osteolysis. We also presented pharmacological evidence to suggest that bone-specific inhibition of NFκB suppresses osteoclast formation and reduces osteoblast inhibition related to breast cancer by engaging β-catenin signalling. When combined with previous studies[32], our

present results raise the possibility that combined targeting of NFκB and β-catenin signalling may be of value in the treatment of breast cancer associated bone disease.

5. Acknowledgements

This work was supported in part by a pump-prime grant from Cancer Research UK (University of Edinburgh) and Breast Cancer Now (University of Sheffield) grants to Aymen I. Idris.

6. Conflict of Interest

Patrick Mollat is an employee of Galapagos SASU (102 Avenue Gaston Roussel, 93230 Romainville, France).

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Figure legends:

Figure 1

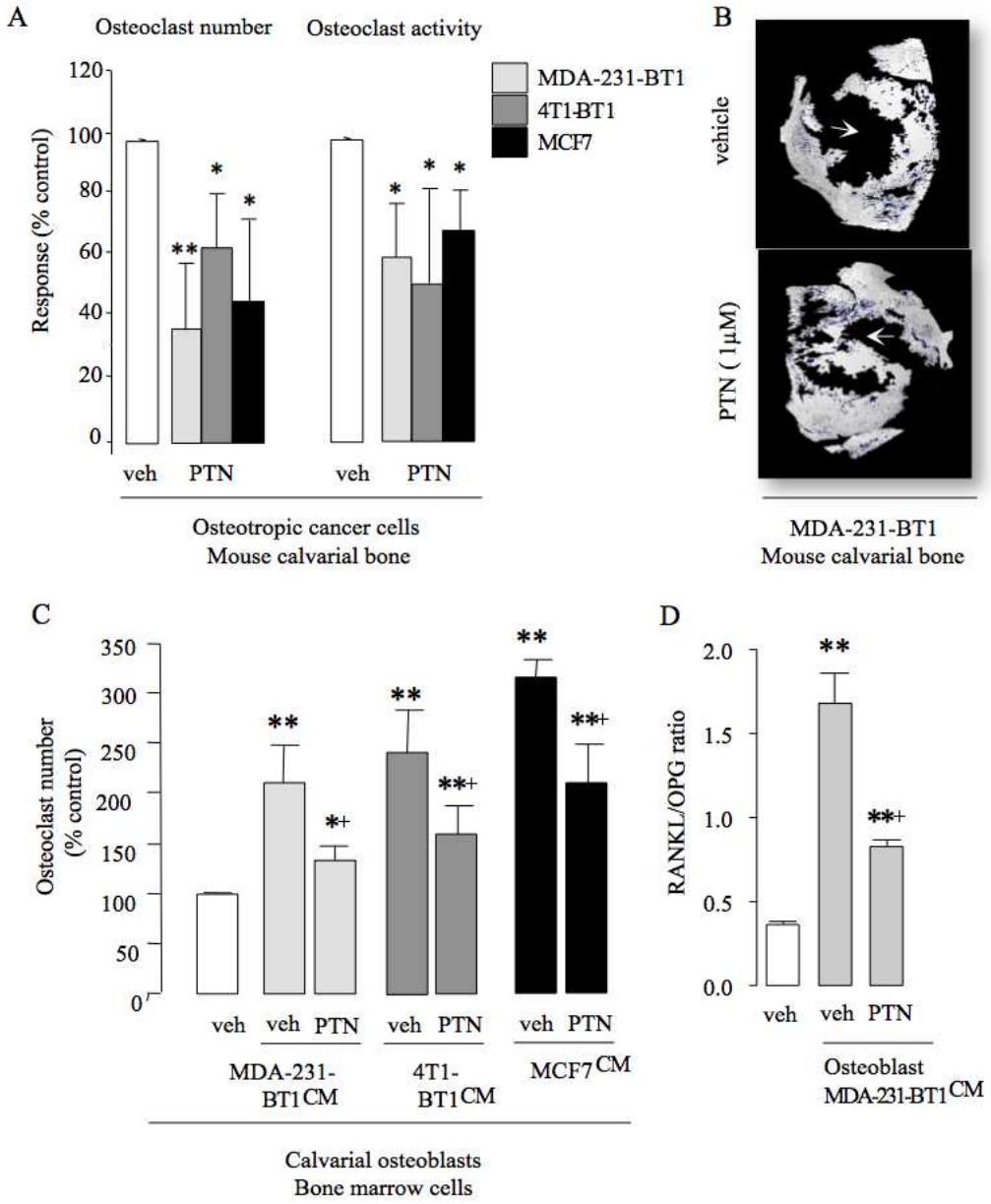

Fig. 1. PTN reduces osteoblast support for osteoclastogenesis. (A) Ex vivo osteoclast number and bone resorption in mouse calvarial organ culture exposed to conditioned medium (20% v/v) from human MDA-231-BT1 and MCF7 or mouse 4T1-BT1 cancer cells in the presence and absence of PTN (1μM). (B) Representative photomicrographs of osteolytic bone damage from the experiment

