Biochemical Pharmacology 116 (2016) 11–21

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Gallium, a promising candidate to disrupt the vicious cycle driving osteolytic metastases

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ARTICLE INFO

Article history:
Received 6 March 2016
Accepted 30 June 2016
Available online 1 July 2016

Keywords:
Breast cancer
Bone metastases
Gallium
Osteoclastogenesis
MDA-MB-231 cells
MDA-231BO cells

ABSTRACT

Bone metastases of breast cancer typically lead to a severe osteolysis due to an excessive osteoclastic activity. On the other hand, the semi-metallic element gallium (Ga) displays an inhibitory action on osteoclasts, and therefore on bone resorption, as well as antitumour properties. Thus, we explored *in vitro* Ga effects on osteoclastogenesis in an aggressive bone metastatic environment based on the culture of pre-osteoclast RAW 264.7 cells with conditioned medium from metastatic breast tumour cells, *i.e.* the breast tumour cell line model MDA-MB-231 and its bone-seeking clone MDA-231BO. We first observed that Ga dose-dependently inhibited the tumour cells-induced osteoclastic differentiation of RAW 264.7 cells. To mimic a more aggressive environment where pro-tumourigenic factors are released from bone matrix due to osteoclastic resorption, metastatic breast tumour cells were stimulated with TGF-β, a mayor cytokine in bone metastasis vicious cycle. In these conditions, we observed that Ga still inhibited cancer cells-driven osteoclastogenesis. Lastly, we evidenced that Ga affected directly and strongly the proliferation/viability of both cancer cell lines, as well as the expression of major osteolytic factors in MDA-231BO cells.

With the exception of two small scale clinical studies from 1980s, this is the first time that antitumour properties of Ga have been specifically studied in the context of bone metastases. Our data strongly suggest that, through its action against the vicious cycle involving bone cells and tumour cells, Ga represents a relevant and promising candidate for the local treatment of bone metastases in patients with breast cancer.

1. Introduction

Bone tissue is one of the most favoured sites for breast tumour metastases, and bone lesions are preferentially localized in spine and pelvic bone [1]. Patients diagnosed with advanced breast cancer mostly develop bone metastases characterized by severe osteolytic lesions [2–4]. Subsequently, bone metastases lead to severe bone pain, bone instability, fractures, spinal cord compression, hypercalcaemia and bone marrow aplasia [5]. As described in a retrospective study including 617 women with breast cancer, 52% experienced at least one of these skeletal-related events (SREs) [6]. Focussing on spinal metastases, about 20% of cases exhibited neurological deficit symptoms due to: (i) mechanical compression of spinal cord directly by the tumour or caused by the displacement of bone fragments, kyphotic deformity; (ii) vascular insufficiency as a consequence of segmental artery occlusion by tumour emboli, venous thrombus, and spinal cord injury due to oedema caused by internal haemorrhage of spinal cord. Consequently, it appears crucial to propose therapeutic solutions for the treatment of SREs since their development considerably affects patients' quality of life and in some cases increases the risk of death [7].

Bone homoeostasis is regulated through a balanced communication between the major cells forming and remodelling bone tissue, *i.e.* osteoclasts, osteoblasts and osteocytes. Breast tumour cells invasion into bone shifts the balance of bone turnover to favour resorption processes through an enhanced osteoclast activity [8]. Indeed, breast cancer cells promote the formation and the activity

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http://dx.doi.org/10.1016/j.bcp.2016.06.020
0006-2952/

of osteoclasts by secreting osteolytic cytokines including parathyroid hormone-related peptide (PTHLH), tumour necrosis factor-α (TNF-α), interleukins (such as IL-8 and IL-11), as well as matrix metalloproteinases that are involved in bone protein matrix destruction [9]. In addition, breast cancer cells negatively affect osteoblasts in terms of proliferation and activity leading to a decrease of bone formation [3]. Consecutively to the development of breast cancer cells-induced osteolytic lesions, regulatory growth factors stored in large quantities within bone tissue are massively released, thus creating a vicious cycle. Among these factors locally available, transforming growth factor-β (TGF-β) is the most implied in tumour cells activation [3,10–12]. As postulated by Stephen Paget, this microenvironment can be considered as a soil that provides a favourable niche for the growth and the development of tumour cells [13]. In turn, tumour cells produce angiogenic factors such as vascular endothelial growth factor (VEGF) [8,14,15]. This phenomenon stimulates tumour neovascularization, which favours oxygen and nutriments supply, as well as tumour cells dissemination. By contrast, it has been demonstrated that VEGF is crucial for bone repair and regeneration by promoting macrophage recruitment and angiogenic responses during the inflammation process [16,17]. Indeed, administering exogenous VEGF has been shown to increase formation of mineralized bone within bone defects [18,19].

In terms of therapeutic options, the disruption of the vicious cycle described above represents a promising therapeutic strategy. This could be achieved by interfering with the crosstalk between cancer cells and osteoclasts, with a view to reduce osteolytic bone lesions and to limit tumour cells growth at the bone metastatic site. In this attempt, the semi-metallic element gallium (Ga) could be a relevant candidate for the prevention or the treatment of bone metastases due to (i) its chemical affinity for biological hydroxyapatite, (ii) its antiresorptive activity [20] and (iii) its clinical application in few patients with cancer [21]. Indeed, Ga significantly decreases osteoclasts differentiation and activity, without negatively interacting with osteoblasts [22], and through the decrease of RANKL-induced initial expression and auto-amplification of NFAT*c*1, the master gene driving osteoclastogenesis [23]. Moreover, through transferrin-dependent and -independent mechanisms, Ga accumulates at sites of accelerated cellular proliferation including malignant tissue [24]. Once internalized into tumour cells, Ga blocks DNA synthesis and alters plasma membrane permeability and mitochondrial functions, these events leading to cell apoptosis [24,25]. Ga antineoplastic activity has been demonstrated in lymphoma and bladder cancer [21]. Among the few studies related to bone tumours, Warrel et al. evaluated the clinical effects of gallium nitrate on biochemical parameters of accelerated bone turnover in 22 patients with bone metastases [26,27]. They showed that subcutaneous administration of Ga nitrate once daily for 2 weeks reduced several bone parameters such as urinary calcium excretion and hydroxyproline. Interestingly, similar findings were also observed in patients with osteoblastic disease related to prostate carcinoma [28]. These first clinical data strongly suggested that Ga can be effective in reducing morbid skeletal events associated with bone metastases, and that may considerably improve the mobility and finally the quality of life of patients. To the best of our knowledge, there are no recent studies evaluating the potential of gallium compounds in bone metastatic context.

Considering Ga antineoplastic potential together with its inhibitory action on osteoclastogenesis and bone resorption, we were interested in evaluating its effects on (i) one of the most aggressive human breast carcinoma cell line (MDA-MB-231 cells), characterized by the absence of expression of receptor of oestrogen and its high ability to metastasize to bone tissue, and (ii) on its specific bone-seeking clone MDA-231BO cells. Thus, taking advantage of a model established by Guo et al. [29], we designed experiments associating cancer cell lines and RAW 264.7 monocytes. Based on this system, we studied Ga impact on the crosstalk occurring between cancer cells prone to spread to bone (MDA-MB-231 and MDA-231BO cells) and precursor cells able to resorb bone tissue upon their differentiation into osteoclasts (RAW 264.7 cells).

2. Materials and methods

2.1. Materials

Alpha Minimum Essential Medium (α-MEM) and Dulbecco's Modified Eagle's Medium (DMEM), glutamine, antibiotics (penicillin: 100 units/mL and streptomycin: 100 μg/mL), Phosphate Buffered Saline (PBS) and trypsin/EDTA were obtained from Gibco (Paisley, UK) and Trypan blue from Invitrogen (Paisley, UK). Hyclone serum for cells culture was obtained from Thermo Scientific (Braunschweig, Germany). Ga nitrate, TGF-β1, dimethyl sulphoxide (DMSO), sodium dodecyl sulphate (SDS), hydrochloric acid (HCl), TRAP staining kit (cat. no. 386) and 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) assay were provided by Sigma (Saint Quentin Fallavier, France). Mouse effector GST-RANKL was produced as previously described [30], and a GST protein was produced and purified using the same protocol and was used as a control. qRT-PCR experiments were performed using: NucleoSpin RNA II kit provided by Macherey-Nagel (Duren, Germany); RT Mix containing primers, oligo dTs, RT buffer and RT enzyme were purchased from Invitrogen Corp. (Paisley, UK); nuclease-free water (Amidon, USA); MasterMix from Applied Biosystems (Foster City, USA). Enzyme-linked immunosorbent assay (ELISA) kits for *in vitro* dosage of MMP-9 (SEA553Hu) and TGFBR1 (SEA397Hu) were provided by Cloud-Clone Corporation (Euromedex, Strasbourg, France).

2.2. Cell culture

The mouse monocyte cell line RAW 264.7 was obtained from ATCC (Ref. # TIB-71; LGC Standards, Molsheim, France). Cells were cultured in DMEM containing 5% Hyclone serum and 1% penicillin/streptomycin. For osteoclast differentiation experiments, RAW 264.7 cells were seeded at 5000 cells/cm^2 in α-MEM containing 5% Hyclone serum and effectors were added immediately. RANKL (Receptor Activator of Nuclear Factor-B Ligand) was used at 20 nM. Cells were cultured for four days with a renewal of the medium at day 2.

