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Research Article

Zoledronic Acid and Leuprorelin Acetate, Alone or in Combination, Similarly Reduce Proliferation and Migration of Prostate Cancer Cells In Vitro

Barbara Mognetti,[1] Giuseppe La Montagna,[1] Maria Giulia Perrelli,[1,3] Silvia Marino,[1] Pasquale Pagliaro,[1,3] Cecilia M. Cracco,[2] and Claudia Penna[1,3]

[1] *Dipartimento di Scienze Cliniche e Biologiche, Università degli Studi di Torino, Regione Gonzole 10, Orbassano, 10043 Torino, Italy*
[2] *S.C. Urologia, Ospedale Cottolengo di Torino, Via San Giuseppe Benedetto Cottolengo 9, 10152 Torino, Italy*
[3] *Istituto Nazionale per le Ricerche Cardiovascolari (INRC), Via Irnerio 48, 40126 Bologna, Italy*
Address correspondence to Barbara Mognetti, barbara.mognetti@unito.it

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Abstract We studied the effects of zoledronic acid (ZA) and leuprorelin acetate (LA) separately and combined on a human cell line derived from a bone metastasis of prostatic adenocarcinoma (PC3). In particular, we focused on the effects that drugs given singularly or in association may play on tumor evolution and metastatization. Cell proliferation, 2D- and 3D-migration were studied in basal conditions and under attractive stimuli exerted by bone marrow mesenchymal stem cells (BM-MSC). Either drug decreased PC3 proliferation, though ZA was much more cytotoxic than LA. However, LA cytotoxic concentrations are higher than those usually reached in vivo. Subtoxic concentration of either drug inhibited migration especially under BM-MSC medium stimuli via an Akt-dependent mechanism. The capability of either drug to inhibit cellular migration is in line with their well-known effect in limiting metastatization. Intriguingly, no additive effect on the antiproliferative activity or in hindering migration is observed when the drugs are administered concomitantly, compared to the effects of each drug alone.

Keywords gonadotropin-releasing hormone analogue; bisphosphonate; prostate cancer; cytotoxicity; metastatization

1. Introduction

GnRH receptors are present both in prostatic malignant tissue and carcinoma derived cell lines, suggesting that GnRH analogues such as leuprorelin acetate (LA) [28], normally used to treat advanced prostate cancer (PCa), can exert a local action [10]. An inhibition of proliferative activity in presence of GnRH agonists has been found in androgen independent and androgen dependent prostatic cancer derived cell lines [23] and in epithelial cells isolated from prostatic tumors [2].

Moreover, LA antagonizes the metastatic behavior of both androgen-independent cell lines, DU145 and PC3, by decreasing their capacity to invade a reconstituted matrix and to migrate in response to haptotactic stimuli [5]. Bone is the preferential site for PCa metastases. Bone metastases likely depend on 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 [14, 22]. In particular, BM-MSC paracrine factors may allow tumor cells to access cellular niches favoring tumor cell survival and growth [14, 22].

Administration of IV bisphosphonates, such as zoledronic acid (ZA), can prevent bone loss and modulate tumor cell migration and invasion [11] in breast and prostate cancer bone metastases in patients treated with GnRH agonists [20].

The beneficial effects of bisphosphonates in PCa patients are attributed to their inhibitory activity on osteoclast-mediated bone resorption, which is markedly increased in lytic and in blastic metastatic bone disease [9]. They likely act on two essential stages of metastatic process: (1) tumor cell adhesion to bone extracellular matrices and (2) invasion through extracellular matrix, via modulation of cell adhesion molecules and extracellular matrix interacting molecules [4].

In addition to their antiosteoclast effects, bisphosphonates directly inhibit in vitro the growth of breast [6], pancreas [30], and prostate cancer cells, in a concentration- and time-dependent manner [17].