described in panel A. (C) In vitro osteoclast formation in mouse calvarial osteoblast - bone marrow cell co-cultures after exposure to conditioned medium (CM, 20% v/v) from human MDA-231-BT1 and MCF7 or mouse 4T1-BT1 cancer cells in the presence and absence of PTN (1μM). (D) mRNA expression of RANKL and OPG in mouse calvarial osteoblasts exposed to conditioned medium (20% v/v) from human MDA-231-BT1 breast cancer cells in the presence and absence of PTN (5μM). Values are mean ± SD; * $p<0.05$ and ** $p<0.01$ from vehicle; + $p<0.05$ from vehicle plus breast cancer cells or conditioned medium.

Figure 2

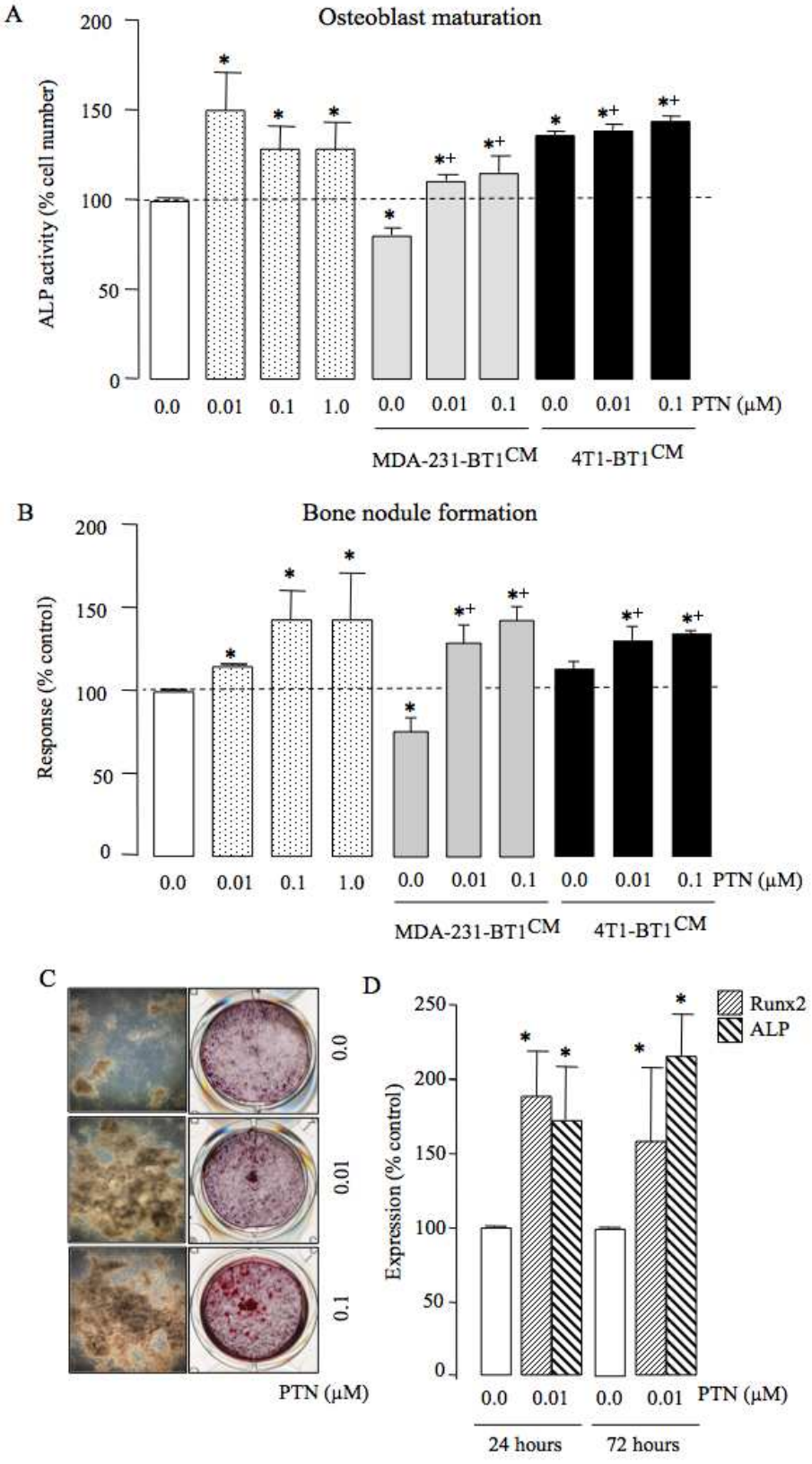
A
Osteoblast maturation
ALP activity (% cell number)
200
150
100
50
0
0.0 0.01 0.1 1.0 0.0 0.01 0.1 0.0 0.01 0.1 PTN (μM)
MDA-231-BT1CM
4T1-BT1CM
B
Bone nodule formation
Response (% control)
200
150
100
50
0
0.0 0.01 0.1 1.0 0.0 0.01 0.1 0.0 0.01 0.1 PTN (μM)
MDA-231-BT1CM
4T1-BT1CM
C
0.0
0.01
0.1
PTN (μM)
D
Runx2
ALP
Expression (% control)
250
200
150
100
50
0
0.0 0.01 0.0 0.01 PTN (μM)
24 hours
72 hours

