Analysis of molecular mechanisms and anti-tumoural effects of zoledronic acid in breast cancer cells

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Abstract

Zoledronic acid (ZOL) is the most potent nitrogen-containing bisphosphonate (N-BPs) that strongly binds to bone mineral and acts as a powerful inhibitor of bone resorption, already clinically available for the treatment of patients with osteolytic metastases. Recent data also suggest that ZOL, used in breast cancer, may provide more than just supportive care modifying the course of the disease, though the possible molecular mechanism of action is still unclear. As breast cancer is one of the primary tumours with high propensity to metastasize to the bone, we investigated, for the first time, differential gene expression profile on Michigan Cancer Foundation-7 (MCF-7) breast cancer cells treated with low doses of ZOL (10 μM). Microarrays analysis was used to identify, describe and summarize evidence regarding the molecular basis of actions of ZOL and of their possible direct anti-tumour effects. We validated gene expression results of specific transcripts involved in major cellular process by Real Time and Western Blot analysis and we observed inhibition of proliferation and migration through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Matrigel assay. We then focused on changes in the cytoskeletal components as fibronectin 1 (FN1), actin, and anti angiogenic compounds as transforming growth factor-β1 (TGF-β1) and thrombospondin 1 (THBS1). The up-regulation of these products may have an important role in inhibiting proliferation, invasion and angiogenesis mediated by ZOL.

Keywords: ZOL ● FN1 ● TGF-β1 ● THBS-1 ● invasion ● breast cancer

Introduction

Breast cancer is the most frequently diagnosed cancer in women around the world and bone is its most common associated site of metastasis [1]. ZOL is a potent N-BPs, inhibitor of bone resorption that reduces the risk of skeletal complications and prevents treatment-induced bone loss [2]. In oncology, its role in metastatic bone disease is well established [3], but there is increasing interest in its potential role in preventing and treating cancer-induced bone loss and its possible anti-tumour effects [4].

N-BP shave been shown to inhibit the mevalonate pathway involved in the synthesis of cholesterol, through inhibition of the enzyme farnesyl diphosphate synthase. This process leads to the decreased production of the isoprenoid lipids farnesyl diphosphate and geranyl geranyl diphosphate both enzymes required for the prenylation of small GTP-ases, such as Rho, Rac, cdc42 and Rab. Small GTP-ases signalling regulates key cellular processes including proliferation, cell motility, angiogenesis, survival and migration, all mechanisms implicated in the development and spreading of many types of cancer including breast cancer [5–8]. Bisphosphonates, ZOL in particular, induce also tumour cell apoptosis and stimulate γδ T cell cytotoxicity against tumour cells. *In vivo*, ZOL inhibits bone metastasis formation and reduces skeletal

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tumour burden in mouse models. This may reflect direct antitumour effects and indirect effects *via* inhibition of bone resorption. In addition, ZOL inhibits experimental angiogenesis *in vitro* and *in vivo* [9]. Data from *in vitro* and pilot studies suggest that ZOL reduces circulating levels of vascular endothelial growth factor (VEGF) in metastatic breast cancer patients [10, 11], suggesting these drugs could interfere with tumour-associated angiogenesis. Evidence *in vivo* already exists that ZOL treatment inhibits tumour-associated angiogenesis by inducing a profound reduction in macrophages infiltrating mammary or cervical carcinoma lesions, associated with decreased VEGF and matrix metalloprotease-9 (MMP-9) levels in the tumour microenvironment [12]. Interactions of cells with their surroundings can have profound influences on gene expression and cellular behaviour [13–15].

Angiogenesis and regulation of tumour environment is essential for cancer growth and progression, and therefore, anti-angiogenesis is one promising strategy to treat cancer [16]. Numerous anti-angiogenic factors have been described as transforming growth factor β-1 (TGF-β1) and relative TGF-β1/SMAD (small mother against decapentaplegic) signalling pathway plays an important role in cancer cells and leads to growth inhibition, differentiation and apoptosis [17]. The TGF-βs represents a family of multifunctional cytokines that modulate the growth and function of many cells, including those with malignant transformation. Their signalling pathways are frequently involved in suppressing the growth of human tumours [18]. Recent data suggest that activation of the TGF-β pathway leads to the induction of apoptosis closely followed by the induction of cytostasis, resulting in different carcinoma regression [19, 20]. An important natural activator of TGF-β1 is Thrombospondin 1 (THBS1), a trimeric glycoprotein strongly bound to the extracellular matrix (ECM) [21] and a potent natural inhibitor of angiogenesis [22]. Its ability to block migration of endothelial and cancer cells *in vitro* has been shown to be independent of the activation of TGF-β1 [23, 24]. THBS1 affects ECM structure and function both through direct interactions and indirect effects on other components that are secreted by the cell [25]. Consider that cell adhesion to ECM is crucial to several steps in tumour progression and metastasis, many studies have demonstrated that THBS1 mediates cellular adhesion of numerous cell types and several transformed cell lines [24, 26]. Inhibition of angiogenesis is also a consequence, in part, of re-organization of the actin cytoskeleton and disassembly of focal adhesions in endothelial cells and to inhibit cellular motility, cellular migration and invasion [27].The molecular and physical composition of the ECM can be affected by tumour cells themselves, as well as multiple stromal cell types. Alterations in the expression of ECM-related genes have been identified in gene expression signatures related to poor prognosis and metastases in breast cancers. Indeed, changes in the cytoskeletal components such as production and organization of fibronectin (FN1), actin and collagen have been implicated in eliciting the transition from dormancy to metastatic growth [3, 28–32].