The oestrogen-independent human breast adenocarcinoma cell line MDA-MB-231 was obtained from ATCC. Cells were cultured in DMEM medium supplemented with 5% of Hyclone serum and 1% of antibiotics [31].

The bone-seeking MDA-231BO clone established from MDA-MB-231 cell line [32] was kindly provided by Dr. Toshiyuki Yoneda (University of Texas Health Science Center at San Antonio). Cells were cultured in glutamine-supplemented DMEM medium plus 5% Hyclone serum and 1% of antibiotics.

2.3. Preparation of conditioned medium (CM)

MDA-MB-231 and MDA-231BO cells were plated in T-75 culture flasks and cultured as described previously. Conditioned media (CMs) were collected from cells stimulated (+) or not (−) with TGF-β1. Briefly, when cells reached confluence, they were deprived of growth factors by an overnight incubation in the presence of 0.1% serum before a 20 h treatment with 10 ng/ml of TGF-β1 (+) or its vehicle (−, control condition). CMs were then collected, filtrated using a 0.22 μm pore size filter and stored at −20 °C.

2.4. RAW 264.7 cells differentiation in the presence of conditioned medium

For differentiation experiments, RAW 264.7 cells were washed with pre-warmed PBS prior to a 4-day treatment with RANKL. Depending on the conditions, cell cultures were supplemented at the same time with 10% CM from MDA-MB-231 or MDA-231BO cell culture, and gallium at various concentrations.

2.5. Tartrate resistant acid phosphatase (TRAP) staining

RAW 264.7 cells were rinsed gently with pre-warmed PBS before fixation, and TRAP staining was performed using the Acid Phosphatase Leucocyte kit according to the manufacturer's instructions to detect the presence of TRAP-positive cells. Stained cells were observed using a light microscope (Axioplan 2, Zeiss, Germany) and TRAP-positive multinucleated cells containing at least three nuclei were counted as osteoclasts. In addition, the area of TRAP-positive multinucleated cells was determined by analysis in ImageJ software. To further quantify the TRAP staining, DMSO (200 µl per well) was added, and the plate was centrifuged for 15 min at 150 rpm at room temperature in dark. Finally, optical density at 562 nm was read.

2.6. Proliferation assay

Bone metastatic MDA-MB-231 and MDA-231BO cells were seeded at a density of 20,000 cells/cm^2 and treated with 100, 200 and 300 µM Ga during three days. Cellular proliferation was quantified by scoring cells manually after Trypan blue staining. Results were expressed as a mean ± SD of three independent counts.

2.7. Viability assay

Bone metastatic MDA-MB-231 and MDA-231BO cells were seeded at a density of 20,000 cells/cm^2 and treated with 100, 200 and 300 µM Ga during three days. At days 1, 2 and 3 of culture, cells were rinsed two times in phosphate-buffered saline (PBS), and incubated in 0.5 mg/ml of 3-(4.5-dimethylthiazol-2-yl)-2.5-di phenyltetrazolium bromide (MTT) for 1 h in cell incubator. MTT was aspirated delicately and 0.3 ml of lysis buffer (sodium dodecyl sulphate-SDS) 10%, HCl 0.01 N) was added/well and left overnight in dark at room temperature. Supernatants were then centrifuged 2 min at 20,000*g*, and absorbance at 562 nm was measured in 100 µl.

2.8. Culture of bone metastatic MDA-MB-231 and MDA-231BO cells for Ga/TGF-β experiments

For PCR experiments, 1.5×10^6 bone metastatic MDA-MB-231 and MDA-231BO cells were seeded in F-75 flasks. For ELISA experiments, 30,000 cells/cm^2 were seeded into mw24 plates. Regardless the experiments, cells were cultured 4 days, with medium renewal at day 2, in the presence of Ga or vehicle. After an overnight starvation period, cells were treated 20 h with TGF-β1 (10 ng/mL) or vehicle. For cells initially treated with Ga, the treatment was maintained during the starvation period and the TGF-β1 treatment.

2.9. Quantitative real-time PCR

For RNA isolation, cells were lysed and RNA was isolated using NucleoSpin RNA II kit according manufacturer's instructions. RNA quantification and samples purity were determined on NanoDrop 1000 (Thermo Scientific, Courtaboeuf, France).

For reverse transcription, 1 µg of isolated RNA, random primers, StrataScript enzyme, oligo dT and nuclease free water were used. Reaction setup was 25 °C for 10 min, 50 °C for 30 min and 85 °C for 5 min (Thermocycler Eppendorf, Le Pecq, France). qRT-PCR was conducted with 25 ng of cDNA, ROX and SYBER GREEN dyes, gene specific primers, and nuclease free water. Controls consisted in samples without cDNA (NTC) and samples without RT enzyme (NRT). The following temperature profile was used: 40 cycles of 30 s at 95 °C, 1 min at 60 °C and 30 s at 72 °C. Amplification curves were analysed using the MxProV3 software (Stratagene). PCR products with Ct over 35 cycles were considered as undetectable. The delta Ct (dCt) (cycle threshold) method was used to calculate relative expression levels. Cycle thresholds were normalized using Hypoxanthine Phosphoribosyltransferase 1 (*HPRT1*) gene expression. Results are expressed as fold change in gene expression relative to control conditions after normalization.

qPCR primers are listed in Table 1. Gene symbols (abbreviations) are as follows: *CXCR4*, Chemokine (C-X-C Motif) Receptor 4; *HPRT1*, Hypoxanthine Phosphoribosyltransferase 1; *MMP9*, Matrix Metalloproteinase 9; *MMP13*, Matrix Metalloproteinase 13; *PTHLH*, Parathyroid Hormone-Like Hormone; *TGFBR1*, Transforming Growth Factor Beta Receptor 1; *VEGFA*, Vascular Endothelial Growth Factor A, *RANKL*, Receptor Activator of Nuclear Factor-B Ligand; *CTSK*, Cathepsin K; *IL-6*, Interleukin-6; *IL-11*, Interleukin-11; *TNF-α*, Tumour necrosis factor-alpha.

2.10. ELISA dosage

For MMP-9, cell culture supernatants were collected from step 2.8 and centrifuged for 20 min at 1000*g*. For TGFBR1, cells were lysed by following the manufacturer's instructions. All samples were stored at −20 °C. Briefly, MMP-9 and TGFBR1 were measured using a sandwich ELISA technique according to the manufacturer's instructions. Detection assay is based on the horseradish peroxidase colorimetric reaction by adding TMB substrate. Absorbance was read at 450 nm immediately.

Table 1
Human primers for qPCR.

Gene	Forward primer (5′–>3′)	Reverse primer (5′–>3′)	GI
CXCR4	GCAGCAGGTAGCAAAGTGAC	GAAGTGTATATACTGATCCCCTCCA	56790928
CTSK	TGAGGCTTCTCTTGGTGTCCATAC	AAAGGGTGTCATTACTGCGGGG	315075295
HPRT1	TGACCTTGATTTATTTTGCATACC	CGAGCAAGACGTTCAGTCCT	164518913
IL6	ATGTAGCCGCCCCACACA	CCAGTGCCTCTTTGCTGCTT	969812508
IL11	AGTTTCCCCAGACCCTCGG	AATCCAGGTTGTGGTCCCC	391353405
MMP9	CCTGGAGACCTGAGAACCAATC	CCACCCGAGTGTAACCATAGC	74272286
MMP13	ACCAGACTTCACGATGGCATT	CCCAGGAGGAAAAGTATGAG	296010793
PTHLH	TCGAGGTTCAAAGGTTTGCCT	CAGGTTGGAGGCGAGTTGAA	299829203
RANKL	CTCAGCCTTTTGCTCATCTCACT	CCAAGAGGACAGACTCACTTTATGG	197927084
TNF	ATCTTCTCGAACCCCGAGTGA	GGAGCTGCCCCTCAGCTT	395132451
TGFBR1	TCCAACTACTGTAAAGTCATCACC	AAGCACACTGGTCCAGCAAT	170014713
VEGFA	GCAGCTTGAGTTAAACGAACG	GGTTCCCGAAACCCTGAG	71051577

2.11. Statistical analysis

Results are expressed as mean ± standard deviation of three determinations, and are representative of at least three independent experiments. The statistical differences between two independent groups were evaluated using the Mann & Whitney test (bi-directional analysis). Comparative analysis of more than two independent groups was performed using the Kruskal–Wallis test (bi-directional analysis). The differences measured were considered to be statistically significant for $p < 0.05$.

3. Results

3.1. Effect of Ga on metastatic breast tumour cells-induced osteoclast formation

3.1.1. RAW 264.7 cells differentiation in basal condition (Fig. 1)

Our preliminary studies indicated that 10% of conditioned medium (CM) from breast cancer cells significantly increased (by 62%) RANKL-induced osteoclast formation as compared to RAW 264.7 cells treated with RANKL alone during 4 days (data not shown). Based on this result, we used in the present study 10% of CM isolated from MDA-MB-231 and MDA-231BO cells culture (Figs. 1 and 2).

