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Zoledronic Acid and Leuprolide Acetate Affect Du-145 Migration towards Stem Cell Conditioned Medium

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Abstract

Prostate cancer (PCa) is the most frequent genitourinary tumour and the third specific death cause, mostly because of bone metastasis. Currently, PCa is treated with GnRH analogues (leuprolide acetate-LA) and bisphosphonates (zoledronic acid-ZA) often in association. Thus, the aim of this work has been to study, in vitro, the effects of these drugs on cell line derived from PCa (DU-145). Particularly, we focused on some crucial aspects that drugs might play in tumor evolution and metastatization, by interfering with vitality, migration and interactions with bone cells. ZA cytotoxicity has been confirmed on DU-145 after 48-hours incubation at 5 μM, while LA cytotoxicity appears only after 72-hours contact at higher concentrations (100 μM). Both sub cytotoxic ZA and LA doses decreased 3D (transwell assay) PCa cell migration rate. pAkt/Akt ratio is diminished by LA and, even if less strikingly, by ZA, in agreement with the respective inhibition migration ratios. Cells underwent migration test in mesenchymal stem cells – MSC – conditioned medium (MSC-CM), which significantly increases the rate of migration (210%± 2.2; $P<0.05$). Addition of both ZA and LA quenched the attractive effect of conditioned medium. Our results suggest that LA and, mostly, ZA have a direct toxic effect on cancer cells. Furthermore, they inhibit cellular migration even under attractive stimuli exerted by MSC, and this might contribute to explain their effect in limiting metastatization.

Keywords: Prostate cancer; Cell migration; Bisphosphonate; GnRH analogues

Abbreviations: PCa: Prostate Cancer; GnRH: Gonadotropin-Releasing Hormone; ZA: Zoledronic Acid; BM-MSCs: Bone Marrow Mesenchymal Stem Cells; MSC-CM: Mesenchymal Stem Cells-Conditioned Medium; LA: Leuprolide Acetate; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; 3D: Three-Dimensional; CDI: Coefficient of Drug Interaction

Introduction

Prostate cancer (PCa) is the most frequent genitourinary tumor, representing 11% of the male malignancies in Europe, and is one of the leading causes of morbidity and mortality in the world [1].

Administration of GnRH agonist or other analogues (such as leuprolide acetate, buserelin, deslorelin, goserelin and istrerelin) is a well-established treatment of prostate cancer inducing a pharmacological castration [2] and resulting in cancer regression. Despite pharmacological treatment, the onset of metastatic dissemination represents, together with the development of androgen-

independent growth, a critical progression step of human prostate cancer that largely determines the clinical course of the disease and survival of the patients [3]. Bone is a preferential site of metastases [4], which produce a crucial impact on patients' functional status and quality of life due to significant pain and high risk of skeletal-related events, including pathologic bone fractures (both vertebral and non-vertebral), spinal cord compression, surgery and radiotherapy to bone [5]. The burden of metastatic disease can be treated by administering a potent inhibitor of osteoclast activity such as zoledronic acid (ZA), a bisphosphonate widely used to treat skeletal complications of malignancy and considered the drug of choice for both the prevention and the treatment of bone mass loss. In vivo, it inhibits the release of growth factors from osteoblasts and bone marrow stromal cells [6]. Moreover, bisphosphonates modulate many other cellular and physiologic processes relevant to bone metabolism and tumor initiation and progression [7].

Prostate cancer cells tropism for the bone is the result of a sequential series of molecular events: bone metastases arise as a result of a crosstalk between metastatic cells, bone matrix, osteoblasts and osteoclasts, and cellular components of the bone marrow microenvironment. Among these, bone marrow mesenchymal stem cells (BM-MSCs) play a paramount role in the so-called metastatic niche [8, 9]. Prostate cancer cells migration can be influenced by MSC-conditioned medium soluble factors [8], and drugs normally used for prostate cancer treatment, such as zoledronic acid and leuprolide acetate, interact with this process [10]. We aimed at examining the effect of such molecules on the behavior of the human prostate cancer cell line DU-145 in an in vitro cell co-culture model of invasion assay. Cells were exposed to the drugs, alone or in combination, prior and/or during the migration test. We previously demonstrated that an up-regulation of Akt phosphorylation may exert a crucial role in DU-145 cell migration under conditioned medium stimulus [9]. Knowing that PI3-K/Akt signaling pathway plays a critical role in cell invasion and in modulation of cell migration [11], we also examined if zoledronic acid and leuprolide acetate modulate AKT level.