Consequently, we studied the potential mechanisms by which ZOL may regulate global gene expression profile, cellular proliferation, invasion and angiogenesis in MCF-7 breast cancer cells, an ideal model of bone metastatizing cells [33], centering our discussion on FN1, actin, TGF-β1 and THBS1, proteins with a central role respectively on cytoskeletal re-organization, cellular motility, invasion and angiogenetic process.

Materials and methods

Cell culture

Human breast cancer cell lines, MCF-7, purchased from the American Type Culture Collection (Rockville, MD, USA) were grown in Dulbecco's modified Eagle's medium Gibco DMEM:F12 (Invitrogen, Carlsbad, CA, USA) containing 10% foetal bovine serum (FBS) and 1% Penicillin/Streptomycin (P/S) (Gibco). Cells were incubated at 37°C in a humidified atmosphere of 5% of CO_2. Eighty per cent confluent cultures were stimulated with either 10 μM of ZOL for 24, 48 and 72 hrs. ZOL was kindly provided by Novartis Pharma AG. The stock solution of ZOL was prepared at a concentration of 4 mg/ml in distilled water, and aliquots were stored at −20°C.

Cell growth assays

Seventy per cent confluent cultures were treated with 10, 50 and 100 μM of ZOL. Cell numbers before and after 1, 2 and 3 days of treatment were determined by counting the cells. All assays were done in triplicate and repeated at least twice.

Cell viability assay

Cell viability in human breast cancer cell lines, MCF-7, treated with 10, 50 and 100 μM of ZOL for 24 hrs, was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described in literature [34] with minor modifications. Briefly, MCF7 cells were seeded in flat-bottomed 96-well plates at a density of 10,000 cells/well. Twenty-four hrs later, growing cells were washed and treated for 24 hrs with the ZOL (10 and 100 μM). Cell viability was measured using MTT at a concentration of 0.5 mg/ml (20 μl/well). After 1 hr incubation at 37°C, cells were solubilised in DMF (Dimethyl formamide) solution (DMF/H_2O, 1:1, pH 4.7) containing 20%SDS for an additional incubation time of 16 hrs at 37°C to dissolve the blue formazan product. Optical density was measured at 570 nm using a 96-well plate reader (EL800; Biotek Instruments, Winooski, VT, USA). All the experiments were run in sextuplicate and repeated twice.

Microarray analysis

Total cellular RNA was isolated from MCF-7 cells treated with ZOL (10 μM) for 24 hrs using the miRNeasy Mini Kit (Qiagen Inc, Valencia, CA, USA) and quantified through 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Five micrograms of total RNA was reverse transcribed using the high capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA) according to vendor's instructions.

Then cDNAs were *in vitro* transcribed for 16 hrs at 37°C using the IVT Labelling Kit (Affymetrix) to produce biotinylated cRNA. Labelled cRNA was isolated using the RNeasy Mini Kit column (QIAGEN). Purified cRNA was fragmented to 200–300 mer using a fragmentation buffer. The quality of total RNA, cDNA synthesis, cRNA amplification and cRNA fragmentation was monitored by capillary electrophoresis (Bioanalizer 2100; Agilent Technologies). Fifteen micrograms of fragmented cRNA was hy-

bridised for 16 hrs at 45°C with constant rotation, using a human oligo-nucleotide array U133 Plus 2.0 (Genechip; Affymetrix, Santa Clara, CA, USA). After hybridization, chips were processed using the Affymetrix Gene Chip Fluidic Station 450 (protocolEukGE-WS2v5_450). Staining was made with streptavidin-conjugated phycoerythrin (SAPE) (Molecular Probes), followed by amplification with abiotinylated anti-streptavidin antibody (Vector Laboratories, Burlingame, CA, USA), and by a second round of SAPE. Chips were scanned using a Gene Chip Scanner 3000 G7 (Affymetrix) enabled for High-Resolution Scanning. Images were extracted with the Gene-Chip Operating Software (Affymetrix GCOS v1.4). Quality control of microarray chips was performed with the Af-fyQCReport software [35]. A comparable quality between microarrays was demanded for all microarrays within each experiment.

Microarray statistical analysis

The background subtraction and normalization of probe set intensities was performed with the method of Robust Multi array Analysis described by Irizarry *et al.* [36]. To identify differentially expressed genes, gene expression intensity was compared using a moderated test and a Bayes smoothing approach developed for a low number of replicates [37]. To correct for the effect of multiple testing, the false discovery rate was estimated from *P*-values derived from the moderated *t*-test statistics [38]. The analysis was performed with the affylmGUI Graphical User Interface for the limma microarray package [39].

