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Zoledronic Acid as an Antimetastatic Agent for Different Human Tumor Cell Lines

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Abstract. *Background: Zoledronic acid effectively reduces skeletal events in patients with metastatic disease. The results of pre-clinical and emerging clinical data suggest an additional activity of zoledronic acid as an antitumor agent, interfering with the growth and dissemination of malignant cells. However, the mechanisms by which zoledronic acid impairs tumor progression are practically unknown. In the present study, we aimed to investigate the impact of zoledronic acid on invasion and colony formation ability of different human tumour cell lines. Materials and Methods: Human ovarian (SKOV3), colonic (HCT116), endometrial (HEC1A and Ishikawa) and breast cancer (MCF-7, MDA-MB-231, HCC1937, SKBR3 and T47D) cell lines were treated with different concentrations (10-100 μM) of zoledronic acid and analyzed using 3D assays to test their invasiveness and their ability to grow anchorage-independently, both hallmarks of aggressive tumor cell behavior. Results: The most intense effect of the drug on tumor invasion was observed on MDA-MB-231 cells, but at high concentrations HEC1A, SKOV3 and SKBR3 cells also exhibited reduced invasion capacity. We also found a significant reduction of colony formation under zoledronic acid treatment in MCF-7, T47-D, HCT116, Ishikawa, HEC1A and SKOV3 cells. Conclusion: Zoledronic acid presents an interesting potential for use as anti-metastatic agent for different solid tumor types, affecting relevant steps of tumor dissemination.*

*Bis*phosphonates are metabolically-stable analogs of pyrophosphates, which preferentially bind mineralized bone matrix around resorbing osteoclasts, blocking their normal cell function and the bone resorption process (1, 2). Significantly, *bis*phosphonates have demonstrated a high efficacy in the treatment of malignant bone disease and now have an important additional role in cancer treatment strategy that can reduce both the symptoms and the complications of bone malignancy (3).

The molecular mechanisms of action proposed for the first generation *bis*phosphonates is the induction of apoptosis by accumulating toxic ATP adducts. Second-generation amino-*bis*phosphonates specifically inhibit farnesyl pyrophosphate synthase, an enzyme of the mevalonate pathway, preventing the protein prenylation of GTPases, Rat sarcoma (RAS) proteins, Ras Homolog family member A (RHOA), rat sarcoma-related protein in brain (RAB) or laminins which regulate cellular polarization and cytoskeleton organization (4, 5). Nowadays, zoledronic acid, a third-generation *bis*phosphonate, is the most potent member of the family and is standard treatment for preventing skeletal complications associated with bone metastases. This drug blocks the synthesis of farnesyl pyrophosphate synthase and its downstream metabolite geranylgeranyl diphosphate. Since the first description of the clinical benefits of zoledronic acid administration in 1998 (6), several trials have been carried out to demonstrate the clinical utility of its combined therapy with anti-hormone or chemotherapy agents (7-9) in order to block tumor progression. The results of these studies are promising but not enough to include zoledronic acid administration as an antitumor agent for patient management. It is, therefore, important to improve our knowledge of the main mechanisms implicated in antitumor effects of zoledronic acid.

In fact, as well as its established effect on the formation of bone metastases, there is promising evidence of broader additional anti-metastatic and anticancer properties (10). For example, its effect on cell proliferation and apoptosis has been reported in several studies. Thus, in experiments in breast cancer, zoledronic acid was shown to promote apoptosis and block cell proliferation *in vitro* (11). This effect was also described in prostate cancer (12) and it seems to occur at both transcriptional and translational levels, especially in highly tumorigenic cancer cell lines, and in a survivin- and caspase-3-or-7-dependent manner (13). In addition, zoledronic acid

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also impairs tumor migration and invasion. For instance, in the human nasopharyngeal carcinoma cell HNE-1 zoledronic acid inhibits cell invasion and migration through the suppression of vascular endothelial growth factor (VEGF) expression and secretion, and by down-regulating the expression of matrix metallopeptidases-2 (MMP2) and -9 (MMP9) (14). Finally, its antiangiogenic activity has also been described. This effect was first suggested by Wood *et al.*, who found dose-dependent inhibition of proliferation of human umbilical vein endothelial cells *in vitro* following exposure to the drug (15) wich was confirmed in later studies (16).

