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The alkynylphosphonate analogue of calcitriol EM1 has potent anti-metastatic effects in breast cancer

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ABSTRACT

The active form of vitamin D_3, calcitriol, plays a major role in maintaining calcium/phosphate homeostasis. In addition, it is a potent antiproliferative and prodifferentiating agent. However, when effective antitumor doses of calcitriol are employed, hypercalcemic effects are observed, thus precluding its therapeutic application. To overcome this problem, structural analogues have been designed with the aim at retaining or even increasing the antitumor effects while decreasing its calcemic activity. This report shows the biological evaluation of an alkynylphosphonate vitamin D less-calcemic analogue in a murine model of breast cancer. We demonstrate that this compound has potent anti-metastatic effects through its action over cellular migration and invasion likely mediated through the up-regulation of E-cadherin expression. Based on the current in vitro and in vivo results, EM1 is a promising candidate as a therapeutic agent in breast cancer.

1. Introduction

The active form of vitamin D_3, 1,25-dihydroxyvitamin D_3 (calcitriol), is mainly known for its effects on calcium and phosphate homeostasis. In addition, non-classical effects have been demonstrated over the last 30 years, including pro-differentiating and antiproliferative effects in cancer cells [1–4]. Moreover, calcitriol has been shown to regulate almost all the processes a tissue undergoes in its progression to malignancy (the hallmarks of cancer) including cellular proliferation and differentiation, cell death, angiogenesis, metastasis, DNA repair, energy metabolism reprogramming, immunomodulation and inflammation [5,6].

Breast cancer (BC) cells have also been shown to be targets of vitamin D compounds [7–10]. Furthermore, numerous in vivo studies demonstrated that vitamin D compounds suppress BC tumor growth and metastasis [4,10–16]. On the basis of this overwhelming evidence of the antitumor action of vitamin D compounds, some clinical trials evaluated the effects of these compounds in the treatment of various malignancies including BC, either alone or in combination. However, important clinical antitumor effects are infrequently observed and dose-limiting toxicity appeared in some cases which precluded the optimal dose for antitumor activity [4]. In other clinical trials, no toxicities have been observed but systemic stable levels of the drug could not be achieved due to the current available formulations of calcitriol [4,17].

The above data indicate the need for designing and synthesizing novel vitamin D analogues with the intention of retaining the potent anticancer effects of calcitriol while being less calcemic. We had previously reported [18] the synthesis of a novel phosphonate analogue of vitamin D (diethyl [(5Z,7E)-(1S,3R)-1,3-dihydroxy-9,10-secochola-5,7,10(19)-trien-23-in-24-yl] phosphonate analogue, named EM1 hereafter). This analogue combines the low

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calcemic properties of phosphonates [19] with decreased metabolic inactivation due to the presence of a triple bond in C-24 [20] and proved to exert antiproliferative effects on various tumor cell lines while lacking hypercalcemic activity in mice [18]. In the present work we demonstrate that EM1 exerts inhibitory effects on the metastatic process in a syngeneic murine model of hormone-independent breast cancer, through its action on cellular migration and invasion.

2. Materials and methods

2.1. Chemicals and reagents

Diethyl[(5Z,7E)-(1S,3R)-1,3-dihydroxy-9,10-secochola-5,7,10 (19)-trien-23-in-24-yl] phosphonate analogue (EM1) was reconstituted in 100% HPLC-grade isopropanol and stored protected from light at −20 °C. The amount of EM1 was determined by UV spectrophotometry between 200 and 300 nm. Drug was dissolved in isopropanol (vehicle) to the concentration of 10^{-3} M and subsequently diluted in the culture medium or in physiologic solution to reach the required concentration or doses [18].

2.2. Animal studies

2.2.1. Studies of the effect of EM1 on tumor burden and the number of metastasis

In vivo studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. 3-month-old virgin female Balb/C mice, each weighing at least 20 g, were purchased from the Facultad de Ciencias Veterinarias (La Plata, Argentina). Animals were given free access to water and food, were housed in a climate controlled room with a 12 h light/12 h dark cycle. 42 animals were injected subcutaneously (s.c.) with LM3 cells (4×10^5 cells in 100 μl of serum-free DMEM) in the right flank using a monojet 200 30-gauge x1/2 needle. When tumors reached 50–70 mm^3 in volume, LM3 tumor-bearing mice were randomly divided in four groups and were subcutaneously injected as previously described [21] as follows: (1) 12 mice with EM1 (20 μg/kg), (2) 12 mice with vehicle (isopropanol, same concentration as in (1), (3)) 9 mice with EM1 (50 μg/kg), (4) 9 mice with vehicle (isopropanol, same concentration as in (3)). Each mouse received 7 injections during two weeks. Tumor growth was blindly measured daily with calipers and tumor volume was calculated as $\pi/6 \times a \times b^2$, where a is the length in millimeters, and b is the width in millimeters. At the end-point animals were sacrificed by cervical dislocation. Tumors were then removed, weighed, measured and put into liquid nitrogen or formaldehyde for further study. Tumor volume was calculated as $\pi/6 \times a \times b \times c$, where a, b, and c are three tumor dimensions. Organs (including lungs, lymph nodes, liver, spleen, pancreas, uterus, ovaries and any other tissues of abnormal appearance) were examined superficially for evidence of macroscopic metastasis and lungs were removed and fixed in Bouin's solution. The number of superficial lung metastases per mouse was counted by an investigator that was unaware of the sample assignment, with the aid of a Stereo Microscope (Nikon SMZ 1500) coupled with High Intensity Illuminator (Nikon NI-150) and a digital camera (Nikon DXM 1200F). After lung metastases count, the organs were processed for hematoxylin and eosin (H&E) staining. Tumors were excised, bisected along the longest axis, fixed for 24 h in 4% formaldehyde in PBS 1X and processed into paraffin by standard procedures. In brief, after paraffin sections were dewaxed, they were rehydrated in a series of ethanol dilutions and either stained with hematoxylin and eosin (H&E) to observe histopathological characteristics.