Matrigel invasion assay

The invasive potential of breast cancer cells was assessed *in vitro* in matrigel-coated invasion Chambers (BD BioCoat Matrigel Invasion Chamber; Becton Dickinson Biosciences, Franklin Lakes, NJ, USA) in accordance with the manufacturer's instructions. Cell invasion experiments were performed with a 24-well companion plate with cell culture inserts containing 8 um pore size filters. Untreated MCF-7 cells and drug-treated MCF-7 cells with ZOL 10 μM for 24 and 48 hrs (5×10^4/ 500 μl) were added to each insert (upper chamber), and the chemoattr-actant (FBS) was placed in each well of a 24-well companion plate (lower chamber). After 22 hrs incubation at 37°C in a 5% CO_2 incuba-tor, the upper surface of the filter was wiped with a cotton-tipped appli-cator to remove non-invading cells. Cells that had migrated through the filter pores and attached on the under surface of the filter were fixed and stained by Diff-Quik staining kit (BD, Becton Dickinson Biosciences, San Jose, CA, USA). The membranes were mounted on glass slides, and the cells from random microscopic fields (×40 magnification) were counted. Five fields per membrane were randomly selected and counted in each group. All experiments were run in duplicate, and the percent-age of invasive cells was calculated as the percentage invasion through the matrigel membrane relative to the migration through the control membrane, as described in the manufacturer's instructions.

Real time-quantitative PCR (Q-PCR)

Total cellular RNA was isolated from MCF-7 cells treated with ZOL (10 μM) for 24hrs using the miRNeasy Mini Kit (Qiagen Inc) and quan-tified through 2100 Bioanalyzer (Agilent Technologies). Five micrograms of total RNA was reverse transcribed using the high capacity cDNA

archive kit (Applied Biosystems), according to vendor's instructions. Five microlitre of the RT products was used to amplify FN1 (hs01549976_m1), ACTIN (hs99999903_m1), TGF-β1 (hs00998133) and Trombospondin 1 (THBS1) (hs00962914) sequences using the Taq-Man gene expression assay (Applied Biosystems). To normalize quanti-tative real-time PCR reactions, parallel TaqMan human Cyclophilin (4326316E) control reagents assays (Applied Biosystems) were run on each sample. Changes in the target mRNA content relative to control mRNA were determined using a comparative Ct method to calculate changes in Ct, and ultimately fold and percent change. An average CT value for RNA was obtained by reactions in triplicate.

Western blotting (WB)

The cells were treated with 10 μM ZOL for 24, 48, 72, and also 96 hrs or left untreated, and then were lysed to obtain total proteins using com-plete Lysis-M reagent (Roche, Mannheim, Germany)Protein concentra-tion was determined by the Bradford method and the expression of proteins was analyzed in 150 μg of total protein lysates. Proteins were separated on a gel with 8 and 10% polyacrylamide under denaturing con-ditions and transferred by electrophoresis to a nitrocellulose membrane. Nonspecific binding was blocked by soaking membranes in $1 \times$ TBS, 5% powdered milk, and 0.05% Tween-20 for at least 60 min. at room temperature. Membranes were incubated with the following primary anti-bodies: p44/42 MAPK (Erk1/2) mouse mAb# 9107 (at 1:2000), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (197G2) Rabbit mAb #4377 (at 1:2000), Akt1 (2H10) Mouse mAb #2967 (at 1:100) and Phospho-Akt (Ser473) (193H12) Rabbit mAb #4058 (at 1:1000) were from Cell Signal-ling Technology, Fibronectin antibody, Rabbit polyclonal antibody to FN1 GTX112794 (at 1:1000), beta Actin [AC-15] antibody, mouse monoclonal GTX26276 (at 1:5000) and TGF beta [TB21] antibody, mouse monoclonal GTX21279 (at 1:1000) were from Gene Tex, Inc. (Irvine, CA, USA), Smad4 (MAB1132 at 1 μg/mL) and anti-Smad2/3 (#07-408 at 1:500) were from Millipore Corporation (Vimodrone MI, Italy), THBS1 mouse monoclonal and GAPDH (6C5): sc-32233 were from Santa Cruz Biotech-nology (Santa Cruz, CA, USA). After the membranes were washed three times with TBS plus 0.05% Tween-20 for 30 min., they were incubated with the following peroxidase (HRP)-conjugated secondary: anti-rabbit, anti-mouse and anti-goat antibody (2030; Santa Cruz Biotechnology) diluted to 1:1000, followed by three washes with TBS plus 0.05% Tween-20. Detection was performed with chemiluminescence detection reagents (ECL; Pierce Biotechnology Inc., Rockford, IL, USA).

Results

ZOL inhibit breast carcinoma cells proliferation

To identify the lower dose of ZOL sufficient to induce an anti-prolifer-ative effect on MCF-7 cell proliferation, we tested different concentra-tions (10–100 μM) of ZOL for 24, 48 or 72 hrs.Cell count showed that cell growth was inhibited by ZOL *versus* control at all concentra-tions used (Fig. 1). In particular, tumour cell growth was reduced to about 40% at a ZOL concentration of 100 μM over a period of incuba-tion of 24 hrs whereas the lower ZOL concentration (10 μM) was slightly less efficient (20%), but effective. Consider that inhibition
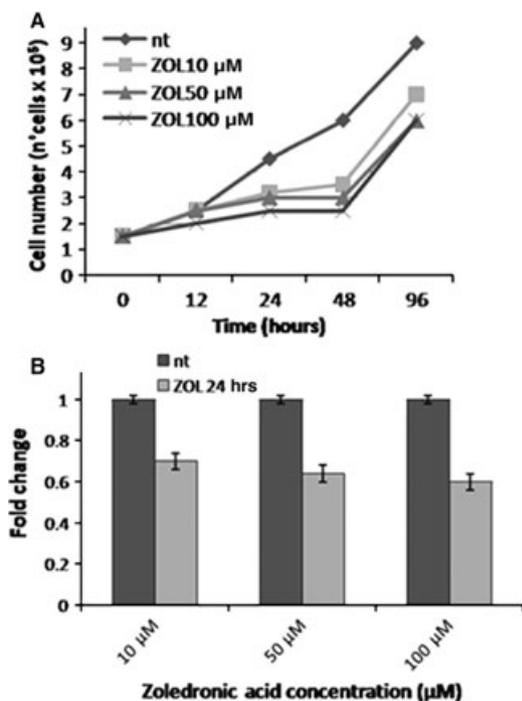
Fig. 1 A, B ZOL inhibits cell growth *in vitro*. About 10^5 and 10^4 cells were cultured in 6-well and 96-well tissue culture plates and exposed to ZOL at a concentration ranging from 10 to 100 μM for different times. Cellular viability was analyzed by cellular count (**A**) and MTT assay (**B**).