For both cell lines, the concomitant addition of 10% CM and 20 nM RANKL led to a significant increase in the number of multinucleated TRAP+ cells as compared to cells treated with 20 nM RANKL alone (Fig. 1A, #). Moreover, for both cell lines again, TRAP+ cells were bigger and contained more nuclei in the presence of CM (Fig. 1B, #).

We next evaluated Ga impact on CM-induced osteoclast differentiation. As shown in Fig. 1C, a 4-day treatment with Ga reduced the number of mature osteoclasts in a dose-dependent manner. For example, as compared to the untreated conditions, 10 μM Ga inhibited the number of multinucleated TRAP+ cells by 18% and 37% in MDA-MB-231 and MDA-231BO cells respectively. This alteration in the number of mature osteoclast was confirmed by the TRAP dosage performed using cell lysates (Fig. 1D).

3.1.2. RAW 264.7 cells differentiation in TGF-β1-stimulated condition (Fig. 2)

With a view to mimic a more aggressive tumour environment, CMs were prepared from TGF-β1-stimulated MDA-MB-231 and MDA-231BO cells, as described in Section 2. For better clarity and ease of explanation, these conditioned media are cited as CM^{TGFb1}. Similarly to the previous observation, treating cells concomitantly with CM^{TGFb1} and 20 nM RANKL significantly stimulated the formation of TRAP+ multinucleated cells as compared to cells treated with 20 nM RANKL alone (Fig. 2A, #). As depicted in Fig. 2B (both panels), mature osteoclasts were bigger in the presence of CM^{TGFb1} from both cancer cell lines.

We next embarked on experiments to document Ga impact on osteoclastic differentiation in the presence of CM^{TGFb1}. The CM^{TGFb1}-induced formation of mature osteoclast cells was significantly reduced in a dose-dependent manner and the number of multinucleated TRAP+ cells was reduced by 34% (MDA-MB-231) and 56% (MDA-231BO) when cells were treated with 10 μM Ga (Fig. 2C). Regardless of the doses tested, Ga deleterious effects were in accordance with TRAP staining quantification in cellular extracts (Fig 2D). Interestingly, cells in this TGF-β1-containing environment seemed more sensitive to Ga effects as compared to cells cultured in the absence of TGF-β1 (Fig. 1C).

3.2. Direct effect of Ga on metastatic breast tumour cells

3.2.1. Effect of Ga on metastatic breast tumour cells proliferation and viability

To determine whether Ga may also directly affect metastatic breast tumour cells, we first explored Ga impact on the viability and the proliferation of both cellular clones. As compared to cells treated with vehicle, Ga treatment induced a dose-dependent decrease of viability of both MDA-MB 231 and MDA-231BO cells (Fig. 3A). These effects were accompanied by a dose-dependent inhibition of cellular proliferation (Fig. 3B), and as expected, they were more pronounced after 72 h of Ga treatment. Regarding the type of cells, we did not observe any difference of sensibility in response to Ga treatment.

3.2.2. Effect of Ga on critical tumoural marker genes expression

Since both cell lines display similar responses to Ga treatment, and with respect to our clinical objective (i.e. bone metastases), we decided to focus our next experiments exclusively on the bone-seeking clone MDA-231BO.

3.2.2.1. Basal condition (absence of TGF-β). To determine Ga impact on MDA-231BO cells osteolytic potential, we quantified the expression level of major tumour cells-produced pro-osteoclastogenic factors, including PTHLH and RANKL. As shown in Fig. 4, *PTHLH* gene expression was reduced by 71% in the presence of 100 μM Ga. By contrast, 100 μM Ga did not affect the expression of *RANKL* gene. We then focussed on a group of genes encoding proteases involved in the degradation of bone tissue organic matrix (*MMP9*, *MMP13* and *CTSK*). We found that 100 μM Ga significantly diminished the expression of *MMP9* by 69% and *CTSK* by 45%. Although we observed a slight decrease in *MMP13* expression as well, the result was not found to be significant. As *VEGFA* is one of the most representative marker of angiogenesis, we next wanted to decipher whether Ga may alter its expression, and we observed that 100 μM Ga significantly enhanced its expression level by approximately 2.2-fold time. Similarly, *CXCR4* receptor gene expression was increased (3-fold) in the presence of Ga, whereas *TGFBR1* receptor expression was inhibited by 79% as compared to untreated cells. Regarding the expression of several cytokines involved in osteoclastogenesis such as IL-6, IL-11 and TNFL, their expression was considerably reduced in the presence of 100 μM Ga.

Finally, by performing an ELISA assay, we verified whether these modifications of gene expression could be observed at the protein level as well, focusing on TGFR1 since its expression was strongly altered upon Ga treatment. In accordance with our RT-PCR results, we measured a 32% reduction of TGFR1 protein expression.

3.2.2.2. TGF-β stimulated condition (CM^{TGFb1}). As explained previously, TGF-β released from bone matrix plays a pivotal role in bone metastases environment. To our knowledge, this is the first time that the impact of TGF-β on MDA-231BO gene profile is documented using RT-qPCR experiments (Fig. 4). Depending on the gene, different situations were observed. In response to TGF-β stimulation, we observed a significant increase of the expression of genes such as *MMP9*, *MMP13*, *VEGFA* and *CXCR4*. By contrast, gene expression of *PTHLH*, *CTSK*, *TGFBR1* and cytokines including *IL-6*, *IL-11* and *TNFL* tended to decrease. Lastly, *RANKL* gene expression was not impacted by TGF-β treatment.

Under these conditions of TGF-β1 stimulation, Ga treatment still inhibited *PTHLH* gene expression by 33%, *MMP9* by 80%, *MMP13* by 35% and *TGFBR1* by 27%, while *RANKL* expression increased and *CXCR4* and *VEGFA* expression remained significantly

