**Title**

# Pharmacological inhibition of the IKKε/TBK-1 axis potentiates the anti-tumour and anti-metastatic effects of Docetaxel in mouse models of breast cancer


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**Running title**

IKKε regulates breast cancer osteolytic metastasis


**Keywords**

IKKε, NFκB, breast cancer, combination treatment, bone metastasis, osteolysis, osteoclast, osteoblast, bone.

## Highlights

- IKKε has been identified as a breast cancer oncogene.
- Pharmacological inhibition and knockdown of IKKε reduced breast cancer-induced osteolysis.
- Combined administration of IKKε inhibitor with Docetaxel reduced breast cancer metastasis and improved survival in mice.
- IKKε inhibitors may be of value in the treatment of advanced triple negative breast cancer.

## Abstract

IκB kinase subunit epsilon (IKKε), a key component of NFκB and interferon signalling, has been identified as a breast cancer oncogene. Here we report that the IKKε/TBK1 axis plays a role in the initiation and progression of breast cancer osteolytic metastasis. Cancer-specific knockdown of IKKε in the human MDA-MB-231-BT cells and treatment with the verified IKKε/TBK1 inhibitor Amlexanox reduced skeletal tumour growth and osteolysis in mice. In addition, combined administration of Amlexanox with Docetaxel reduced mammary tumour growth of syngeneic 4T1 cells, inhibited metastases and improved survival in mice after removal of the primary tumour. Functional and mechanistic studies in breast cancer cells, osteoclasts and osteoblasts revealed that IKKε inhibition reduces the ability of breast cancer cells to grow, move and enhance osteoclastogenesis by engaging both IRF and NFκB signalling pathways. Thus, therapeutic targeting of the IKKε/TBK1 axis may be of value in the treatment of advanced triple negative breast cancer.

## Introduction

The nuclear factor-κB (NFκB) signaling is implicated in inflammation and cancer[1-5]. IκB kinase subunit epsilon (IKKε), a key component of NFκB signalling, has been identified as a breast cancer oncogene[6]. A number of studies have shown that IKKε is overexpressed in mammary breast tumours and various human breast cancer cell lines[6, 7], and its inhibition reduced the development of primary breast cancer in mice[8]. IKKε has also been found to contribute to Tamoxifen resistance in oestrogen receptor positive (ER+) breast cancers[9]. Binding of IKKε to the TANK-binding kinase 1 (TBK1) leads to the activation of NFκB, interferon regulatory factor (IRF) signalling pathway[10, 11] as well as various cancer-driving factors including, protein kinase B (Akt), tumour necrosis factor receptor (TNFR)-associated factor 2 (TRAF2), and inhibition of tumour suppressors such as cylindramatosis tumour suppressor (CYLD) and Forkhead box O 3a (FoxO3a)[9-15]. Although the mechanisms through which IKKε contributes to cellular transformation and primary tumour growth have been established[6], the role of IKKε in breast cancer metastasis is not fully understood.

Breast cancer cells preferentially metastasise to bone and skeletal related events are diagnosed in approximately 70% of advanced breast cancer cases[16]. NFκB activation enhances excessive osteoclastic activity and causes bone loss, and various studies have implicated IKKε in bone loss associated with oestrogen deficiency, rheumatoid arthritis and Paget's disease[17-20]. However, the role of IKKε in excessive bone loss associated with metastatic breast cancer has not been investigated. Encouraged by a recent report that pharmacological inhibition of IKKε, using the verified IKKε/TBK1 inhibitor Amlexanox[21], inhibited bone loss in a mouse model of osteoporosis[22], we tested the effects of over-expression/knockdown and pharmacological inhibition of IKKε on the initiation, progression and metastasis of breast cancer. Using mouse models of syngeneic and human breast cancer, we showed that cancer-specific knockdown of IKKε in human MDA-MB-231 and administration of the IKKε/TBK1 inhibitor Amlexanox in immune-competent mice inoculated with syngeneic 4T1 cells inhibited skeletal tumour burden and reduced osteolytic bone damage. Administration of Amlexanox alone in mice reduced the growth of the syngeneic 4T1 breast cancer cells in the mammary fat pads and it significantly enhanced the anti-tumour and anti-metastatic properties of the FDA-approved Docetaxel[23] in mice after removal of the primary tumour. Collectively, these findings suggest that, due to the combined anti-tumour and anti-resorptive effects, IKKε inhibitors, alone and in combination with

chemotherapeutic agents, may be of value in the treatment of skeletal and non-skeletal complications associated with advanced triple negative breast cancer.

## Materials and Methods

### Reagents

The IKKε/TBK1 inhibitor Amlexanox was purchased from Abcam (Cambridge, UK). Parental MDA-MB-231, 4T1, Saos-2 osteoblast-like cells and mouse RAW 264.7 macrophages-like cells were purchased from ATCC (Manassas, VA). The human triple negative bone tropic MDA-MB-231 were generated by Dr Nadia Rucci at the Univeristy of L'Aquila, Italy. Single cell clones of MDA-MB-231 were expanded and injected in to the left ventricle of 4-week-old female BALB/c-nu/nu mice a clone that generated bone only lesions with significant osteolysis in 8-weeks was used.[24-26]. Murine 4T1-luc2 cells were a kind gift from Dr. Munitta Muthana (Sheffield, UK) and were transfected to express Luciferase. Tissue culture medium (DMEM and alpha-MEM) was obtained from ThermoFisher (Leiceistershire, UK). All primary antibodies were obtained from Cell Signalling Technology (MA, USA) excluding rabbit anti-actin, which was purchased from Sigma-Aldrich (Dorset, UK). Mouse macrophage colony stimulating factor (M-CSF) was obtained from R&D Systems (Abingdon, UK) and receptor activator of NF□B ligand (RANKL) was a gift from Patrick Mollat (Galapagos SASU, France).

### Cell lines and conditioned medium

Human MDA-MB-231 breast cancer cells, Saos-2 osteoblast-like cells, mouse 4T1 breast cancer and RAW.267 macrophage-like cells were cultured in a standard D-MEM supplemented with 10% fetal calf serum (FCS), glutamine (2mM), penicillin (100U/ml), and streptomycin (100µg/ml). Human MCF-7, T47D, SK-BR-3, ZR-75-1 were cultured in a standard RPMI supplemented with 10% fetal calf serum (FCS), glutamine (2mM), penicillin (100U/ml), and streptomycin (100µg/ml).

### Lentiviral infection and generation of stable cell lines

In order to generate IKKε-shRNA expressing lentivirus, HEK 293T-packaging cells were transfected with the 5 µg pLKO.1 lentiviral vector (empty or containing IKKε-shRNA [TRCN-735, -736](Dharmacon)), psPAX2 vector, pCMV-VSV-G vector and 40 µl polyethylenimine, Lentiviral supernatant and polybrene (5 µg/ml; Sigma-Aldrich Dorset, UK) were were incubated with the MDA-MB-231-BT cells. Cells were selected with 1 µg/ml puromycin. To generate IKKε overexpressing cells, a lentiviral delivered CRISPR activation system was used (Santa-Cruz Biotechnology) and cells were transected according to

manufacturer's instructions.. The transfected MDA-MB-231 cells were maintained in selection for at least two passages until knockdown/overexpression was confirmed via western blotting.

