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Research Article

# RANKL enhances the effect of an antagonist of inhibitor of apoptosis proteins (cIAPs) in RANK-positive breast cancer cells

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ABSTRACT

*Objective:* Between 65% and 75% of patients with metastatic breast cancer will have decreased 5-year survival and increased morbidity due to cancer relapse in bone. At this stage of disease treatment is palliative, but tumor-targeted compounds could add to the benefits of anti-resorptive agents, improving clinical outcome. Inhibitor-of-apoptosis proteins (IAPs) are overexpressed in many tumors and second mitochondria-derived activator of caspases (Smac) mimetics have been designed to antagonize IAPs. In this work we explored the use of AT-406, a Smac mimetic, to target the tumor compartment of bone metastases.
*Methods:* Effect of AT-406 on cancer cells apoptosis, expression of IAPs and osteogenic potential was addressed *in vitro* using the RANK-positive MDA-MB-231 breast cancer cell line. Effect of AT-406 on osteoclastogenesis was determined by inducing the differentiation of the RAW 264.7 mouse monocytic cell line. Osteoclastogenesis was measured by TRAP staining and TRACP 5b quantification.
*Results:* AT-406 increased apoptosis in MDA-MB-231 breast cancer cells *in vitro*, and activation of RANK-pathway improved this effect. RANKL stimuli induced a strong increase in c-IAP2. AT-406 increased osteoclast differentiation and activity, by up-regulating the osteogenic transcription factor *Nfatc1*, but also increased the apoptosis of mature osteoclasts in the absence of RANKL.
*Conclusions:* Our results indicate that despite the anti-tumoral effect of AT-406, its use in the context of bone metastatic disease needs to be carefully monitored for the induction of increased bone resorption. We also hypothesize that the combination of AT-406 with anti-RANKL directed therapies could have a beneficial effect, especially in RANK-positive tumors.

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## 1. Introduction

Between 65% and 75% of patients with metastatic breast cancer will have decreased 5-year survival and increased morbidity due to cancer relapse in bone [1,2]. Bone metastases phenotype results from an unbalanced bone remodeling, where in a "vicious cycle" the tumor cells stimulate osteoclast activity, and have increased proliferation due to growth factors released from bone matrix upon bone resorption [3].

Currently available therapeutics for bone metastatic disease are palliative and target mainly the bone component, with the use of anti-resorptive agents that will decrease bone osteolysis and associated morbidity. The tumor itself is only indirectly affected by the decrease in growth factors, and tumor burden often progresses. Therefore, the use of anti-tumoral agents is appealing at this stage of disease.

Re-establishing the apoptotic program in tumor cells is a promising strategy for cancer therapy. Inhibitor-of-apoptosis proteins (IAPs) are inhibitors of caspases' pathways that prevent cells from undergoing apoptosis. IAPs are frequently overexpressed in cancer and contribute to tumor cell survival, chemoresistance, disease progression and poor prognosis. IAPs are also potent regulators of nuclear factor k-B (NF-kB) and tumor-necrosis factor (TNF) receptor signaling pathways [4–6].

Cells have natural IAP antagonists, like Smac (second mitochondria-derived activator of caspases)/DIABLO in mammals, that bind to caspases preventing further IAPs binding [7,8]. Based on this, Smac mimetics have been designed to antagonize IAPs and cause cancer cells to undergo apoptosis [9–11]. Also, it has been shown that IAPs are direct activators of tumor cell motility and metastatic genes independently of their roles in cytoprotection, suggesting that IAP antagonists could provide antimetastatic therapies for patients with cancer [12].

AT-406 (formerly SM-406) is a potent and orally bioavailable Smac mimetic and an antagonist of IAPs [11]. This compound binds to XIAP, cIAP1, and cIAP2 proteins, induces rapid degradation of IAPs, and inhibits cancer cell growth in various human cancer cell lines. AT-406 is currently in phase I clinical trial for the treatment of human cancer (NCT01078649).

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In this work we explored the use of AT-406 to target the tumor compartment of bone metastases, using an *in vitro* model of breast cancer. Since cIAP1 and cIAP2 were originally identified through their ability to interact directly with TNF-family [13], and MDA-MB-231 breast cancer cells express the receptor activator of NF-κB (RANK) [14–18] and are sensitive to RANK ligand (RANKL) that induces the activation of RANK–TRAF-dependent pathways [14–16,19,20], we also explored the activation of RANKL–RANK pathway in these cells and its significance on AT-406 effect. Given that IAPs also play a role in osteoclastogenesis, the effect of IAP antagonists on osteoclasts needs to be addressed if AT-406 is used in the context of bone metastatic disease. Recently, it was demonstrated that IAPs negatively regulate osteoclastogenesis by inhibiting *NFATc1* mRNA expression [21]. It was also shown that IAP antagonists induce high turnover osteoporosis characterized by enhanced osteoclast and osteoblast activities, in mice, and may increase tumor growth and metastasis in the bone by stabilizing NF-κB inducing kinase (NIK) and activating the alternative NF-κB pathway in osteoclasts [22]. Therefore we also addressed the effects of AT-406 in osteoclastogenesis and osteoclast activity *in vitro*.