Fig. 2. PTN enhances osteoblast differentiation and protects against osteoblast inhibition by breast cancer cells. (A-B) In vitro osteoblast differentiation (A) and bone nodule formation (B) in mouse calvarial osteoblast cultures exposed to standard or conditioned medium (20%v/v) from human MDA-231-BT1 or mouse 4T1-BT1 cancer cells in the presence or absence of PTN for 24 hours. (C) In vitro bone nodule formation in mouse calvarial osteoblasts (21 days) and Saos2 (10 days) treated with PTN at the indicated concentration in the presence and absence of conditioned medium from MDA-231-BT1 and 4T1-BT1. Osteoblast differentiation and bone nodule formation were assessed by Alkaline phosphatase (ALP) and Alazarin Red assays. (C) Representative photomicrographs of osteoblasts and bone nodule formation from the experiment described in panel B. (D) mRNA expression of ALP and Runx2 in mouse calvarial osteoblasts after 24 and 72 hours in the presence or absence of PTN (0.1μM). Values are mean ± SD; * $p<0.05$ and + $p<0.05$ from vehicle plus breast cancer cell conditioned medium.

Figure 3

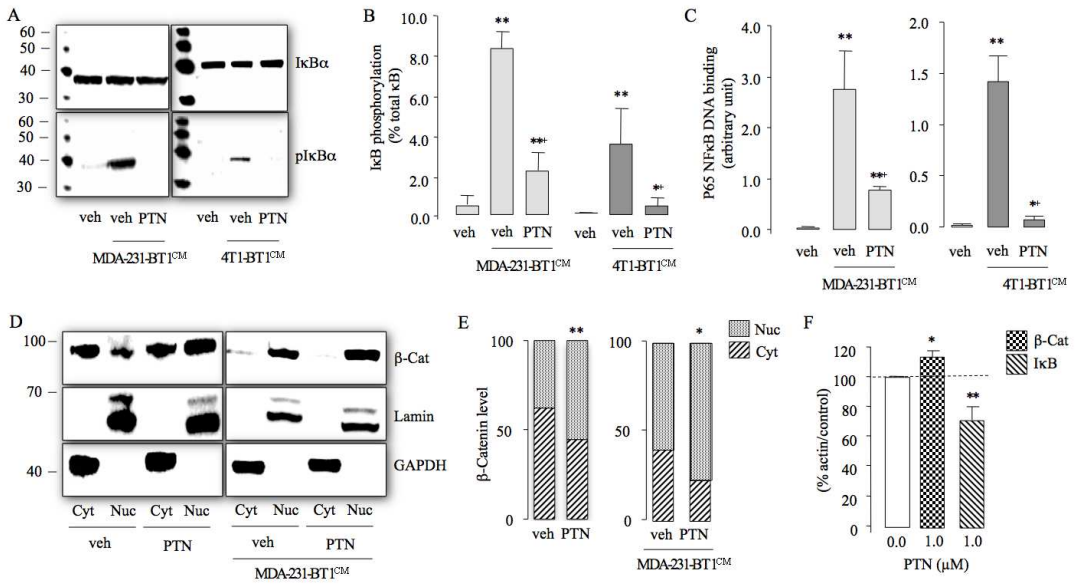

Fig. 3. PTN activates β-catenin signalling in osteoblasts. (A-B) Western blot analysis (A) and quantification (B) of total and phosphorylated IκB in mouse calvarial osteoblast cultures pretreated with vehicle or PTN (5μM) for 1 hour and exposed to conditioned medium 20%v/v) from human MDA-231-BT1 or