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RESEARCH ARTICLE

Zoledronic Acid Inhibits the RhoA-mediated Amoeboid Motility of Prostate Cancer Cells

Laura Pietrovito[1,#], Giuseppina Comito[1,#], Matteo Parri[1], Elisa Giannoni[1], Paola Chiarugi[1,#] and Maria Letizia Taddei[1,2,3,*]

[1]*Dipartimento di Scienze Biomediche Sperimentali e Cliniche, Università degli Studi di Firenze, Viale Morgagni 50, 50142 Firenze;* [2]*Dipartimento di Medicina Sperimentale e Clinica, Università degli Studi di Firenze, Viale Morgagni 50, 50142 Firenze;* [3]*Tuscany Tumor Institute and "Center for Research, Transfer and High Education DenoTHE", 50134 Florence, Italy*

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Abstract: ***Background***: The bisphosphonate Zoledronic acid (ZA) is a potent osteoclast inhibitor currently used in the clinic to reduce osteoporosis and cancer-induced osteolysis. Moreover, ZA exerts an anti-tumor effect in several tumors. Despite this evidence, the relevance of ZA in prostate cancer (PCa) is not completely understood.

Objective: To investigate the effect of ZA administration on the invasive properties of PC3 cells, which are characterised by RhoA-dependent amoeboid motility.

Methods: The effect of ZA administration on the *in vitro* invasive properties of PC3 cells was evaluated by cell migration in 3D collagen matrices, immunofluorescence and Boyden assays or transendothelial migration. Lung retention and colonization assays were performed to assess the efficacy of ZA administration *in vivo*.

Results: PC3 cells are characterised by RhoA-dependent amoeboid motility. We now report a clear inhibition of *in vitro* PC3 cell invasion and RhoA activity upon ZA treatment. Moreover, to confirm a specific role of ZA in the inhibition of amoeboid motility of PC3 cells, we demonstrate that ZA interferes only partially with PC3 cells showing a mesenchymal phenotype due to both treatment with conditioned medium of cancer associated fibroblasts or to the acquisition of chemoresistance. Furthermore, we demonstrate that ZA impairs adhesion to endothelial cells and the trans-endothelial cell migration, two essential properties characterising amoeboid motility and PC3 metastatic dissemination. *In vivo* experiments prove the ability of ZA to inhibit the metastatic process of PC3 cells as shown by the decrease in lung colonization.

Conclusion: This study demonstrates that ZA inhibits Rho-dependent amoeboid motility of PC3 cells, thus suggesting ZA as a potential therapy to impede the metastatic dissemination of PC3 cells.

Keywords: Prostate cancer, zoledronic acid, amoeboid motility, RhoA, metastasis, endothelium.

1. INTRODUCTION

Zoledronic acid (ZA) is an aminobisphosphonate proven to be useful in the clinic for the treatment of bone metastasis in breast, prostate, lung cancer and melanomas [1-4]. Indeed, many cancers are osteotropic and bone is the most common site of metastasis. Indeed the majority of cancer patients develop skeletal lesions during disease progression. Patients with bone metastases have an increased risk of developing fractures due to the enhancement of the osteoclastic action, bone pain, decreased haematopoiesis hence resulting in anaemia as well as hypercalcemia [5]. Skeletal-related events (SREs) are a common impediment of bone metastases and bisphosphonates are effective in preventing/delaying SREs, preserving the patients' quality of life. Actually, bisphosphonates decrease the rate of bone resorption by promoting apoptosis of osteoclasts [6]. Therefore, they are the most commonly recommended drugs to treat several pathologies such as osteoporosis, but also for Paget's disease of bone, bone metastasis, multiple myeloma, primary hyperparathyroidism, osteogenesis imperfecta, fibrous dysplasia, and other conditions that exhibit bone fragility [7]. Beside the inhibitory effect of bisphosphonates on osteoclasts [8], recently a direct anti-tumor effect of ZA as well has been reported, through the inhibition of proliferation, angiogenesis, cell invasion and migration of cancer cells [9].

ZA is a specific inhibitor of farnesyl pyrophosphate (FPP) synthase, an enzyme involved in the mevalonate pathway, which produces isoprenoids and cholesterol [10]. Isoprenoid compounds, such as FPP and/or geranylgeranyl pyrophosphate are required for post-translation prenylation of several regulatory proteins. Among them are the small

*Address correspondence to this author at the Department of Experimental and Clinical Medicine, University of Florence, Viale Morgagni 50, 50134 Firenze, Italy; Tel: -39-055-2751246; E-mail: marialetizia.taddei@unifi.it
[#]*These authors have contributed equally to this work*

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GTPases of the Ras and Rho family, which are farnesylated and geranylgeranylated, respectively [11-13]. Both members of the Ras and Rho family have been reported to be involved in tumorigenesis and metastasis [14, 15]. Therefore, the hypothesis we want to test in the current study is that the anti-invasive properties of ZA may be due in part to the inhibition of RhoA activation, a key player in the organization of actin cytoskeleton and cell motility [16]. In our laboratory, we have already demonstrated a strict involvement of RhoA activation in prostate cancer cell motility. Indeed PC3 cells, following Ephrin Receptor A2 (EphA2) activation, elicit a repulsive response that is fulfilled through a Rho-dependent actino/myosin contractility activation, leading to cell body retraction [17]. RhoA activation is involved in amoeboid motility, hence allowing trans-endothelial migration and metastatic colonization of PC3 cells [18, 19]. An impairment of RhoA activation, due to EphA2 silencing, strongly decreases the tumorigenic potential of PC3 cells [20].