2.3. Study of EM1 effects on plasma calcium levels

To determinate plasma calcium levels of a 50 μg/kg body weight dose of the EM1 we used female Balb/C mice from Facultad de Ciencias Veterinarias (La Plata, Argentina), 20 g of weight and 12 weeks of age. EM1 and vehicle were administrated by an intraperitoneal injection three times a week during a total period of 15 days. Animals were maintained in a specific pathogen-free environment under controlled conditions of light and humidity for several weeks. General toxicity was assessed by clinical measures, such as weight loss, changes in appearance and behaviour, lethargy and death. Animal weight was evaluated three times a week. Blood samples were collected from mice at the end of the treatment period and plasma calcium levels were performed as previously described [18]. Additionally, the hematocrit for each mouse was analyzed following treatments.

2.4. Liver and kidney histological analysis

Dissected livers and kidneys from animals treated with EM1 and vehicle at 50 μg/kg body weight were fixed in 4% formaldehyde during 24 h and gradually dehydrated using serial ethanol concentrations of 70%, 90%, and 100%. Dehydrated organs were cleared two hours using xylol and then included in paraffin. Sections (5 μm thick) were prepared using a rotary microtome (Leica, RM 2155), were attached to clean slides and stained with H&E for histological examination by an expert pathologist. For each slide, 10 fields at ×100 and ×400 magnification were analyzed.

2.5. Cell culture studies

HC11 non-malignant murine mammary epithelial cell line and LM3 murine breast tumor cell line derived from a murine mammary adenocarcinoma that spontaneously arose in Balb/C mice were generous gifts from E. Bal de Kier Joffé (Instituto de Oncología Ángel Roffo, Buenos Aires, Argentina) and Edith Kordon (IFIBYNE-CONICET), respectively. HC11 cells were maintained in RPMI (Sigma) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Gibco), Insulin (5 μ/ml, Gibco), L-glutamine (5 mM, Gibco), penicillin (100 U/ml, Gibco), and streptomycin (100 μg/ml, Gibco). LM3 cells were maintained in DMEM (Sigma) supplemented with 5% (v/v) Fetal Bovine Serum (FBS) (Gibco), L-glutamine (5 mM, Gibco), penicillin (100 U/ml, Gibco), and streptomycin (100 μg/ml, Gibco). Both cell lines were maintained at 37 °C in a humidified 5% CO_2 air atmosphere.

2.6. Cell migration

Cell migration was studied by employing the "wound healing" assay as previously described [21]. Briefly, the cells were seeded in 35 mm Petri dishes and cultured until confluence. The cells were treated with EM1 (100 nM) or vehicle and they were scraped with a 200 μl micropipette tip, denuding a strip of the monolayer. Then they were observed and photographed for 17 h. Images were captured with an inverted microscope (Nikon Eclipse TE2000-S), equipped with a digital camera (Nikon Coolpix S4, 6.0 Mpix, 10× zoom). The uncovered wound area was measured and quantified at different intervals for 17 h with ImageJ 1.37 v (NIH).

2.7. Cell invasion

LM3 cells were analyzed for invasion through Matrigel chambers as previously described [21]. In brief, single cell suspensions containing 12.500 LM3 cells/well in 0.5 ml DMEM medium (Sigma) with EM1 (100 nM) or vehicle were plated into 24-well inserts (Falcon cell culture inserts, 12 μm pore size) with