**Small RNA interference**

Human MDA-MB-231-BT cells were transfected with siRNA (5 nM) using Dharmafect 1 reagent (Dharmacon, CO, USA), according to the manufacturer's instructions. A pool of four annealed double – stranded RNA oligonucleotides against TBK1 (M-003788-02-0005), IKKβ (M-003503-03-0005), p65 (M-003533-02-0005), IRF3 (M-006875-02-0005) or non-targeting pool no. 1 (D-001206-13-05) siRNAs were purchased from Dharmacon and used according to the manufacturer's instructions. The cells were cultured for 48 hours in antibiotic free complete medium with the transfection reagent. The efficiency of IRF3, TBK1, IKKβ and p65 knockdown was assessed by Western blot analysis.

**Assessment of osteoblast differentiation and bone nodule formation**

Primary osteoblasts were isolated from the calvarial bones of 2-day-old mice by sequential collagenase digestion as previously described[27]. Osteoblasts were maintained in standard alpha-MEM. For early osteoblast differentiation assays, primary osteoblasts were plated ($7x10^3$ cells/ well). Test compounds and conditioned medium was added the following day. Osteoblast differentiation was measured after 48 hours. For bone nodule assays, Saos-2 osteoblast-like cells were seeded into 12-well and treated with L-ascorbic acid (50 µg/ml), breast cancer cell conditioned medium (20% v/v), compounds at stated doses. The medium was refreshed every 48 hours after two treatments, β-glycerophosphate (10mM) was also added. The cells were cultured for up to 15 days. At the end of the culture period, osteoblast number, differentiation and bone nodule formation were determined by the AlamarBlue assay, alkaline phosphatase (Alk Phos) assay and alizarin red (ALZ) staining [27, 28].

**Assessment of osteoclast formation and activity**

Osteoclast formation was studied using RANKL and M-CSF generated human and mouse osteoclasts[29] or the macrophage-like cell line RAW.267. For human osteoclasts, $CD14^+$ monocytes were isolated by using positive selection beads (Miltenyi Biotec) from peripheral blood mononuclear cells of healthy volunteers following separation using Ficoll-Pacque. Following isolation, $CD14^+$ monocytes were plated with 25ng/ml MCSF to generate macrophages. Mouse osteoclasts were generated as previously described[30]. For cultures involving conditioned medium, conditioned medium was prepared as previously described [31] and added to cultures at 10% (v/v) with M-CSF, RANKL and test compounds for up to 9 days with medium refreshed every 48 hours. In macrophage-like RAW cell cultures, MCSF

was omitted. Osteoclasts were identified using Tartrate-resistant Acid Phosphatase (TRAcP) staining[31].

**Assessment of cell motility**

The migration of human MDA-MB-231 cells was assessed by wound healing assay [32]. The confluent cell monolayer was scratched and cellular migration was assessed over time using time-lapse microscopy and T-scratch analysis program. . The invasive phenotype of human MDA-MB-231 cells was assayed using the transwell migration assay. Briefly, matrigel (Corning, UK) was diluted to a final concentration of 1.5mg/ml in serum-free DMEM. A volume of 20µl of diluted matrigel was added to 0.8 µm Transwell inserts (Corning, UK). A cell suspension in serum free DMEM containing $10x10^3$ cells and test compounds was added to each insert. Standard DMEM (500µl) was used as the chemoattractant. After 72 hours, invasive cells on the underside of the membrane were fixed and stained with haemotoxylin and eosin. Invasive cells were quantified using ImageJ.

**Western Blotting**

Western blot analysis was used to detect protein expression and phosphorylation in cultured bone and breast cancer cells. Briefly, cells were seeded in 12 well plates and maintained in standard media until confluency. Prior to stimulation with test agents or vehicle, cells were incubated in serum free tissue culture medium 16 hours. Test agents or vehicle were prepared in serum free media and were then added for the desired period of time. Cells were lysed and protein amount was quantified as previously described [33]. Total protein 50-100µg was resolved by SDS-PAGE on 12% polyacrylamide SDS gels, transferred onto PVDF membranes (BioRAD, UK) and immunoblotted with appropriate antibodies according to manufacturer's instructions, using horseradish peroxidase-conjugated secondary antibody (Jackson labs, UK), and then visualised using chemiluminescence (Amersham, UK) on a Biorad ChemiDocMP imaging system.

**Measurement of levels of tumour-derived factors**

Level of tumour-derived factors in conditioned medium from human MDA-MB-231 breast cancer cells was determined by Proteome Profiler Human XL Cytokine Array Kit (ARY022, R&D Systems, Abingdon, UK), according to the manufacturer's instructions.

**Animal experiments**

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

**Intra-tibial injection in mice**

The effects of cancer-specific inhibition and pharmacological inhibition of IKKε on skeletal tumour growth and osteolysis were investigated through intra-tibial injection of either human MDA-MB-231 or syngeneic 4T1 breast cancer cells. Briefly, 7 four week-old female BALB/c-nu/nu athymic mice were randomly allocated in groups received intra-tibial injection of human MDA-MB-231-BT IKKε-shRNA or IKKε-mock ($4\times10^3$ cells) in the left tibia. In the second intra-tibial experiment, 14 female BALB/c 10-week-old mice received intra-tibial injection of mouse 4T1 breast cancer cells ($4\times10^3$ cells) in the left leg or a sham injection of PBS into their right leg. Animals were sacrificed after 21 days (MDA-MB-231) or 14 days (4T1). Animals were divided into two groups and received intraperitoneal injection of either vehicle (PBS) or Amlexanox (20mg/kg/daily). Following sacrifice, bones were analyzed by micro–computed tomography (microCT, Skyscan 1172 scanner). Skeletal tumor growth was measured on 2D microCT images using Image J and results were expressed as a percentage of total metaphyseal area (4T1) or on H&E stained sections using the OsteoMeasure software at 10x magnification.

**Intracardiac injection of murine 4T1 breast cancer cells**

We studied the effects of treatment with Amlexanox on the formation of bone metastasis and following intracardiac injection of syngeneic mouse breast cancer cells. Briefly, 8 week-old female BALB/c mice were randomly allocated into groups, anesthetized and single-cell suspension of $1 \times 10^5$ 4T1-Luc2 cells /100 µL PBS was injected into the left cardiac ventricle. The following day mice were divided in to groups (8 mice per group) and treated with either daily i.p. injection of vehicle (5% DMSO in PBS) or Amlexanox (35 mg/kg/day). Animals were monitored daily for cachexia (evaluated by body weight) and behavior. Bone metastases were monitored tri-weekly using the IVIS system and animals were sacrificed by cervical dislocation on day 11.

**Orthotopic injection of murine 4T1 breast cancer cells**

We studied the effects of treatment with Amlexanox, Docetaxel or a combination treatment on primary tumour growth of 4T1-Luc2 after orthotopic injection. Briefly, 8 week-old female BALB/c mice were randomly allocated into groups and anesthetized. 4T1-luc2 ($10x10^6$/ml) were re-suspended in 60% PBS, 39% growth factor reduced matrigel (Corning) and 1% trypan blue (Sigma Aldrich). A volume of 100µl of the cell suspension was injected into the fat pad of both the left and right inguinal breast (10 mice per group). Animals were monitored daily for cachexia (evaluated by body weight) and behavior. The size of each tumour was measured externally three times a week by measuring their two axes using a caliper and

applying the formula of the volume of a sphere [$4/3\pi r^3$ where r is the mean of the two axes]. Once tumour volume had reached the maximum volume of $1cm^3$, the tumours were resected. The development of metastases was monitored biweekly using the IVIS system. The primary tumours and the lungs, brain, spleen and hind limbs of the animals were removed and ex vivo IVIS imaging was performed before formalin fixation.