To obtain a better understanding of the impact of zoledronic acid on the metastasic potential of tumor cells, in the present study, we explored its effects on cell invasion and colonization ability. We examined the effect of zoledronic acid treatment on a great variety of breast, colonic, endometrial and ovarian tumor cell lines in an *in vitro* 3-D assay which mimics the first steps of the tumor invasion process. In addition, the impact of zoledronic acid on tumor cell colony formation as a requirement for tumor implantation in a different location was also analyzed. Our results demonstrated the usefulness of zoledronic acid in impairing key steps of tumor invasion and metastasis, and revealed different patterns of response dependent on the type of the tumor cell line analyzed.

Materials and Methods

Cell lines and reagents. Human ovarian SKOV3, colonic cancer HCT116, endometrial cancer HEC1A and Ishikawa cell lines were provided by the group of Dr. Reventós (Vall d' Hebron Hospital, Barcelona, Spain). The breast cancer cell lines MCF-7, MDA-MB-231, HCC1937, SKBR3 and T47D were obtained from the group of Dr. Perez Fernández (Faculty of Physiology, University of Santiago de Compostela, Spain). All breast cancer cell lines were routinely cultured in Dulbecco's modified Eagle's medium (DMEM), Ishikawa cell line in F12K-DMEM, and remaining cell lines in McCoy's medium. All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin, and the cells were maintained at 37˚C in a humidified atmosphere with 5% CO_2. Zoledronic acid was a generous gift from Novartis Pharmaceuticals Inc. (Basel, Switzerland) and the stock solution (1 mM) was prepared with phosphate buffered saline (PBS).

Cell inverted invasion assay. Invasion assay was performed as described previously (17). Cells (5×10^4) were seeded directly in the lower face of 6.5-mm polycarbonate transwell inserts with a 8-μm pore size membrane (Corning, NY, USA), and incubated 5 h to allow cells to attach to the membrane. Inserts were then turned right-side-up and dipped into serum-free medium, 200 μl of growth factor-reduced Matrigel (BD Bioscience, Bedford, MA, USA) diluted 1:3 in medium without fetal calf serum were placed over the upper well, and complete medium applied to the top of the Matrigel as chemoattractant. Zoledronic acid (10 and 100 μM) was added to the lower and the upper face of the membrane. The culture medium was changed every three days, and after 15 days of culture, living cells were stained with 4 μM of calcein-acetoxymethyl ester (Invitrogen, Carlsbad, CA, USA) in serum free-medium and visualized by TCS SP2 confocal microscopy (Leica Microsystems GmbH, Germany) using a ×10 objective. Optical sections were scanned at 5 μm intervals moving up from the underside of the membrane into the Matrigel to produce serial images. The fluorescence from each optical section was analyzed with LCS Lite software (Leica Microsystems, GmbH).

Cells invading into the Matrigel were isolated by homogenization in 800 μl PBS and centrifugation at 600g for 5 min at 4˚C. The cellular pellet was then subjected to TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) extraction and purification using the RNeasy kit (Qiagen, Valencia, CA, USA). cDNA synthesis was performed using MuLV reverse transcriptase (AppliedBiosystems, Carlsbad, CA, USA) according to the manufacturer's protocol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels (as a housekeeping gene for the quantification of invading cells) were measured by using the TaqMan Gene Expression Assays (Applied Biosystems) containing specific primers and TaqMan MGB probe on a 7500 quantitative Real-time PCR Machine and SDS software (Applied Biosystems).

Colony formation assays. Colony formation assays were performed in 96-well plates by seeding 3×10^3 cells resuspended in 100 μl of an agar-medium solution (0.3%) on top of a more concentrated agar-medium (0.6%) layer. Zoledronic acid (10 or 50 μM) was then added on top of the 0.3% agar-medium layer; PBS was used as the vehicle control. Cells were then incubated for seven days at 37˚C with 5% CO_2, renewing the top medium at the middle of the assay. Colony formation was evaluated after three hours of incubation with Alamar Blue reagent (Invitrogen), at 590 nm fluorescence emission. All assays were carried out at least in triplicate and data represent the percentage of fluorescence compared to vehicle-treated controls.