## 2. Methods

### 2.1. Cell culture, cell proliferation and cell apoptosis assays

MCF 10A (CRL-10317, ATCC) normal mammary epithelial cells were cultured in mammary epithelial cell basal medium (MEGM) containing 0.4% bovine pituitary extract (BPE), 0.1% human epidermal growth factor (hEGF), 0.1% hydrocortisone, 0.1% gentamicin/amphotericin B (GA-1000), 0.1% insulin, and 100 ng/ml cholera toxin. MDA-MB-231 (HTB-26, ATCC) breast cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were maintained at 37 °C with 5% $CO_2$ in a humidified chamber.

MDA-MB-231 conditioned media (CM) were obtained as follows: $2 \times 10^4$ cells were plated in 35 mm plates and allowed to adhere for 12 h. Cells were treated with 1 μM AT-406, 2.5 μg/ml RANKL, or AT-406+RANKL, for 24 h. Medium was collected, centrifuged, and supernatant aliquots were stored at −80 °C.

For proliferation and apoptosis assays, MDA-MB-231 cells were plated in 96-well plates ($3 \times 10^3$ cells/well, assayed in triplicate) and incubated for 24 h, in the presence or absence of RANKL (2.5 μg/ml). Cells were treated with different concentrations of AT-406 (Ascenta; prepared in DMSO), or 0.1% DMSO for control, and incubated for 24–48 h. Cell proliferation was analyzed by alamarBlue assay (Invitrogen). Medium was replaced by 200 μl fresh medium with 1:10 alamarBlue. After a 1 h incubation at 37 °C with 5% $CO_2$, fluorescence was measured at 560/590 nm (excitation/emission). Cell apoptosis was analyzed by measuring caspase-3 and -7 activities using the Caspase-Glo 3/7 kit (Promega), according to manufacturer's instructions.

### 2.2. Osteoclastogenesis assays

RAW 264.7 mouse monocytic cells were cultured in DMEM containing 10% heat inactivated FBS and 1% penicillin/streptomycin. For differentiation cells were plated in 96-well plates (950 cells/well) in DMEM containing 10% heat inactivated FBS, 1% penicillin/streptomycin, and 100 ng/ml RANKL (day 0). Medium supplemented with AT-406 (10, 100, or 1000 nM) or 60% MDA-MB-231 CM (obtained as described above) was changed at day 3. Osteoclastogenesis was measured at day 5 by counting the number of TRAP positive multinucleated cells/well after TRAP staining (Sigma-Aldrich) and TRACP 5b quantification by solid phase immunofixed enzyme activity assay (IDS). For osteoclast survival assay, differentiated osteoclasts were deprived from RANKL at day 5 and incubated with 100 nM AT-406 for 24 h under standard conditions, before TRAP staining or TRACP 5b quantification. TRAP5b activity rate and TRAP+ multinucleated cells are expressed as a percentage of TRAP5b activity or TRAP+ multinucleated cells, respectively, without RANKL deprivation at day 6.

### 2.3. RT-qPCR

$2 \times 10^4$ MDA-MB-231 cells were plated in 35 mm plates, allowed to adhere for 12 h, and treated with 2.5 μg/ml RANKL for 60 min. $5 \times 10^4$ RAW 264.7 mouse monocytic cells were plated in 35 mm plates, in differentiation medium as described above, supplemented with 1 μM AT-406 for 72 h. Samples were collected at different time points. Cells were rinsed in 1× PBS and RNeasy mini spin column (Qiagen) were used for total RNA isolation according to manufacturer's instructions. DNase I (Promega) treatment was performed to remove genomic DNA contamination and RNA concentration and purity were assessed in a NanoDrop spectrophotometer (Thermo Fisher Scientific). RNA (500 ng per sample) was reverse transcribed using Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions with anchored oligo(dT) primer. cDNAs were amplified by semi-quantitative real-time PCR (qPCR) using Power SYBR Green PCR Master Mix (Applied Biosystems) and specific primers for human *RANK* (PPH01102B), human *cIAP1* (PPH07279B), human *cIAP2* (PPH00326B), human *XIAP* (PPH00323A), and human *GADPH* (PPH00150E) (SABiosciences, Quiagen); and mouse *Birc2*, mouse *Birc3*, mouse *Nfatc1*, and mouse *β-actin* [21], in a Rotor Gene 6000 (Corbett, Quiagen), for 40 cycles (95 °C for 15 s, 55 °C for 40 s, and 72 °C for 30 s) after an initial incubation at 95 °C for 10 min. Reactions were performed in triplicate. Target gene expression was normalized against the housekeeping gene *GAPDH* (human) or *β-actin* (mouse), using the mean value of the three replicates.