**Analysis of clinical data**

Retrospective analysis of IKKε and TBK1 expression with respect to primary tumour growth and bone relapse was conducted using published cohorts of data. The NKI295 dataset was chosen to assess the association of IKKε bone metastasis, which did not initially probe for TBK1 expression.The CB560 dataset was used for analysis of TBK1 association with bone metastasis. Exhaustive survival analysis was performed by considering all possible cut-points using the survivALL package. P values were derived from Wilcoxon test and were two-tailed.

**Statistical Analysis**

Results were reported as mean ± standard deviation (SD) unless otherwise stated. A p-value value of 0.05 or below was considered statistically significant. All statistical analyses carried out between two groups were students T-test and between three groups analysis of variance (ANOVA) was used. All tests were conducted in GraphPad prism 7.0 or Statistical analyses were performed using IBM (Armonk, NY, USA) SPSS Statistics, for windows. The concentration that produced 50%of response IC50 was calculated using GraphPad Prism 7 for Windows.

**Results**

**IKKε inhibition reduces breast cancer cell growth in vitro and in vivo**

Previous studies have implicated that IKKε in breast cancer initiation and bone disease[6, 17-20]. In our studies, we have observed that IKKε expression is upregulated in highly metastatic human triple-negative breast cancer cell lines including MDA-MB-231, when compared to hormone-sensitive MCF7 and their non-transformed MCF10 control (Fig. S1A). With this in mind and the observation that TBK1 expression is associated with higher risk for bone metastasis (Fig. S2), we tested the effects of the verified IKKε/TBK1 inhibitor Amlexanox on a panel on the viability of a panel of ER+ and ER- breast cancer cell lines with different metastatic abilities to metastasise to the skeleton. As shown in Table 1, Amlexanox (0 – 100μM) significantly reduced the viability of breast cancer cells, however the ER- breast cancer cell cohort had a significantly lower IC50 value. In view of this, we stably knocked

down and over-expressed IKKε in the sub-clone MDA-MB-231-BT (Fig. S3), and assessed the growth and metastatic behaviour of these cells in vitro and in vivo. As shown in Figure 1, over-expression of IKKε in MDA-MB-231-BT enhanced cell migration within 6 hours, invasion within 48 hours and growth with 72 hours, whereas knockdown and pharmacological inhibition of IKKε using the IKKε/TBK1 inhibitor Amlexanox were inhibitory (Fig. 1A-E). In vivo, both immuno-deficient mice inoculated with MDA-MB-231-BT cells deficient in IKKε and immuno-competent mice which received the IKKε/TBK1 inhibitor Amlexanox after inoculation with the syngeneic 4T1 cells exhibited significant reduction in the growth of tumour cells in the tibial metaphysis of mice (Fig. 1F).

**IKKε inhibition reduced breast cancer-induced osteolysis in vivo**

To test if IKKε is implicated in the ability of cells to cause osteolytic bone loss, we assessed bone volume and architecture in mice after intra-tibial injection of breast cancer cells. Detailed micro-computed tomography (microCT) analysis of bone volume at the tibial metaphysis of immuno-deficient mice inoculated with human MDA-MB-231-BT cells deficient in IKKε (Fig. 2A) showed significant increase in total bone volume (BV/TV; Fig. 2B), increased trabecular connectivity (Conn, Fig. 2C) and reduced porosity (Po, Fig. 2D) yet had no effect on trabecular thickness (Tb.Th, Fig 2E). Histomorphometric analysis of histological samples from these mice demonstrated that cancer-specific knockdown of IKKε reduced osteoclast number (Fig. 2F), but had no effect on the number of osteoblasts (Fig. 2G). Using the syngeneic model of 4T1-BT (Fig. 3A), we went on to demonstrate that treatment of immuno-component mice with the IKKε/TBK1 inhibitor Amlexanox modestly but significantly increased total bone volume (BV/TV; Fig. 3B) and enhanced trabecular thickness (Fig. 3C), reduced bone porosity (Fig. 3D) connectivity (Fig. 3E). Histomorphometric analysis of histological samples from these mice revealed that administration of the IKKε/TBK1 inhibitor Amlexanox reduced osteoclast number (Fig. 3F) without affecting osteoblast number (Fig. 3G). Similar effects on osteoclast and osteoblast differentiation were obtained in in vitro experiments using RANKL-stimulated osteoclasts and calvarial osteoblasts. As shown in Figure 4, over-expression of IKKε significantly increased the ability of in the MDA-MB-231-BT to enhance RANKL-induced osteoclast formation, whereas exposure to the IKKε/TBK1 inhibitor Amlexanox inhibited this effect (Fig. 4A, left panels). Consistently, exposure of osteoclast precursors to MDA-MB-231-BT (Fig. S4) or conditioned medium from MDA-MB-231-BT deficient in IKKε or treated with Amlexanox reduced RANKL-induced osteoclast formation in vitro (Fig. 4B-C). Exposure of calvarial osteoblasts to conditioned medium from MDA-MB-231-BT deficient in IKKε or

treated with Amlexanox had no effects on alkaline phosphatase activity (Fig. 4D) or bone nodule formation (Fig. 4E-F). These results demonstrate that expression of IKKε by breast cancer cells contributes to their osteolytic potential without affecting osteoblast number.

**IKKε regulates MDA-MB-231 behaviour by IRF3/NFκB activation**

IRF3/NFκB signalling plays a key role in IKKε/TBK1 activity in primary cancer cells [6, 34]. To examine whether IRF3/NFκB signalling contributes to the osteotropic behaviour of breast cancer cells in our models, we knocked down, TBK1, IKKβ, p65 or IRF3 in MDA-MB-231-BT cells overexpressing IKKε (Fig. S5A-B), and we studied the metastatic and osteolytic behaviour of these cells in vitro. First, we observed that knockdown of TBK1, IKKβ, IRF3 or p65 in these cells significantly inhibited IKKε-driven cell migration (Fig. 5A) and reduced the ability of these cells to support RANKL-induced osteoclast formation in bone marrow cell line cultures (Fig. 5B). In light of this, analysis of protein level of human cytokines and chemokines in conditioned medium revealed that IKKε overexpression in the MDA-MB-231-BT cells is associated with upregulation by greater than 2-fold of 11 secreted proteins (Fig. 5C). The identified proteins are common tumour-derived factors that have previously been found to be involved in the regulation of inflammation, angiogenesis, and tumorigenesis and osteolysis including lipocalin-2 (LPN2) and interleukins –1 (IL-1), -6 (IL-6) and -8 (IL-8) (Fig. 5D), Furthermore, only silencing of p65 reduced the expression of these proteins to below basal levels of MDA-MB-231 mock cells, implicating the NFκB signalling pathway in the regulation of IKKε/TBK1 driven osteotropic behaviour of the model described. Consistent with this, we also show that knockdown of IKKβ or p65 – but not IRF3 or TBK1 - reduced the viability of MDA-MB-231 cells over-expressing IKKε (Fig. S5C).