mouse 4T1-BT1 cancer cells for 15 minutes. (C) NFκB DNA binding in mouse calvarial osteoblasts cultures pretreated with vehicle or PTN (5μM) for 1 hour and exposed to conditioned medium (20% v/v) from human MDA-231-BT1 (left) or mouse 4T1-BT1 (right) cancer cells for 45 minutes. (D-E) Western blot analysis (D) and quantification (E) of β-catenin expression in cytoplasm (Cyt) and nuclear (Nuc) fractions obtained from mouse calvarial osteoblasts cultures pretreated with vehicle or PTN (5μM) for 1 hour and exposed to standard medium or conditioned medium (20%v/v) from human MDA-231-BT1 breast cancer cells for 3 hours. (F) Differential expression of β-Catenin and IκB obtained from mouse calvarial osteoblast cultured in the presence or absence of PTN at the indicated concentration for 24 hours. Values are mean ± SD; * $p<0.05$ and ** $p<0.01$ from vehicle; + $p<0.05$ from vehicle plus breast cancer cell conditioned medium.

Figure 4

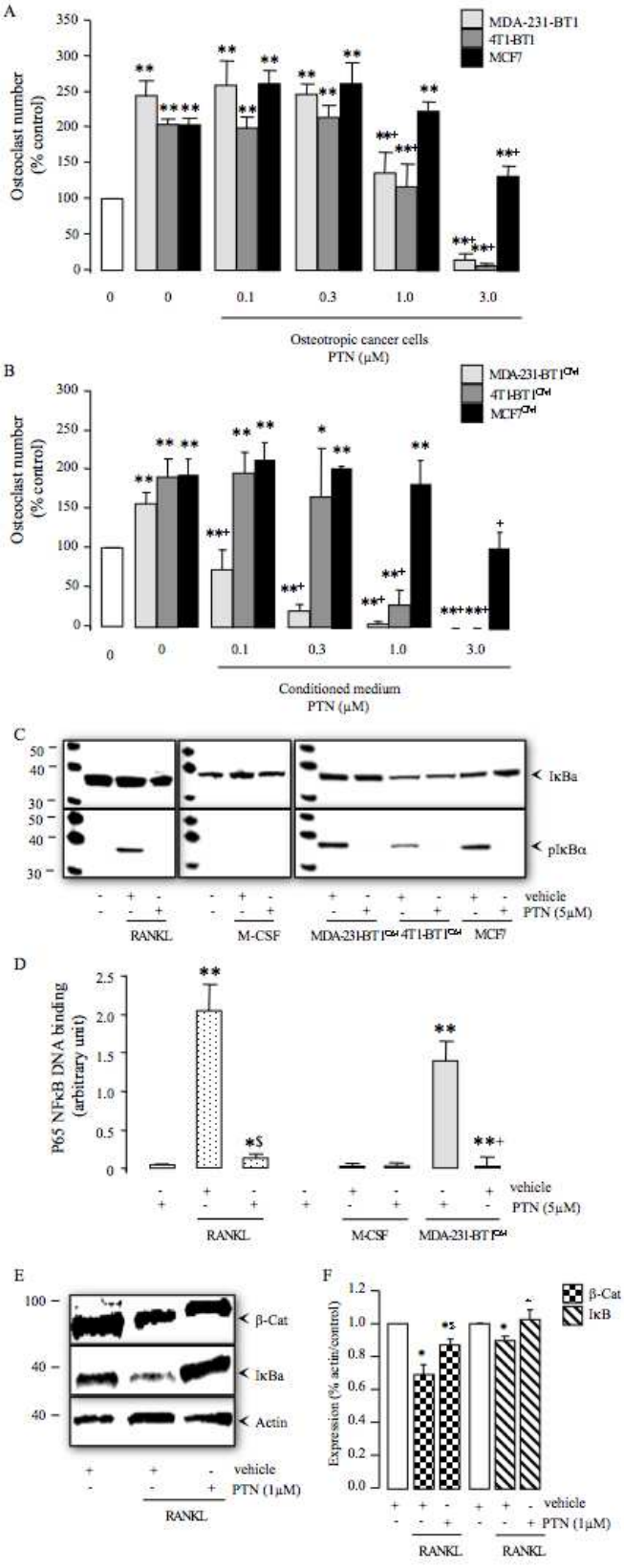
A
Osteoclast number (% control)
MDA-231-BT1
4T1-BT1
MCF7
Osteotropic cancer cells
PTN (μM)
B
Osteoclast number (% control)
MDA-231-BT1CM
4T1-BT1CM
MCF7CM
Conditioned medium
PTN (μM)
C
IκBa
pIκBα
vehicle
PTN (5μM)
RANKL
M-CSF
MDA-231-BT1CM
4T1-BT1CM
MCF7
D
P65 NFκB DNA binding (arbitrary unit)
vehicle
PTN (5μM)
RANKL
M-CSF
MDA-231-BT1CM
E
β-Cat
IκBa
Actin
vehicle
PTN (1μM)
RANKL
F
Expression (% actin/control)
β-Cat
IκB
vehicle
PTN (1μM)
RANKL
RANKL