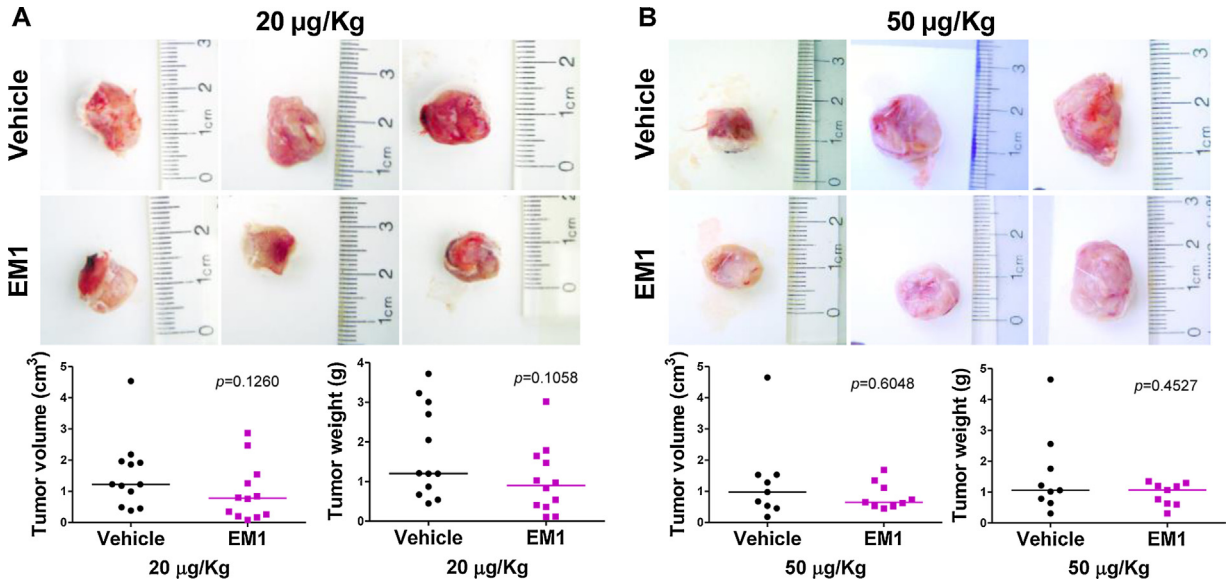

Fig. 1. Effect of EM1 analogue in a murine model of breast cancer. LM3 cells (4×10^5) were inoculated subcutaneously in Balb/C mice. When the tumor reached 50 to 70 mm^3, subcutaneous injections of EM1 or vehicle were administered. The animals were treated with 7 doses of (A) 20 µg/kg ($N = 24$) or (B) 50 µg/kg ($N = 18$) of EM1 or vehicle. Mann–Whitney test was applied to analyze the possible differences in the tumor burden between groups.

Matrigel (BD). The lower chamber was filled with 0.6 ml of medium. After incubation for 17 h at 37 °C, the cells on the upper side of transwell membrane were removed by cotton swab and rinsed with PBS 1X. Cells migrating to the lower side of the membrane were fixed in 100% methanol for 10 min at room temperature, stained with crystal violet for 5 min (Sigma), photographed, and counted.

2.8. Phalloidin staining and fluorescence confocal imaging

LM3 cells were seeded on glass coverslips in 35 mm petri dishes and cultured until 50% confluence. They were treated with EM1 (100 nM) for 21 h. After treatment, they were washed three times with PBS 1X and fixed with 4% paraformaldehyde (PFA) in PBS 1X for 1 h. The cells were then permeabilized in 0.1% triton in PBS 1X

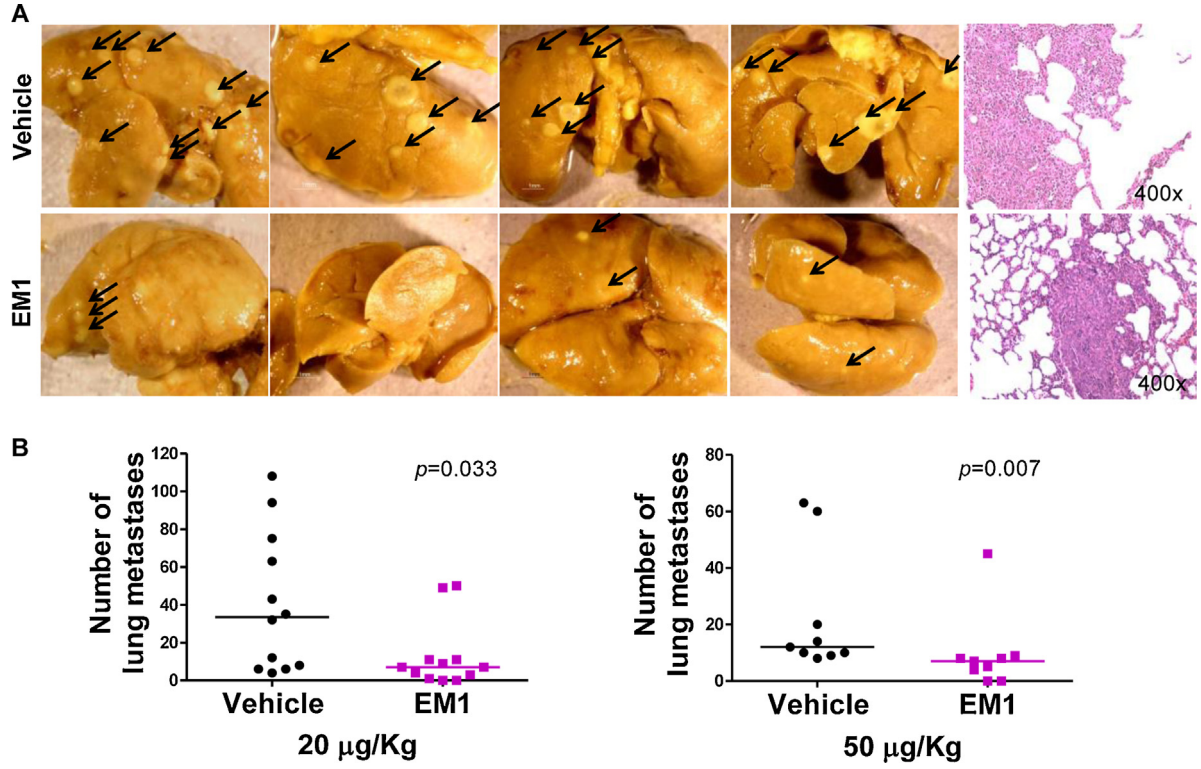

Fig. 2. EM1 effects on the number of lung metastases in a murine model of breast cancer. The animals were treated with 7 doses of 20 µg/kg or 50 µg/kg of EM1 or vehicle. (A) Representative pictures of the lung metastases observed in lungs of mice treated with EM1 or vehicle at 50 µg/kg and representative micrographs of H&E of the lung histological change. (B) Quantification of lung metastases at doses of 20 and 50 µg/kg. $p < 0.05$, Mann–Whitney test was applied for the comparison of the number of metastases between groups.

for 15 min and after that they were washed three times with PBS 1X. Then they were incubated with rhodamine–phalloidin (1:100) in PBS 1X for 30 min and To-pro3 1/1000 in PBS 1X for 5 min. After that they were washed three times with PBS 1X. Glass coverslips were mounted on glass microscope slides, confocal images were acquired with the Leica confocal microscopy TSP2 and analyzed with ImageJ 1.37v (NIH). Counting of 500/600 cells in ×400 random fields was done in order to study the proportion of cells containing cortical localization of actin fibers.