Recently, our group has also shown that ZA is able to impair both M2 macrophage polarization and cancer-associated fibroblast (CAF) activation, thus affecting the microenvironment of tumor cells [21]. The aim of our work was to investigate the possible direct effect of ZA administration on the invasive properties of PC3 cells, characterised by Rho-dependent amoeboid motility.

2. MATERIALS AND METHODS

2.1. Materials

Unless specified, all reagents were obtained from Sigma (St. Louis, MO, USA) and all the antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) except for anti-phospho MLC (Ser 19) (Cell Signalling, Danvers, MA, USA). Matrigel Matrix was purchased from BD Biosciences (San Jose, CA, USA). ZOMETA (Zoledronic acid) was from Novartis (Basel, Switzerland). The invasion chambers were from Corning Costar (NY, USA). Ilomastat was from Chemicon International (Temecula, CA, USA). Type I collagen was from RD Systems (Minneapolis, MN, USA). Fluorescein isothiocyanate (FITC)-Phalloidin was from Molecular Probes (Eugene, OR, USA). Secondary antibodies conjugated with Alexa Fluor 488 and CellTraceTM Carboxyfluorescein succinimidyl ester (CFSE) were from Life Technologies Invitrogen (Carlsbad, CA, USA). CellTracker™ Orange (5-(and-6)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine) (CMTMR) Dye was from Thermo Fisher Scientific (Waltham, MA, USA). Glutathione S-transferase (GST)-Rhotekin was from Peprotech (London, UK) .

2.2. Cell Cultures

The PC3 human prostate cancer cell line derived from bone metastasis of a grade IV prostatic adenocarcinoma and human umbilical vein endothelial cells (HUVECs) were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). PC3 docetaxel resistant cells (PC3-DR) were obtained from our laboratory as previously described [22]. CAFs were isolated from surgical explant after patients' informed consent as previously described [23]. PC3 cells and CAFs were cultured in DMEM containing 10% Foetal Bovine Serum (FBS). HUVECs were cultured as previously reported [24]. Preparation of conditioned medium (CM): CM was obtained from CAFs grown to sub-confluence and then maintained in serum-free medium for 48 h.

2.3. Western Blot Analysis

Cells were lysed in Radioimmunoprecipitation Assay (RIPA) buffer and 20-50 µg of total proteins were loaded on precast SDS-PAGE gels (BioRad, Hercules, CA, USA). Western blot analysis was performed as previously described [25].

2.4. RhoA Activity Assay

Cells were directly lysed in RIPA buffer, the lysates were clarified by centrifugation and RhoA-GTP was quantified as previously described [20].

2.5. Cell Fractionation

To detect the isoprenylated/active membrane-associated RhoA protein and the non-isoprenylated cytosolic form, cells were lysed with magnesium-containing lysis buffer (MLB) buffer (125 mmol/L Tris-HCl, 750 mmol/l NaCl, 1% v/v NP40, 10% v/v glycerol, 50 mmol/L MgCl2, 5 mmol/L EDTA, 25 mmol/L NaF, 1mmol/L NaVO4, 10 µg/ml leupeptina, 10 µg/ml pepstatin, 10 µg/ml aproteinin, 1 mmol/L phenyl-methylsulfonyl fluoride, pH 7.5) and centrifuged at 13000 xg for 10' at 4°C according to Riganti *et al.* [26].

2.6. Invasion Assay

Cells were serum starved and treated or not with 25 µM ZA for 48 h and then 8 x 10^4 cells were seeded onto Matrigel-precoated Boyden chamber (8 mm pore size, 6.5 mm diameter, 12.5 µg Matrigel/filter) with or without 50 µM Ilomastat. In the lower chamber, complete medium was added as chemoattractant. Invaded cells were quantified as previously reported [21].

2.7. Gelatin Zymography

Serum-free medium from a monolayer of cells was collected and 20 µl were added to sample buffer (SDS 0.4%, 2% glycerol, 10 mM Tris-HCl, pH 6.8, 0.001% bromphenol blue). The sample were run and then incubated as previously reported [27].

2.8. Cell Adhesion to Endothelium

Adhesion to endothelium was performed as previously described [28]. Briefly, 6 x 10^4 CSFE (360 ng/mL) labelled PC3 cells were directly seeded for 2 h onto a monolayer of HUVEC cells. The adherent cells were fixed in methanol, photographed using an inverted fluorescent microscope and then quantified by counting the CSFE-positive cells.

2.9. Trans-endothelial Cell Migration

HUVECs were grown to confluence on the separating filter of a Transwell and activated for 90' with 10 ng/ml TNFα. 8 x 10^4 CSFE (360ng/ml) labelled PC3 were seeded onto HUVEC cells monolayer. Trans-endothelial cell migration was performed as previously described [18]. Complete me-

dium was added as chemoattractant. Following 16 h, non-invading cells on the upper surface were removed with a cotton swab. Migrated cells were photographed using an inverted fluorescent microscope and then quantified by counting the CSFE-positive cells.