Despite the absence, to our knowledge, of in vitro and achieved preclinical studies investigating any potential interaction in the coadministration of GnRH analogues and bisphosphonates, this association is considered the gold standard of metastatic PCa therapy. Particularly, pharmacodynamic drug interactions could be the result of overlapping mechanisms of action. Therefore, we wondered whether

drug interactions can positively or negatively modify both drug effect and/or toxicity. A better understanding of these potential interplays could be used to increase the therapeutic effect of a combination regimen or to minimize its toxicity. We, therefore, examined the antiproliferative and antimigratory effect of a GnRH analogue, LA, and of a bisphosphonate, ZA, alone or in combination, on PC3 cells in vitro, in basal and stimulated conditions, obtained by exposing PC3 cells to the medium conditioned by BM-MSCs. Moreover, we analyzed the role of these two drugs on Akt phosphorylation, which is one of the most notable mechanisms involved in cell proliferation and migration [16]. PC3 cells, isolated from bone metastases of PCa, were chosen because they mimic the castration-resistant phase of PCa patients treated with both GnRH analogues and bisphosphonates [15] and because they are thoroughly recognized as a representative of a highly metastatic PCa cell line, on account of, among other aspects, their lack of AC-tubulin [29] and increased levels of β1 integrins and integrin-induced autophosphorylation of FAK [18]. PC3 cells are frequently used to study PCa bone metastasis both in vivo and in vitro [12, 13].

With this in vitro study, besides confirming the direct cytostatic effect of either drugs (ZA or LA), we also show, for the first time, that both drugs limit cell migration induced by BM-MSCs and that there is no additive effect when these drugs are used concomitantly. Therefore, we suggest that it is worthwhile to consider a therapeutic regimen in which the two classes of drugs are included in early stages when there are not yet signs of bone metastases in patients with PCa.

2. Materials and methods

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 [1-hydroxy-2-(1H-imidazol-1-yl)ethane-1,1-diyl]bis(phosphonic acid) was generously provided by Novartis (Surrey, UK) and leuprorelin acetate

(N-[1-[[1-[[1-[[1-[[1-[[1-[[5-(diaminomethylideneamino)-1-[2-(ethylcarbamoyl)pyrrolidin-1-yl]-1-oxo-pentan-2-yl]carbamoyl]-3-methyl-butyl]carbamoyl]-3-methyl-butyl]carbamoyl]-2-(4-hydroxyphenyl)ethyl]carbamoyl]-2-hydroxy-ethyl]carbamoyl]-2-(1H-indol-3-yl)ethyl]carbamoyl]-2-(3H-imidazol-4-yl)ethyl]-5-oxo-pyrrolidine-2-carboxamide).

was from Takeda (Osaka, Japan)

2.1. Cell culture

PC3 cells (human androgen-independent cells derived from a bone metastasis of prostatic adenocarcinoma) 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) decomplemented for 30 min at 55 °C.

2.2. Bone marrow mesenchymal stem cells (BM-MSC) isolation and production of BM-MSC conditioned medium

Bone marrow cells were harvested from femurs of adult rats as detailed in the work of Raimondo et al. [25]. Animals received care in accordance with the Italian law (DL-116, 27 January 1992), which complies with the Guide for the Care and Use of Laboratory Animals by the US National Research Council.

We previously demonstrated that BM-MSCs isolated with this procedure are $CD90^+$ and $CD34/CD45^-$ and that under opportune stimuli they can differentiate [7, 25]. We also previously reported that BM-MSCs stimulate PCa cell migration [22]. BM-MSCs were therefore included in the present study according to the described protocols.

BM-MSC conditioned medium was collected after three days of culture, centrifuged to eliminate cells and cellular debris, and freshly used or frozen [22].

2.3. Proliferation assay

PC3 cells were seeded into flat-bottomed 96-well microplates (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) [19] and 0.5 μM to 100 μM (LA) [23] for 24–96 h, according to protocols.

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed as described by Mognetti et al. [21]. Data (mean $\pm$ standard errors) 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.

2.4. 2D migration assay-wound healing

PC3 cells were seeded in a 6-well plate at 30,000 cells/well. When confluent, we performed a cross "wound" in each well with a p1000 tip [8], then wells were washed thrice with PBS and medium replaced without (control) or with single (ZA 5 μM and LA 100 μM) or combined drugs (ZA 5 μM plus LA 100 μM).

We photographed "wounds" on time-laps every 45 min to highlight migration until a maximum of 6 h and, as a final time point, at 24 h.