Fig. 4. PTN inhibits breast cancer cell- and RANKL-induced osteoclast formation. (A-B) In vitro osteoclastogenesis of murine M-CSF (25ng/ml) and RANKL (100ng/ml) stimulated bone marrow treated with vehicle or PTN (5μM) in the presence and absence of breast cancer cells (A) or their conditioned medium (20%v/v) (B) for 48 hours. (C) Western blot analysis of total and phosphorylated IκB in cultures of M-CSF (25ng/ml) dependent pre-osteoclasts after exposure to human RANKL (100ng/ml), human M-CSF (25ng/ml) or conditioned medium (20% v/v) from human MDA-231-BT1, 4T1-BT1 and MCF7 cancer cells in the presence and absence of PTN (5μM). (D) NFκB DNA binding in cultures of M-CSF (25ng/ml) dependent pre-osteoclasts described in panel C. (E-F) Differential expression of β-Catenin and IκB in mouse RAW 264.7 macrophage (pre-osteoclasts) pretreated with vehicle or PTN (1μM) for 1 hour and cultured in the presence or absence of RANKL (100ng/ml) for 48 hours. Values are mean ± SD; * $p<0.05$ and ** $p<0.01$ from vehicle; + $p < 0.05$ from vehicle plus breast cancer cells or conditioned medium; $ $p<0.05$ from vehicle and RANKL treated cultures.