2.9. Immunofluorescence

Immunofluorescence (IF) was performed as previously described [21]. Briefly, LM3 cells were seeded on glass coverslips in 35 mm petri dishes and cultured until 50% confluence. They were treated with EM1 (100 nM) or vehicle for 17 h. After treatment, they were washed three times with PBS 1X and fixed with PFA 4% in PBS 1X. The cells were then permeabilized with 0.2 % triton in PBS 1X and blocked with 1% bovine serum albumin (BSA) in PBS 1X. Then they were incubated with E-cadherin (Santa Cruz Biotechnology, 1:100) in 1% BSA in PBS 1X for 1 h. After incubation with the primary antibody, the cells were washed with PBS 1X and incubated with anti-rabbit Alexa 566 fluoro-conjugated antibodies (Molecular Probes, Invitrogen) for 1 h. Then they were washed and mounted. The cell nucleus were stained with DAPI (1:10,000). The cells with expression of E-cadherin were counted by analyzing 10 images randomly for each condition.

2.9.1. Western blot

Cells were seeded in plates with complete medium and treated with EM1 (100 nM) or vehicle for 17 h and then 75 μg of protein lysates was separated by SDS-PAGE on 12% gel, transferred onto nitrocellulose membrane, blocked with 5% non-fat dry milk for 30 min, then incubated with a primary antibody, washed, incubated further with horseradish peroxidase-conjugated secondary antibodies, and reactions were detected by enhanced chemiluminescence (ECL) following the manufacturer's directions (Amersham, ECL Plus Western Blotting Detection Reagents, GE Healthcare). Primary antibodies used were rabbit polyclonal anti-E-cadherin (H-108) (Santa Cruz Biotechnology, sc-7870), rabbit polyclonal anti-VDR (C-20) (Santa Cruz Biotechnology, sc-1008), and goat polyclonal anti-β-actin (C-11) (Santa Cruz Biotechnologies, sc-1615) was used as internal control for protein loading and analysis. T-47D cells were used as positive control of E-cadherin expression [22].

2.10. Statistical analysis

The GraphPad Prism software package, version 5.00 was used for collection, processing and statistical analysis of all data. Comparison of the tumor burden, lymphocytic infiltration, porcentage of necrosis and number of lung metastases among different groups was analysed by the non-parametrical Mann–Whitney *U* test. Effects on plasma calcium levels, hematocrit and body weight of the animals was analized by unpaired *t* test. Migration and invasion assays, effects on actin cytoskeleton and E-cadherin expression were analyzed with unpaired *t* test. $^{*}p < 0.05$ was considered significant.

3. Results

3.1. Effects of EM1 on the tumor burden of a murine model of breast cancer

We had previously reported the synthesis and preliminary biological evaluation of EM1 alkynylphosphonate [18]. Taking into account that the EM1 analogue, in addition to lacking calcemic effects at supra-physiological doses, showed to decrease the cell viability of various cancer cell lines including breast cancer ones [18], we proposed to investigate the antitumoral activity of this analogue in breast cancer. To this end we conducted animal studies using the subcutaneous implant of hormone-independent [23] breast adenocarcinoma LM3 cells in syngeneic female Balb/C mice (Fig. 1) which produces lung metastases [24] and therefore it is a good model to study the effects of EM1 on both the primary tumor growth and the metastatic process. In the first term we evaluated the effect of EM1 on the primary tumor volume and weight. As shown in Fig. 1, although there is a tendency to a reduction in tumor burden with EM1 treatment, no statistically significant differences in tumour volumes (vehicle = 1.221 cm^3 vs EM1 = 0.777 cm^3; $p = 0.1260$) or tumor weight (vehicle = 1.205 g vs EM1= 0.905 g; $p = 0.1058$) were found between groups after animal treatment with the dose of 20 μg/kg. Increasing the amount of EM1 injected to 50 μg/kg did not result in significant primary tumor growth reduction either (vehicle = 0.971 cm^3 vs EM1 = 0.650 cm^3; $p = 0.6048$ and vehicle = 1.060 g vs EM1 = 1.070 g; $p = 0.4527$).

3.2. Effect of EM1 on distant organ metastasis

The occurrence of metastasis is responsible for more than 90% of the mortality associated with cancer and is the least known event of the pathogenesis of this disease [25]. Moreover, since primary tumors are already developed at diagnosis, potential therapeutic agents should preferably target the metastatic process. On the other hand, calcitriol and analogues have shown to modulate the metastatic process rather than the primary tumor growth in breast cancers lacking hormone receptors [10]. Therefore, the next step in the biological evaluation of EM1 in breast cancer was to analyse the compound effect on the metastatic process.

For this purpose, the lungs of mice treated with EM1 and vehicle were examined to assess the number of metastases that they presented. As shown in Fig. 2, we found highly significant differences in the number of pulmonary metastases between groups at both 20 μg/kg (vehicle = 33.5 vs EM1 = 7.0; $^{*}p = 0.033$) and 50 μg/kg (vehicle = 12.0 vs EM1 = 7.0; $^{**}p = 0.007$) doses.

3.3. Evaluation of the effect of EM1 on tumor lymphocytic infiltrates

Taking into account the in vivo results showing a reduction in the number of lung metastases with EM1 treatment, we decided to study the mechanisms underlying the antimetastatic activity of EM1.