Materials and Methods

Materials

All reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. Tissue culture plasticware was from Falcon (Franklin Lakes, NJ, USA). Zoledronic acid (ZA) [1-hydroxy-2-(1H-imidazol-1-yl)ethane-1,1-diyl]bis (phosphonic acid) was generously provided by Novartis (Surrey, UK) and leuprolide acetate (LA) (N-[1-[[1-[[1-[[1-[[1-[[5-(diaminomethylideneamino)-1-[2-(ethylcarbamoyl)pyrrolidin-1-yl]-1-oxo-pentan-2-yl] carbamoyl]-3-methyl-butyl]carbamoyl]-3-methylbutyl] carbamoyl]-2-(4hydroxyphenyl)ethyl] carbamoyl]-2-hydroxy-ethyl]carbamoyl]-2-(1H-indol-3-yl)ethyl] carbamoyl]-2-(3H-imidazol-4-yl)ethyl]-5-oxopyrrolidine-2-carboxamide) was from Takeda (Osaka, Japan).

Prostate cancer cell culture

Human androgen independent DU-145 prostate cancer cells were purchased from ATCC (Rockville, MD, USA). Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere in RPMI 1640 containing 10 mL/L penicillin and streptomycin solution, $NaHCO_3$ 2 g/L (7.5% w/v), 10% Fetal Bovine Serum (FBS).

Proliferation assay

DU-145 cells were seeded into flat-bottomed 96-well micro plates (1,000/100 µL culture medium/well) and allowed to attach overnight in complete medium before drugs addition. Drugs were added to culture medium, alone or in combination, testing various concentrations from 2.5 µM to 50 µM (ZA) [12] and from 0.5 µM to 100 µM (LA) [13] for 24–96 hours, according to protocols. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed as previously described [14]. Data (mean ± standard deviation) were the average values of 8 replicates. Each experiment was repeated thrice. Cell viability was expressed as percentage of living cells with respect to controls.

Mesenchymal stem cells isolation and MSC-CM collection

Bone marrow cells were obtained from femurs of adult rats as described in Mognetti et al, 2013. They were grown in complete αMEM containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg /mL streptomycin at 37 °C and 5% $CO_2$for 3 days as previously described [9].

For migration assay, conditioned medium was collected after three days of culture, centrifuged at 4000 rpm for 5 minutes at 4°C in order to eliminate cells and cellular debris, and used for migration assays or frozen.

Three-dimensional migration assay

Three-dimensional (3D) migration assay was used to measure the invasiveness of DU-145 cells in response

to various stimuli. Migration assays were performed in transwells (BD Falcon cell culture inserts incorporating polyethylene terephthalate – PET – membrane with 8.0 μM pores, $6\pm2\times10^4$ pores /cm^2) as previously described [9].

Briefly, 10^5 cells were resuspended in 200 μL of RPMI containing 2% FBS with or without drugs (zoledronic acid 20 μM, leuprolide acetate 100 μM or both, representing non-toxic concentration at 24 hours as per the described viability assay) and then seeded in the upper chamber of a transwell; in the lower chamber, we added RPMI or MSC-CM as detailed in Table 1. In some cases, before seeding cells were grown for 18 hours in presence of zoledronic acid 20 μM, leuprolide acetate 100 μM or both (Table 1).

Table 1: Experimental conditions for migration assay

Pre-incubation 18 hours	Migration test 6 hours
none	RPMI
	ZA
	LA
LA	RPMI
	LA
	ZA
ZA	RPMI
	ZA
	LA
ZA + LA	RPMI
none	MSC-CM
	MSC-CM + ZA
	MSC-CM + LA

LA= leuprolide acetate 100 μM
ZA= zoledronic acid 20 μM

Transwells were placed in the incubator at 37 °C and 5% CO_2 for 6 hours and finally treated as detailed by Mognetti et al.[9].