Experiments were repeated three times and every time five different points for each experimental condition were considered.

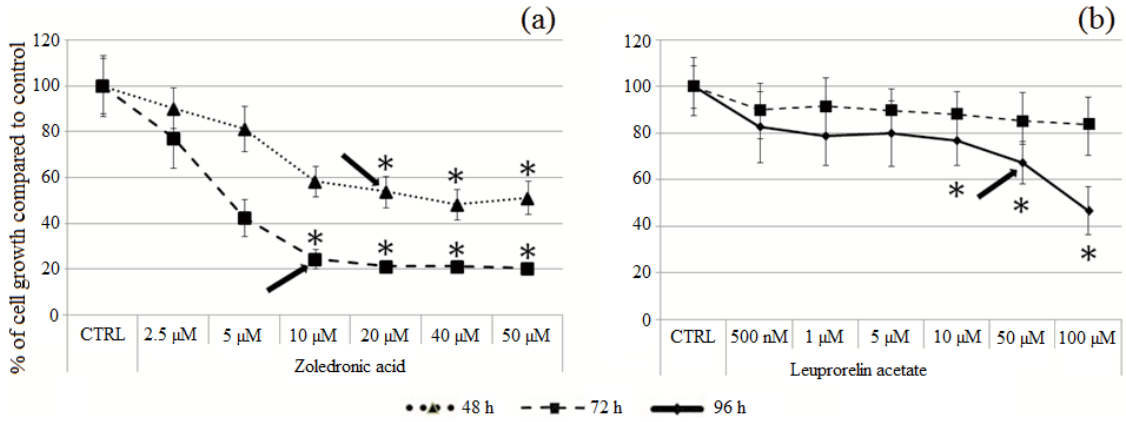


Figure 1: *Proliferation assay.* Effect of ZA (a) and LA (b) on PC3 proliferative activity. Arrows show IC50. $* = P < .05$ versus control conditions.

Images were analyzed using ImageJ software: migration was evaluated considering the reduction of wound area, and then compared to controls.

2.5. 3D migration assay

The transwell migration assay was used to measure the three-dimensional movements of cells. Migration assays were performed in transwells (BD Falcon cell culture inserts incorporating polyethylene terephthalate (PET) membranes with 8.0 μM pores, 1×10^5 pores/cm^2).

When assessing spontaneous migration, culture medium was RPMI. When assessing migration in stimulated conditions, control medium was αMEM. When tests were performed in the presence of Wortmannine, cells were preincubated for 30 min at 37 °C with 100 nM.

Cells (10^5) resuspended in 200 μL of culture medium containing 2% FBS were seeded in the upper chamber of a transwell. The lower chamber (a 12-well plate) was filled with fresh or conditioned culture medium containing 2% FBS and placed in the incubator. After 6 h, cells were treated as detailed by Gambarotta et al. [8]. Wells were photographed using a BRESSER MikroCam 3 Mpx camera, with an optical microscope (Leica DC 100) at 100×. Five pictures were randomly chosen per well, and used to count the migrated cells with ImageJ software using cell-counter plug-in. Results from different experiments (performed at least three times in duplicate) were expressed as mean ± SE.

In order to avoid any cytotoxic effect of potentially confounding migration results, we performed a cytotoxicity test as previously described at the same time and same conditions of every migration test.

2.6. Western blotting

Since Akt phosphorylation plays a role of paramount importance in cell migration [16], we tested the effects of ZA or LA on the phosphorylation levels of Akt (expressed as pAkt/Akt ratio) in the presence of studied drugs and in the presence of Wortmannine (100 nM), used as a positive control.

Cells were seeded in 10 cm diameter Petri dishes, cultured until subconfluence, when drugs were added (ZA 5 μM and LA 100 μM). After 6 h incubation, cells were collected and treated as detailed by Mognetti et al. [22] and immunoblotted according to Penna et al. [24]. In parallel proliferation tests—as previously described—performed in the same culture conditions in order to exclude any confounding element, no significant cytotoxicity was appreciable at the chosen concentration after 6 h incubation.