**Amlexanox reduces breast cancer osteolytic metastasis**

Previous research has implicated IKKε in the initiation of primary tumour growth[6, 8]. However, the effect of IKKε inhibition on the initiation and development of breast cancer osteolytic metastasis has not been investigated. As shown in Figure 6, administration of Amlexanox (35mg/kg/day) in mice reduced the homing and growth of the syngeneic 4T1 breast cancer cells as evidenced by in vivo bioluminescent imaging (Fig. 6A-C).

**Combination of Amlexanox and Docetaxel reduces the growth and metastasis of mammary tumour cells**

Previous studies have implicated members of the NFκB pathway in increased resistance to various chemotherapies[9, 35-37]. Of all the chemotherapeutic agents tested (Docetaxel, Cyclophosphamide, Doxorubicin, Rapamycin, 5-Fluorouracil and Tamoxifen; data not

shown), only Docetaxel significantly enhanced IKKε expression in parental MDA-MB-231 breast cancer cells (Fig. S6A) and treatment of these cells with Amlexanox enhanced the anti-tumour effects of Docetaxel by up to 50% (Fig. S6B-D, $p<0.05$). With this in mind, we next sought to determine whether Amlexanox sensitizes the parental and syngeneic 4T1 breast cancer cells to Docetaxel in mammary orthotopic mouse model. As shown in Figure 6D, combined administration of Amlexanox (35mg/kg/day) and Docetaxel (15mg/kg/week) reduced mammary tumour growth (59.7%, $p<0.001$) in mice inoculated with the 4T1-luc2 cells. After removal of tumours, Amlexanox and Docetaxel reduced incidence of overt metastases (Fig. 7A-C) and improved survival (Fig. 7D, 1.82 fold, $p<0.005$) ($p<0.05$), when compared to vehicle or individual treatments.

**Discussion**

Although previous studies have implicated both the canonical and non-canonical IKK/NFκB pathways in breast cancer[38, 39], IKKε is the only IKK family member that has been deemed a breast cancer oncogene[6]. Here we provide evidence for a previously unknown role of IKKε in the regulation of the initiation and progression of breast cancer osteolytic metastasis. The evidence for this comes from the experiments that showed that cancer-specific knockdown of IKKε in the human triple negative MDA-MB-231 breast cancer cells reduced cell growth and motility in vitro and both cancer-specific knockdown and pharmacological inhibition of IKKε suppressed the growth of human MDA-MB-231 and mouse 4T1 cells in the long bones of mice, thereby indicating an inhibitory effects on skeletal tumour burden.

Previous studies reported that the activation of IKK/NFκB signalling plays a critical role in breast cancer bone metastasis and osteolysis[39-42]. Our current data compliment these studies and demonstrate that systemic administration of the pharmacological inhibitor of IKKε/TBK1 suppressed the ability of the syngeneic 4T1 breast cancer cells to home, grow and to enhance osteoclast number and osteolysis in mice. These data also expand on previous observations and suggest that the osteoprotective effect associated with IKKε/TBK1 inhibition is mediated not only by the effects on breast cancer cells but also by inhibition of IKKε/TBK1 in bone cells of the tumour microenvironment in the skeleton. Evidence for this comes from the in vivo observations that pharmacological inhibition of IKKε/TBK1 reduced osteoclast number and the in vitro data that showed that IKKε/TBK1 in either breast cancer cells or osteoclast precursors significantly reduced the ability to support osteoclast formation.

Pro-inflammatory mediators secreted by breast cancer and bone cells in the tumour microenvironment in the skeleton are essential for the development of breast cancer related bone disease[1-5, 43]. The activation of NFκB and IRF signal transduction pathways by tumour-derived factors is know to regulate osteoclastogenesis, skeletal tumour growth and bone remodelling and bone loss[9-15, 17, 44]. Mechanistic studies in human breast cancer cell and osteoclast cultures revealed that the IKKε-driven pro-migratory and pro-osteolytic capacities in our models were abrogated by siRNA-mediated knockdown of TBK1, IRF3 or NFκB, consistent with the involvement of both the IRF and NFκB signalling pathways[10, 11]. Collectively, these results provide a novel insight into the role of IKKε/TBK1 signalling in the reciprocal interaction between bone and breast cancer cells in the skeleton. When combined with previous studies that reported the role of IKKε in breast cancer initiation and development[8, 9] – these results implicate cancer and bone-cell autonomous expression of IKKε in primary and secondary breast cancer to bone.

The IKKε/TBK1 inhibitor Amlexanox enhanced the cytotoxic effects of a panel of chemotherapeutic agents including Docetaxel (Fig. S6 and data not shown), and continuous exposure of human breast cancer cells to Docetaxel enhanced IKKε. Consistent with this we have demonstrated that combined administration of Amlexanox and Docetaxel exerted a synergistic effect characterised by significant reduction of primary tumour growth, overt metastases coupled with approximately a 2-fold improvement in metastasis-free survival when compared to vehicle or either treatment as a single-agent. It is important to note that Amlexanox - as a single agent – also reduced primary tumour growth in these experiments but it had no effect on overt metastases or enhance metastasis-free survival of mice following removal of the primary tumour. Our interpretation of these results is that IKKε/TBK1 inhibition is sufficient to reduce mammary and skeletal tumour growth and osteolysis but failed to reduce general metastasis in the 4T1 models described.

Although the work presented here indicates that IKKε inhibition may be of value in the reduction of breast cancer-induced osteolysis, our studies were restricted to the MDA-MB-231 and 4T1 in vivo models. It is also important to note that administration of Amlexanox alone or in combination with Docetaxel had no significant effect on the frequency of bone metastasis (Fig. 5C) and the expression of TBK1 – but not IKKε – is associated with higher risk for bone metastasis in the dataset tested (Fig. S2). Therefore, further studies will be required to determine if inhibition of IKKE, TBK1 or both are effective in reducing the development and progression of bone metastases.

In summary, the results of the present study demonstrate that IKKε plays an essential role in the regulation of primary and skeletal tumour growth, osteolysis and enhanced bone cell activity associated with advanced breast cancer (Figure S7). These results offer new insight into the role of the NFκB and IRF signalling pathways in the crosstalk between breast cancer cells and bone cells of the tumour microenvironment, and provide evidence to show that disruption of IKKε/TBK1, alone or in combination with clinically relevant chemotherapies such as Taxanes, may have potential therapeutic efficacy in advanced triple negative breast cancer. The potential benefit of combined administration of IKKε/TBK1 inhibitors and anti-resorptive agents such as bisphosphonates and Denosumab in models of in breast cancer bone metastasis needs to be explored. For that, further in vivo studies are warranted.

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**Conflict of interest**

The authors declare no conflict of interest.