### 2.4. Western blot

For Western blot analysis of protein expression, cells were cultured as described above. Neutralized RANKL was obtained by incubating 2.5 μg/ml RANKL with an anti-RANKL antibody (2.5 μg/ml; R&D), in culture medium, at 37 °C for 1 h. Cells were washed once with PBS, lysed in 200 μl 2× SDS-loading buffer, and heated to 95 °C for 10 min. Samples were loaded onto a 10% polyacrylamide gel and electrophoresis was performed using a Mini-PROTEAN Tetra cell (BioRad). Proteins were transferred onto a Protran BA85 nitrocellulose membrane (Whatman) using a Mini-PROTEAN Tetra Cell transfer system (BioRad). Membranes were blocked in PBST, 5% skim milk for 1 h, incubated overnight with the primary antibody and for 2 h with the secondary antibody. Antibody detection was performed using SuperSignal West Pico Chemiluminescent HRP Substrate (Pierce) according to the manufacturer's directions and signal was visualized on radiographic film. Antibodies used include anti-cIAP1 (1E1-1-10, Enzo Life sciences), anti-cIAP-2 (Clone 315304, R&D), and anti-NFATc1 (H-110, Santa Cruz); β-actin (Abcam) was used as control. Secondary antibodies conjugated to peroxidase were purchased from Santa Cruz.

### 2.5. Statistical analysis

Data were analyzed with the use of Graphpad Prism v5.0 software. Samples were analyzed in triplicate for apoptosis, osteoclastogenesis-related assays and RT-qPCR. Statistics were analyzed by one-way ANOVA and Newman–Keuls or Dunnett's

multiple comparison tests. Results are expressed as mean ± SEM and $p < 0.05$ was considered significant.

## 3. Results

### 3.1. Effect of AT-406 in MDA-MB-231 breast cancer cells

To assess the effect of the IAP antagonist AT-406 on the breast cancer cell line MDA-MB-231 we measured apoptosis upon treatment with different doses of AT-406 (Fig. 1a). AT-406 increased apoptosis as early as 24 h after treatment at the highest concentration ($p < 0.001$). The effect was shown to be dose-dependent at 48 h, when also the lower concentrations of AT-406 have a significant effect ($p < 0.05$ to $p < 0.001$). An increase in apoptosis was observed in control cells treated with 0.1% DMSO, 48 h after treatment, due to cell proliferation and confluence. As expected, AT-406 induced the degradation of c-IAP1 and c-IAP2 (Fig. 1b).

It was previously reported by us and other authors that MDA-MB-231 cells express RANK [14]; therefore we aimed to determine if the activation of RANKL–RANK pathway could alter the effect of AT-406 on apoptosis induction. RANKL treatment of RANK expressing cells (Fig. 1c) increased the effect of AT-406 on apoptosis, by 24% at the highest concentration ($p < 0.001$) (Fig. 1d).

To explore the mechanism by which RANKL improved the AT-406 effect we analyzed whether RANKL affected the expression of IAPs. RANKL induced a strong increase in *c-IAP2* mRNA (up to 4-fold, $p < 0.001$), a modest increase in *c-IAP1* mRNA, and had no effect on *XIAP* (Fig. 2a). Increase in c-IAPs was also shown at the protein level (Fig. 2b).

### 3.2. Effect of AT-406 in osteoclast differentiation in vitro

The possibility to use AT-406 for the therapy of bone metastatic disease, and the fact that IAPs play a role in osteoclast differentiation and survival, led us to address a possible effect of AT-406 in osteoclastogenesis. RAW264.7 murine monocytic cells were cultured in osteoclast differentiation medium in the presence of AT-406 at different doses. AT-406 increased osteoclast differentiation and activity in a dose-dependent manner ($p < 0.05$ to $p < 0.001$) (Fig. 3a–c).

Next we aimed to determine the effect of AT-406 on the survival of mature osteoclasts. To address this question, mature osteoclasts were deprived from RANKL for 24 h, and treated with 100 nM AT-406. AT-406 significantly decreased TRAP5b activity and osteoclast number ($p < 0.01$ and $p < 0.05$, respectively) (Fig. 3d and e).

To investigate the means by which AT-406 could increase osteoclast differentiation, we analyzed its effect on the expression of IAPs and the osteogenic transcription factor Nfatc1. Although AT-406 had no effect on IAPs expression at the mRNA level, *c-IAP2* was shown to be down-regulated during osteoclast differentiation (Fig. 4a). However, AT-406 up-regulated *Nfatc1* in the early stages of differentiation ($p < 0.05$) (Fig. 4a). After 48 h, the levels of *Nfatc1* were independent of AT-406. These results were confirmed at the protein level (Fig. 4b).