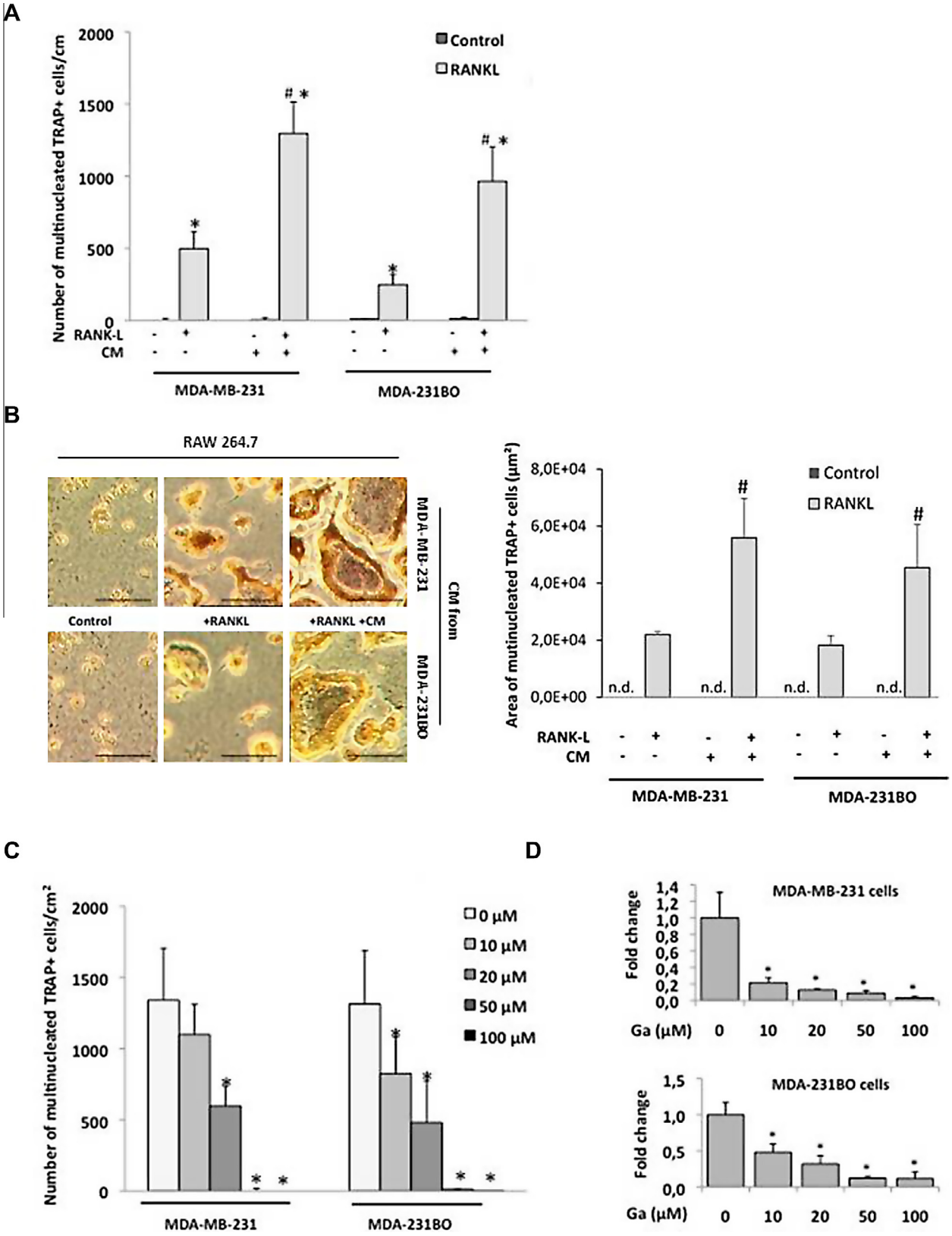

Fig. 1. Ga effect on RAW 264.7 cells differentiation in the presence of conditioned medium from breast tumour cells: basal condition. (A) RAW 264.7 cells were treated during 4 days with 20 nM RANKL (+) or its vehicle (−), concomitantly treated with (+) or without (−) 10% conditioned medium (CM) previously isolated from MDA-MB-231 cells or MDA231-BO cells. Multinucleated TRAP+ cells that contained at least 3 nuclei were considered as mature osteoclast. (B) Left panel: TRAP staining of RAW 264.7 cells treated during 4 days w/o 20 nM RANKL and 10% CM (scale bar = 200 µm). Right panel: ImageJ quantitative analysis of the area of multinucleated TRAP+ cells (µm^2). (C) Ga effect on RAW 264.7 cells differentiation. Cells were cultured for 4 days with increased doses of Ga and in presence of 20 nM RANKL and 10% CM previously isolated from untreated MDA-MB-231 cells and MDA231-BO cells. (D) TRAP dosage of cellular extracts. Results are normalized according to TRAP+ cell number in 0 µM Ga condition. $^{*}p < 0.05$, statistically significant compared to Ga-untreated cells; $^{\#}p < 0.05$, statistically significant compared to cells treated with 20 nM RANKL alone.

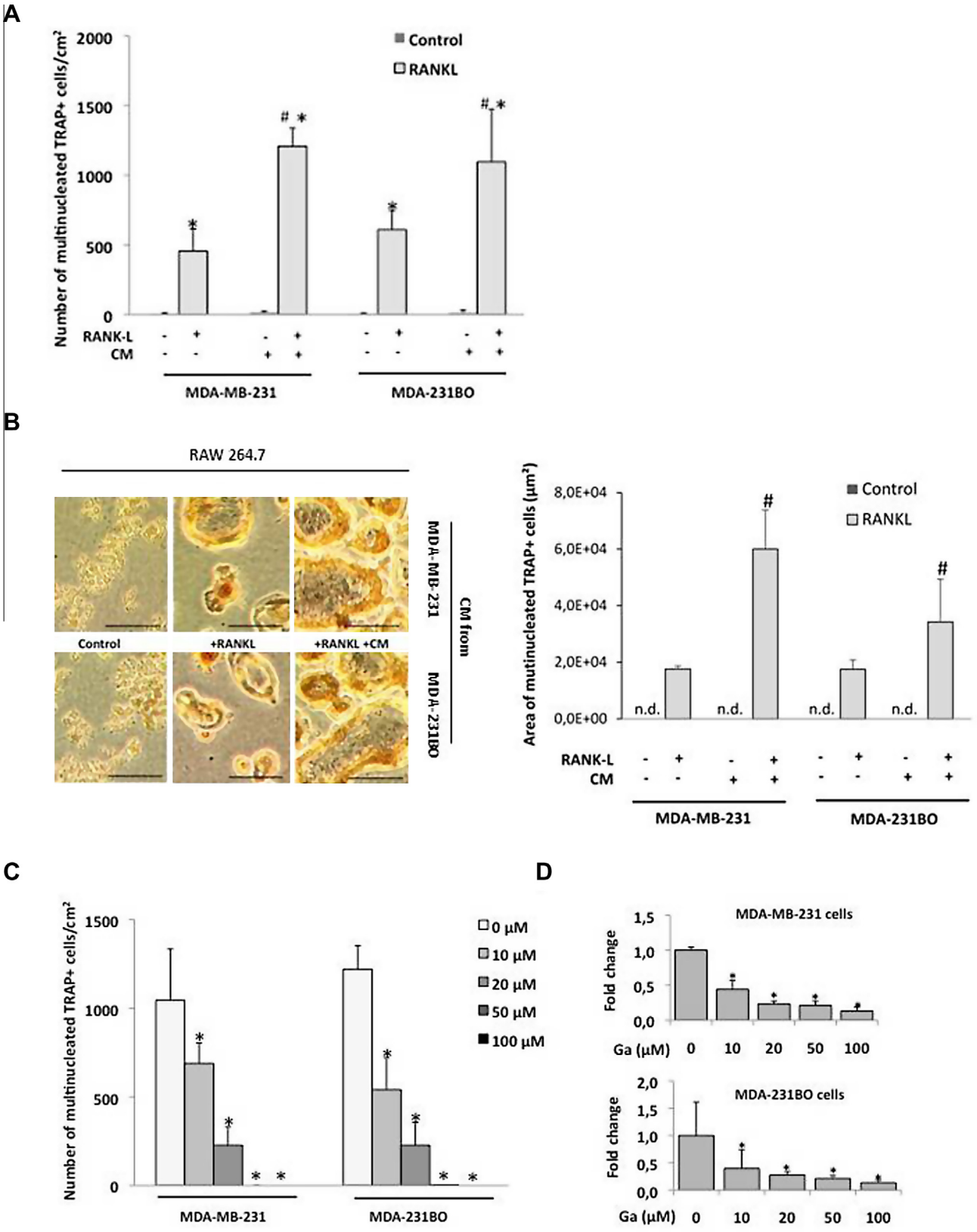

Fig. 2. Ga effect on RAW 264.7 cells differentiation in the presence of conditioned medium from breast tumour cells: TGF-$\beta 1$-stimulated condition (CM^{TGFb1}). (A) RAW 264.7 cells were treated during 4 days with 20 nM RANKL (+) or its vehicle (−), concomitantly treated with (+) or without (−) 10% conditioned medium previously isolated from 10 ng/mL TGF-$\beta 1$-stimulated MDA-MB-231 cells or MDA231-BO cells (CM^{TGFb1}). Multinucleated TRAP+ cells that contained at least 3 nuclei were considered as mature osteoclast. (B) Left panel: TRAP staining of RAW 264.7 cells treated during 4 days w/o 20 nM RANKL and 10% CM (scale bar = 200 µm). Right panel: ImageJ quantitative analysis of the area of the multinucleated TRAP+ cells (μm^2). (C) Ga effect on RAW 264.7 cells differentiation. Cells were cultured for 4 days with increased doses of Ga in the presence of 20 nM RANKL and 10% CM^{TGFb1}. (D) TRAP dosage of cellular extracts. Results are normalized according to TRAP+ cell number in 0 µM Ga condition. $^{*}p < 0.05$, statistically significant compared to untreated cells; $^{\#}p < 0.05$, statistically significant compared to cells treated with 20 nM RANKL alone.