Fig. 2 Effects of ZOL addition on MAPK and Akt-dependent pathways on MCF-7 cells. Cells were treated with 10 μM ZOL for 24, 48, 72 and 96 hrs. Thereafter, both the expression and activity of MAPK p44/42 and AKT were evaluated. Determination of the expression and phosphorylation of MAPK p44/42 and AKT evaluated as described in Materials and methods. Expression of the house-keeping protein GAPDH, used as loading control.

rates of 10 and 100 μM of ZOL were not shown a significant difference, we can assert that the treatment at lower concentration for only 24 hrs is sufficient to induce an inhibition of proliferation, also confirmed by determination of number of metabolically active cells by MTT assay (Fig. 1A and B). On the basis of these data, we have selected this concentration of ZOL for all the subsequent experiments.

To elucidate the mechanisms by which cell proliferation is suppressed, we have analysed the effects of ZOL on specific proliferative pathways. Time-course experiments were performed using WB to determine phosphorylation and thus activation of MAPK and AKT pathways. We found that phosphorilation of MAPK and AKT was decreased significantly after both 24 hrs and 48 hrs exposure to 10 μM ZOL (Fig. 2).

Thus, as expected and previously reported with higher doses [40], also low doses of ZOL induced decrease of both MAPK and Akt activity, by which ZOL inhibits the cell proliferation and the ability of tumour cells to expand once they colonize bone [41–44].

Gene expression profile of MCF-7 breast cancer cells treated with low doses of zoledronic acid

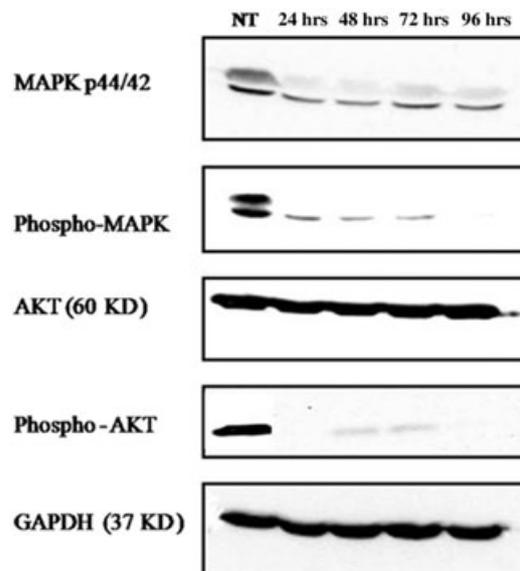The main aim of this study was to investigate the molecular mechanisms by which low doses of ZOL exert their antitumour effects in breast cancer cells. Though ZOL have clearly demonstrated to inhibit proliferation and induce apoptosis in cancer cell lines by interfering with the mevalonate pathway [5–8], the type and pattern of downstream genes modulated by ZOL treatment are still unknown.

To investigate molecular basis of anti-tumoural effect of low doses of ZOL on breast cancer cells, we have evaluated the expression profiling of MCF-7 treated with 10 μM of ZOL for 24 hrs *versus* untreated, using a cDNA microarray platform Affymetrix. Of the 33,000 independent features on the microarrays, 126 were found to be differentially expressed after 24 hrs of treatment. In particular, 17 genes were downregulated (-1.57 to -2.88), and 109 genes were upregulated ($+1.52$ a $+5.27$). For following analysis we considered only the genes with fold change >2 and with statistical difference of expression of each gene was at least $P < 0.001$ (Fig. 3A).

We grouped genes related to biological process, molecular function categories and finally in cellular component categories, that have changed in a statistically significant manner (*P*-value ≤ 0.05) after treatment with ZOL (Fig. 3B–D). The most significant changes in biological processes confirmed the involvement of ZOL in metabolic processes, in fact 38 genes are differentially regulated. Other changes were observed in the cellular localization (24 genes regulates), cell communication (20 genes regulated) and in cell proliferation pathways (eight genes) (Fig. 3B).