Figure 5

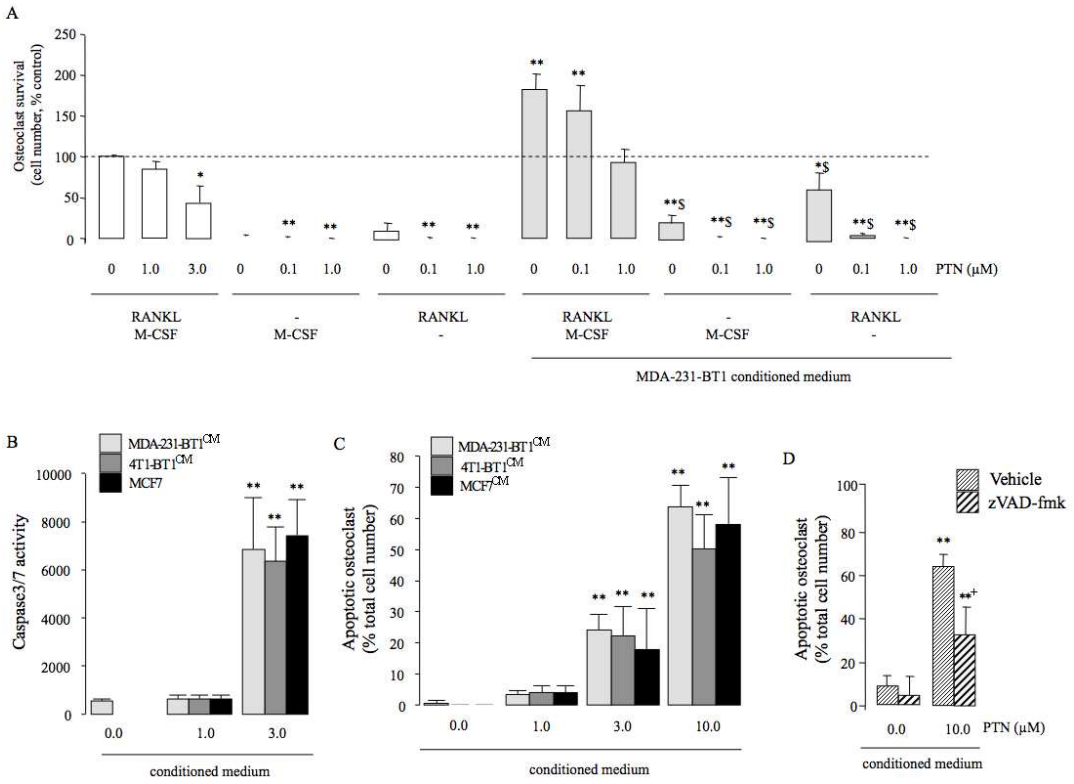

Fig. 5. PTN induces osteoclast apoptosis. (A) In vitro survival of mature osteoclasts after withdrawal of human RANKL (100ng/ml), M-CSF (25ng/ml), or both followed by exposure to MDA-231-BT1 conditioned medium (20%v/v) in the presence or absence of PTN for 24 hours. (B-C) In vitro caspase-3/7 activation (B) DNA fragmentation (C) in mature osteoclasts from the experiment described in panel A after 6 and 24 hours, respectively. (D) In vitro osteoclast apoptosis in cultures treated with PTN (10μM) in the presence of absence of the caspase inhibitor zVAD-fmk (20μM). Values are mean ± SD; * $p<0.05$ and ** $p<0.01$ from vehicle; $ $p<0.05$ from vehicle plus breast cancer conditioned medium; + $p < 0.05$ from PTN plus caspase inhibitor zVAD-fmk.