Statistical analysis. Statistical analyses were performed using SPSS v15.0 (SPSS Inc., Chicago, IL, USA). Non-parametric test were used to determine the differences between experimental conditions. A p-value of <0.05 was used to indicate statistical significance.

Results

Effect of zoledronic acid on tumor invasion ability. We tested the impact of zoledronic acid upon the ability of malignant cells to invade through a basement membrane and an extracellular matrix (Matrigel). Although physiological conditions imply a more complex cellular context, this *in vitro* assay mimics the tumor invasion process as a key mechanism in the process of dissemination and metastasis.

As shown in Figure 1, at 10 μM zoledronic acid we observed a significant decrease of invasion only in the MDA-MB-231 cell line. This inhibitory effect was more intense at 100 μM of zoledronic acid, with a 0,74-fold decrease with respect to the untreated control. At 100 μM, we also observed a significant decrease of invasion in the SKBR3 cell line. Although the extent of inhibition was less than that observed for the MDA-MB-231cell line, zoledronic acid also significantly impaired SKOV3 and HEC1A invasion at the maximal concentration. HCC1937 (data not shown) and HCT116 cells exhibited no change in invasion on zoledronic acid treatment conditions. Finally, MCF7, Ishikawa, and T47D cells were not able to invade under basal conditions (data not shown).