Since MDA-MB-231 breast cancer cells are able to stimulate osteoclastogenesis, we wanted to analyze the effect of AT-406 on the osteogenic potential of these cells. As expected, enrichment of osteoclast differentiation medium with 60% conditioned medium (CM) from MDA-MB-231 cells increased osteoclast differentiation, and this effect was independent of RANKL stimuli to breast cancer cells ($p < 0.001$) (Fig. 5). Our data show that the treatment of

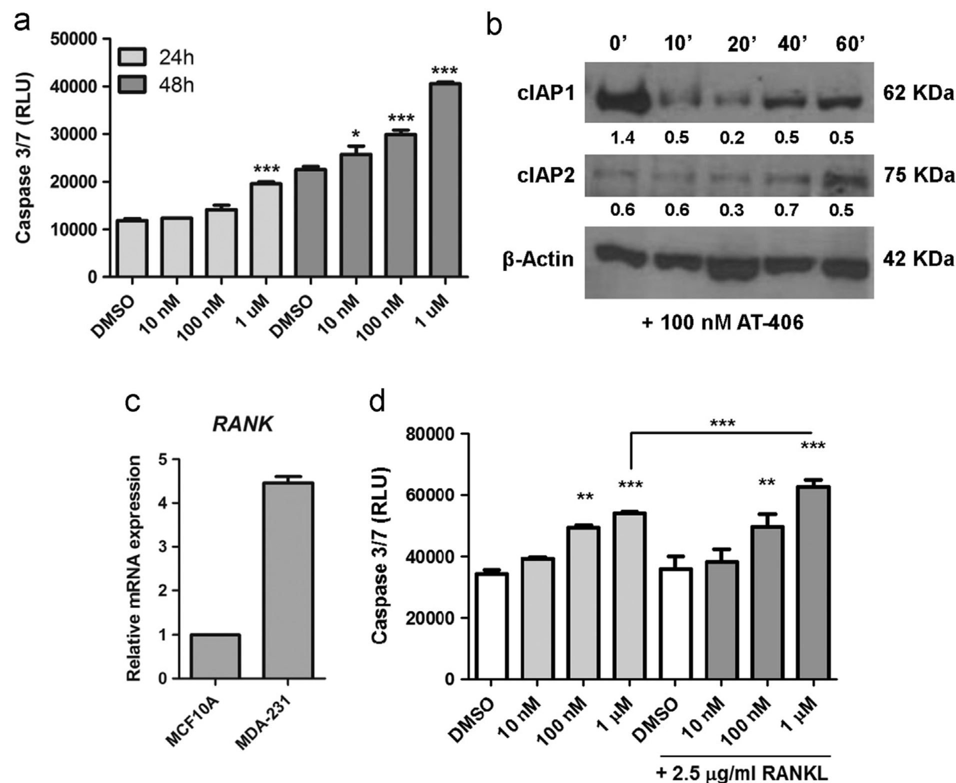


**Fig. 1.** Effect of AT-406 in survival of MDA-MB-231 breast cancer cells. Cell apoptosis was analyzed by measuring caspase-3 and -7 activities. 0.1% DMSO was used as control. MDA-MB-231 cells are sensitive to AT-406 in a dose-dependent manner (a). AT-406 (100 nM) induced the depletion of cIAP1 and 2 as analyzed by Western blot. cIAP1 and cIAP2 protein levels were normalized by densitometry to β-actin levels (b). *RANK* expression was analyzed by RT-qPCR. MDA-MB-231 breast cancer cells show increased expression of RANK (c). Cells were plated for 24 h, in the presence or absence of RANKL (2.5 μg/ml), prior to 48 h treatment with AT-406. RANKL stimulus increased the AT-406-induced apoptosis (d). $n=3$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, using one-way ANOVA with a Newman–Keuls multiple comparison test.