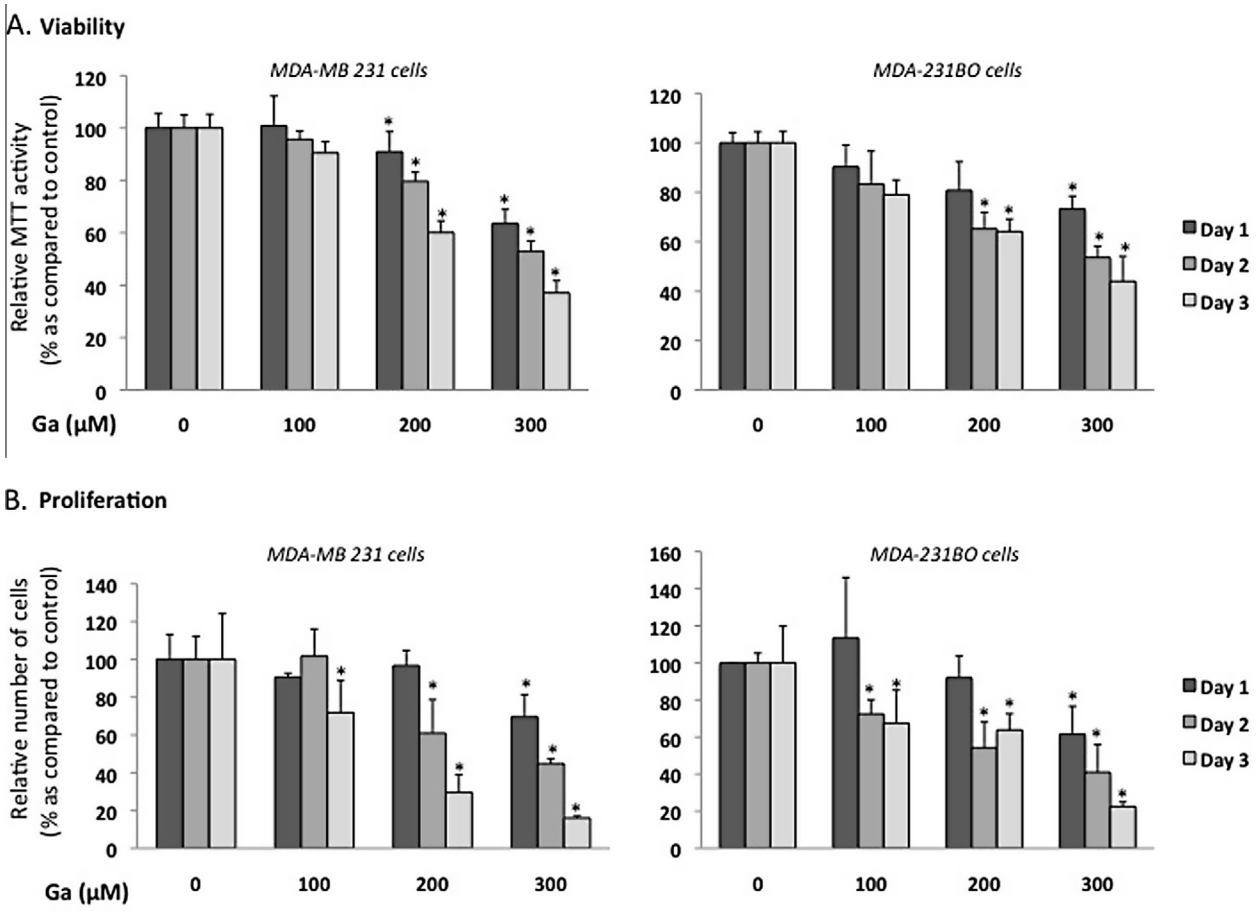

Fig. 3. Ga effect on breast tumour cells viability and proliferation. Effects of increasing doses of Ga on viability (A) and proliferation (B) of MDA-MB-231 and MDA231-BO cells. Cells were treated with 100, 200 and 300 μM Ga or vehicle for 24, 48 and 72 h. Results are normalized according to the untreated condition. $^{*}p < 0.05$, statistically significant compared to untreated cells.

stimulated. By contract, Ga effects on *CTSK* and cytokines (*IL-6*, *IL-11* and *TNFL*) genes were reversed, and we observed an upregulation of their expression.

Finally, since it appeared to be strongly down-regulated, an ELISA assay was conducted to quantify MMP-9 protein content in TGF-β1 stimulated condition. As shown in Fig. 4, MMP-9 protein expression was reduced by 44% in presence of 100 μM Ga.

4. Discussion

Bone metastases of breast cancer typically lead to a severe osteolysis due to an excessive osteoclast activity. Considering its bone affinity, as well as its inhibitory action on bone resorption, we decided to explore the *in vitro* Ga effects on osteoclastogenesis in a bone metastatic environment. Experiments were conducted using the breast tumour cell line model MDA-MB-231, and its bone-seeking clone MDA-231BO. We stimulated RAW 264.7 cells with breast cancer-derived factors by using conditioned medium (CM) isolated from breast tumour cell culture and, similarly to Guo et al. [29], we observed that factors derived from breast cancer cells supported mature osteoclasts formation from RANKL-primed precursors in the absence of supporting cell types. According to Gallet et al., MDA-MB-231 cells release several soluble factors such as macrophage colony-stimulating factor (M-CSF) and parathyroid hormone-related protein (PTHLH), which both act on osteoclastogenesis and mature osteoclast survival [33]. We demonstrated here that, under these conditions, Ga inhibited in a dose-dependent manner the tumour cell-induced formation of mature osteoclasts. With a view to mimic a more aggressive environment where pro-tumourigenic factors are released from bone matrix due to osteoclastic resorption, metastatic breast tumour cells were stimulated with TGF-β. In these conditions, we observed that Ga still inhibited cancer cells-driven osteoclast differentiation. To summarize, our results clearly evidence that Ga may disturb the vicious circle between bone cells and breast cancer cells by preventing the release of pro-tumourigenic factors from bone matrix (Fig. 5).

We were next interested in deciphering whether Ga could impact directly metastatic breast cancer cells; and we focussed our experiments on the bone-seeking clone MDA-231BO. We first confirmed previous results published by Yoneda et al. [32] concerning the greater production of PTHLH by MDA-231BO as compared to the parental MDA-MB-231 clone (data not shown). Further supporting the interest of using this cell clone in experiments related to a bone metastases context, this phenotypic change allows breast cancer cells to survive, proliferate into bone and promote osteoclastic resorption, which in turn leads to the establishment of severe osteolytic bone metastases. Importantly, we observed that Ga inhibited in a dose-dependent manner the proliferation and the viability of these bone metastatic breast tumour cells.

To ascertain whether Ga may also affect the activity of these aggressive cells in terms of angiogenesis, invasion, and osteolysis, we next embarked on quantifying the expression of critical marker genes. We evaluated Ga impact on the major osteolytic marker PTHLH, which is mainly produced by bone metastatic cells. In fact, while 60% of primary breast tumours express PTHLH, 90% of their

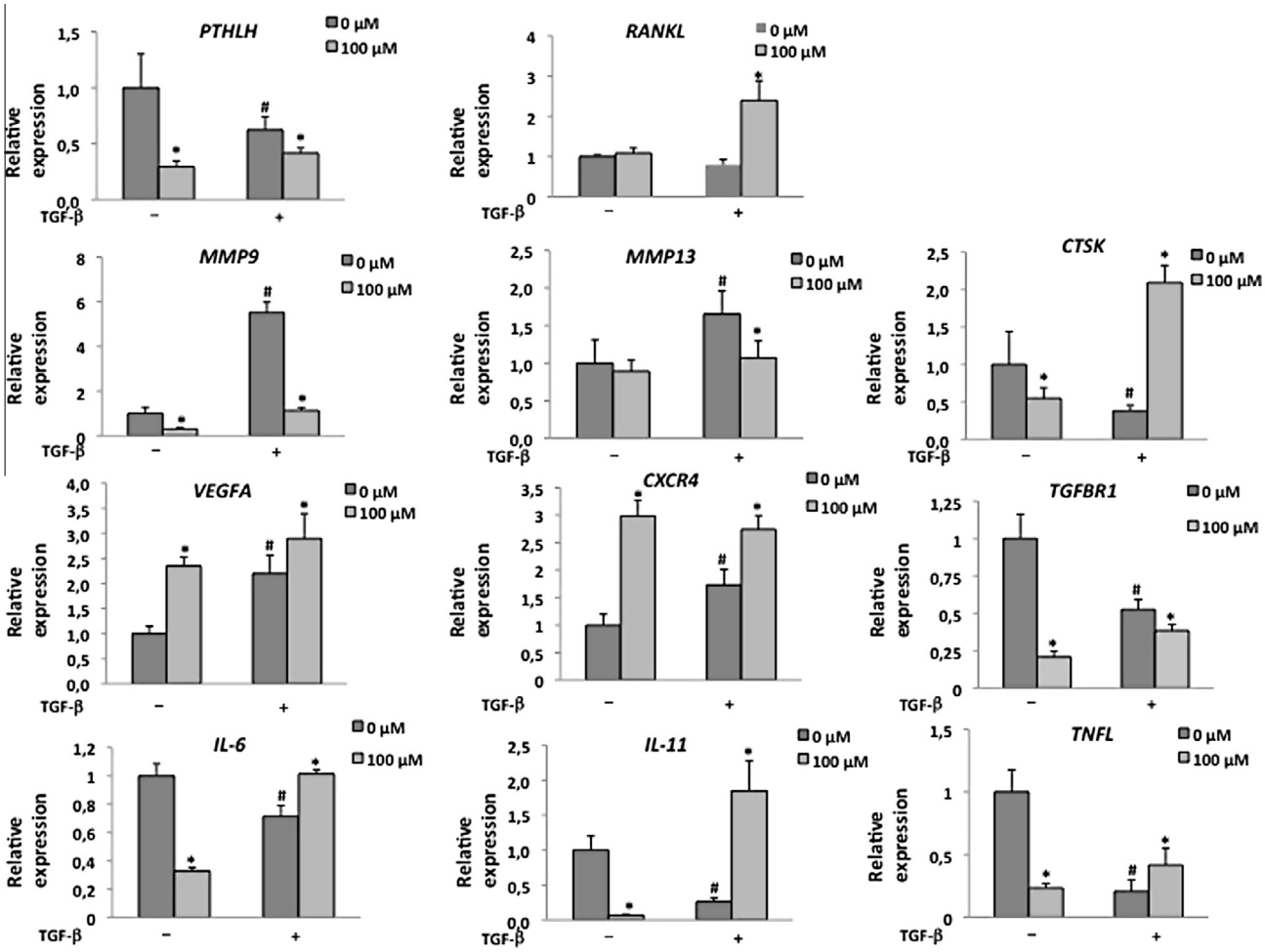

Fig. 4. Ga effect on gene expression in bone-seeking MDA-231BO tumour cells MDA-231BO cells were cultured 4 days in the presence of 100 µM Ga or its vehicle, with medium renewal at day 2, then serum-starved overnight and treated 20 h with TGF-β1 (10 ng/mL) or its vehicle. For cells initially treated with Ga, the treatment was maintained during starvation and TGF-β1 treatment. Results are reported as fold change in gene expression relative to untreated cells (0 µM Ga/-TGF-β1) after normalization against HPRT-1. $^{*}p < 0.05$, statistically significant compared to untreated cells; $^{\#}p < 0.05$, statistically significant compared to untreated and TGF-β1-unstimulated cells.