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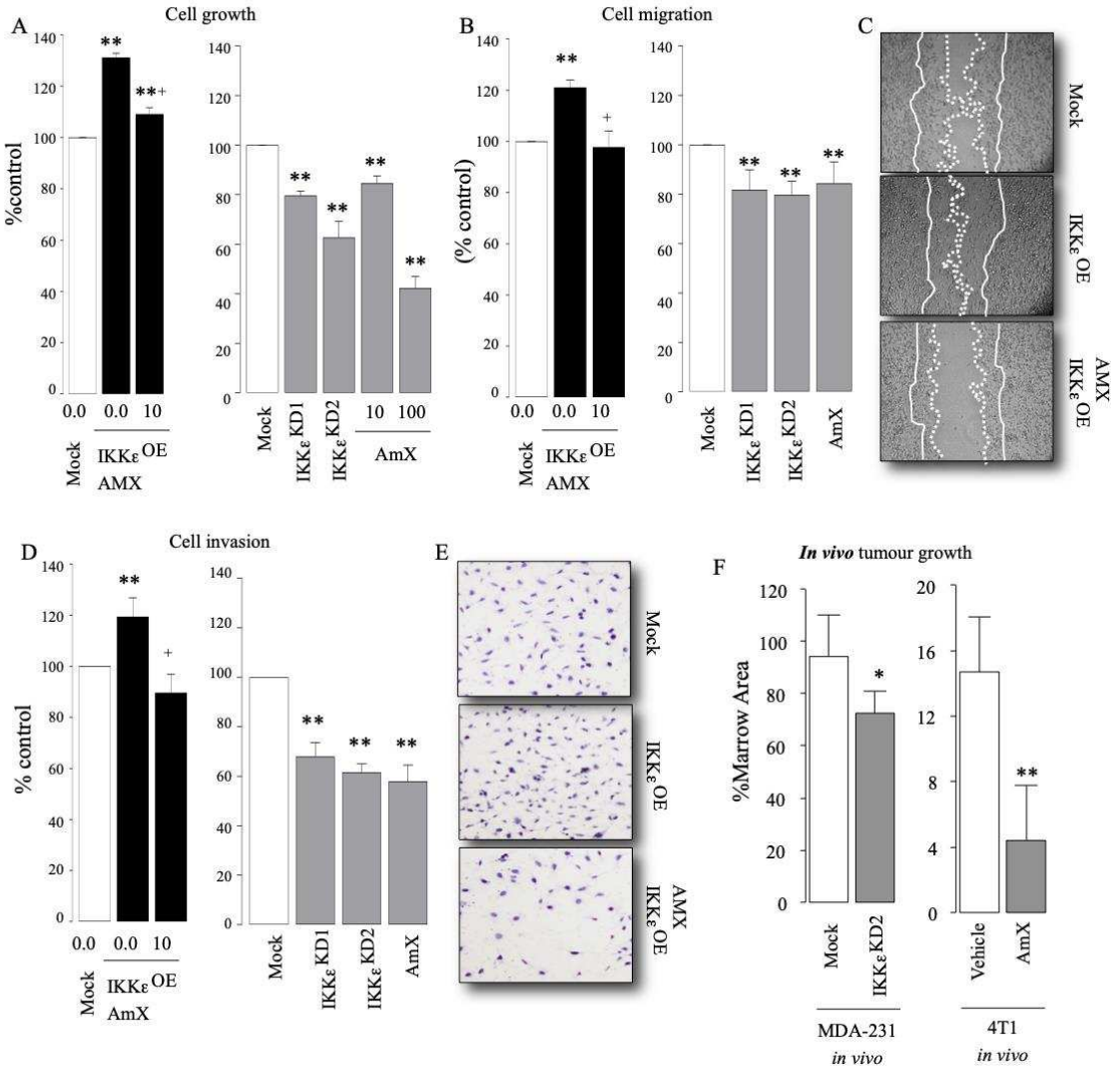
Tables

**Table 1. Effects of Amlexanox on the viability of a panel of ER+ and ER- breast cancer cell lines with different metastatic abilities to metastasise to the skeleton.**

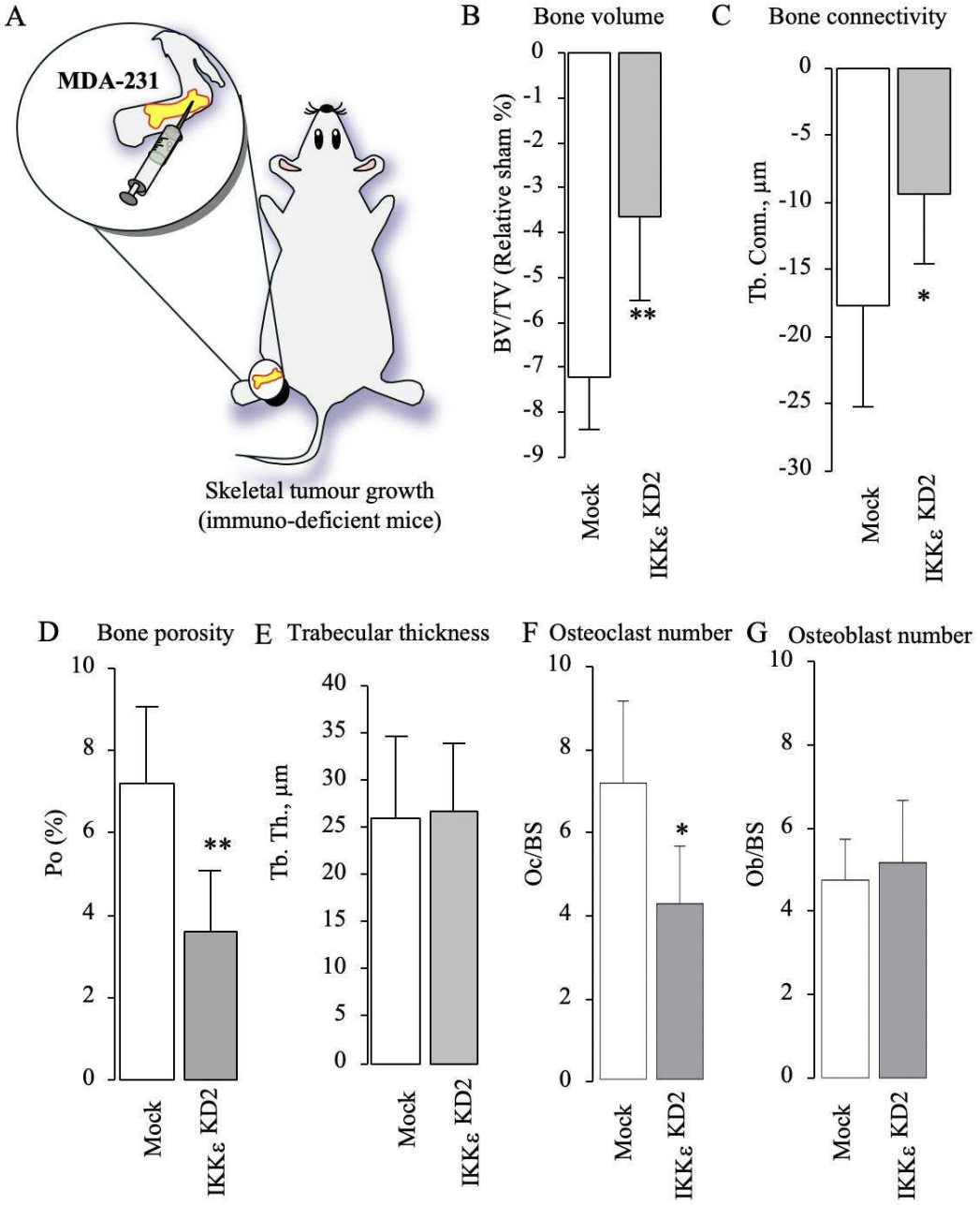
| Receptor Status | Breast Cancer Cell line | $IC_{50}$ (μM ±SD) |
|---|---|---|
| ER+ | MCF7 | 159.6 ± 1.4 |
| | T47D | 330.4 ± 1.6 |
| | ZR-75-1 | 142.6 ± 1.3 |
| ER- | SKBR3 | 98.9 ± 1.2 |
| | MDA-MB-468 | 101.9 ± 1.2 |
| | MDA-MB-231 | 108.1 ± 1.1 |
| | MDA-MB-BT1 | 73.4 ± 2.5 |
| | 4T1-Luc2 | 30.0 ± 1.1 |

Cell viability was measured after 48 hour of continuous exposure to Amlexanox 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 3 independent experiments. BT denotes osteotropic cells.