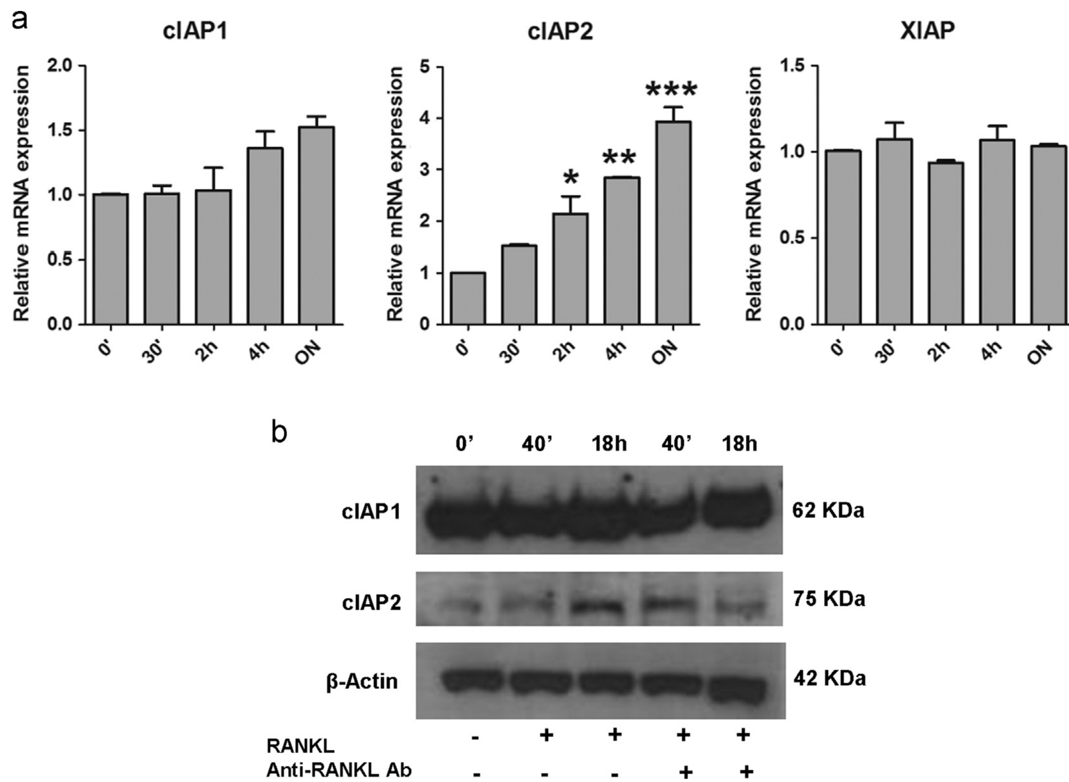


**Fig. 2.** Effect of RANKL in IAPs expression in MDA-MB-231 breast cancer cells. *cIAP1*, *cIAP2* and *XIAP* expression were analyzed by RT-qPCR (a) and Western blot (b). Approx. 18 h post-treatment with RANKL (2.5 μg/ml), MDA-MB-231 breast cancer cells show a modest increase in *cIAP1* expression, no effect on *XIAP* expression, but a highly significant increase in *cIAP2* expression. RANKL neutralization with an anti-RANKL antibody abrogated this effect, as analyzed by Western blot. $n=3$, *$p<0.05$, **$p<0.01$, ***$p<0.005$, using one-way ANOVA with a Dunnett multiple comparison test.

MDA-MB-231 cells with AT-406, with or without RANKL, decreased the effect of the conditioned medium in osteoclastogenesis (Fig. 5).

## 4. Discussion

In this work we explore the potential of AT-406 for the treatment of breast cancer at advanced stages of disease, namely in patients with bone metastases. Although at this stage of disease therapy is only palliative, treatment should not only prevent and decrease the secondary effects of disease, like increased bone resorption, but also decrease tumor burden.

One of the hallmarks of cancer is the ability of cancer cells to evade apoptosis. Inhibition of apoptosis not only enhances the survival of cancer cells but also facilitates their escape from chemotherapy [5]. Therefore, apoptosis regulatory mechanisms are promising targets for anti-cancer therapy.

Inhibitors of apoptosis proteins (IAPs) are able to abrogate death-inducing signals, by direct inhibition of caspases and modulation of NF-κB [23]. IAP antagonists are being tested in the clinical setting for their ability to restore apoptosis in cancer cells.

In this work we confirmed that MDA-MB-231 breast cancer cells are sensitive to AT-406 by restoring apoptosis in this cell line at nanomolar concentrations [11].

It was previously shown by us and other authors that RANK (receptor activator of NF-κB) is expressed *in vitro* by several human breast cancer cell lines, including MDA-MB-231 [15–18,24]. In RANK-positive cells, RANKL (RANK ligand) induces the activation of RANK-dependent pathway, increases migration and invasion, and induces epithelia–mesenchymal transition, favoring an invasive phenotype [14–16,19,20,25]. This fact is extremely relevant in the context of bone metastases since the molecular triad RANK–RANKL–OPG (osteoprotegerin) controls bone remodeling. In bone metastatic disease there is an unbalanced bone remodeling. Tumor cells interact with the bone microenvironment and stimulate osteoclastogenesis by releasing important factors like PTHrP (parathyroid hormone-related protein). This leads to an increased amount of growth factors released from bone matrix that further promote tumor growth, in a "vicious cycle" [3,26]. In this work we show that RANK-positive breast cancer cells show an up-regulation of cIAP-2 when activated by RANKL, and are more sensitive to AT-406, when using non-limiting concentrations of the drug. In fact, it was previously reported that *cIAP2* is one of the most-upregulated genes in PC-3 prostate cancer cell line, which expresses the highest levels of RANK, when stimulated with RANKL [16]. Pharmacokinetics analysis of AT-406 on SCID mice bearing MDA-MB-231 tumors has shown that AT-406 achieves a maximum concentration in plasma between 1.5 μM and 9.9 μM, and a maximum concentration in tumor tissue between 0.5 μM and 18.2 μM, depending on the dose and administration route (intravenous *versus* oral gavage) [11]. Therefore, the described effects of AT-406 concentrations ranging from 100 nM to 1 μM may be further increased *in vivo*.