Wells were photographed using a BRESSER Mikro Cam 3 Mpx camera, with an optical microscope (Leica DC 100) at 100 x. Five pictures were randomly chosen per well, and used to count the migrated cells with Image J software using cell-counter plug-in. Results from different experiments (performed at least three times in duplicate) were expressed as mean ± standard deviation. In order to avoid any cytotoxic effect potentially confounding migration results, we performed a cytotoxicity test at the same time and same conditions of every migration test.

Immunoassay to detect cytokine content in MSC-CM

Cytokine profiles in MSC-CM were determined using the Human Cytokine/ Chemokine Magnetic Bead Panel protocol from the "Milliplex®Human Cytokine 5 Plex" kit (Billerica, MA). The procedure was conducted according to the manufacturer's protocol.

Briefly, the assay plates were washed with washing buffer, and shaken on an orbital plate shaker for 10 minutes at room temperature. The washing buffer was decanted and the standards, assay buffer, or samples/ controls were mixed with serum matrix in each well, incubated overnight at 4°C on an orbital shaker with specific antibody to detect GM-CSF, MCP1/CCL2, IL-10, IFNγ, TNFα; well contents were then removed and wells washed. Biotinylated detection antibodies were then added and incubated for 1 hour at room temperature while shaking. After incubation, well contents were removed and streptavidin-phycoerythrin was added and incubated for 30 minutes at room temperature, then washed and resuspended in Sheath Fluid. Plates were read on the LuminexMagPix® machine and data were collected using the LuminexxPONENT® software (v.4.2); data analysis was performed using the Milliplex® Analyst software (v. 5.1).

Western blotting

Cells were seeded in 10 cm diameter Petri dishes, cultured until sub-confluence and then drugs were added (ZA 20 μM and LA 100 μM, to reproduce the same conditions of migration assay). After 6 hours incubation, cells were collected, treated and immunoblotted as detailed according to Mognetti et al. [9].

Blots were probed with primary polyclonal antibodies (Cell Signaling Technology, Danvers, MA, USA) suspended in TBS Tween 0.1% as follows: anti-Akt (mouse, 1:800), anti-pAkt (Ser473, rabbit, 1:500), anti-α5β1 (rabbit, 1:500, kindly provided by Prof. L. Primo, University of Turin) and anti-vinculin (developed in rabbit, Sigma). Vinculin was used as an internal control.

HRP-conjugated anti-mouse (Amersham-GE Healthcare, Buckinghamshire, UK) and anti-rabbit (Santa Cruz Biotechnology) were diluted (1:6000 and 1:8000, resp.) in TBS Tween 0.025%. Bands were quantified using the ImageJ software.

Results from different experiments (performed at

least three times in duplicate) were expressed as mean ± standard deviation.

Phosphorylation levels of Akt were expressed as ratio pAkt/Akt. All data were expressed as percentage modification relative to control conditions.

Focal Adhesion

Cells were grown on Lab-Tek chamber slide (Nalge Nunc International, Rochester, NY, USA) until approximately 50-60%confluent, then drugs were added (ZA 20 μM and LA 100 μM, to reproduce the same conditions of migration assay). After 6 hours cells were fixed with 4% paraformaldehyde in 1x PBS for 15-20 minutes at room temperature and then treated with the Actin Cytoskeleton and Focal Adhesion Staining Kit (Millipore, Burlington, MA, USA) according to the manufacturer instructions. The samples were finally mounted with a Dako fluorescent mounting medium (DAKO) and analyzed by aLSM 510 confocal laser microscopy system (Zeiss, Jena, Germany).

Statistical analysis

All the data in this study were shown as the mean ± standard deviation. Statistical analyses were performed by One-way ANOVA, with Dunnett's post-tests, or two-way ANOVA using Graph Pad Prism version 5.00 for Windows (Graph Pad Software, San Diego California USA). Coefficient of Drug Interaction (CDI) was used to define the type of interactions between the employed drugs. CDI was calculated by means of the equation: CDI = AB/(A × B), where AB is the relative cell migration of the combination; A or B, relative cell migration of the single agent. CDI < 1 indicates a synergistic effect; CDI = 1 indicates an additive effect; CDI > 1 indicates an antagonistic effect.

Results

Effect of zoledronic acid, leuprolide acetate and combination of both on cell proliferation

Incubation up to 24 hours with 20 and 40 μM zoledronic acid induced a weak cytotoxic effect on DU-145 (Figure1A). A statistically significant cytotoxicity appears after 48 hours of incubation, already at the 5μM concentration.