2.10. Cell Migration in Three-dimensional Collagen Matrices

Reconstruction by time-lapse video microscopy and confocal microscopy was performed on PC3 cells treated or not with 25 µM ZA for 48 h. Cells were detached by EDTA (2 mM), washed, incorporated into three-dimensional collagen lattice (1.67 mg/mL; native dermal bovine type I collagen) and monitored by time-lapse video microscopy . Cells within the lattice were labelled by CFSE (360 ng/mL), and monitored as previously described [24].

2.11. Immunofluorescence

Confocal analysis was performed as previously reported [24]. Briefly, PC3 cells were fixed in p-formaldehyde (4% v/v in PBS) for 20', permeabilized in Triton X-100 (0.5% v/v in PBS) for 5', then washed twice with bovine serum albumin (BSA) (1% v/v) and FBS (5% v/v in PBS) solution and incubated overnight at 4°C with primary antibodies against phospho-MLC (1:100). After two washes with PBS, the cells were incubated with anti-rabbit AlexaFluor 488 antibodies (1:1000). Incubation with FITC-Phalloidin was performed for 1 h at room temperature in the dark. The coverslips were mounted in Gel Mount™ Aqueous Mounting Medium (Sigma-Aldrich, St. Louis, MO, USA). A Nikon Eclipse TE2000-U (Nikon, Tokio, Japan) confocal microscope was used for data acquisition.

2.12. *In vivo* Experiments

In vivo experiments were performed in accordance with national guidelines and approved by the ethical committee of Animal Welfare Office of Italian Work Ministry and conformed to the legal mandates and Italian guidelines for the care and maintenance of laboratory animals. Lung retention assay. 0.5 x 10^6 cells, treated or not with 25 µM ZA for 48 h in serum-free medium, were labelled with CellTracker™ Orange CMTMR Dye and injected into the tail vein of 6- to 8-week-old male SCID bg/bg mice (Charles River Laboratories International, Wilmington, MA, USA). Mice were sacrificed at 24 h and lung fixed for 24 h in 4% p-formaldehyde. Lungs were then analysed under the inverted Leica SP5 confocal microscope (Leica, Wetzlar, Germania) (and 10 randomly chosen fields taken across the whole lung as previously reported [29]. Lung colonization assay. 0.5 x 10^6 PC3 cells or PC3 cells treated with 25 µM ZA for 48 h in serum-free medium were injected into the tail vein of SCID bg/bg mice. 6 Male SCID-bg/bg mice (6 to 8 weeks old) per group were used. Mice were assessed weekly for weight (weight of mice at the beginning of treatment: mean 19,8 gr). ZA 100 µg/kg/mouse was administered intraperitoneally once a week. Mice were sacrificed after 8 weeks and the lungs were inspected for metastatic nodules by histological analyses as previously reported [18]. The bar graph reports the number of metastatic nodules and paraffin-embedded tissue sections from lung metastases stained with Hematoxylin and Eosin stain (H&E) are shown.

2.13. Histopathological Sample Preparation

Excised lungs were fixed overnight in 4% p-formaldehyde for 24 h, washed in PBS and embedded in paraffin for H&E staining: 4µm tissues sections were stained with Hematoxylin (Polyscience, Inc Warrington, PA) for 40'' and with Eosin (Sigma, St. Louis, MO, USA) for 30''. Tissues sections were examined under a light microscope Leica DM3000 (Leica, Wetzlar, Germania).

2.14. Statistical Analysis

Data are presented as means ± SD from at least three independent experiments with three independent technical replicates. Statistical analysis of the data was performed with Microsoft Office Excel 2016 (Microsoft Corporation, Redmond, WA, USA) using two tails homoscedastic Student's t test. P values of ≤ 0.05 were considered statistically significant.

3. RESULTS

3.1. ZA Inhibits Invasion of PC3 Cells and RhoA Activity

We have previously reported that PC3 cells invasion is characterised by amoeboid motility, a process that is dependent on the activation of the small GTPase RhoA [17, 19]. Our aim was to investigate whether ZA administration could have a role in controlling both *in vitro* cell invasion and RhoA activity. The PC3 amoeboid motility style is shown by live imaging of cells in three-dimensional collagen lattice. Untreated PC3 cells, when exposed to three-dimensional matrices of type I collagen, move through a round-shape squeezing movement, independent of matrix degradation, forming blebs, proving a typical amoeboid motility. Conversely, ZA treatment strongly prevents PC3 movement (Fig. **1A** and movies 1-2). Impairment of ZA-treated PC3 cells was confirmed by a statistically significant decrease in cell invasion (Student t-test, * $p < 0.001$ ZA treated *vs* PC3) as well as in RhoA activation (Fig. **1B**-**C**). We excluded a toxic effect of the drug through a dose-dependence cell-survival curve (data not shown). ZA is a specific inhibitor of farnesyl pyrophosphate (FPP) synthase in the mevalonate pathway, which produces isoprenoids and cholesterol [10]. Isoprenoids are required for post-translation prenylation of several regulatory proteins, and also for the geranyl-geranylation of RhoA [30]. Through a cell fractionation analysis, we demonstrated that ZA treatment deeply decreases the level of the prenylated, membrane bound pool of RhoA, consistent with the inhibition of its activity (Fig. **1D**). Finally, we analysed the cellular localization of the phospho-myosin light chain protein (P-MLC), a known substrate of RhoA/Rho-associated protein kinase (ROCK) signalling as well as the cytoskeleton organization by means of Phalloidin immunostaining. As shown by confocal images, ZA strongly inhibits the cortical distribution of P-MLC and causes actin depolarization with loss of the cortical ring, suggesting an impairment of RhoA dependent amoeboid motility (Fig. **1E**-**F**).