It was formerly shown in an animal model of breast cancer bone metastases that RANKL inhibition inhibits osteolysis induced by MDA-MB-231 cells, but also enhances the ability of rhApo2L/TRAIL to reduce skeletal tumor burden *in vivo* [27]. Therefore, it may be important to address if AT-406 could be used in combination with an anti-RANKL therapy in the context of bone metastatic disease.

It is known that IAPs also play a role in osteoclast differentiation and survival. Osteoclast differentiation and function are stimulated by activation of the alternative NF-κB pathway [28]. In monocytes, cIAP1 and 2, that have E3 ligase activity, are

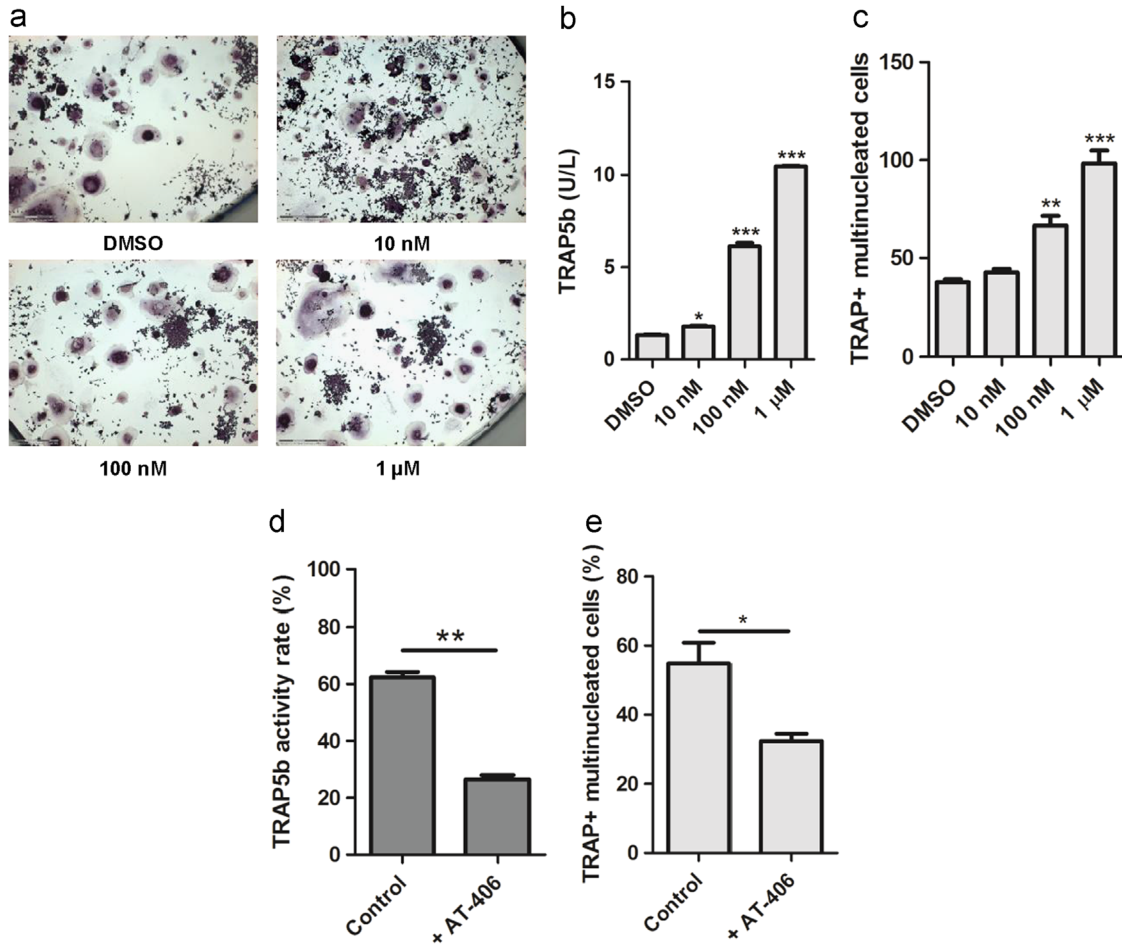


**Fig. 3.** Effect of AT-406 in osteoclastogenesis. Osteoclasts were cultured in differentiation medium, with 0.1% DMSO or AT-406, for 5 days, and visualized after TRAP staining, 50 × (a). Osteoclastogenesis was quantified at day 5 by measuring TRAP5b activity and counting the number of TRAP+ multinucleated cells/well. Osteoclasts differentiated in the presence of AT-406 show increased