Figure 6

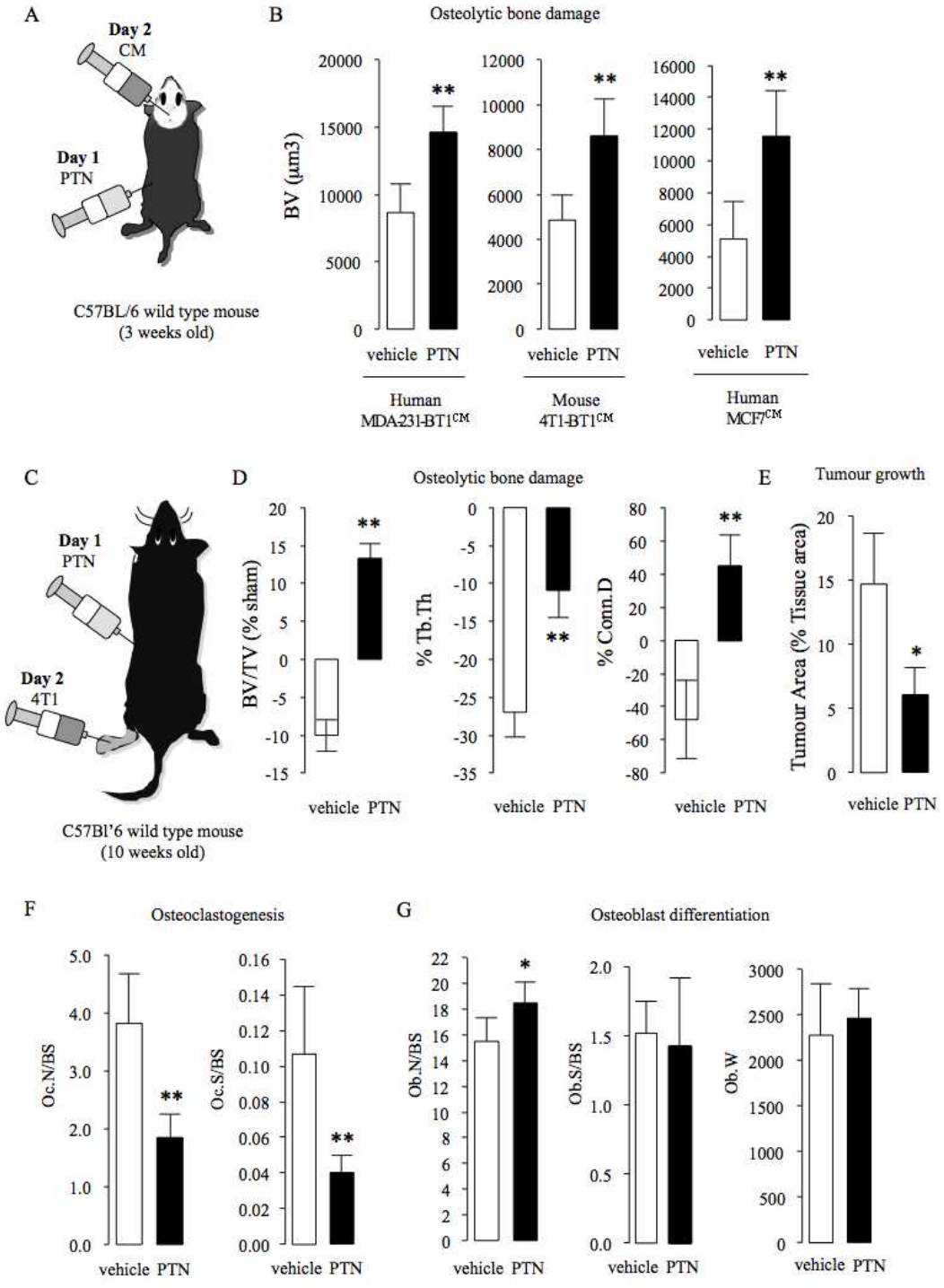

Fig. 6. PTN inhibits skeletal tumour growth and reduces breast cancer-induced osteolysis. (A) Graphic representation of supracalvarial injection of conditioned medium (CM) from human MDA-

231-BT1 and MCF7 and mouse 4T1-BT1 breast cancer cells in immunocompetent mice pretreated with vehicle or PTN (3mg/kg/day) for 3 days. (B) Total bone volume from the experiment described in panel A (n = 7). (C) Graphic representation of intra-tibial injection of the mouse breast cancer osteotropic 4T1-BT1 in immunocompetent BALB/c mice pretreated with vehicle (DMSO/water: 0.1:10) or PTN (10mg/kg/day) for 12 days. (D) Total bone volume (BV/TV), trabecular thickness (Tb.Th.) and connectedness density (Conn.D) in tibial metaphysis of mice from the experiment described in panel C. (E) Tumour size from the experiment described in panels C-D. (F) In vivo osteoclast formation (Oc.N, left panel) and activity (Oc.S/BS, right panel) from the experiment described in panels C-D. (G) In vivo osteoblast number (Ob.N, left panel), osteoid surface (Ob.S/BS, middle panel) and osteoid width (Ob.W, right panel) from the experiment described in panels C-F. Values are mean ± SD; * $p<0.05$ and ** $p<0.01$ from vehicle plus breast cancer cells or conditioned medium.

Tables:

Table I

Effects of Parthenolide on the growth of osteotropic breast cancer cells in vitro.

	Cell viability (IC_{50}, μM)
MDA-231	10.57 ± 0.15
MDA-231-BT1	8.17 ± 0.62
MDA-231-BT2	7.82 ± 0.69
MCF7	12.7 ± 1.10
4T1	6.79 ± 0.18
4T1-BT1	5.72 ± 0.61
4T1-BT2	1.90 ± 0.56
Pre-Osteoclasts	> 10
Osteoblasts	> 10

Cell viability was measured after 48 hours of continuous exposure to PTN by AlamarBlue assay. Calculation of half maximal inhibitory concentrations (IC50) has been performed as described under “Materials and

Methods". Values are mean ± SD and are obtained from 5 independent experiments. BT denotes osteotropic tropic cells, and pre-osteoclasts refers to M-CSF generated osteoclast precursor cells.