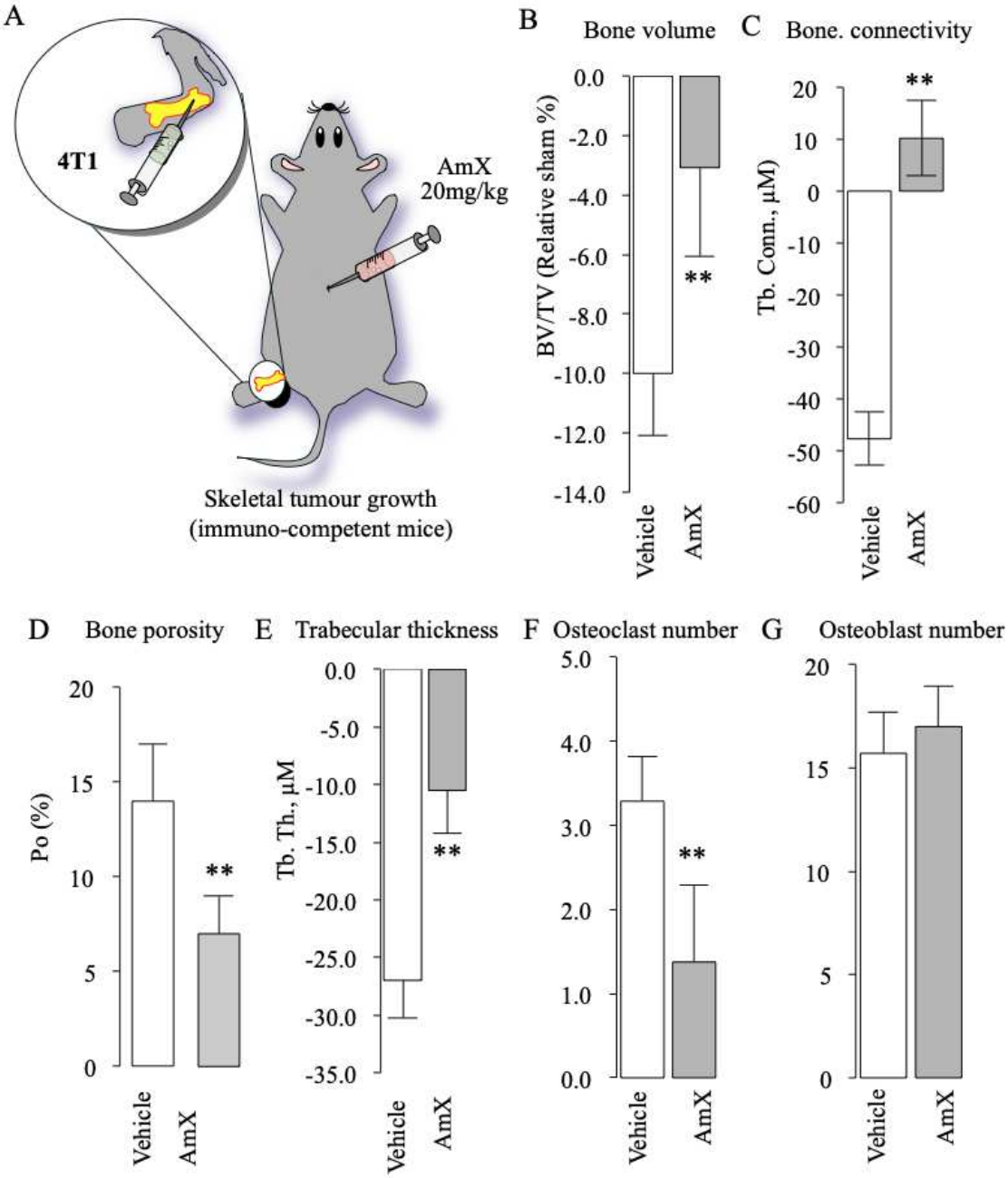
**Figure 1.**


A
Cell growth
%control
Mock
IKKε OE
AMX
AmX
B
Cell migration
(% control)
C
Mock
IKKε OE
AMX
IKKε OE
D
Cell invasion
% control
E
F
*In vivo* tumour growth
%Marrow Area
MDA-231
*in vivo*
4T1
*in vivo*
Vehicle

# Figure 2.


A
MDA-231
Skeletal tumour growth
(immuno-deficient mice)
B
Bone volume
BV/TV (Relative sham %)
0
-1
-2
-3
-4
-5
-6
-7
-8
-9
**
Mock
IKKε KD2
C
Bone connectivity
Tb. Conn., µm
0
-5
-10
-15
-20
-25
-30
*
Mock
IKKε KD2
D
Bone porosity
Po (%)
10
8
6
4
2
0
**
Mock
IKKε KD2
E
Trabecular thickness
Tb. Th., µm
40
35
30
25
20
15
10
5
0
Mock
IKKε KD2
F
Osteoclast number
Oc/BS
10
8
6
4
2
0
*
Mock
IKKε KD2
G
Osteoblast number
Ob/BS
10
8
6
4
2
0
Mock
IKKε KD2

# Figure 3.


A
4T1
AmX
20mg/kg
Skeletal tumour growth
(immuno-competent mice)
B
Bone volume
BV/TV (Relative sham %)
0.0
-2.0
-4.0
-6.0
-8.0
-10.0
-12.0
-14.0
**
Vehicle
AmX
C
Bone. connectivity
Tb. Conn., µM)
20
10
0
-10
-20
-30
-40
-50
-60
**
Vehicle
AmX
D
Bone porosity
Po (%)
20
15
10
5
0
**
Vehicle
AmX
E
Trabecular thickness
Tb. Th., µM
0.0
-5.0
-10.0
-15.0
-20.0
-25.0
-30.0
-35.0
**
Vehicle
AmX
F
Osteoclast number
5.0
4.0
3.0
2.0
1.0
0.0
**
Vehicle
AmX
G
Osteoblast number
20
15
10
5
0
Vehicle
AmX