TRAP5b activity in a dose-dependent manner (b), and increased number of TRAP+ multinucleated cells (c). Mature osteoclasts were deprived from RANKL and cultured with 0.1% DMSO or 100 nM AT-406 for 24 h. TRAP5b activity rate and TRAP+ multinucleated cells/well are expressed as a percentage of TRAP5b activity or TRAP-positive cells, measured for mature osteoclasts without RANKL deprivation. AT-406 increased osteoclasts apoptosis in the absence of RANKL (d and e). $n=3$, *$p<0.05$, **$p<0.01$, ***$p<0.005$, using one-way ANOVA with a Newman–Keuls multiple comparison test.

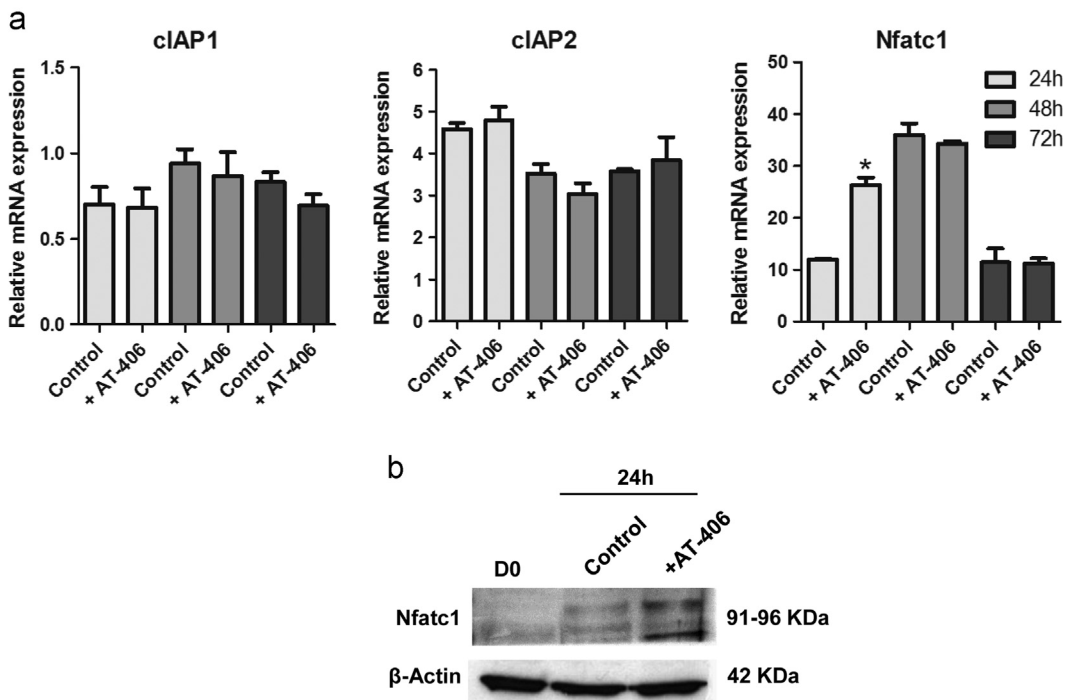


**Fig. 4.** Effect of AT-406 in IAPs and Nfatc1 expression in osteoclasts. *cIAP1*, *cIAP2* and *Nfatc1* expression were analyzed by RT-qPCR (a) and Nfatc1expression was also analyzed by Western blot (b). 24 h post-treatment, RAW264.7 cells treated with AT-406 (1 μM) show a significant increase in *Nfatc1* expression at both mRNA and protein level. $n=3$, *$p<0.05$, **$p<0.01$, *** $p<0.005$, using one-way ANOVA with a Dunnett multiple comparison test.