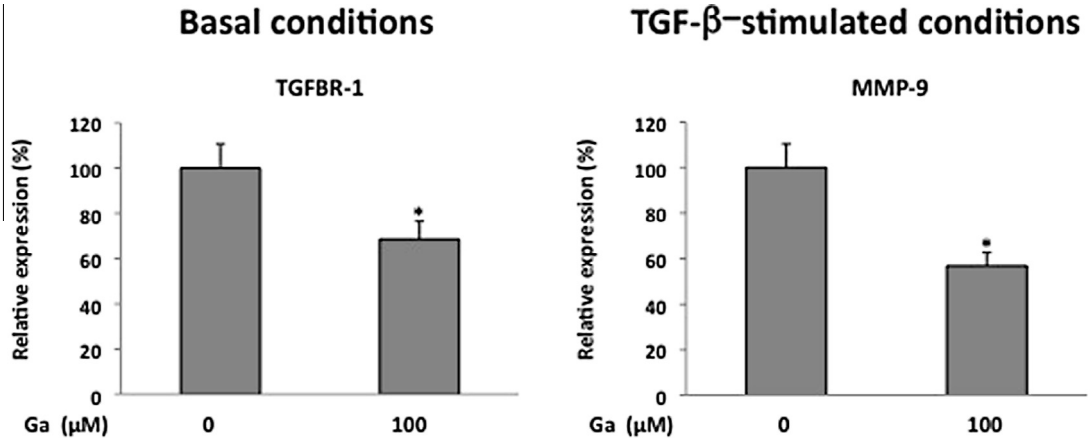

Fig. 5. Ga effect on protein expression in bone-seeking MDA-231BO tumour cells. MDA-231BO cells were cultured 4 days in the presence of 100 µM Ga or its vehicle, with medium renewal at day 2, then serum-starved overnight and treated 20 h with TGF-β1 (10 ng/mL) or its vehicle. For cells initially treated with Ga, the treatment was maintained during starvation and TGF-β1 treatment. Results are normalized according to the untreated condition. $^{*}p < 0.05$, statistically significant compared to untreated cells.

bone metastases display PTHLH expression, compared to 17% when they are located at non-bony sites [34]. As evidenced by our results, Ga may directly impact MDA-231BO cells osteolytic properties through the reduction of *PTHLH* (71%) gene expression in basal conditions. Indeed, PTHLH upregulates the expression of RANKL in bone marrow stromal cells, which in turn stimulates the

differentiation and activation of osteoclasts, hence favouring tumour progression and bone destruction [35]. Consequently, TGF-β that is among the most abundant growth factors stored in bone [36], is released continually into the bone marrow cavity in the active form. In response to TGF-β, increased PTHLH production may be one of the most critical properties for breast cancer cells to accelerate osteolytic bone metastasis [15,37]. Mimicking these conditions, we stimulated MDA-231BO cells with TGF-β, and we found that the expression of *PTHLH* gene was still inhibited by 100 μM Ga. Thus, Ga antiosteolytic activity could be mediated in part through the downregulation of *PTHLH* gene expression by tumoural cells, and in other part by disturbing the RANKL signalling pathways in osteoclasts as described in our previous study [23].

In addition, interleukin-6 (IL-6), interleukin-11 (IL-11) and tumour necrosis factor-alpha (TNF-alpha), which are frequently produced by breast cancer cells, promote osteoclast development and resorption activity at the site of breast cancer bone metastases [38]. We evidenced that Ga blocked the expression of these cytokines in basal condition, while their expression was upregulated in TGF-β-stimulated condition. Importantly, as evidenced by results depicted in Fig. 2, these cytokines up-regulation in TGF-β stimulated conditions was not sufficient on its own to counteract Ga action and to enhance osteoclastogenesis. As a whole, these data strongly support the notion that PTHLH can be considered as a major osteolytic factor, which inhibition takes priority over the upregulation of IL-6, IL-11 and TNF-alpha cytokines.

By hydrolysing components of the extracellular matrix (ECM), Matrix MetalloProteinases (MMPs) are zinc-dependent endopeptidases, which play a pivotal role in the migration, the invasion and the development within bone tissue of breast carcinoma cells [15]. In accordance with this, it has been shown that MMP9 expression was much higher in the MDA-231BO bone-seeking clone as compared to its parental clone MDA-MB-231 cells [32,39,40]. In preliminary experiments, we confirmed that MDA-231BO cells expressed higher level of MMP9 and MMP13 as compared to parental cells (data not shown). We evidence here for MMP9 and MMP13 that Ga is able to decrease their expression by 80% and 35% respectively under TGF-β1 treatment. Among MMPs, we focused our attention on the expression profile of MMP9 since MMP9 has been described to play a crucial role in bone resorption [41]. Moreover increased MMP-9 expression has been correlated with the metastatic potential of many tumours [42], and MMP-9 positivity has been correlated with early recurrence in patients with oestrogen receptor-negative breast cancer [43]. Consequently, by reducing MMP9 expression in both basal and more aggressive conditions (TGF-β treatment), Ga might contribute to prevent the development of massive osteolytic lesions and to improve the prognostic for patients with bone metastases. We were also interested in studying the expression of CTSK by tumour cells as this protease is known to degrade extracellular matrix including bone matrix proteins [44]. CTSK expression was down-regulated in presence of Ga in basal condition whereas its expression was strongly enhanced by Ga treatment under TGF-β conditions. Before concluding that might be prejudicial for patients with aggressive bone metastases, it is relevant to considering that CTSK is mainly produced by osteoclasts as compared to tumour cells. Indeed, immunolocalization of cathepsin K in breast tumour bone metastases revealed that the invading breast cancer cells expressed this protease at a lower intensity than osteoclasts [44]. Subsequently, bone degradation due to the contribution of CTSK secretion by tumour cells might be not so important compared to Ga action on osteoclastic differentiation and activity.

Anti-angiogenic therapies have raised major interest and promises in cancer therapy in general, and more specifically for the treatment of hyper-vascularized skeletal metastases that are observed in patients with renal carcinoma. Indeed, cutting the blood supply to a tumour is a pertinent approach. Ga induces *VEGFA* expression regardless of the condition (basal or TGF-β-stimulated), and this could be considered as prejudicial for the patient. Nevertheless, taking into account that surgical management of bone metastases requires bone reconstruction after tumour resection, a sufficient vascularization is essential to favour resorption/substitution of the implanted bone substitutes leading to the formation of new bone tissue. As demonstrated by Hu et al., VEGF plays critical role in bone repair since angiogenesis and osteogenesis are highly coupled [17]. In fact, VEGF is required for the early angiogenesis response and macrophage infiltration during the initial inflammation phase. Maintaining a high local concentration of VEGF might promote angiogenesis, osteoblast differentiation and consequently bone formation at the repair site. Interestingly, mesenchymal stem cells from aged mice and from osteoporotic mice produced lesser VEGF as compared to the healthy mice [45,46]. Similarly, associations between VEGF production and spinal bone mineral density in postmenopausal women have been evidenced [47]. Based on these clinical observations, local VEGF supplementation may be useful in treating the impaired bone healing as a consequence of bone metastases. Thus, by increasing VEGF content in bone tissue, Ga might positively contribute to bone repair following metastases resection.

Similarly to anti-angiogenic therapies, targeting TGF-β has also been used for cancer treatment [12,48]. Given that TGF-β has been described to stimulate angiogenesis and to suppress immune surveillance of tumour cells [11,49], "anti-TGF-β" strategies may be efficient to reduce bone invasion by tumour cells. Accordingly, different classes of TGF-β inhibitors, including monoclonal neutralizing TGF-β antibodies, have been tested in clinical trials for the treatment of bone metastases [10,50]. Considering this, we wanted to decipher whether Ga could disturb TGF-β signalling, and we observed that Ga action could be beneficial since it down regulated *TGFBR1* gene expression. Interestingly, this result is in accordance with the decrease of *PTHLH* expression we observed. Indeed, it has been evidenced by Kakonen et al. that blocking TGF-β signalling pathway through the inhibition of Smad and mitogen-activated protein kinase reduced TGF-β-stimulated PTHLH secretion [51].

Lastly, cellular interactions between breast tumour cells and the bone microenvironment are important to support the establishment of bone metastases. Among chemokine receptors expressed by osteotropic cancer cells to mediate their adhesion to endothelium, chemokine (C-X-C motif) receptor 4 (CXCR4), the receptor for SDF-1 (stromal cell derived factor-1, also named CXCL12) has been shown to play a pivotal role in tumour cell homing to bone [52,53]. Ga significantly increased CXCR4 expression in both conditions thus possibly promoting the anchorage of metastatic cells in the bone niche. By modulating the expression of CXCR4, Ga would favour tumour cells retention within CXCL12-rich bone microenvironment, this preventing their dissemination towards other organs. And as an additional benefit, keeping metastatic cells in close contact with Ga, which displays a strong affinity for bone mineral, should favour Ga antitumour actions on cancer cells that we described in this report, and which are summarized in Fig. 6.