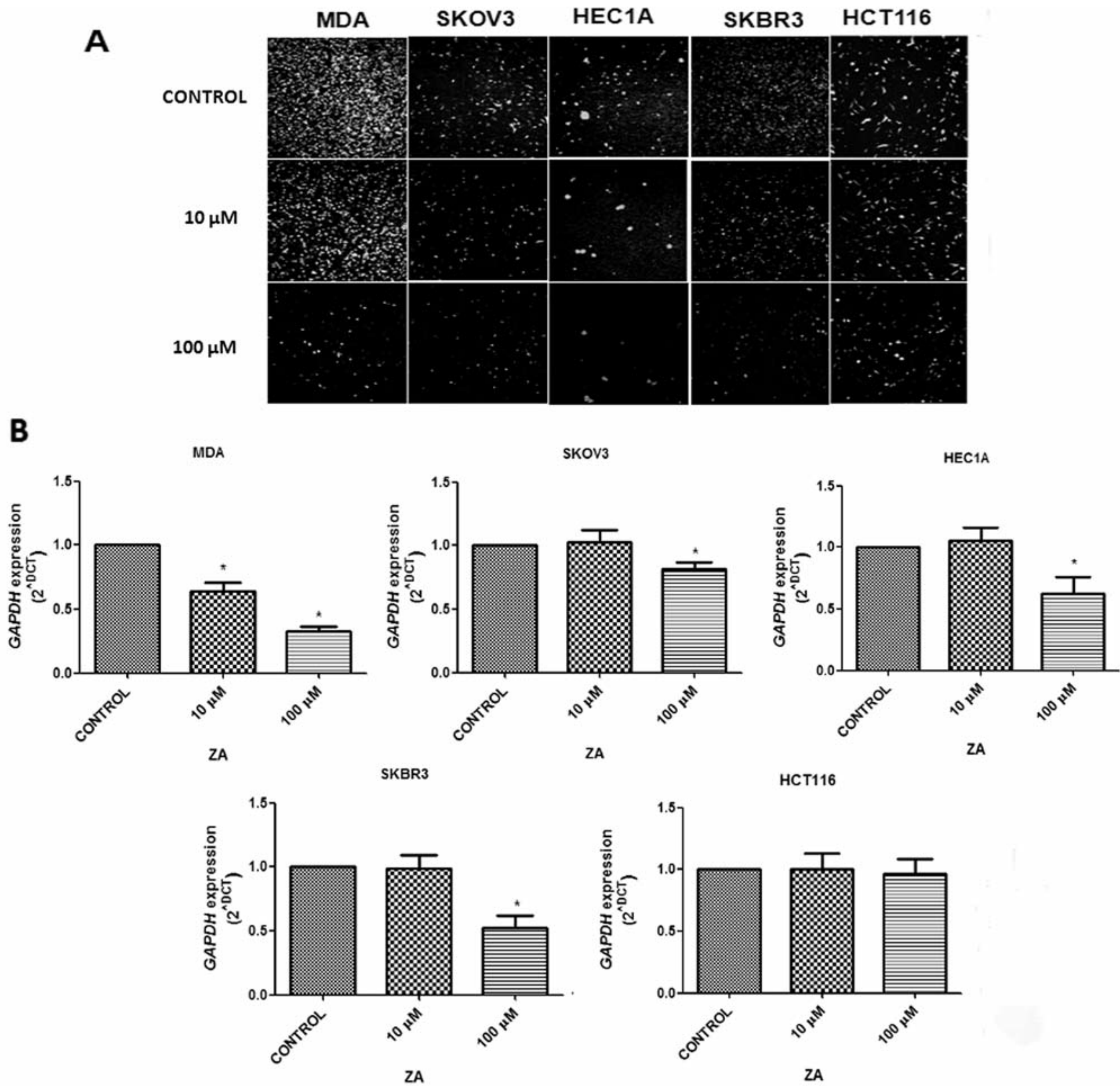

Figure 1. *Zoledronic acid impairs invasion in different tumor cell lines. A: Confocal microscopy section from the upper side of the transwell membrane in an inverted invasion assay. Cells were treated with 10 and 100 µM of zoledronic acid. B: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels represent indirect quantification of cells inavading into the Matrigel. Data are represented as fold change (2-ΔCT) in gene expression relative to that of the control. Bars=SEM; *p-value ≤0.05.*

Effect of zoledronic acid on colony formation. In order to test the impact of zoledronic acid on the ability of tumor cells to grow anchorage independently, a hallmark of transformed and aggressive tumor cells, we performed colony formation assays. We found a significant reduction in colony formation under the treatment with 50 µM zoledronic acid in SKOV3, HEC1A, HCT116, Ishikawa, T47D and MCF-7 cells (Figure 2). For the breast cancer cell lines MCF-7 and T47D and the colonic cancer cell line HCT116, there was a 50% reduction compared with the control in colony growth. For MCF-7, T47D and SKOV3 colony formation inhibition was also significantly reduced at 10 µM. For the remaining cell lines, no statistically significant differences were observed, neither with 10 nor with 50 µM zoledronic acid.