Blots were probed with primary polyclonal antibody (Cell Signaling Technology, Danvers, MA, USA) suspended in TBS Tween 0.1%: anti-Akt (mouse, 1:800), anti-pAkt (Ser473, rabbit, 1:500), 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.

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

2.7. Statistics

Statistical analyses were performed by one-way or two-way ANOVA, and $P < .05$ was considered significant. If not differently specified, data are expressed as percentage mean ± standard deviation compared to control.

3. Results

3.1. Inhibition of proliferative activity

ZA strongly inhibited cell proliferation (Figure 1(a)); cell growth was inhibited after 48 h already by 20 μM ZA, and even more strikingly after 72 h culture.

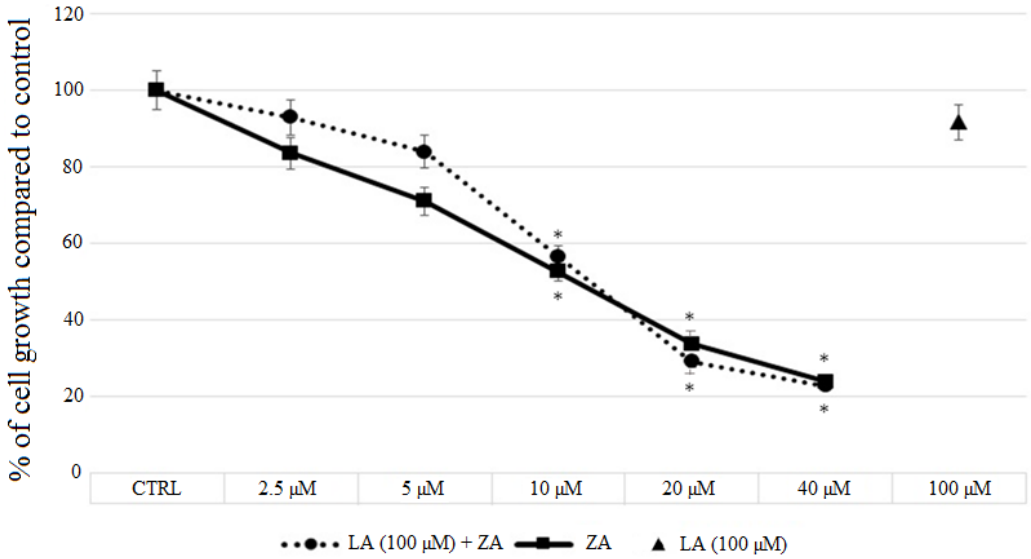


Figure 2: *Proliferation assay.* Effect of the simultaneous administration of LA (100 μM, constant concentration) plus ZA (2.5 to 40 μM, dotted line) on PC3 proliferation compared to cytotoxicity of 100 μM LA alone (triangle) and ZA alone (2.5 to 40 μM, black line). Results are expressed as percentage of control $\pm$SE.

Yet, no significant effect on cell proliferation was induced by LA at any concentration tested in the first 72 h (Figure 1(b)). A significant inhibition was induced by concentrations greater than 10 μM only after 96 h treatment (Figure 1(b)).

The simultaneous presence of the two drugs neither increased nor decreased the effect of the single drugs at any considered time point. An example is reported in Figure 2, where a constant amount of LA (100 μM, i.e., the highest concentration tested) was added to increasing concentrations of ZA. As in Figure 1, LA (100 μM) alone did not induce appreciable variation of cell proliferation (triangle in Figure 2).

To further analyze drug interactions, the concentrations of LA (from 1 μM to 100 μM) were increased in the presence of 5 μM ZA, and cells were grown for 72 h in the presence of the two drugs. Also in this case no significant variation of cell growth was observed after the addition of increasing concentrations of LA (data not shown).

3.2. Inhibition of migratory activity

Two-dimensional migration was inhibited both by ZA and LA, with significant effects already visible after 1.5 h (Figure 3). The simultaneous presence of the two drugs did not relevantly change the cell behavior, except between 2 and 3 h incubation, when it significantly quenched LA effects. On the other hand, no significant difference was evidenced between the drug combination and ZA alone, or between the two single drugs. The same trend is respected after 24 h incubation, when the three conditions are significantly different from control but not different between each other.