Based on the observation that for some markers Ga treatment provoked a different response depending on the culture conditions, stimulated or not with TGF-β, it is difficult to conclude about their possible *in vivo* implications in the bone metastatic environment. Nevertheless, the exhibited inhibition of tumour cell proliferation and viability, coupled to a decrease in osteoclastogenesis, promote the hypothesis that Ga may disrupt the vicious cycle by interfering with the cross talk between breast cancer cells and osteoclasts, which consequently may reduce osteolytic bone lesions and affect tumour cells growth in bone metastases. Thus, Ga represents a

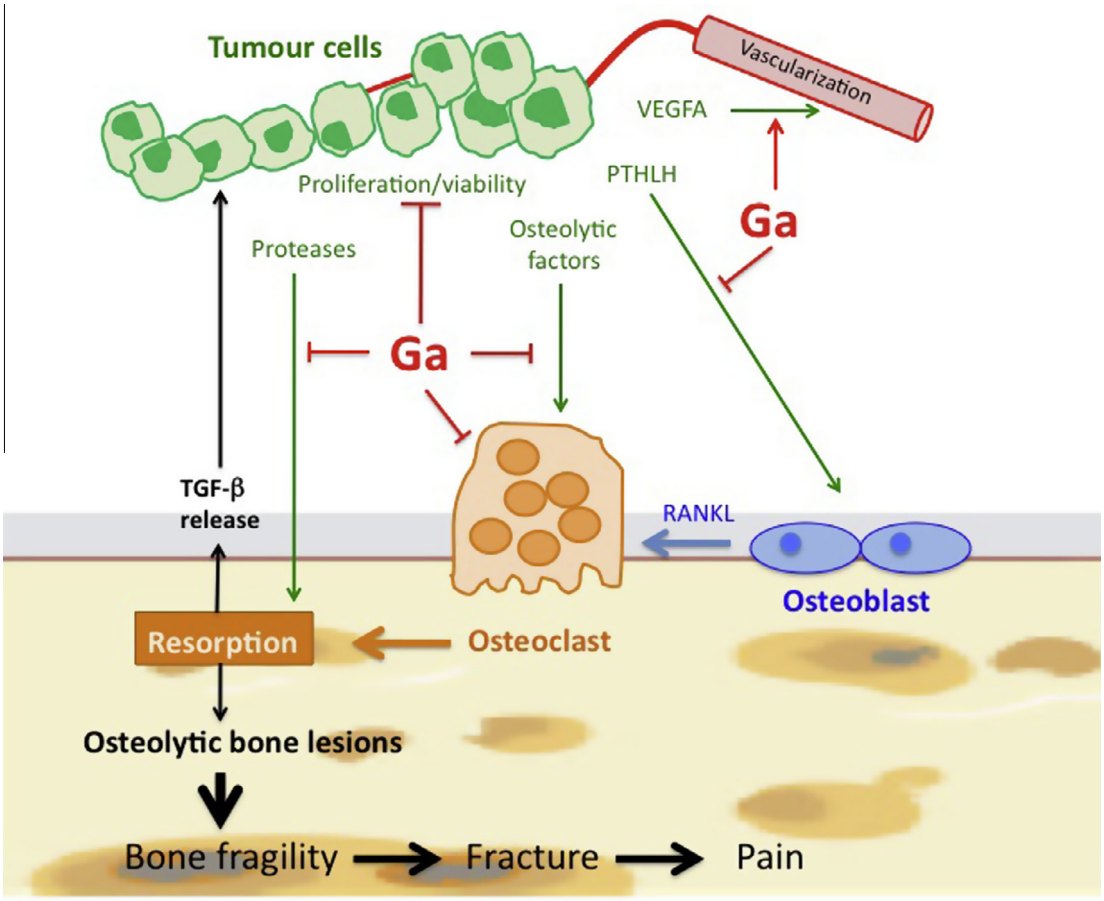

Fig. 6. Ga action in the context of bone metastases. Ga disrupts the vicious cycle driving osteolytic metastases by directly inhibiting tumour cells-stimulated osteoclastogenesis, which in turn reduces considerably the release of TGF-β from the bone matrix. In addition, Ga impacts the expression of secreted factors (proteases, osteolytic cytokines) of tumour cells, involved in the degradation of bone tissue. By altering the secretion of PTHLH from tumour cells, Ga may alter the RANKL communication between osteoblasts and osteoclasts. Lastly, Ga may affect neovascularization induced by tumour cells.

promising candidate for the local treatment of bone metastases in patients with breast cancer.

Considering the biological doses of Ga required and its pharmacokinetic constraints, a smart gallium-delivery system should be designed to optimize the bioavailability into bone tissue. In this attempt, we developed an injectable calcium phosphate (CaP) biomaterial loaded with Ga [54]. In the field of bone metastases, CaP based materials such as cement are well adapted since they are bioactive and have an added value as drug carriers for bone tissue due to their unique ability to adsorb chemical species such as Ga [55,56]. Once injected into the bone tissue, the CaP cement will harden *in situ* without providing exothermic reaction and will fill perfectly into the bone defect. This innovative local approach could be interesting for the following reasons. First, an appropriate CaP biomaterial implanted after bone tumour resection (i) can immediately reinforce the mechanical properties of the weakened bone, (ii) relieve severe bone pain and (iii) will act as a bone substitute, which will serve as a support for new bone formation. In addition, a Ga-desired concentration can be maintained even in inaccessible bone sites or after bone structure modification due to surgery. Consequently, a Ga released *in situ* will provide both effective antitumour activity and tolerance as compared to systemic administration.

To the best of our knowledge, with the exception of two small scale clinical studies from 1980s, this is the first time that antitumour properties of Ga have been specifically studied in the context of bone metastases. Taken together, our data strongly suggest that Ga may disrupt the vicious cycle by interfering with the cross talk between breast cancer cells and osteoclasts, which consequently may reduce osteolytic bone lesions and affect tumour cells growth in bone metastases. Thus, Ga represents a promising candidate for the local treatment of bone metastases in patients with breast cancer.

References

[1] E. Verron, H. Schmid-Antomarchi, H. Pascal-Mousselard, A. Schmid-Alliana, J.C. Scimeca, J.M. Bouler, Therapeutic strategies for treating osteolytic bone metastases, Drug Discov. Today 19 (2014) 1419–1426.
[2] G.R. Mundy, Metastasis to bone: causes, consequences and therapeutic opportunities, Nat. Rev. Cancer 2 (2002) 584–593.
[3] Y.C. Chen, D.M. Sosnoski, A.M. Mastro, Breast cancer metastasis to the bone: mechanisms of bone loss, Breast Cancer Res. 12 (2010) 215.
[4] P. Clezardin, Therapeutic targets for bone metastases in breast cancer, Breast Cancer Res. 13 (2011) 207.
[5] R.E. Coleman, Metastatic bone disease: clinical features, pathophysiology and treatment strategies, Cancer Treat. Rev. 27 (2001) 165–176.
[6] T. Delea, J. McKiernan, J. Brandman, J. Edelsberg, J. Sung, M. Raut, et al., Retrospective study of the effect of skeletal complications on total medical care costs in patients with bone metastases of breast cancer seen in typical clinical practice, J. Support. Oncol. 4 (2006) 341–347.
[7] F. Saad, A. Lipton, R. Cook, Y.M. Chen, M. Smith, R. Coleman, Pathologic fractures correlate with reduced survival in patients with malignant bone disease, Cancer 110 (2007) 1860–1867.
[8] G.D. Roodman, Mechanisms of bone metastasis, Discov. Med. 4 (2004) 144–148.