Leuprolide acetate displayed no cytotoxicity on DU-145 cells (Figure1B), within the concentration range tested, after 24 and 48 hours of incubation.

A significant toxicity is appreciable only after 72 hours of incubation from 5 μM on.

Addition of leuprolide acetate 100 μM did not increase zoledronic acid cytotoxicity at any concentration after 48 hours incubation (Figure 1C).

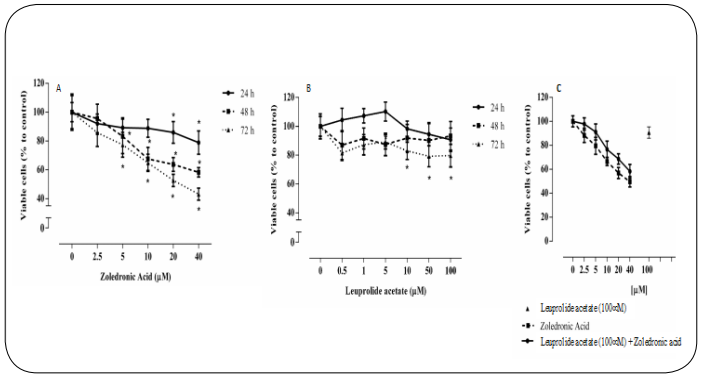

Figure 1: Effect of zoledronic acid and leuprolide acetate on DU-145 growth. (A) Proliferation assay after 24, 48 and 72 hours culture in presence of increasing concentration of zoledronic acid (2.5-50 μM). (B) Proliferation assay after 24, 48 and 72 hours culture in presence of increasing concentration of leuprolide acetate (0.5-100 μM). (C) Effect of LA 100 μM and ZA (0-40 μM), alone or in combination, on DU-145 cell after 48 hours incubation.
*=P<0.05 vs control.

3D migration assay

Both drugs (ZA and LA) decreased migration rate of DU-145 cells when compared to control (Figure 2). At these conditions, leuprolide acetate was significantly more effective than ZA in inhibiting cell migration. The simultaneous presence of the two drugs influenced migration less than each single drug; association decreased the efficiency of leuprolide acetate alone and did not seem to differ from incubation with zoledronic acid alone.

Single drug pre-incubation did not potentiate the effect of simple incubation, in any case (Figure 2).The effects of any single drug were not significantly potentiated by any pre-incubation, even, in some cases, abolished (pre-incubation with ZA or LA and pre-incubation with ZA followed by migration with ZA).

When cells were pre-incubated with the single drugs before migration test performed in control medium, migration was not inhibited at all. On the other hand, when cells were pre-incubated with both drugs, migration, even if occurring in RPMI, was inhibited as much as when

cells were not pretreated but incubated with both drugs simultaneously. MSC-CM significantly increased the rate of migration towards control medium, and the addition of both drugs quenched the attractive effect of conditioned medium (Figure 2).

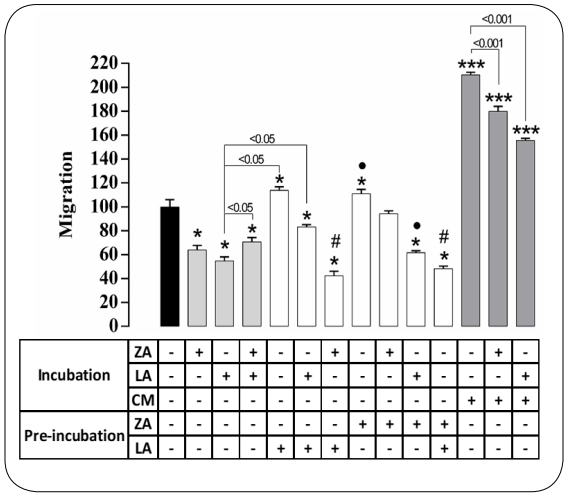

Figure 2: Three-dimensional migration. Migration rate after incubation or pre-incubation with drugs and/or in conditioned media (CM) compared to control. Migration is expressed in arbitrary units. *=P<0.05 vs control; ***= P<0.001 vs control; •=P<0.001 vs ZA; #=P<0.001 vs LA+ZA.