# Figure 4.

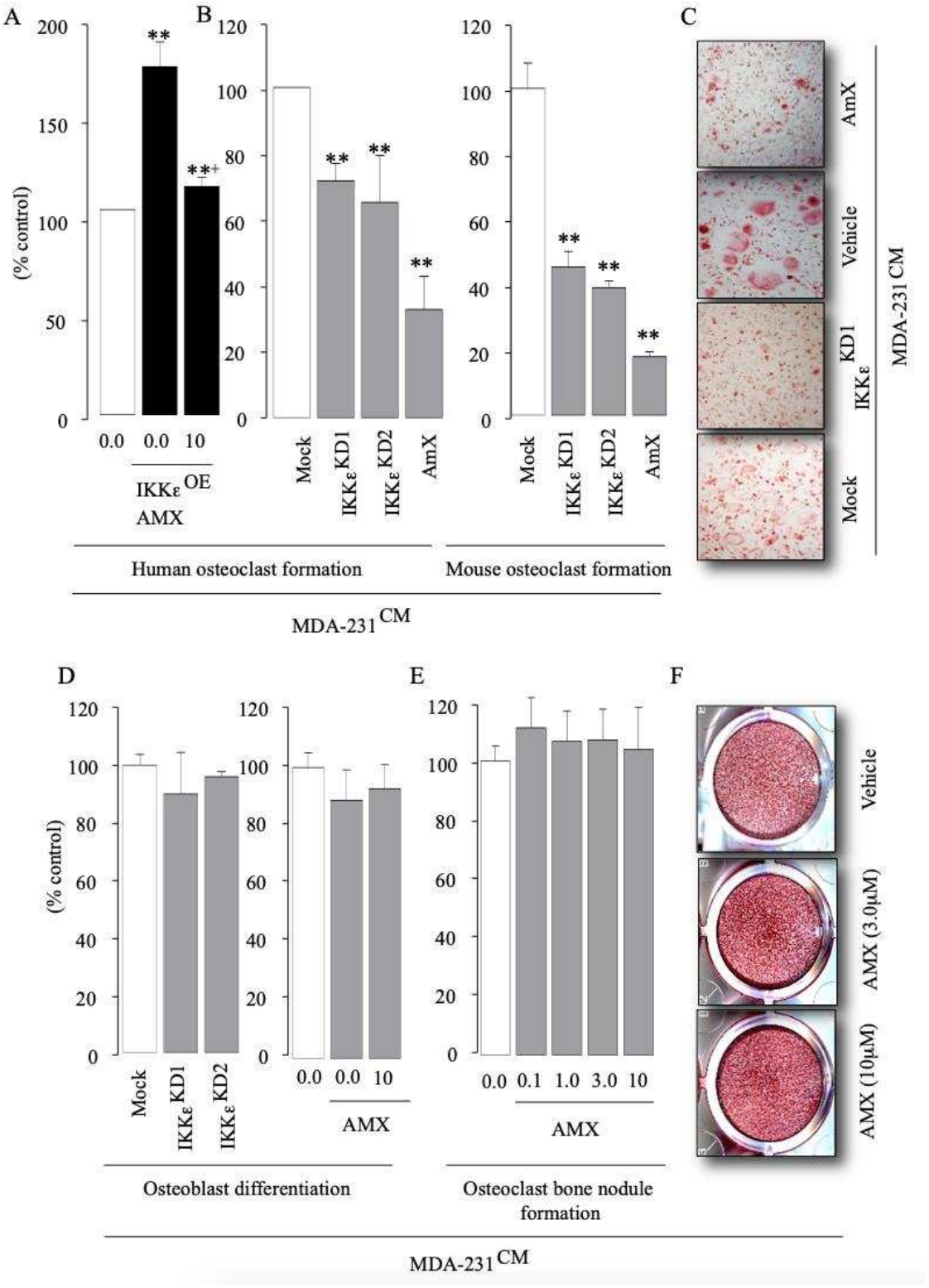

A
B
C
(% control)
IKKε OE
AMX
Mock
IKKε KD1
IKKε KD2
AmX
Human osteoclast formation
Mouse osteoclast formation
MDA-231 CM
Vehicle
KD1
D
E
F
Osteoblast differentiation
Osteoclast bone nodule formation
AMX (3.0μM)
AMX (10μM)