[9] M. Akhtari, J. Mansuri, K.A. Newman, T.M. Guise, P. Seth, Biology of breast cancer bone metastasis, Cancer Biol. Ther. 7 (2008) 3–9.
[10] P. Juarez, T.A. Guise, TGF-beta in cancer and bone: implications for treatment of bone metastases, Bone 48 (2011) 23–29.
[11] J.T. Buijs, K.R. Stayrook, T.A. Guise, TGF-beta in the bone microenvironment: role in breast cancer metastases, Cancer Microenviron. 4 (2011) 261–281.
[12] J.T. Buijs, P. Juarez, T.A. Guise, Therapeutic strategies to target TGF-beta in the treatment of bone metastases, Curr. Pharm. Biotechnol. 12 (2011) 2121–2137.
[13] D. Ribatti, G. Mangialardi, A. Vacca, Stephen Paget and the 'seed and soil' theory of metastatic dissemination, Clin. Exp. Med. 6 (2006) 145–149.
[14] J.A. Sterling, J.R. Edwards, T.J. Martin, G.R. Mundy, Advances in the biology of bone metastasis: how the skeleton affects tumor behavior, Bone 48 (2011) 6–15.
[15] S.M. Kakonen, G.R. Mundy, Mechanisms of osteolytic bone metastases in breast carcinoma, Cancer 97 (2003) 834–839.
[16] J. Street, M. Bao, L. deGuzman, S. Bunting, F.V. Peale Jr., N. Ferrara, et al., Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 9656–9661.
[17] K. Hu, B.R. Olsen, Osteoblast-derived VEGF regulates osteoblast differentiation and bone formation during bone repair, J. Clin. Invest. (2016).
[18] B. Emad, M. Sherif el, G.M. Basma, R.W. Wong, M. Bendeus, A.B. Rabie, Vascular endothelial growth factor augments the healing of demineralized bone matrix grafts, Int. J. Surg. 4 (2006) 160–166.
[19] B. Behr, P. Leucht, M.T. Longaker, N. Quarto, Fgf-9 is required for angiogenesis and osteogenesis in long bone repair, Proc. Natl. Acad. Sci. U.S.A. 107 (2010) 11853–11858.
[20] E. Verron, J.M. Bouler, J.C. Scimeca, Gallium as a potential candidate for treatment of osteoporosis, Drug Discov. Today 17 (2012) 1127–1132.
[21] C.R. Chitambar, Gallium-containing anticancer compounds, Future Med. Chem. 4 (2012) 1257–1272.
[22] E. Verron, M. Masson, S. Khoshniat, L. Duplomb, Y. Wittrant, M. Baud'huin, et al., Gallium modulates osteoclastic bone resorption in vitro without affecting osteoblasts, Br. J. Pharmacol. 159 (2010) 1681–1692.
[23] E. Verron, A. Loubat, G.F. Carle, C. Vignes-Colombeix, I. Strazic, J. Guicheux, et al., Molecular effects of gallium on osteoclastic differentiation of mouse and human monocytes, Biochem. Pharmacol. 83 (2012) 671–679.
[24] L.R. Bernstein, Mechanisms of therapeutic activity for gallium, Pharmacol. Rev. 50 (1998) 665–682.
[25] P. Collery, B. Keppler, C. Madoulet, B. Desoize, Gallium in cancer treatment, Crit. Rev. Oncol. Hematol. 42 (2002) 283–296.
[26] R.P. Warrell Jr., N.W. Alcock, R.S. Bockman, Gallium nitrate inhibits accelerated bone turnover in patients with bone metastases, J. Clin. Oncol. 5 (1987) 292–298.
[27] R.P. Warrell Jr., Gallium nitrate for the treatment of bone metastases, Cancer 80 (1997) 1680–1685.
[28] H.I. Scher, T. Curley, N. Geller, D. Dershaw, E. Chan, J. Nisselbaum, et al., Gallium nitrate in prostatic cancer: evaluation of antitumor activity and effects on bone turnover, Cancer Treat. Rep. 71 (1987) 887–893.
[29] Y. Guo, K. Tiedemann, J.A. Khalil, C. Russo, P.M. Siegel, S.V. Komarova, Osteoclast precursors acquire sensitivity to breast cancer derived factors early in differentiation, Bone 43 (2008) 386–393.
[30] G.E. Beranger, D. Momier, J.M. Guigonis, M. Samson, G.F. Carle, J.C. Scimeca, Differential binding of poly(ADP-Ribose) polymerase-1 and JunD/Fra2 accounts for RANKL-induced Tcirg1 gene expression during osteoclastogenesis, J. Bone Miner. Res. 22 (2007) 975–983.
[31] C.R. Chen, Y. Kang, J. Massague, Defective repression of c-myc in breast cancer cells: a loss at the core of the transforming growth factor beta growth arrest program, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 992–999.
[32] T. Yoneda, P.J. Williams, T. Hiraga, M. Niewolna, R. Nishimura, A bone-seeking clone exhibits different biological properties from the MDA-MB-231 parental human breast cancer cells and a brain-seeking clone in vivo and in vitro, J. Bone Miner. Res. 16 (2001) 1486–1495.
[33] M. Gallet, N. Sevenet, C. Dupont, M. Brazier, S. Kamel, Breast cancer cell line MDA-MB 231 exerts a potent and direct anti-apoptotic effect on mature osteoclasts, Biochem. Biophys. Res. Commun. 319 (2004) 690–696.
[34] G.J. Powell, J. Southby, J.A. Danks, R.G. Stillwell, J.A. Hayman, M.A. Henderson, et al., Localization of parathyroid hormone-related protein in breast cancer metastases: increased incidence in bone compared with other sites, Cancer Res. 51 (1991) 3059–3061.
[35] H. Nakamura, T. Hiraga, T. Ninomiya, A. Hosoya, N. Fujisaki, T. Yoneda, et al., Involvement of cell-cell and cell-matrix interactions in bone destruction induced by metastatic MDA-MB-231 human breast cancer cells in nude mice, J. Bone Miner. Metab. 26 (2008) 642–647.
[36] P.V. Hauschka, A.E. Mavrakos, M.D. Iafrati, S.E. Doleman, M. Klagsbrun, Growth factors in bone matrix. Isolation of multiple types by affinity chromatography on heparin-Sepharose, J. Biol. Chem. 261 (1986) 12665–12674.
[37] J.J. Yin, K. Selander, J.M. Chirgwin, M. Dallas, B.G. Grubbs, R. Wieser, et al., TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development, J. Clin. Invest. 103 (1999) 197–206.
[38] C. Suarez-Cuervo, K.W. Harris, L. Kallman, H.K. Vaananen, K.S. Selander, Tumor necrosis factor-alpha induces interleukin-6 production via extracellular-regulated kinase 1 activation in breast cancer cells, Breast Cancer Res. Treat. 80 (2003) 71–78.
[39] B.E. Bachmeier, A.G. Nerlich, R. Lichtinghagen, C.P. Sommerhoff, Matrix metalloproteinases (MMPs) in breast cancer cell lines of different tumorigenicity, Anticancer Res. 21 (2001) 3821–3828.
[40] J.M. Barrett, M.A. Puglia, G. Singh, R.G. Tozer, Expression of Ets-related transcription factors and matrix metalloproteinase genes in human breast cancer cells, Breast Cancer Res. Treat. 72 (2002) 227–232.
[41] J.P. Witty, S.A. Foster, G.P. Stricklin, L.M. Matrisian, P.H. Stern, Parathyroid hormone-induced resorption in fetal rat limb bones is associated with production of the metalloproteinases collagenase and gelatinase B, J. Bone Miner. Res. 11 (1996) 72–78.
[42] S.M. Arnold, A.B. Young, R.K. Munn, R.A. Patchell, N. Nanayakkara, W.R. Markesbery, Expression of p53, bcl-2, E-cadherin, matrix metalloproteinase-9, and tissue inhibitor of metalloproteinases-1 in paired primary tumors and brain metastasis, Clin. Cancer Res. 5 (1999) 4028–4033.
[43] E. Rahko, A. Jukkola, J. Melkko, P. Paavo, R. Bloigu, A. Talvensaari-Mattila, et al., Matrix metalloproteinase-9 (MMP-9) immunoreactive protein has modest prognostic value in locally advanced breast carcinoma patients treated with an adjuvant antiestrogen therapy, Anticancer Res. 24 (2004) 4247–4253.
[44] A.J. Littlewood-Evans, G. Bilbe, W.B. Bowler, D. Farley, B. Wlodarski, T. Kokubo, et al., The osteoclast-associated protease cathepsin K is expressed in human breast carcinoma, Cancer Res. 57 (1997) 5386–5390.
[45] A.S. Maharaj, P.A. D'Amore, Roles for VEGF in the adult, Microvasc. Res. 74 (2007) 100–113.
[46] A. Wilson, L.A. Shehadeh, H. Yu, K.A. Webster, Age-related molecular genetic changes of murine bone marrow mesenchymal stem cells, BMC Genomics 11 (2010) 229.
[47] N. Costa, S. Paramanathan, D. Mac Donald, A.S. Wierzbicki, G. Hampson, Factors regulating circulating vascular endothelial growth factor (VEGF): association with bone mineral density (BMD) in post-menopausal osteoporosis, Cytokine 46 (2009) 376–381.
[48] M. Korpal, Y. Kang, Targeting the transforming growth factor-beta signalling pathway in metastatic cancer, Eur. J. Cancer 46 (2010) 1232–1240.
[49] D. Padua, J. Massague, Roles of TGFbeta in metastasis, Cell Res. 19 (2009) 89–102.
[50] J.R. Edwards, J.S. Nyman, S.T. Lwin, M.M. Moore, J. Esparza, E.C. O'Quinn, et al., Inhibition of TGF-beta signaling by 1D11 antibody treatment increases bone mass and quality in vivo, J. Bone Miner. Res. 25 (2010) 2419–2426.
[51] S.M. Kakonen, K.S. Selander, J.M. Chirgwin, J.J. Yin, S. Burns, W.A. Rankin, et al., Transforming growth factor-beta stimulates parathyroid hormone-related protein and osteolytic metastases via Smad and mitogen-activated protein kinase signaling pathways, J. Biol. Chem. 277 (2002) 24571–24578.
[52] M.C. Smith, K.E. Luker, J.R. Garbow, J.L. Prior, E. Jackson, D. Piwnica-Worms, et al., CXCR4 regulates growth of both primary and metastatic breast cancer, Cancer Res. 64 (2004) 8604–8612.
[53] A. Muller, B. Homey, H. Soto, N. Ge, D. Catron, M.E. Buchanan, et al., Involvement of chemokine receptors in breast cancer metastasis, Nature 410 (2001) 50–56.
[54] C. Mellier, F. Fayon, F. Boukhechba, E. Verron, M. LeFerrec, G. Montavon, et al., Design and properties of novel gallium-doped injectable apatitic cements, Acta Biomater. 24 (2015) 322–332.
[55] E. Verron, I. Khairoun, J. Guicheux, J.M. Bouler, Calcium phosphate biomaterials as bone drug delivery systems: a review, Drug Discov. Today 15 (2010) 547–552.
[56] E. Verron, J.M. Bouler, J. Guicheux, Controlling the biological function of calcium phosphate bone substitutes with drugs, Acta Biomater. 8 (2012) 3541–3551.