Several studies conducted in patients with breast cancer and other tissues have shown that the lymphocytic infiltration of the tumors is associated with a better prognosis [26]. More precisely, the presence of lymphocytic infiltrate was associated independently with the survival of the patients of BC [27–29]. On the other hand, it is known that calcitriol modulates the tumoral immune response [30,31]. Therefore, we proposed to study whether treatment with EM1 produced some effect on the lymphocytic infiltration in this murine model of breast cancer, which could explain the reduction on the number of metastases. The histological evaluation of the lymphocytic infiltration in the peritumoral area showed no differences between the control group (16% of inflammatory response) and the EM1-treated group (33% of inflammatory response; $p = 0.345$). The classification of the peritumoral area of the tumors that presented lymphocyte response in adjacent and distant did not show differences, as in all the tumors we observed presence of lymphocytes in both areas. The evaluation of the intratumoral lymphocytic infiltrate could not

be assessed due to the presence of extensive areas of tumor necrosis. The degree of necrosis was similar in both groups ($p = 0.857$). In conclusion, tumor lymphocytic infiltration was not affected by EM1.

3.4. Evaluation of the effects of EM1 on cellular migration and invasion.

Given that EM1 reduced the progression of the disease by decreasing the number of lung metastases and knowing that calcitriol and analogues modulate the processes of cell migration and invasion in various tumor types, we proposed to assess EM1 effects in these processes. For this purpose, we evaluated the effect of EM1 on cell migration through a wound closure assay in the LM3 breast adenocarcinoma cell line, further comparing the effect on a non-malignant mammary cell line (HC11). Importantly, whereas EM1 retarded the closure of the wound in the malignant LM3 cell line (Fig. 3 A, percentage of wound closed: vehicle = 87.51 ± 6.43% vs EM1 = 59.23 ± 3.47%; $^{**}p = 0.0031$) it had no effect in the non-malignant HC11 mammary cell line (Fig. 3B). In order to test if the differential response in migratory rates between the malignant LM3 and the non-malignant counterpart HC11 could be the result of different VDR levels, western-blot analyses were conducted. As shown in Fig. 3C and D, no differences in basal VDR levels were observed between both cell lines. In addition, following EM1 treatment no differential up-regulation of VDR was observed between HC11 and LM3 cells.

We then conducted invasion cell assays using Matrigel-coated transwell inserts to confirm and extend the studies of EM1 effects on the invasive potential of breast cancer cells. As observed in Fig. 4, EM1 treatment significantly reduced the number of LM3 cells that had invaded through the Matrigel when compared with vehicle-treated cells (number of invading cells: vehicle = 89.50 ± 2.688 vs EM1 = 59.73 ± 2.6; $^{***}p < 0.001$).

3.5. Study of the effect of EM1 on the actin cytoskeleton in cultured LM3 cells

To be able to invade nearby tissues, the cancer cell must acquire the ability to migrate and invade, processes that EM1 analogue was shown to affect. It is well known that the reorganization of the cytoskeleton and concomitant change in cell morphology is an event related to the invasive cell capacity. For this purpose, we studied the actin cytoskeleton by phalloidin staining of the F-actin filaments in EM1- and vehicle-treated LM3 cells. As shown in Fig. 5, after treatment with EM1 the actin filaments were rearranged, reducing the amount of stress fibers and increasing the cortical localization of actin filaments (percentage of cells with cortical actin: vehicle = 5.75 ± 0.25 vs EM1 = 91.75 ± 3.55; $^{**}p = 0.003$).

3.6. Evaluation of the effect of EM1 on E-cadherin expression in LM3 cell line

E-cadherin is a transmembrane protein and its expression is inversely related to the invasion and migration ability of tumor cells. E-cadherin is also a mammary differentiation marker [32–34] and has been demonstrated to be upregulated by calcitriol or analogues [33,35–37]. We therefore analysed E-cadherin expression in LM3 cells treated with EM1 or vehicle. As shown in Fig. 6, E-cadherin protein expression was increased after the cells were