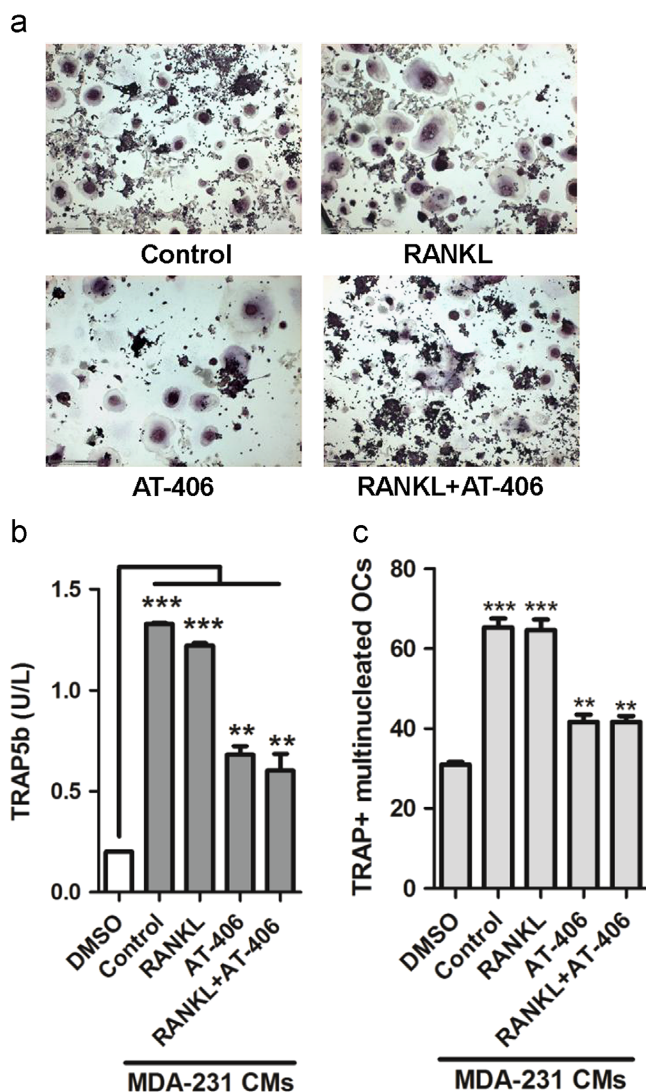


**Fig. 5.** Representative images of osteoclastogenesis assays after TRAP staining, 50× (a). Osteoclastogenesis was quantified by measuring TRAP5b activity and counting the number of TRAP+ multinucleated cells/well. The positive effect of MDA-MB-231 conditioned medium (CM) in stimulating osteoclastogenesis was lower if breast cancer cells were exposed to AT-406 (1 μM). The effect of breast cancer cells CMs was independent of RANKL stimulus (2.5 μg/ml) (a and b). $n=3$, *$p<0.01$, ***$p<0.005$, using one-way ANOVA with a Newman–Keuls multiple comparison test.

recruited by TRAF3 upon binding to NIK (NF-κB inducing kinase), targeting it to the proteosome [29,30]. RANK activation induces release of NIK and activation of the non-canonical NF-κB pathway, leading to the up-regulation of osteogenic transcription factors like Nfatc1 and osteoclast differentiation [29,30]. On the other hand, activation of the canonical NF-κB pathway, via RANKL–RANK–TRAF6, up-regulates cIAP1 and 2 and inhibits cell death [23,31]. This dual effect of IAPs in osteoclastogenesis and osteoclast survival led us to assess the effect of AT-406 on osteoclastogenesis. We found that AT-406 increases osteoclast differentiation in a dose-dependent manner, but independent of IAPs up-regulation. However, AT-406 induced a 2.5-fold increase in Nfactc1, at the early stages of osteoclast differentiation. Upon removal of RANKL mature osteoclasts enter apoptosis, which was increased by AT-406. We also demonstrate that breast cancer cells exposed to AT-406 are less osteogenic. Although the conditioned medium from breast cancer cells treated with AT-406 still increased osteoclastogenesis (3-fold), it was significantly lower than the effect of the conditioned medium from untreated cells. This effect is most probably due to breast cancer derived factors, and not to AT-406, since mouse monocytes were cultured with RANKL, impairing AT-406-induced apoptosis.

Pharmacokinetics analysis of AT-406 on SCID mice bearing MDA-MB-231 tumors has shown that AT-406 achieves a maximum concentration in plasma between 1.5 μM and 9.9 μM, and a maximum concentration in tumor tissue between 0.5 μM and 18.2 μM, depending on the dose and administration route (intravenous *versus* oral gavage) [11]. Therefore, the described effects of AT-406 concentrations ranging from 100 nM to 1 μM may be further increased *in vivo*.

Results from this work indicate that despite the anti-tumoral effect of AT-406, its use in the context of bone metastatic disease needs to be carefully monitored for the induction of increased bone resorption. In this case, the use of AT-406 with an anti-resorptive agent could be beneficial. The combination of AT-406 with bisphosphonates for example could compensate for the increase in bone resorption. We also hypothesize that the combination of AT-406 with anti-RANKL directed therapies could have a beneficial effect, especially in RANK-positive tumors. In these cases, the lack of available RANKL could prevent the induction of metastatic phenotype of cancer cells triggered by RANK activation, decrease the *de novo* osteoclastogenesis, and increase the apoptosis of mature osteoclasts via AT-406.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

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