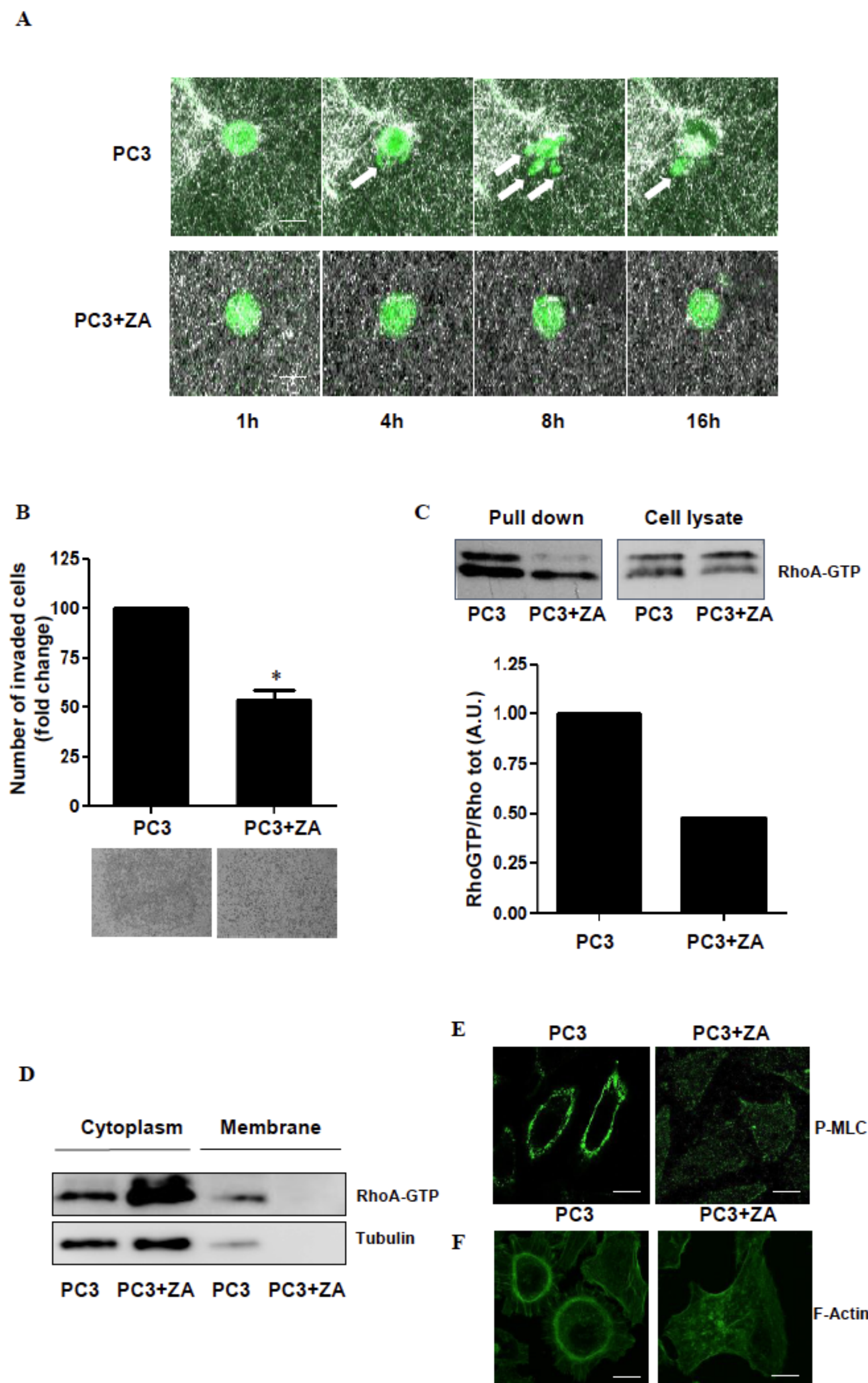

Fig. (1). ZA decreases PC3 cell invasion and RhoA activation: A) Live imaging of PC3 cells migration in three-dimensional collagen lattice. CFSE-loaded PC3 cells, treated or not with 25 mM for 48 h, were incorporated into the collagen matrix and monitored by confocal fluorescence-reflection video microscopy. Tumor cells are visualized in green, while the back scatter signal of the collagen I is reported in white. Arrows indicate the round-shape of the cells squeezing across collagen I fibers. Scale bar: 10 µm. **B)** Boyden cell invasion assay: cells were treated as in A) and then 8 x 10^4 cells were seeded into the upper compartment of Boyden chamber and allowed to invade through the Matrigel coated filter towards complete medium. Cell invasion was evaluated after Diff-Quick staining by counting cells in six randomly chosen fields. The results are representative of three experiments with similar results. Student t-test, * $p < 0.001$ ZA treated *vs* PC3. **C)** PC3 cells were treated with 25 µM ZA for 48 h in serum free medium. After treatment, RhoA-GTP was analysed by pull-down assay from cell lysates. The total amount of RhoA was quantified by anti-RhoA immunoblot. The bar graphs obtained from densitometry analysis of the immunoblot is shown. **D)** Cell Fractionation: cells were treated as in A and then cytosolic and membrane fractions were isolated. Anti-RhoA and tubulin immunoblots were performed on total cell lysates, cytoplasmic and membrane fractions. **E)** Confocal microscopy images of anti-phospho-MLC immunohistochemistry of PC3 cells treated as in A. Scale bar: 5 µm. **F)** Representative confocal microscopy images of F-actin (FITC-Phalloidin) staining of PC3 cells treated as in A) Scale bar: 5 µm. The images are representative of three independent experiments with similar results.