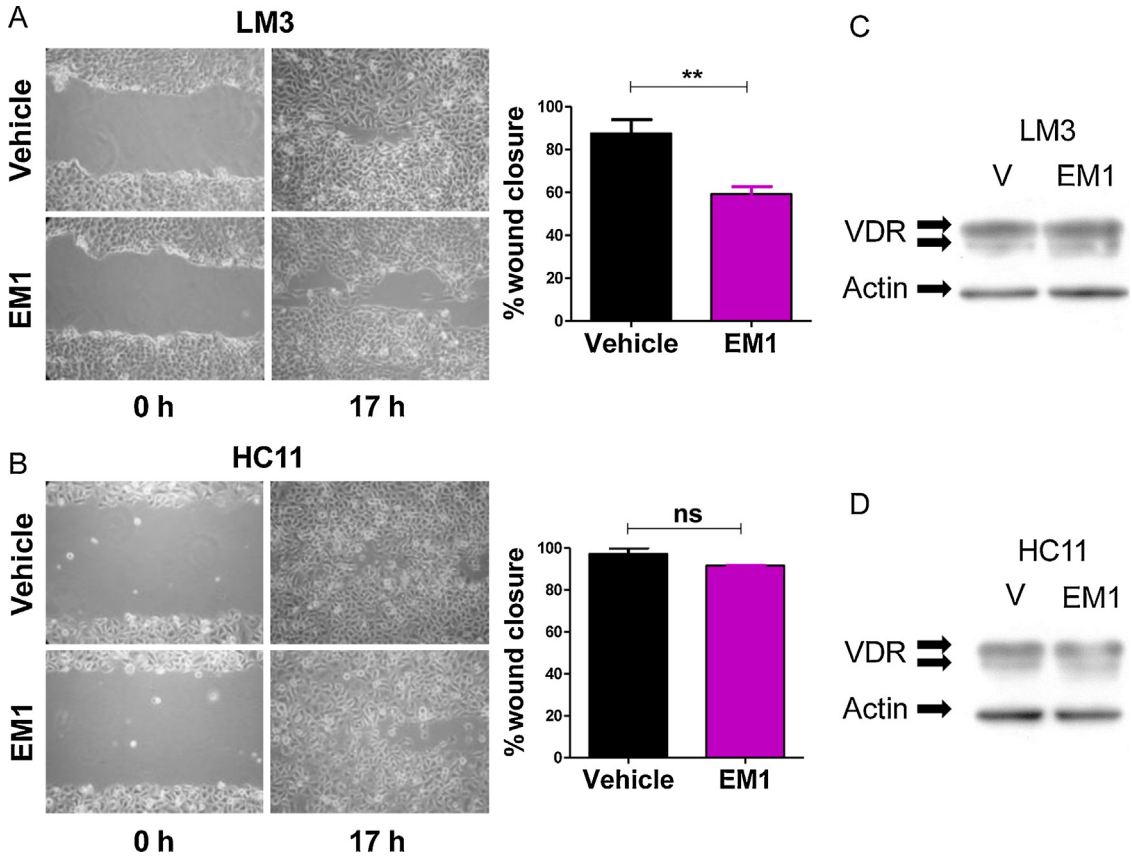

Fig. 3. Wound healing assay of the (A) breast adenocarcinoma LM3 and (B) non-malignant mammary HC11 cell lines at 17 h of EM1 treatment. Cells were scratched by a 200ul pipette tip and wound closure was monitored every hour for 17 h. The uncovered wound area was measured and quantified at different time intervals with Image J 1.37 v. The experiment has been conducted three times. Unpaired t test was applied. $^{**}p < 0.01$. Western blots showing VDR levels in LM3 (C) and HC11 (D) cell lines following 17 h treatment with EM1.

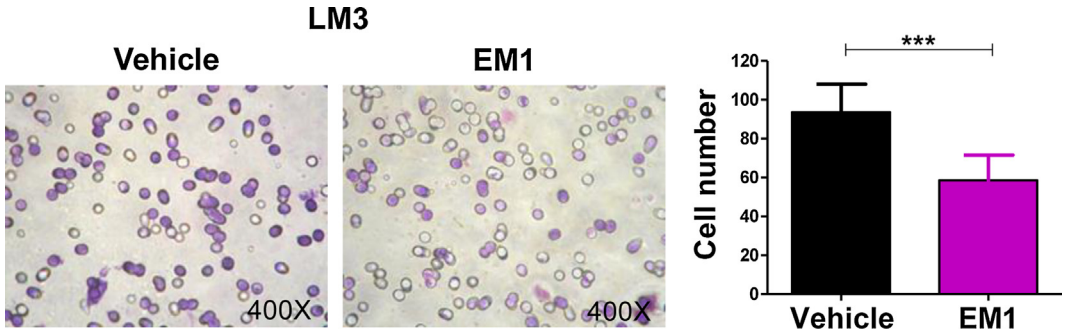

Fig. 4. Cell invasion assay of the LM3 cell line at 17 h of EM1 treatment. LM3 cells were treated with EM1 or vehicle and cell invasion was measured using Matrigel-coated transwell inserts. Cells that invaded to the underside of the inserts were counted by light microscopy. Representative photographs from two independent experiments are shown. Each bar represents the average of 10 randomly chosen fields. Unpaired t test was applied. $^{***}p < 0.001$.

treated with EM1 during 17 h, as observed by immunofluorescence (A) and western-blot (B). We concluded that EM1 have the ability to upregulate E-cadherin expression in LM3 cells, which may lead to the observed inhibition in migration and invasion ability shown in Figs. 4 and 5.

3.7. Study of the calcemic activity of EM1 in mice

We had previously demonstrated that 5-days treatment of CF1 mice with EM1 (5 μg/kg) did not result in hypercalcemia or toxic effects over the entire administration period (5 days) whereas the same calcitriol concentration induced both hypercalcemia and the death of the animals during the period treatment [18]. We have now followed with the toxicologic and calcemic studies analysing longer times, higher doses and different mouse strains (N:NIH(S)-Fox1^{nu} and Balb/C). The Balb/C strain was chosen because the animal model used to analyse EM1 antitumoral effects in breast cancer consisted in a sub-cutaneous implant of syngeneic LM3 cell line in Balb/C mice (results shown in Figs. 1 and 2). The second strain was chosen because additional investigations of the antitumoral effects of EM1 would employ xenograft implants of human cell lines in N:NIH(S)-Fox1^{nu} mice. As observed in Fig. 7A, the animal weight in Balb/C mice did not show variations during all the EM1 treatment period (seven injections of 50 μg/kg three times a week), with respect to vehicle treatment. The blood calcium levels (Fig. 7B) and the hematocrit (Fig. 7C) remained within the normal levels. Similarly, the analysis of H&E-stained slides of liver and kidney (Fig. 7D) evidenced no anomalies in histology. Similar results were obtain in N:NIH(S)-Fox1^{nu} mice (data not shown).

4. Discussion and conclusions

Although much progress in both diagnostic and therapeutic approaches has been made in the treatment of breast cancer, metastases remains the leading cause of death of this disease. Therefore, development of novel therapeutic regimens to prevent or treat tumor metastases is critically important in breast cancer therapeutics. Vitamin D plays an essential role in the normal development of the mammary gland and in the progression of breast cancer [8,38]. Moreover, vitamin D compounds have consistently shown to display strong antitumor activity in in vivo breast cancer models [9]. However, the active form of vitamin D, calcitriol, can cause hypercalcemic side effects at the doses required to exert antitumor activity and therefore is not suitable as a therapeutic agent in cancer treatment.