Analysis also showed a regulation of molecular function categories, as protein (37 genes) and ion binding (27 genes), and transporter activity (11 genes) affected by ZOL (Fig. 3C). Cellular
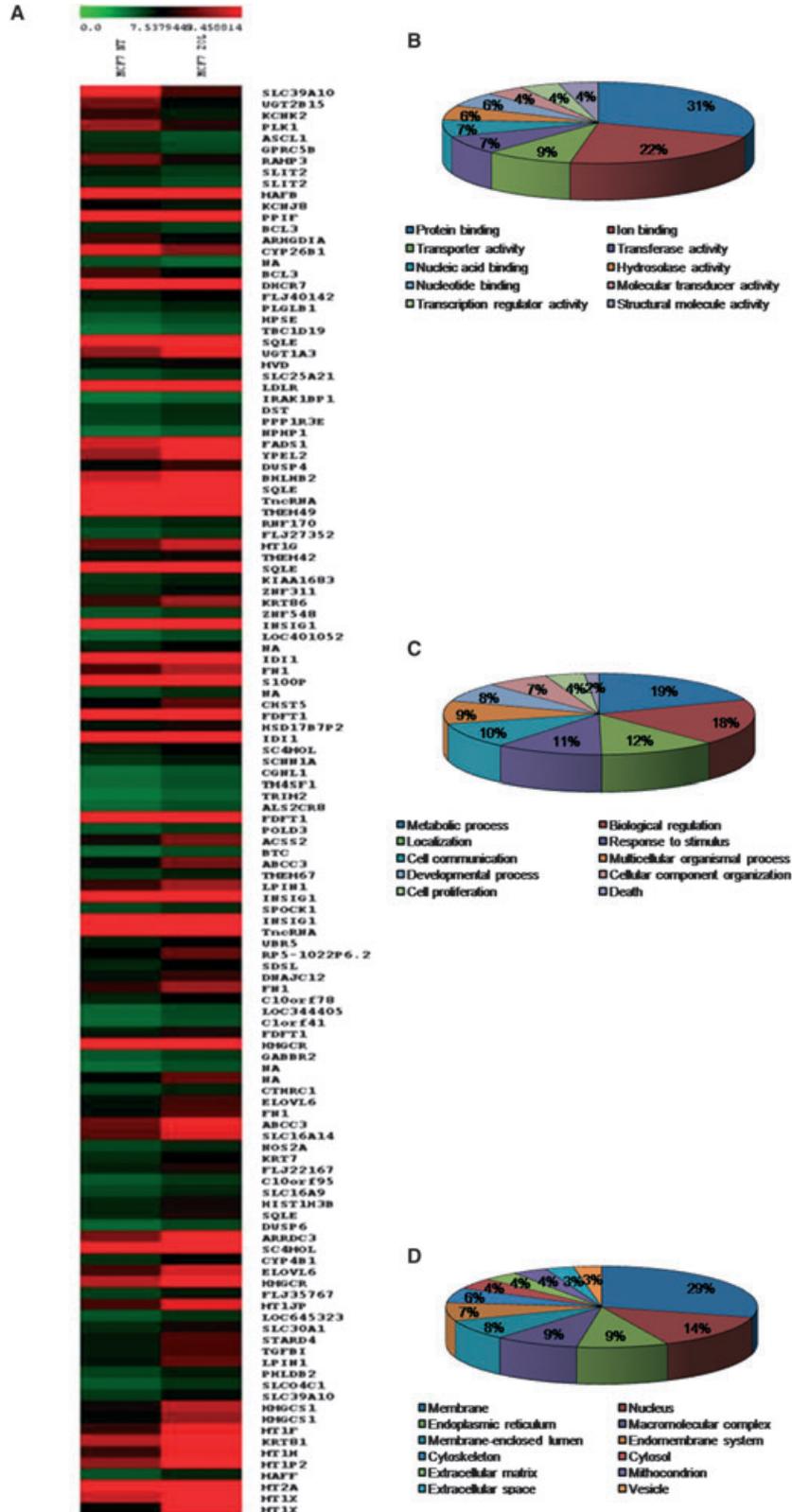
Fig. 3 (**A**) Treatment with ZOL globally affects gene expression profile in MCF-7 cells. (**B, C, D**) Corrected microarray signal values of genes involved in different biological process, clustered by specific functions (Biological process, Cellular function, Cellular component) of MCF-7 cells treated for 24 hrs with 10 μM ZOL in comparison to control cells.

component categories that result differentially regulated by gene expression profile included components of membrane and cytoskeletal (48 genes), nucleus (19 genes) and of endoplasmic reticulum (13 genes) (Fig. 3D).

Alterations in gene expression identified by microarray analysis show modification of possible early-response genes as the treatment with ZOL was carried out for only 24 hrs, and were further investigated by real-time quantitative reverse transcription-PCR.

Effects of ZOL on breast carcinoma cells invasion

In light of previous observation, we hypothesized that the inhibitory effect of ZOL on cellular growth and deregulation of cytoskeletal component observed by analysis of gene expression, could result in inhibition of tumour cell invasion. To address this question, alterations in gene expression, identified by microarray analysis, were further investigated by real-time quantitative reverse transcription-PCR and WB analysis to investigate whether alterations in gene expression were translated into corresponding changes in protein levels.

We found that treatment with ZOL induces transcription and protein expression of some matrix and cytoskeletal components, such as Fibronectin and actin, involved in cancer microenvironment. In particular, the up-regulation of gene coding for FN1 shown by microarray (fold change of 1.93) was confirmed by Real Time RT-PCR with a fold change of 2.3 compare with control (Fig. 4A) and mRNA expression of actin, analysed by Real time RT-PCR, showed a fold change of 1.5. Interestingly, a high protein expression is maintained even at longer treatment (at 96 hrs), and with the most activating effect in the protein products, indicating the potential consequences of ZOL treatment on the morphology and cell motility, considered the cellular roles of FN1 and actin as factors that can change the ECM (Fig. 4B).