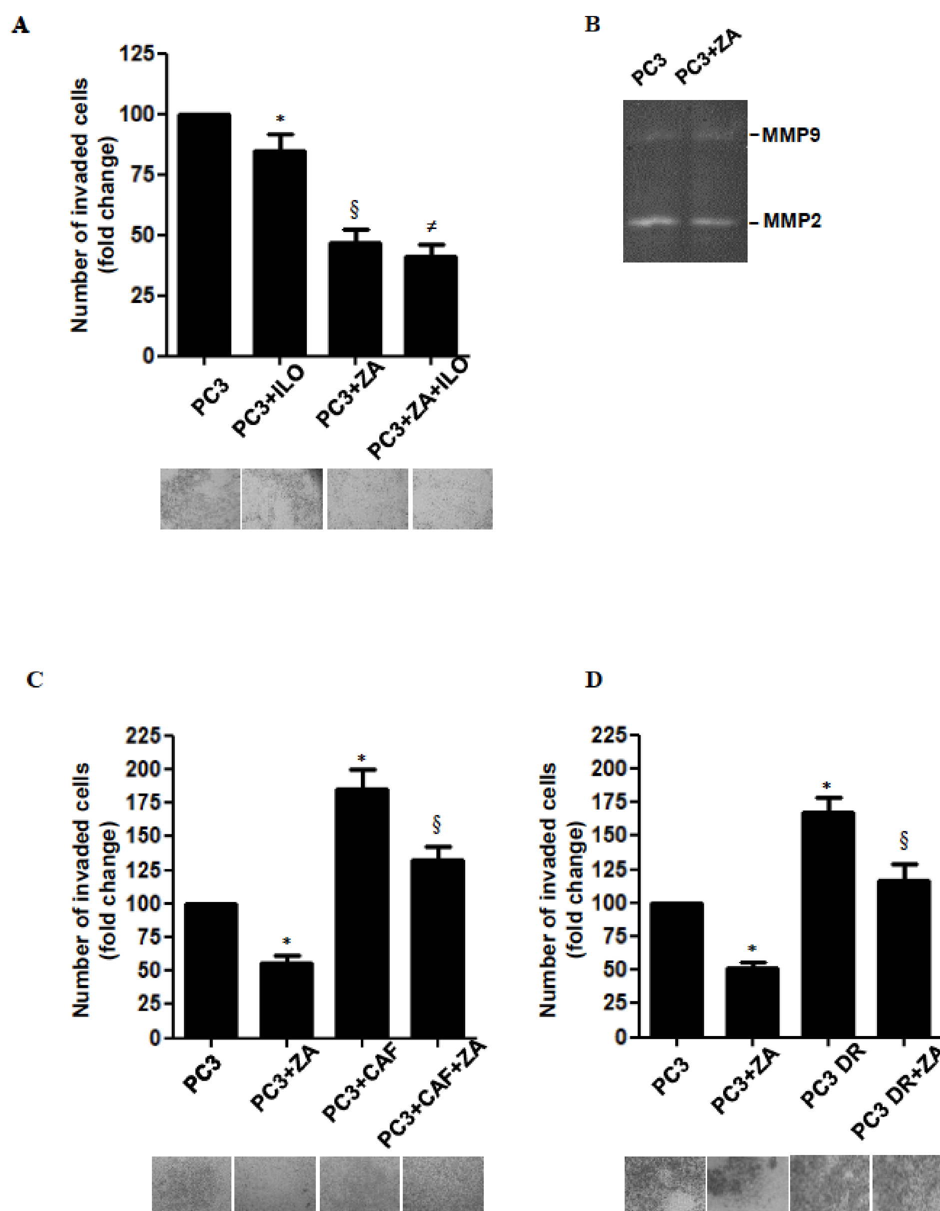

Fig. (2). ZA inhibits specifically amoeboid motility of PC3 cells: A) Boyden cell invasion assay. PC3 cells were treated or not with 25 µM ZA for 48 h in serum-free medium and then 8 x 10^4 cells were seeded into the upper compartment of Boyden chamber with or without the MMPs inhibitor Ilomastat 50 µmol/L. Cells were allowed to migrate through the filter coated with Matrigel toward the lower compartment filled with complete medium. Cell invasion was evaluated after Diff-Quick staining by counting cells in 6 randomly chosen fields. Student t-test, * $p < 0.05$ *vs* PC3; (; ≠ $p < 0.001$ ZA + Ilomastat *vs* Ilomastat **B)** Analysis of MMP activity. Media from a confluent monolayer of cells treated or not with 25 µM ZA for 48 h in serum-free medium were collected and analysed by gelatin zymography. The clear bands represent areas of gelatinase activity. The results shown are representative of three experiments. **C)** PC3 cells were treated or not with CAF-conditioned medium (CM) for 72 h to induce the EMT. Then cells were treated as in A) and a Boyden cell invasion assay was performed. Student t-test, * $p < 0.001$ *vs* PC3;§ $p < 0.01$ CAF+ZA *vs* CAF **D)** PC3 cells or mesenchymal PC3-DR cells were treated as in A and Boyden cell invasion assays were performed. Student t-test, * $p < 0.001$ *vs* PC3; § $p < 0.01$ DR+ZA *vs* DR.

3.2. ZA Inhibits Specifically Amoeboid Motility of PC3 Cells

To verify the hypothesis that ZA actually affects *in vitro* amoeboid motility of PC3 cells we performed invasion assays in the presence of both ZA and Ilomastat. Ilomastat is a broad-range inhibitor of metallo-proteinases (MMPs) thus able to interfere with the motility-types of invasion that depend on extracellular matrix degradation. Results show that PC3 cells, which move by amoeboid motility, are only slightly sensitive to Ilomastat treatment (* $p < 0.05$ PC3+ILO *vs* PC3) while are efficiently blocked in their invasion by ZA administration (§ $p < 0.001$ PC3 + ZA *vs* PC3) (Fig. **2A**). These data further corroborate the specificity of ZA in the inhibition of the amoeboid motility. Furthermore, we demonstrated that ZA does not affect MMPs activity as shown by gelatin zymography of culture media recovered from PC3 cells that have been subjected to ZA administration (Fig. **2B**). Moreover, to confirm this effect, we assessed