Since no difference in the rate of migration was evidenced when experiments were performed in RPMI or αMEM, we pooled data and globally referred to them as control.

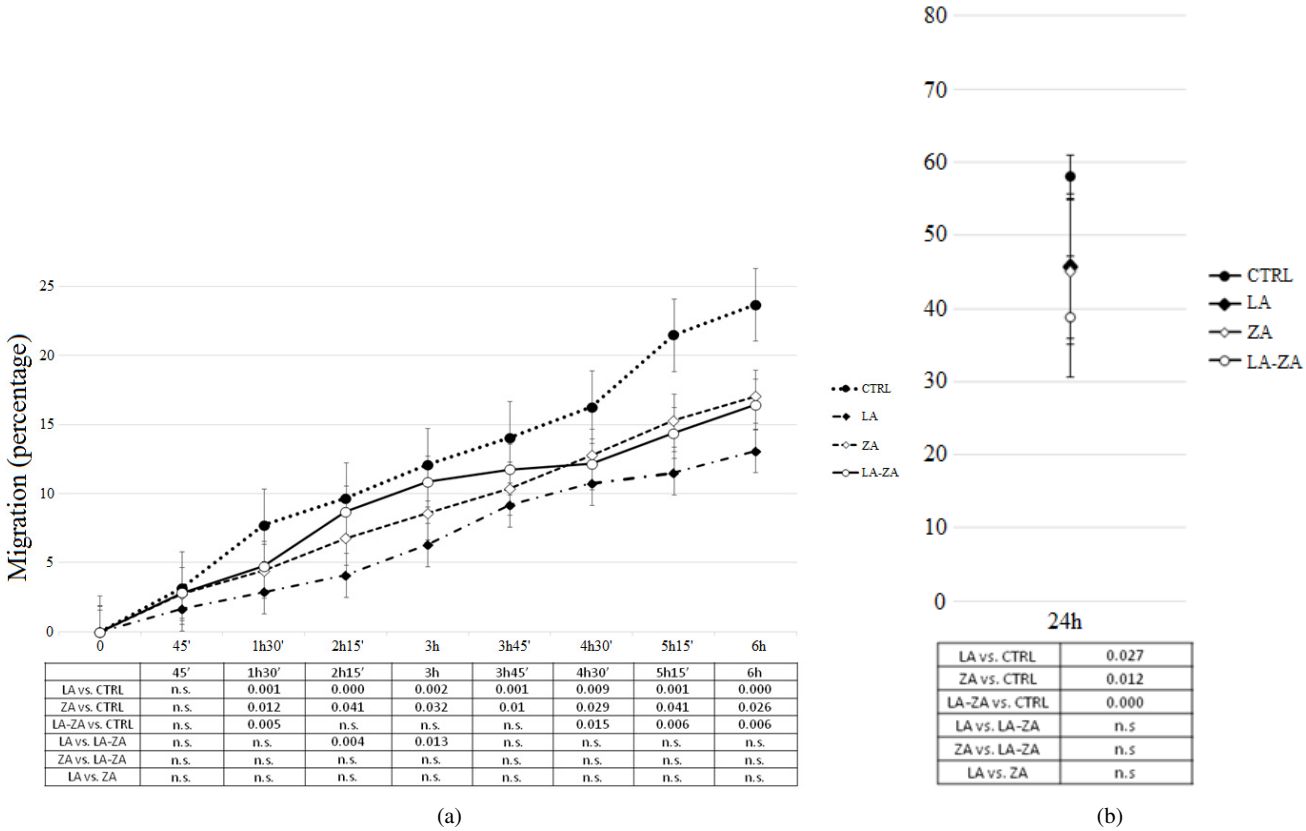
Three-dimensional migration was significantly inhibited after 3 h (data not shown) and 6 h (Figure 4) by ZA and by LA, both in basal and in stimulated conditions. Again, the concurrent presence of the two drugs did not increase their inhibitory effect (even, it quenched LA effect).

In the same conditions, we measured Akt phosphorylation and revealed that both ZA and LA decrease it in basal and stimulated conditions.