We had previously demonstrated that the novel phosphonate analogue of calcitriol EM1 exerts considerable antiproliferative activity in various tumor cell lines corresponding to different cancer types, including breast cancer ones, while lacking hypercalcemic or toxic effects in mice [18]. In this work we have now focused on the antitumoral effects in breast cancer. Interestingly, although EM1 had no effect on decreasing the tumor burden it reduced the number of metastasis in the LM3-Balb/C

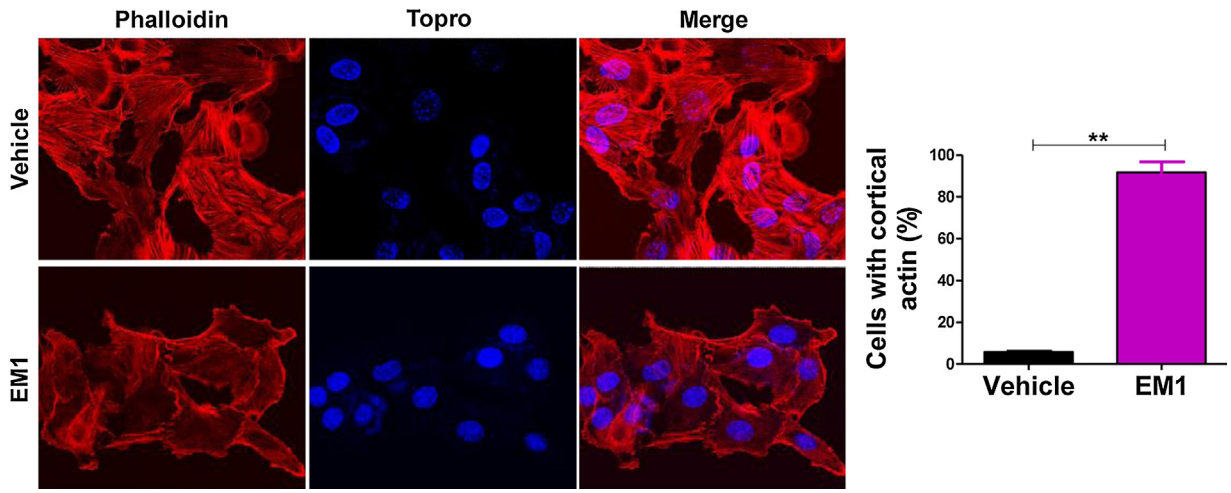

Fig. 5. Study of the effect of EM1 on the reorganization of actin fibers in LM3 cell line. The cells were treated for 21 h with EM1 or vehicle. Amplification: ×100. The graph shows the percentage of cells with cortical localization of the actin fibers. Each bar represents the average of 10 randomly chosen fields. The experiment has been conducted two times. Unpaired t test was applied, $^{**}p < 0.01$.

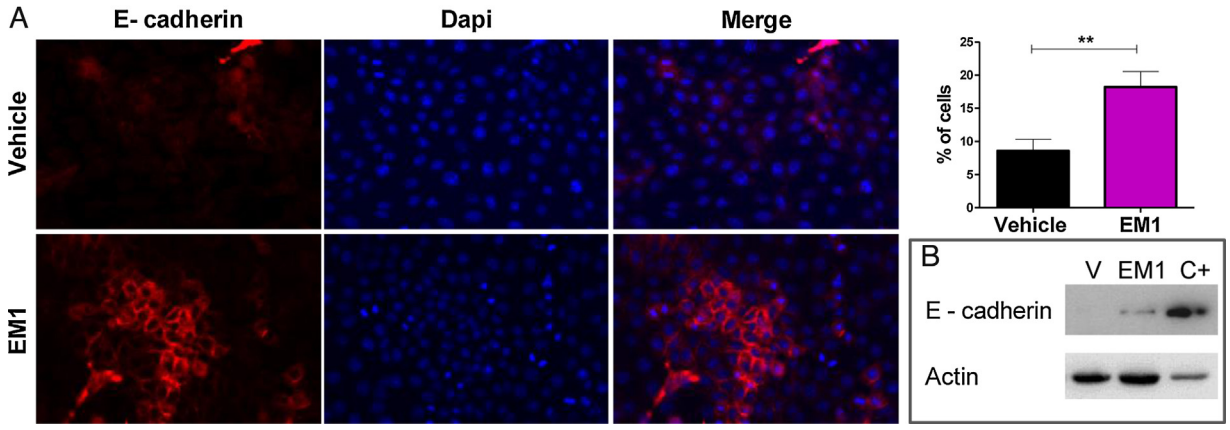

Fig. 6. Effect of EM1 on E-cadherin expression in LM3 cell line. The cells were treated with EM1 or vehicle over 17h. Amplification: ×400. (A) E-cadherin expression by immunofluorescence. The graph shows the percentage of cells with E-cadherin expression. Each bar represents the average of 10 randomly chosen fields. B) E-cadherin expression by Western blot. The experiments have been conducted two times. Unpaired *t* test was applied, $^{**}p < 0.01$.