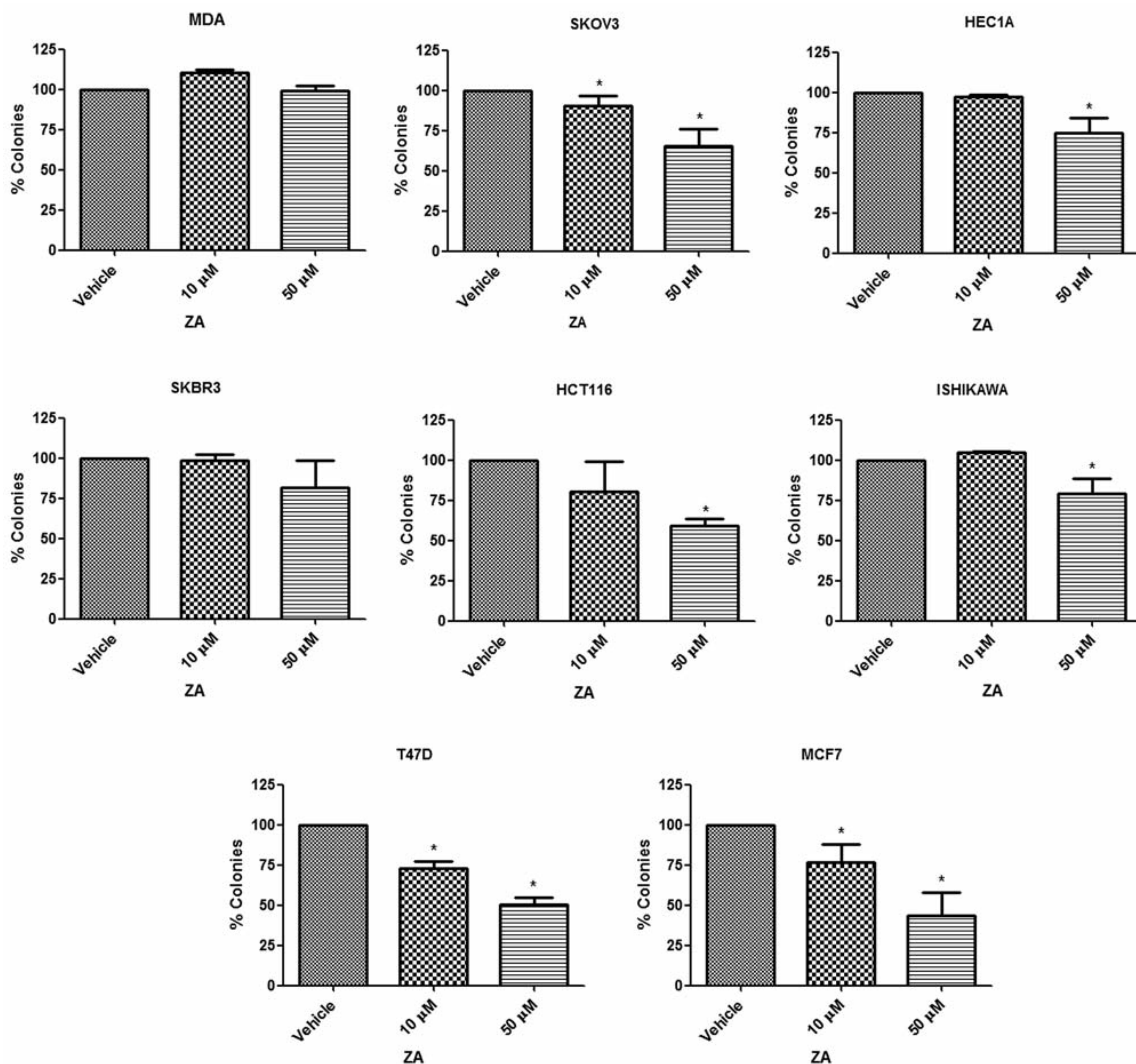

Figure 2. *Zoledronic acid blocks colony formation in different cell lines. Cells were transferred to soft-agar plates and incubated with 10 and 50 µM zoledronic acid for seven days. Colony formation was evaluated after three hours of incubation with Alamar Blue reagent, as fluorescence emission at 590 nm. Data are represented as the percentage of fluorescence compared to that of vehicle-treated controls. Bars=SEM; *p-value ≤0.05.*

Discussion

Tumor metastasis is a multistep process involving tumor growth, migration, adhesion, invasion, and induction of neoangiogenesis (18). Pre-clinical studies have described that *bis*phosphonates, such as zoledronic acid, in addition to their ability to block osteoclast-mediated bone resorption, can inhibit tumor proliferation and some of the critical mechanisms of tumor dissemination. The clinical benefit of this type of drug in blocking tumor progression in bone has also been demonstrated in different clinical studies (7, 8). Therefore, the therapeutic use of *bis*phosphonates is not only related to the prevention of specific clinical complications of bone metastasis (hypercalcemia, bone fractures and pain), but also to its additional potential as an antimetastatic agent (19). In the present study, we tried to clarify the impact of zoledronic acid on the metastatic potential of a representative pool of cell lines from different cancer types with diverse histology and aggressive phenotype, in order to determine the most suitable tumor types for the treatment with this drug.

The development of metastasis implies that malignant cells break away from the primary tumor, invade local tissues crossing the basal lamina, enter the circulation, and establish new cellular colonies at distant sites (20). We used an *in vitro* assay to analyze the impact of the zoledronic acid in the first steps of tumor invasion. We found that the MDA-MB-231 cell line exhibited a significant decrease in their invasion ability at a low concentration of zoledronic acid (10 μM). At high concentration (100 μM), the drug also exerts anti-invasive activity on HEC1A, SKBR3 and SKOV3 cell lines. Previous studies have described a decrease in MDA-MB-231 motility and migration under zoledronic acid treatment, however as far as we are aware of we describe this effect for the first time in an endometrial cancer cell line, suggesting that zoledronic acid would be a choice in the treatment of high-risk endometrial carcinoma.