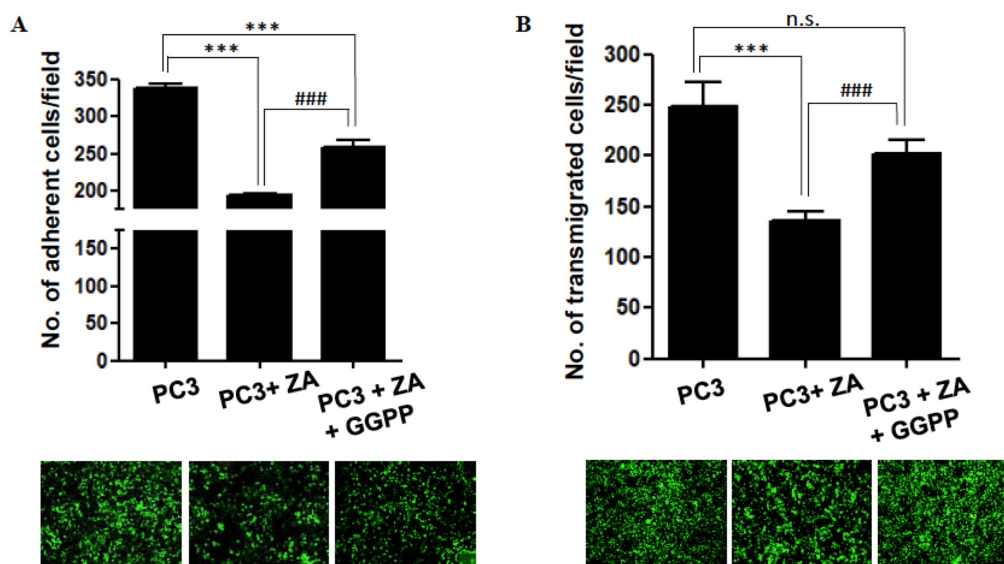

Fig. (3). ZA inhibits adhesion of PC3 and their trans-endothelial migration through the endothelium: Adhesion through endothelium: 6 x 10^4 CSFE labelled PC3 cells treated or not with 25 μM ZA alone or in combination with 20 μM GGPP in serum-free medium for 48 h were directly seeded for 2 h onto a monolayer of HUVEC cells. The adherent cells were photographed using an inverted fluorescent microscope and then quantified by counting the CSFE-positive cells. Results are present as mean of three biological replicates ± SEM. Student t-test, *** $p < 0.001$ *vs* St Med; ### $p < 0.001$ ZA + GGPP *vs* ZA. **B)** 8 x 10^4 CSFE labelled PC3 cells treated or not with 25 μM ZA alone or in combination with 20 μM GGPP in serum- free medium for 48 h were plated onto a monolayer of HUVECs and let trans-migrate over-night. Migrated cells were photographed using an inverted fluorescent microscope and quantified. Results are present as the mean of three biological replicates ± SEM. Student t-test, *** $p < 0.001$ *vs* St Med; ### $p < 0.001$ ZA + GGPP *vs* ZA.

the sensitivity of mesenchymal addicted PC3 cells to ZA. To this purpose we used two different models of PC3 cells that are invading with a mesenchymal-type of movement: PC3 cells treated with conditioned medium derived from CAF [25] or docetaxel resistant PC3 cells (PC3-DR), which have achieved mesenchymal features following the acquisition of drug resistance [31]. As shown, both models of PC3 invading through a mesenchymal motility, show a statistically significant lower sensitivity to the ZA treatment with respect to PC3 cells that are invading with an amoeboid type of movement, suggesting a strong action of ZA on amoeboid motility inhibition (Fig. **2C**-**D**).