Drug interaction

CDI results suggest an antagonistic effect in the incubation LA + ZA, slightly additive for pre-incubation ZA + incubation LA and synergistic for pre-incubation LA + incubation ZA, as reported in Table 2.

Table 2: Drug interactions. CDI < 1 indicates a synergistic effect; CDI = 1 indicates an additive effect; CDI > 1 indicates an antagonistic effect.

AB	A	B	CDI
Incubation LA+ ZA	incubation ZA	incubation LA	2,02
Pre-incubation ZA + incubation LA	Pre- incubation ZA	incubation LA	1,02
Pre-incubation LA + incubation ZA	Pre-incubation LA	incubation ZA	0,58

Cytokines content in MSC-CM

Cytokines were detected in MSC-CM as follows:
GM-CSF: 93.40 pg/ml
MCP1/CCL2: 12.95 µg/ml
IL-10: 1.81 pg/ml
IFNγ: 30.75 pg/ml
TNFα: 40.34 pg/ml

pAkt/Akt Ratio after Drugs Incubation

LA slightly inhibited Akt phosphorylation compared to control (Figure 3) after 6 hours incubation, though ZA effect was more evident.

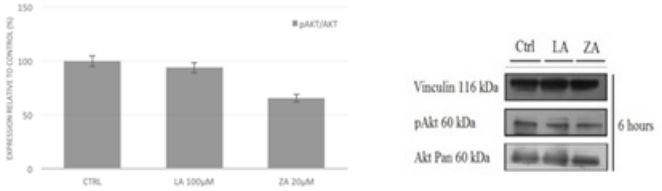

Figure 3: Drugs effect on pAkt/Akt in DU-145 cell line after 6 hours of incubation with LA 100 µM or ZA 20 µM, as detected by western blot (one representative experiment). Vinculin as internal control.

Adhesion Molecules Expression

The expression of α5β1 as detected by western blot analysis was not modified by 6 hours incubation in ZA 20 µM or LA 100 µM, as shown in figure 4.

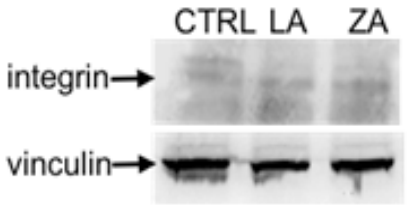

Figure 4: Drugs effect on α5β1 expression in DU-145 cell line after 6 hours of incubation with LA 100 µM or ZA 20 µM, as detected by western blot. Vinculin as internal control.

Immunocytochemistry analysis was performed after 6 hours of culture to qualitatively evaluate cell adhesion and morphology. In order to obtain a more detailed evaluation of cell adhesion, the actin cytoskeleton and focal adhesion complex were stained using TRITC-conjugated phalloidin and antivinculin antibody, respectively.

No gross difference was detectable in the three different culture conditions, in terms of dimension, orientation of the actin cytoskeleton or exact location of focal adhesion sites. Vinculin-positive sites were observed both on control and treated cells, and their distribution

was alike (data not shown).

Discussion

In this work, we demonstrate that ZA and LA inhibits, in vitro, the proliferation of human prostate cancer cells: this is particularly true for ZA, whose cytotoxicity appears already after 48- hours incubation and at lower concentrations than those necessary to observe a LA-induced cytotoxic effect. Since patients often receive both drugs, we searched for eventual interactions between the two molecules *in vitro*. Therefore, we incubated DU-145 cells with both drugs contemporaneously (at variable concentrations of ZA). No synergistic effect was induced by the simultaneous incubation with ZA and LA on cells.

Besides cytotoxicity, we wondered if ZA and LA could have other effects on prostate cancer cells in vitro. Therefore, because of its relevance on metastasis phenomenon, we decided to investigate tridimensional migration by means of the transwell assay and the evaluation of Akt phosphorylation level.

Both ZA and LA decreased in Akt phosphorylation in 6 hours, but while LA inhibition was faint and far from significance, ZA effect was irrefutable. The timing and the effect, greater than LA- induced, are both in agreement with what observed in inhibition of cell migration (Figure 2).