Cell migration is strongly associated with RAS and the GTP-binding proteins RHO, (RAS-related C3 botulinum toxin substrate) RAC and RAB. These molecules are implicated in cytoskeletal organization, intracellular signaling and cellular motility (21). The absence of prenylation in these molecules has been reported to be critical in suppressing tumor invasion and metastasis. Thus, zoledronic acid at low concentrations prevents the translocation of RHOA from the cytoplasm to the cell membrane of MDA-MB-231 cells, promoting the disorganisation of the actin cytoskeleton and a loss of stress fibers, reducing their motility and blocking invasion (16). This interference with the critical RHOA signaling mechanism may also occur in prostate cancer cells, although this was not measured specifically in the experiments reported here. Additionally, some studies have reported the impact of zoledronic acid on the expression of the MMPs and their direct inhibitors. Thus, in PC3 prostate cancer cells the levels of MMP7 and TIMP metallopeptidase inhibitor-2 (TIMP2) are strongly regulated by zoledronic acid it seems that a decrease in MMP7 and an increase in TIMP2 levels are responsible for invasion inhibition observed in this cell line after zoledronic acid treatment (22). In the human nasopharyngeal carcinoma cell line HNE-1, the inhibition of migration and invasion observed after treatment with zoledronic acid was associated with down-regulation of MMP2 and MMP9 expression (14). Interestingly, in MDA-MB-231cells, this inhibition is not associated with a decrease in MMP secretion as it was observed for concentrations higher than those required for cell invasion inhibition (23). In contrast, urokinase-type plasminogen activator (u-PAR) expressed on the cell surface of MDA-MB-231 cells was dramatically reduced by zoledronic acid at low concentrations, becoming a candidate player in inhibition of cell invasion (16).

During the metastatic process, after invading through the basement membrane, tumor cells need to grow in the absence of anchorage to their native matrix environment in order to develop a new tumor. We observed a statistical decrease in colony formation in MCF7, SKOV3 and T47D cells at low concentration of zoledronic acid and at medium concentrations, HCT116, HEC1A and Ishikawa cells exhibited a decrease of clonogenicy ability. Thus, zoledronic acid seems to block the late steps of the metastatic process in these cell lines, when the tumor cells reach a new organ and need to proliferate to develop a new tumour.

In HCT-116 cells inhibition of the colony formation under zoledronic acid treatment has already been described at as even lower concentration (24). Regarding the molecular mechanism implicated in this inhibition, it is well-known that zoledronic acid suppresses the downstream signaling cascades of RAS proteins, such as protein kinase B or the Mitogen-Activated Protein Kinase (ERK) strongly implicated in the activation of cell proliferation (25). Moreover, the coordinated expression of several cell-cycle regulators, such as cyclins, is modified by zoledronic acid (26). However, the net effect of the drug will depend on the balance between growth inhibiting and growth survival signals. For example, it seems that *bis*phosphonates can activate the p38 pathway and this activation might confer resistance to the drug in some cell lines (27). On the other hand, it has been reported that zoledronic acid-induced inhibition of cell proliferation in MCF-7 and HCT-116 cells is dependent on the induction of caspases while in other cell lines this dependence is less important (24, 28). Zoledronic acid also exerts its antiproliferative activity *via* p53-independent induction of apoptosis, being an attractive drug for the treatment of tumors with loss of p53 function (26).

Interestingly, the most sensitive cell line in the invasion assay, MDA-MB-231, maintained its colony formation ability at high concentrations. The low effect of zoledronic acid on MDA-MB-231 proliferation was previously reported (28). However, the drug seems to block key mechanisms for tumor spread in this cell line, being a good option to inhibit the first steps of metastasis in triple-negative breast cancer.

In summary, our results demonstrated that zoledronic acid has antiproliferative and antiinvasive activity on a wide variety of human carcinoma cell lines. These findings lend weight to the proposal that this compound has significant potential for use as an antimetastatic agent in breast cancer but also in other tumor types where the effect of the drug has been less described such as endometrial cancer. Finally, our results confirmed the need for further *in vivo* studies to clarify the molecular mechanisms of the antiinvasive activity of zoledronic acid.

Competing Interests

There are no competing interests.

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