**Figure 5.**

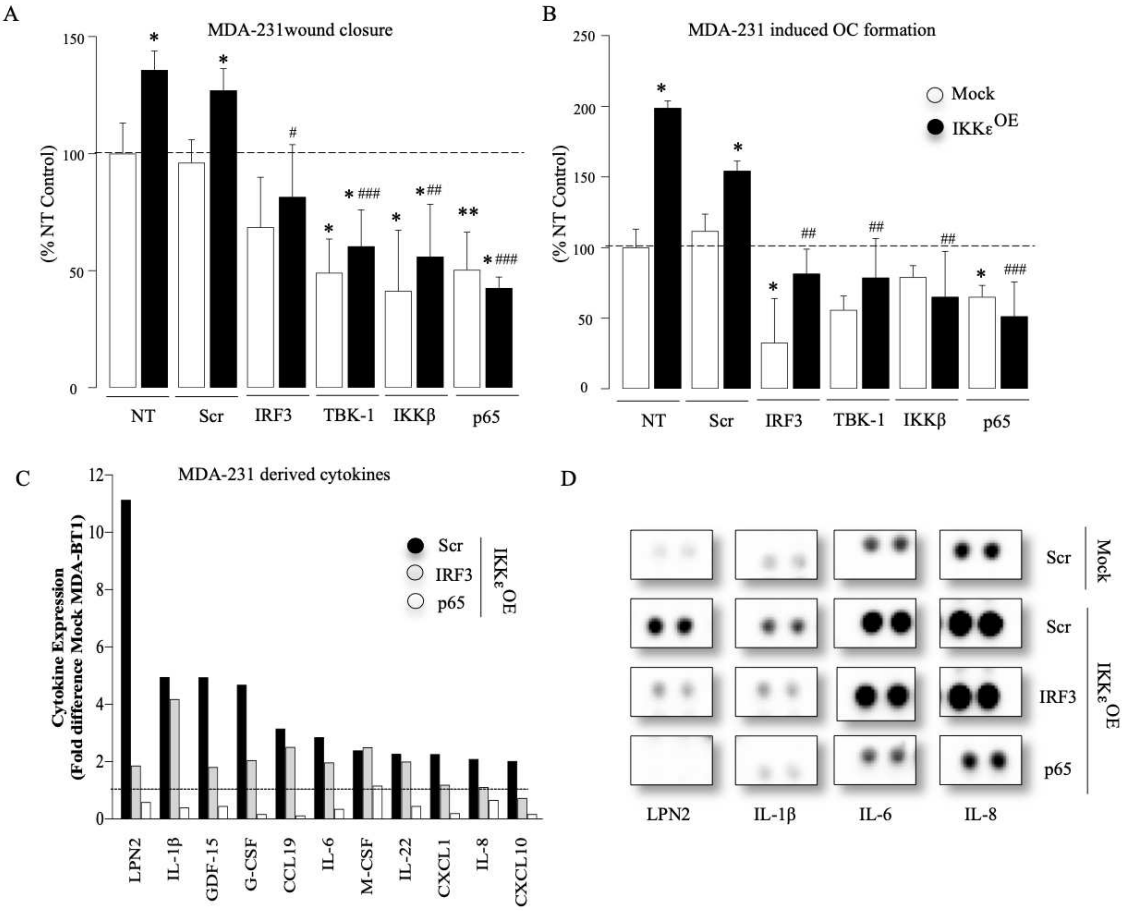
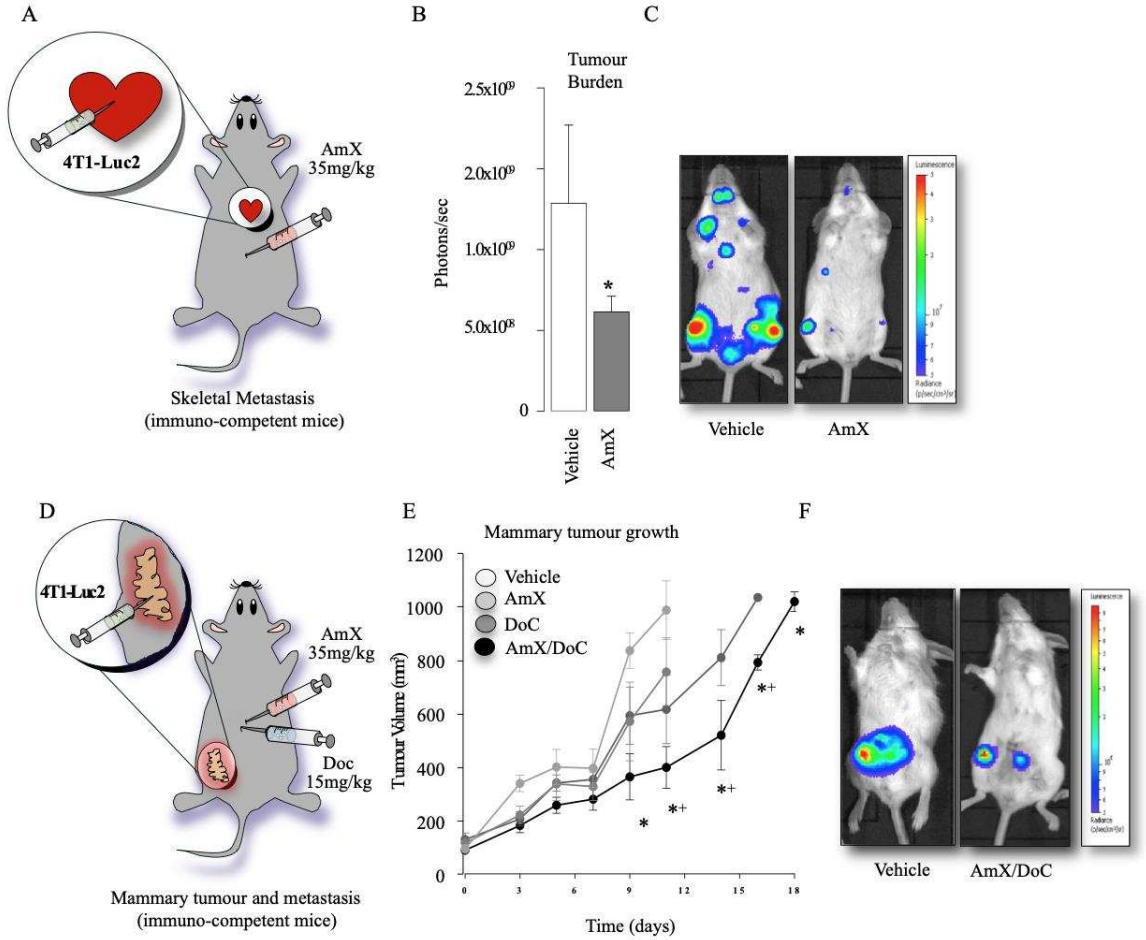
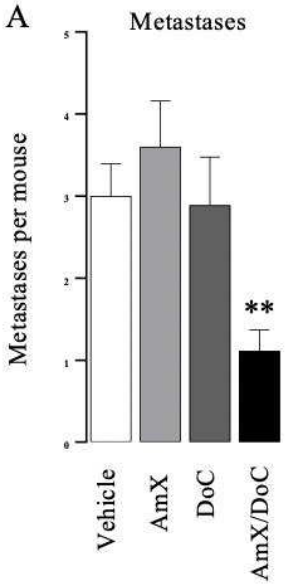

A
MDA-231wound closure
(% NT Control)
NT
Scr
IRF3
TBK-1
IKKβ
p65
B
MDA-231 induced OC formation
Mock
IKKε OE
C
MDA-231 derived cytokines
Cytokine Expression
(Fold difference Mock MDA-BT1)
Scr
IRF3
p65
IKKε OE
LPN2
IL-1β
GDF-15
G-CSF
CCL19
IL-6
M-CSF
IL-22
CXCL1
IL-8
CXCL10
D
Mock
Scr
IKKε OE
IRF3
p65
LPN2
IL-1β
IL-6
IL-8

**Figure 6.**

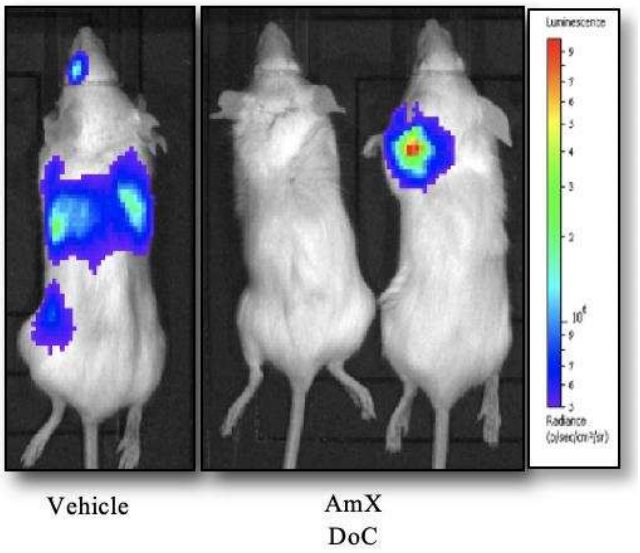

A
4T1-Luc2
AmX
35mg/kg
Skeletal Metastasis
(immuno-competent mice)
B
Tumour
Burden
2.5x10⁹
2.0x10⁹
1.0x10⁹
5.0x10⁸
0
Photons/sec
*
Vehicle
AmX
C
Vehicle
AmX
D
4T1-Luc2
AmX
35mg/kg
Doc
15mg/kg
Mammary tumour and metastasis
(immuno-competent mice)
E
Mammary tumour growth
Vehicle
AmX
DoC
AmX/DoC
1200
1000
800
600
400
200
0
Tumour Volume (mm³)
0
3
6
9
12
15
18
Time (days)
*
*+
F
Vehicle
AmX/DoC

# Figure 7.

A




B




C

| | Frequency of Metastasis | | | |
|---|---|---|---|---|
| | - - | AmX - | - Doc | **AmX Doc** |
| **Lung** | 10/10 | 9/10 | 10/10 | **6/9*** |
| **Bone** | 3/40 | 7/40 | 5/40 | **0/36** |
| **Brain** | 1/10 | 3/10 | 2/10 | **0/9** |
| **Spleen** | 3/10 | 6/10 | 5/10 | **0/9#$** |
| **Visceral** | 9/10 | 9/10 | 6/10 | **4/9*** |

D