Inhibition of Akt phosphorylation and that of 3D migration are not proportional (no direct correlation could be established), nevertheless the trend is clearly the same. Importantly, 3D migration rate was decreased by the inhibition of Akt phosphorylation (100 nM Wortmannine), both in basal and stimulated conditions.

4. Discussion

A major source of morbidity in patient with prostate cancer is the widespread bone metastases, which often grow in a castration-resistant manner and still are a major topic in the research for novel treatment strategies. Monotypic in vitro cultures are commonly employed to study drug effects on cell proliferation and migration. Cell lines are still the main experimental model used in many publications, despite the fact that animal models have become widely available in recent years. PC3 cells are androgen independent, proliferate normally in androgen deprived media in vitro and possess high migratory capability. In addition, PC3 xenograft



	45′	1h30′	2h15′	3h	3h45′	4h30′	5h15′	6h
LA vs. CTRL	n.s.	0.001	0.000	0.002	0.001	0.009	0.001	0.000
ZA vs. CTRL	n.s.	0.012	0.041	0.032	0.01	0.029	0.041	0.026
LA-ZA vs. CTRL	n.s.	0.005	n.s.	n.s.	n.s.	0.015	0.006	0.006
LA vs. LA-ZA	n.s.	n.s.	0.004	0.013	n.s.	n.s.	n.s.	n.s.
ZA vs. LA-ZA	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
LA vs. ZA	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

(a)

LA vs. CTRL	0.027
ZA vs. CTRL	0.012
LA-ZA vs. CTRL	0.000
LA vs. LA-ZA	n.s
ZA vs. LA-ZA	n.s
LA vs. ZA	n.s

(b)

Figure 3: *Two-dimensional migration.* The wound healing was quantified every 45 min in time laps up to 6 h (a) and at 24 h (b), and is displayed as basal conditions (CTRL) or as the effect of 5 μM ZA, 100 μM LA or a combination of both drugs (5 μM ZA + 100 μM LA). Data are expressed as relative to control conditions $\pm$SE. Experiments were performed three times in duplicate. P values are reported in the underlying table; n.s. means difference being not statistically significant.

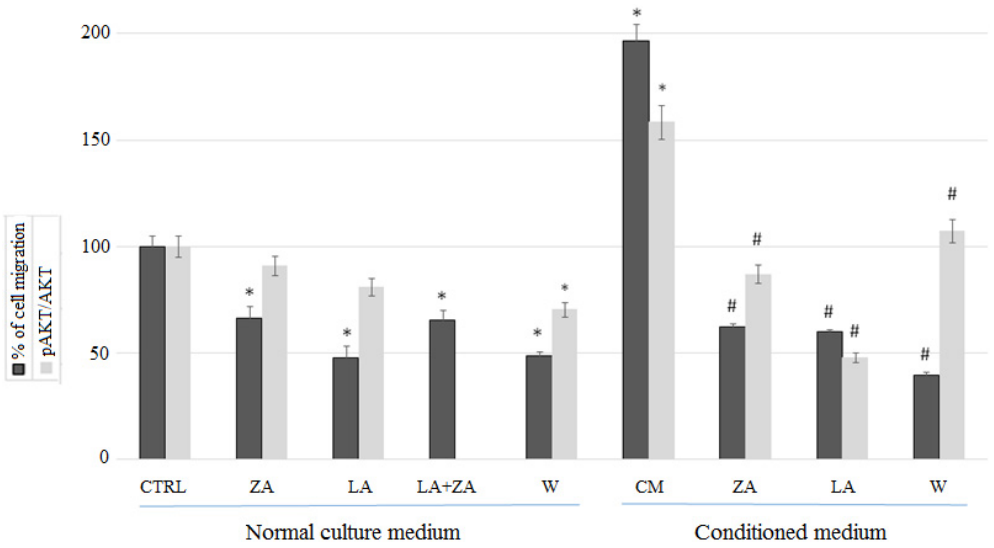


Figure 4: *Three-dimensional cell migration and Akt phosphorylation.* Effect of 5 μM ZA, 100 μM LA, the combination of both drugs (5 μM ZA + 100 μM LA) and 100 nM Wortmannine (W) on PC3 3D migration (black bars) and Akt phosphorylation (grey bars) in basal (CTRL) and stimulated (CM) conditions. Data are expressed as percentage towards basal conditions (CTRL). $* = P < .05$ versus control conditions. $\# = P < .05$ versus conditioned medium (CM).

tumors proliferate rapidly and are quite invasive. For those reasons, PC3 cells are thoroughly recognized as an appropriate model for the study of the more aggressive forms of PCa.