Because of its important role in driving the chemotactic affinity of prostate cancer cells to human bone marrow mesenchymal stromal cells [15] we decided to check if ZA and LA had an effect in modulating the expression of α5β1. According to our western blot analysis neither drug affected α5β1expression in 6 hours; analogously, neither drug modified the expression or localization of exact location of focal adhesion sites, nor orientation of the actin cytoskeleton. Hence, this is probably not the way by which LA and (even more) ZA hamper DU-145 migration.

Furthermore, co-incubation with ZA and LA raises no synergistic effect (Table 2); rather, LA effect seems to be significantly reduced by the presence of ZA. On the other hand, pre-incubation with LA (but not with ZA) significantly potentiates inhibitory migration properties of both LA and ZA in a synergistic manner. Nevertheless, the simultaneous presence of the drugs has to induce some effect on cell migratory properties, since when cells were pre-incubated with the single drugs their migration in culture medium was not inhibited at all, while 18-hours pre-incubation with ZA and LA significantly decreased their migration, even in RPMI. This might suggest that incubation with each drug singularly can cause not relevant/reversible changes, which cells are able to repair, while specific pre-incubation and/or specific drugs sequences (in particularly pre-incubation with LA followed by incubation with ZA) could lead to hard reversible changes affecting cell migration. We do not know, at present, how to explain this phenomenon, but a durable effect of the simultaneous presence of ZA and LA is probably worth further investigations.

About the ability to migrate more towards MSC-CM, it has been shown that migration and formation of metastases by prostate cancer cells in vivo is largely influenced by several factors produced by bone cells [16,17,18,19,20]. We observed a massive migratory increase under MSC-CM stimulus. It is interesting to note that both ZA and LA inhibit DU-145 3D-migration at the same magnitude also when cells undergo MSC-CM stimuli (Figure 2). According to the literature, we found that the MSC-CM contains several factors involved in cancer cell survival and migration, such as TNFα, MCP1/CCL2 [16-18] and GM-CSF [21]. Aggressive cancer cell lines such as DU-145 and PC3 express a higher amount of CCL2-specific receptor CCR2 compared with the less aggressive cancer cells such as LNCaP or non-neoplastic PrEC and RWPE-1 cells [22]; a positive correlation has also been established between CCR2 expression and prostate cancer progression [18]. Furthermore, Rivas and coll. [21] shown that prostate cancer cells express functional high-affinity GM-CSF receptors and therefore this hematopoietic growth factor may have an effect on prostate carcinoma cells. The increased expression of GM-CSF receptors in prostatic hypertrophy and neoplastic prostate ephitelium suggests a relationship between prostatic epithelial cell growth and GM-CSF [21]. At least, as shown by Gao and coll [23], TNFα factor in endothelial cells may increase the activation and ligation of αvβ3 integrins [23] to facilitate cell migration, and regarding prostate cancer activation have a central role in prostate cancer metastatization [24].

As previously reported in literature, most of these factors are affected by action of ZA and LA [25,26,27,28,29,30]. In particular, a direct correlation between GM-CSF and MCP-1 decreased levels and cell invasiveness has been demonstrated [25,28]. On the basis of these observations, we could speculate that, as demonstrated in other systems, it can be confirmed in our own that the action of ZA and LA in decreasing migration could be both directed to the tumor cells and

indirectly on potential metastatic niche.

Conclusions

Our results suggest that: (i) drugs in use in vivo for prostate cancer treatment have a direct effect on prostate cancer cells proliferation, and this could contribute to justify the results obtained in vivo; (ii) effect of drugs overlapping is difficult to predict a priori; we are unable, with data at our disposal, to identify a regimen that clearly enhances the effect of the two drugs. However, we have shown that specific co-incubation prolongs inhibitory effect on prostate cancer cells migration; (iii) drugs affect migratory ability of prostate cancer cells, and this could contribute to justify their limiting effect of in vivo metastases (mostly due to LA). This inhibition, though, does not seem to be mediated by a change in the expression of α5β1 or focal adhesion molecules distribution; (iv) in the same way ZA and LA diminish the chemoattractive effect of the bone marrow mesenchymal stem cells and, hence, the potential role that they can play in the phenomenon of metastasis; (v) ZA-induced decrease in cell migration, compatible with action on the PI3K/Akt pathway [31].

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Conflict of Interest

The authors confirm that there are no conflicts of interest.

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