Then, the effects of ZOL, on the *in vitro* invasion of MCF-7 were investigated by Matrigel assays. We observed that cells treated even with only 10 μM of the drug, resulted in a reduction in invasion in a time dependent manner, reaching 60–90% inhibition after 24 hrs (Fig. 5).

These results demonstrate that ZOL treatment has a strong inhibitory effect not only on MCF-7 cells growth but also on invasiveness and that possibly the alteration of FN1 and actin expression, could be involved in invasion of human breast cancer cell lines.

ZOL increases expression of anti angiogenetic factors in breast carcinoma cell lines

ZOL can inhibit angiogenesis of tumour cells and emerging evidence suggests that the use of this agent may impede the development of bone metastases [45, 46].THBS1, TGF-β1 and its signalling effectors regulate many aspects of tumour cell biology, such as growth arrest and cell motility, the latter of which is important for the metastatic dissemination of tumour cells from their primary location to lymph or blood vessels [47–49].
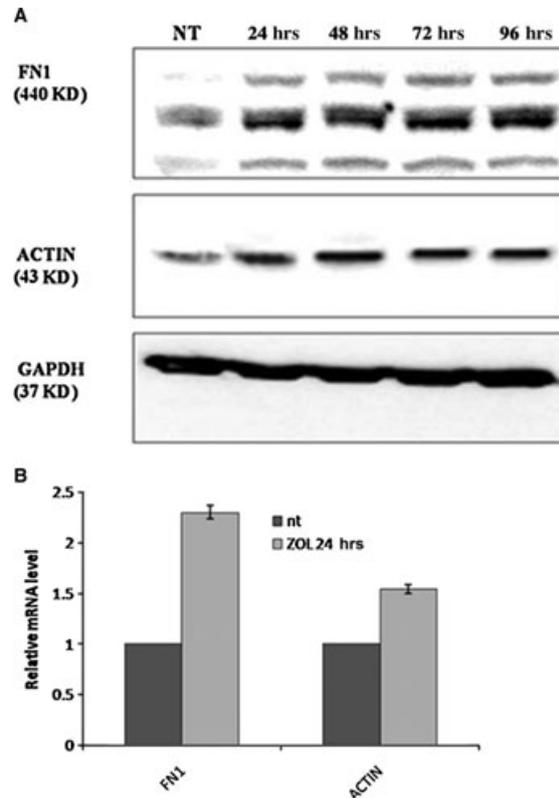

Fig. 4 Effect of ZOL on the mRNA expression and protein levels of FN1 and ACTIN. (**A**) Effect of ZOL 10 μM on the mRNA expression of FN1 and ACTIN, as quantified by real time PCR in MCF-7 cells. (**B**) Effect of ZOL on FN1 and ACTIN protein levels. MCF-7 cells were incubated with low concentration of ZOL for different times, and protein expression were examined by Western blot developing with the enhanced chemoluminescence reagent (ECL). Each membrane was also probed with GAPDH to confirm equal loading.

To investigate the effects on angiogenesis induced by low dose of ZOL, we observed specific mRNA expression and protein levels of TGF-β1and THBS1, to confirm overexpression observed by microarrays analysis, in particular, TGF-β1showed a fold change of +2.3 and THBS1 a fold change of +2.6 compare with untreated control. After only 24 hrs exposed to ZOL, both transcription and protein expression was significantly increased (Fig. 6), indicating possible implication of these two protein in anti-angiogenetic process mediated by low doses of ZOL.

Moreover, as classic TGF-β signalling involves the activation of Smad2/3 and Smad4, direct mediators that accumulate into the nucleus, we examined Smad expression using WB method. Smad complexes interact with transcription factors, co-activators and co-repressors where they participate in the regulation of different target gene expression [50].

