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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.

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: 2×10^4 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 (3×10^3 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

2×10^4 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. 5×10^4 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