3.3. ZA Inhibits both the Adhesion of PC3 to the Endothelium and Trans-endothelial Cell Migration Affecting PC3 Metastatic Dissemination

PC3 cells possess a high plasticity in cell motility: we have previously demonstrated that amoeboid motility confers to PC3 cells the ability to effectively adhere to the endothelium and to increase their trans-endothelial migration capacity thus sustaining the metastatic process [18]. We have now tested the *in vitro* ability of ZA to inhibit these two phenomena that are dependent on amoeboid motility. CSFE labelled PC3 cells treated with or without ZA were allowed to adhere (Fig. **3A**) or to migrate through (Fig. **3B**) a monolayer of endothelial HUVEC cells. Representative pictures show that both these key phenomena involved in the metastatic process, are inhibited by ZA administration. According to the dependence of these phenomena on amoeboid motility reliant on RhoA activation, the treatment with geranyl-geranyl pyrophosphate (GGPP), which restores the prenylation of GTPase signalling proteins [21], partially restores both adhesion and trans-endothelial migration of PC3 cells (Fig. **3A**-**B**).

Finally, to prove whether ZA-mediated decrease in tumor-endothelial cell interaction could exert a reduction of PC3 *in vivo* metastatic potential, we performed a lung retention assay: PC3 cells and ZA treated PC3 cells were labelled and injected into the tail vein of SCID bg/bg mice. As shown in Fig. (**4A**), ZA impairs lung retention suggesting a role in the inhibition of the metastatic process. To further corroborate this hypothesis we performed an experimental metastasis assay in SCID bg/bg mice. PC3 cells were injected into the tail vein of SCID bg/bg mice and ZA administrated intraperitoneally once a week. Lungs inspection shows that ZA treatment statistically inhibits lung colonization thus confirming a crucial role of ZA-mediated inhibition of amoeboid motility in the *in vivo* metastatic process of PC3 cells.

4. DISCUSSION

Among the members of the bisphosphonate family, ZA has been until recently the only standard of care for the prevention of SREs in metastatic prostatic cancer, multiple myeloma and metastatic breast cancer [7, 32, 33]. ZA inhibits the FPP synthase. FPP synthase is a ubiquitously expressed enzyme in mammalian cells, nevertheless, ZA-induced apoptosis only occurs in osteoclasts. This is likely due to the high affinity of bisphosphonates to selectively adhere to the bone and being here retained, reach a high local concentration before osteoclasts-mediated endocytosis. Once within the osteoclasts, the inhibition of post-translational modification of proteins such as Rac, Rab and Rho, which have a central role in the regulation of stress fibres assembly, membrane ruffling and cell survival, leads finally to osteoclast apoptosis [6, 34].