Treated MCF-7 cells exhibited a substantial increase in Smad 2/3 at 24 hrs whereas Smad4 peaked at 24 hrs and began to decrease
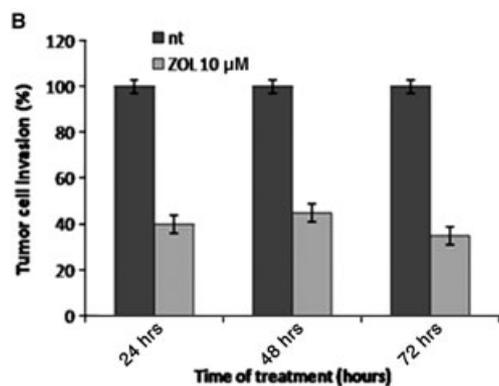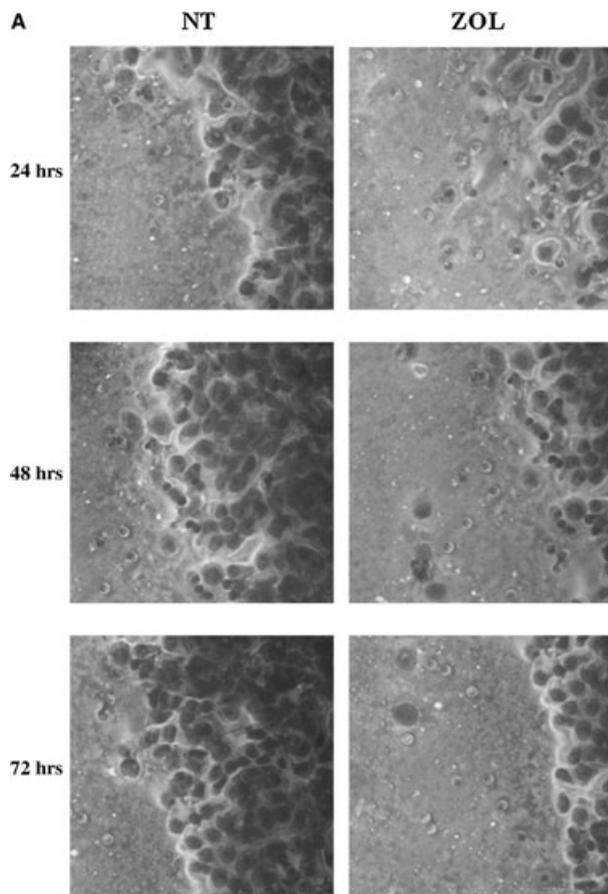
Fig. 6 Effect of ZOL on the transcript and protein levels of THBS1 and TGFβ1. A. mRNA expression of THBS1 and TGFβ1, as quantified by real time PCR in MCF-7 cells treated. (**B**) Effect of ZOL on THBS1 and TGFβ1 protein levels. MCF-7 cells were incubated with low concentration of ZOL for different times, and protein expression were examined by Western blot. The house-keeping protein GAPDH was used as loading control. The experiments were performed at least three different times, and the results were always similar.
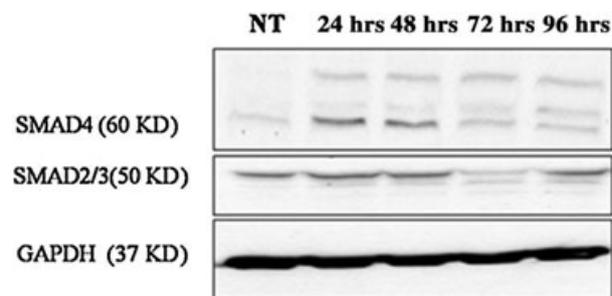
Fig. 7 Effects of ZOL on TGF-β1-dependent pathway and Smad protein expression. Cells were treated with 10 μM ZOL for 24, 48, 72 and 96 hrs. Determination of the expression SMAD2/3 and SMAD4 evaluated after blotting with specific antibodies, as described in Materials and methods. Expression of the house-keeping protein GAPDH, used as loading control. The experiments were performed at least three different times, and the results were always similar.

after 72 hrs (Fig. 7), indicating that MCF-7 cells possibly contain sufficient quantities of receptors and Smads to signal in response to TGF-β 1 (Fig. 7).

Fig. 5 ZOL decrease the invasive potential of human breast cancer cells. Effect of ZOL on the invasion of MCF-7 cells. Treated or not with ZOL 10 μM for 24 hrs, were plated onto Matrigel invasion chambers as described in Materials and methods, and the cell invasion was evaluated. The invaded cells for each insert were stained and quantified. The results are expressed as a percentage of MCF-7 not treated cells (**B**). The experiments were performed at least three different times, and the results were always similar. Data are represented as percentage of control (100%).
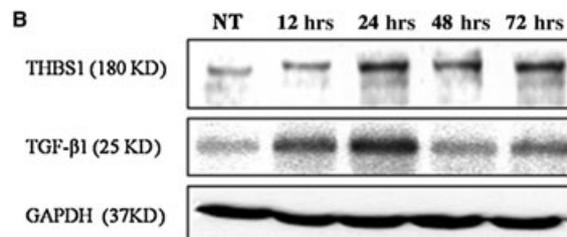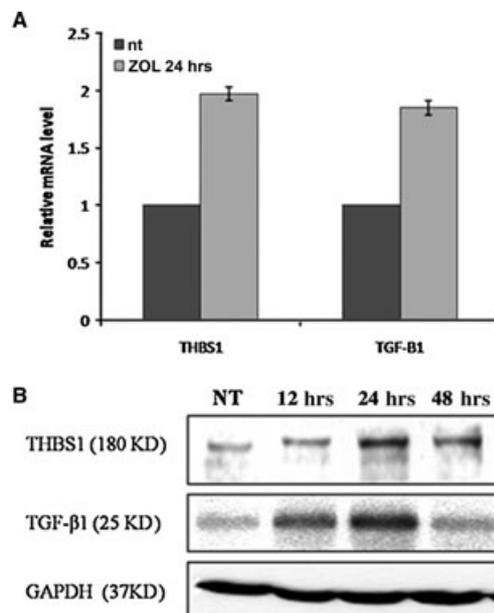
Discussion

Preclinical studies have demonstrated that ZOL can inhibit proliferation, invasion, migration and angiogenesis of tumour cells. Emerging evidence also suggests that the use of this agent may impede the development of bone metastases in mouse models [45, 46]. The mechanism by which ZOL exerts its anti-cancer properties have already been investigated, and its direct effect on cancer cells, as well as the inhibitory effect on tumour angiogenesis, has been confirmed [51, 52]. Several studies have demonstrated that, *in vitro*, the binding of breast and prostate cancer cells to bone surfaces is inhibited by ZOL, that this treatment also has an inhibitory effect on cell proliferation and that a decrease of cellular migration was observed when prostate and breast cancer cell lines were cultured with ZOL [4, 53, 54]. This mechanism seems to be mediated by the effects on the cytoskeleton through Rho A [5].