The use of complementary assays, such as wound healing and transwell motility assays, is commonly used to assess in vitro metastatic potentials [31]. Moreover, our coculture system permits to study the drug effects on the migration of PCa cells in conditions that more resemble the in vivo tumor microenvironment, by providing the tumor cells with contextual signals. This model has already been validated [22] and proved to be reliable in reproducing the chemotactic effect exerted by BM-derived cells on PCa.

This in vitro study strongly supports that ZA has cytotoxic effects on a PCa cell line derived from a bone metastases, an effect that is much less evident for LA. Yet, either drug limits cell migration in a similar extent. Intriguingly, no relevant additive effects can be appreciated between the two drugs in affecting cell migration and proliferation. The lack of additive effects as well as the similar effects on Akt phosphorylation suggests that diminution of the effect of every single drug is highly unlikely in vivo.

A direct cytotoxicity of ZA has been already described [17], though never in combination with LA. In our hands, ZA toxicity was already significant after 48 h culture at 20 μM, regardless of LA cotreatment. In a previous study [19], the toxicity of ZA was observed at lower concentrations probably because of different experimental conditions. It is of note that, in vivo, the effective local concentrations of bisphosphonates at sites of active bone resorption are much higher than serum levels and may reach up to 10^{-3} M in the resorption lacunae [27]. It is possible therefore to infer that in treated patients the close association of cancer cells with actively resorbing osteoclasts and the bone matrix will result in exposure of tumor cells to bisphosphonates concentrations sufficient to induce their apoptosis [17].

It is much more difficult, on the other hand, to expect, in vivo, a direct cytotoxicity attributable to LA, since concentrations needed, as defined by our study and by other groups [5,23], cannot be reached with therapeutical dosages.

When used at subtoxic concentration, both ZA and LA significantly inhibit 2D migration, already after 1.5 h. This property is even more striking when cells are exposed to a chemotactic stimulus. Intriguingly, this phenomenon is probably very close to what happens in vivo, where bone microenvironment strongly influences cancer cells motility [3,32].

Caraglia et al. [1] described a decrease in the activation of PI3K/Akt pathway following treatment with ZA, suggesting that it may act upstream of the metastasis, by inhibiting PCa cell migration towards metastatic sites through interference with this pathway mostly implicated in cell migration [16]. Our study concurs with that of Caraglia et al. by suggesting that inhibition of migration provoked by ZA and LA might be mediated by inhibition of Akt phosphorylation, which, on the other hand, is increased when cells are exposed to a chemotactic stimulus fostering their motility.

We conclude that ZA is more cytotoxic than LA in vitro. However, due to the effective dosage of the two drugs, we suggest that such an effect is likely, in vivo, for ZA only. Nevertheless, both compounds strongly reduce basal and BM-MSC stimulated cell motility in vitro. Therefore, they are likely implicated in reduction of PCa cells motility, with great fall out on bone metastases formation. This antitumoral activity is in line with the recently demonstrated beneficial effects of these two compounds in PCa patients with bone metastases [26].

The clinical implications might be the possible increase in GnRH analogues dosage in castration-resistant patients, to increase its antiproliferative effect, and an early use of ZA before the appearance of the metastasis, with the aim of inhibiting PCa cell proliferation, promoting apoptosis and preventing cell migration.

Importantly, when these two drugs are used in association we do not observe unfavorable interactions between them on the studied parameters. However, we cannot rule out that association treatment regimen that enhances or inhibits the effect of the two drugs exists in vivo. The experience with this association has scarcely been studied in vivo under a pharmacological point of view; therefore studies such as ours are needed.

Conflict of interest The authors declare that they have no conflict of interest.

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