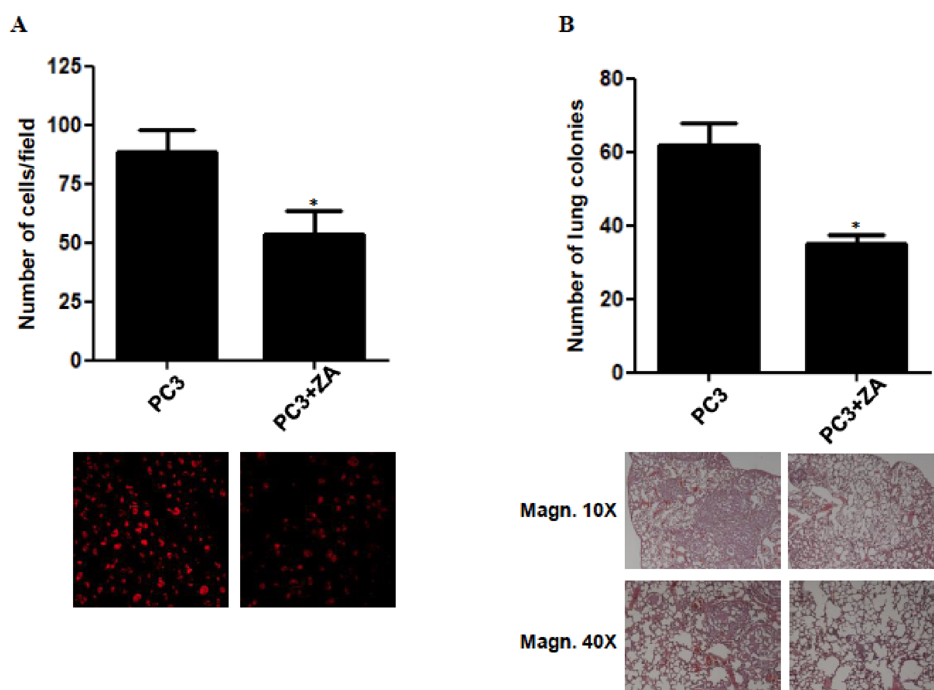

Fig. (4). ZA impairs retention and lung colonization in SCID bg/bg mice: A) Lung retention assay: 0.5 x 10^6 PC3 cells or PC3 cells treated with 25 µM ZA for 48 h in serum-free medium were labelled with CellTracker™ Orange CMTMR Dye and then injected into the tail vein of SCID bg/bg mice. After 24 h mice were sacrificed and the lungs examined under the inverted Zeiss microscope and 10 randomly chosen fields taken across the whole lung. Student t-test, * $p < 0.05$ ZA treated *vs* PC3 **B)** Lung colonization assay: mice were injected into the lateral tail vein with 0.5 x 10^6 PC3 cells treated as in A). ZA 100 µg/kg/mouse was administered intraperitoneally once a week. Mice were sacrificed after 8 weeks and the lungs were inspected for metastatic nodules by histological analyses. The bar graph reports the number of metastatic nodules and paraffin-embedded tissue sections from lung metastases stained with H&E are shown. Student t-test, * $p < 0.005$ ZA treated *vs* PC3.

Although ZA is not administrated to prevent tumor progression, it has been reported that it can affect several tumor-related functions, such as angiogenesis, by inhibiting the viability of endothelial cells [35, 36] and immune-modulation by sustaining maturation and activation of γδT cells [37-39]. Moreover, along with others, we have shown the ability of ZA to affect macrophage differentiation and fibroblast activation [1, 21], both stromal cells known to play an active role in tumorigenesis and tumor progression. In addition, ZA exhibits also a direct anti-tumor activity by inhibiting cancer cells proliferation, by synergistically acting with cytotoxic agents and by decreasing cell motility [9, 40]. A direct action of ZA in *in vitro* growth, migration and invasive properties of prostate cancer cells has already been described [41] but we have now further characterised the role that ZA exerts to decrease PC3-amoeboid migration which represents one of the possible invasion and motility styles of PC3 cells. Indeed, PC3 cells possess great plasticity in cell motility and adapt their invasive strategy depending on the different extracellular stimuli. Indeed, PC3 cells can shift from mesenchymal to amoeboid motility and *vice versa,* adapting their motility according to the microenvironmental changes in order to optimize and finalize the metastatic process [42]. Inhibition of mesenchymal motility by administration of integrin and/or protease inhibitors, causes a clear and dramatic shift to amoeboid movements, thereby allowing cancer cells to escape pharmacological treatments [43]. Here we show that ZA inhibits *in vitro* RhoA activity and PC3 cell invasion that is mainly mediated by amoeboid motility, as previously described [17, 19]. Interestingly, ZA administration not only inhibits amoeboid motility but also prevents the shift towards mesenchymal motility, thus blocking the overall plasticity of PC3 cells. This evidence suggests that treatment with ZA may have a broad-spectrum significance thus preventing PC3 metastatic spreading.

Furthermore, ZA impedes efficiently two *in vitro* phenomena strictly correlated with amoeboid cell motility, namely adhesion to endothelium and trans-endothelial migration. Overall, we report that ZA impairs the metastatic potential of PC3 cells as shown by inhibition of both lung retention and lung colonization in mice. These evidences are actually remarkable since ZA is a drug able to interfere not only with the physiological function of stromal cells that compose the tumor-microenvironment (endothelial cells, macrophages, CAFs) and that are involved in cancer progression but also directly with tumor cells by decreasing their motility. Thus, differently from other pharmacological therapy, ZA could hit, at the same time, these two tumor components, inhibiting efficiently tumor progression. Indeed, it is now largely accepted that the tumor evolution depends on the active cross-talk between cancer cells and their surrounding stromal cells and both concur to successful tumor evolution [44]; thus the power of ZA is intrinsic to its dual mode of action. Furthermore, another advantage of ZA is that it could be administered not only to metastatic but also to primary cancers. Indeed, the administration of ZA to PCa patients could overcome the simple skeletal-associated side

effect impairing directly tumor progression. The use of ZA in the treatment of SREs has been established since 2002 when a randomized trial showed that ZA reduced SRE in prostate cancer patients with bone metastases even if there was no increase in overall survival [45]. However, in accordance with our proposal, a clinical trial on early breast cancer showed a 36% decrease in disease progression in ZA arms with respect to controls, by strengthening its potential use as direct antitumor drug [46]. Indeed, the role of ZA is still ambiguous and several trials have yielded discordant results, thus further studies are needed. Nevertheless, our data suggest a possible use of this drug to directly impair tumor progression.

CONCLUSION

Our results show that ZA inhibits PC3 cells amoeboid motility and hence their invasion and trans-endothelial migration, thus suggesting ZA as a potential therapy to impede the metastatic dissemination of PC3 cells.

ABBREVIATIONS

BSA	=	Bovine serum albumin
CAF	=	Cancer associated fibroblast
CFSE	=	Carboxyfluorescein succinimidyl ester
CMTMR	=	(5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine)
EphA2	=	Ephrin receptor A2
FITC	=	Fluorescein isothiocyanate
FPP	=	Farnesyl pyrophosphate
GGPP	=	Geranyl-geranyl pyrophosphate
GST	=	Glutathione S-transferase
MLB	=	Magnesium-containing lysis buffer
MMPs	=	Matrix metallo-proteinases
PCa	=	Prostate cancer
P-MLC	=	Phospho-myosin light chain
RIPA	=	Radioimmunoprecipitation Assay
ROCK	=	Rho-associated protein kinase
ZA	=	Zoledronic acid

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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