The main aim of our study was to investigate the molecular mechanisms by which ZOL exerts its antitumour effects in breast cancer cells by Microarray analysis.

To identify the lower dose of ZOL sufficient to induce a moderate anti-proliferative effect on MCF-7 cell proliferation, we first performed cell proliferation assays, by cellular count and MTT. We tested different concentrations (10-100 μM) of ZOL for 24, 48 or 72 hrs, and we found that the highest inhibitive rate reached to nearly 50%. Considering that 10 μM of ZOL had shown a sufficient inhibitory effect, we have selected this concentration of ZOL for all the subsequent experiments.

Data obtained from observation of the activation of major cellular pathways are indicative of mechanisms by which this drug is able to block cellular proliferation. In particular, we confirm, also with low doses, the inhibition of the phosphorylation state of AKT and MAPK protein [40], responsible for key cellular pathways.

To deeply investigate the molecular mechanism by which ZOL acts as antitumour drug, we have performed a gene expression profiling of MCF-7 breast cancer cells treated with low doses of ZOL, and we have demonstrated that ZOL induce differential expression of 126 genes with a strongly up-regulation of different cytoskeletal and ECM component. Based on these results, we also hypothesized that low concentrations of ZOL may affect the processes of invasiveness in cancer cells by altering their ability to invade the tumour microenvironment and thus inhibit their metastatic potential.

As tumour cell invasion requires both cell migration and digestion of the basement membrane, we hypothesized that ZOL inhibited MCF-7 tumour cell invasion was mostly dependent on the cell surface activity driven by FN1 expression and on remodelling of cytosckeletal components. Several studies suggest that FN1 is related to tumour invasion and metastasis [55, 56] playing a key role in the tissue remodelling and cell migration events that occur during normal development; it has been thought to have an important role in both tumour invasion and metastasis. In particular, FN1 is a major constituent of the cell surface of many cultured cells, and it is either eliminated or reduced on the surface of oncogenically transformed cells [55]. Many reports have suggested that there is a correlation between the loss of cell surface FN1 and the ability of a cell to metastasize [44].

In our study, after treatment with10 μM of ZOL, FN1 and actin result up-regulated both by Real Time RT-PCR and WB, indicating their possible involvement in cytoskeletal re-organization induced by ZOL.

On the basis of these considerations, we have performed a Matrigel assay of MCF-7 breast cancer cells treated with ZOL at 10 μM for 24 hrs, and we have demonstrated that ZOL strongly inhibits invasion of these cells. These data agreed with some earlier research *in vitro* [18, 56]. However, the regulatory mechanism of FN1 expression of breast carcinoma is not clear. It is thought it could be regulated by a variety of growth factors such as TGF-β1 frequently involved in suppressing the growth of human tumours [18].

In fact our analysis confirmed that ZOL treatment have induced an up-regulation of transcription and of protein product of TGF-β1, letting us to speculate its involvement in transcriptional control of FN1. As classic TGF-β signalling involves the activation of Smad2/3 and Smad4, we also demonstrated that ZOL induce, at 24 hrs, an increase of Smad2/3 and Smad4 as direct mediators of TGF-β signalling in final activation of anti angiogenetic effects of ZOL. ZOL can also inhibit angiogenesis of tumour cells and emerging evidence suggests that the use of this agent may impede the development of bone metastases [45, 46].

We also found that low dose of ZOL, increased expression of THBS1, a factor involved in the angiogenesis process [55, 56], but also in the regulation of FN1 and actin. THBS1, TGF-β1 and its signalling effectors regulate many aspects of tumour cell biology, such as growth arrest and cell motility, the latter of which is important for the metastatic dissemination of tumour cells from their primary location to lymph or blood vessels [47, 48].

Finally, our results suggested that ZOL showed anti-proliferative and anti-invasive effects in MCF-7 cells and that these data may depend on the activator effect of ZOL in the expression of ECM, cytoskeletal component, and anti-angiogenc factors found in this study. On the basis of this preliminary results *in vitro*, it could be interesting to develop molecular therapeutic strategies based on the specific activation of the expression of particular component for inhibit tumoural growth and angiogenesis, or to evaluate in particular specific roles of FN1 and actin, blocking their expression, in inducing effect antiproliferative and anti-invasive of ZOL in human breast cancer cells.

This study strongly encourage the new experimental design for treatment of breast cancer based on administration of ZOL and to discover their target molecular in cancer cells for future more effective synergistic treatments.

In conclusion, in the present studies, we investigated the role of ZOL in the regulation of breast cancer cell invasion. Our results demonstrated that ZOL, *via* cytoskeletal remodelling, plays an inhibitory role in breast cancer cell invasion, possibly by specifically up-regulating the TGF-β1/Smad signalling pathway, and the downstream activity of FN1 and ACTIN.

On the basis of these results, future work has been hypothesized, it could be interesting to develop molecular therapeutic strategies based on the specific regulation of expression and/or function of cytoskeletal components.

Therefore in future works, will be evaluated the activity of ectopic regulation of FN1 mRNA expression to study effective potential anti-proliferative and anti-invasive of ZOL in human breast cancer cells, focusing on the other factors or protein families that influence invasive potential of MCF-7 tumour cells.

Finally, these data strongly encourage the design of clinical trials based on the concomitant administration of ZOL and ectopic additional expression of matrix proteins for efficacy testing.

Conflicts of interest

There are no conflicts of interest in relation to this work.

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