animal model of breast cancer, which forms tumors lacking ER and PR expression [23]. These results were obtained from two independent experiments using either 20 μg/kg or 50 μg/kg doses of EM1. Although the number of metastasis per animal showed variation in the isopropanol-treated groups between the 20 μg/kg and the 50 μg/kg experiments, this is not statistically significant ($p = 0.618$) and reflects the variation among independent experiments. This result was expected in light of our previous results showing that EM1 decreases viability of ER-positive breast cancer cell lines but not of ER-negative ones [18]. Instead, EM1 modulates LM3 migration and invasion processes. This is also in accordance with published data showing that calcitriol and analogues impair the metastatic process in ER-negative breast cancer cells whereas they inhibit the growth of the primary tumor in breast cancers that express hormone receptors [10]. Genomic profiling of calcitriol-treated MCF-7 and MDA-MB231 cells performed by Feldman's group showed that, in the hormone-responsive cell line, calcitriol regulated genes that were involved in growth factor signalling, cell cycle, apoptosis and immune responses, whereas in the ER-negative MDA-MB-231 cell line calcitriol regulated genes related to disease progression (i.e.,invasion and angiogenesis) [39]. Another work also showed that calcitriol down-regulated genes involved in breast cancer invasion and metastasis in triple-negative breast cancer [40]. Of note, EM1 retarded the migration process in a breast cancer cell line, while having null effects on the non-malignant counterpart HC11. The differential response cannot be ascribed to differences in VDR levels as both cell lines contain similar vehicle- or EM1- treated VDR protein expression. In addition, EM1 had no

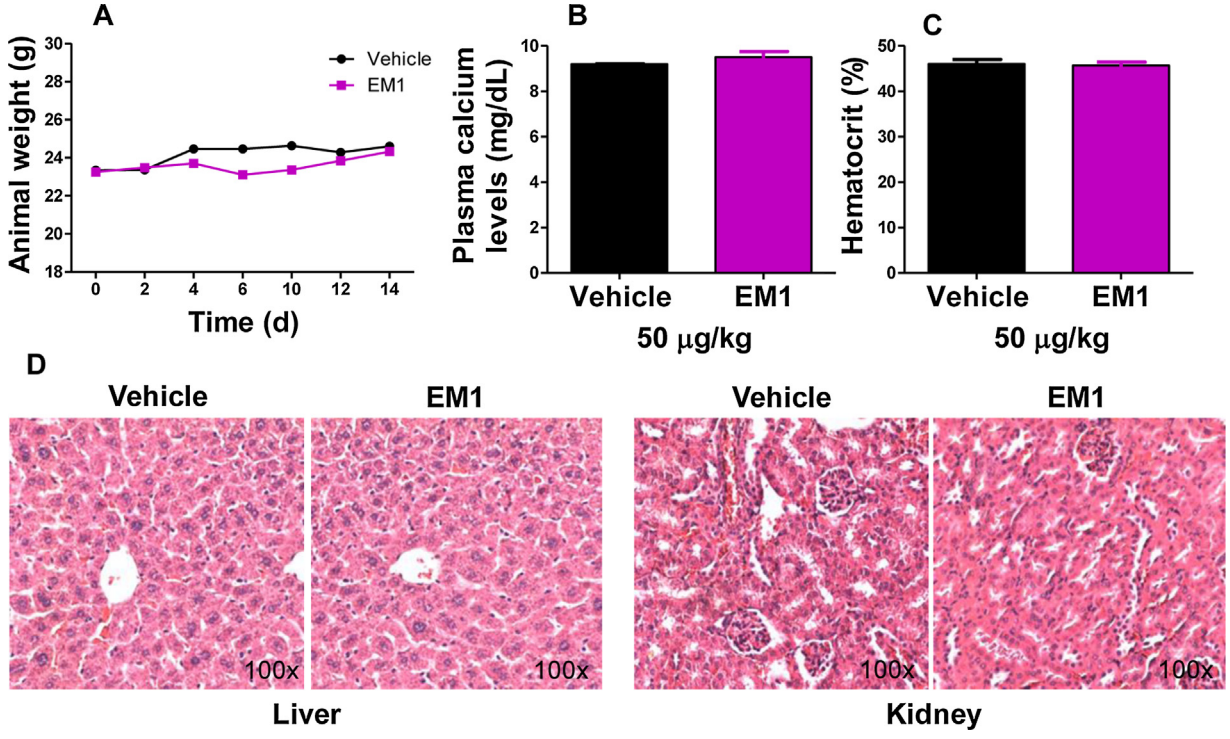

Fig. 7. EM1 effects on calcemic activity, animal weight, hematocrit and histological examination of livers and kidneys in Balb/C mice. Animals were treated with 7 doses of 50 μg/kg over 15 days of EM1 or vehicle. (A) Animal weight during treatments, (B) Plasma calcium levels, (C) Hematocrit and (D) H&E staining of livers and kidneys from animals treated with EM1 and vehicle at 50 μg/kg body weight. ANOVA and Bonferroni post test and unpaired *t* test analysis were applied.

effect on the viability of HC11 (data not shown). This difference in the response of malignant and non-malignant cells to EM1 is of mounting importance in cancer therapeutics.

Cytoskeletal reorganization is essential for changing the cell shape, the cell migration, and other processes [41]. Furthermore, calcitriol and analogues have shown to alter the reorganization of the cytoskeleton [42–44]. Specifically for breast cancer, it has been demonstrated that calcitriol induces a change from a rounded to a flattened morphology and increase lamellipodia and fillopodia formation [44]. In this regard, we also demonstrated that EM1 induces reorganization of the actin cytoskeleton and concomitant change in cell morphology, which could account for the reduction of migration and invasion of LM3 cells. Also, the antimetastatic effects observed with EM1 administration may be secondary to an increase in E-cadherin expression. In support of these results many other groups have recently described calcitriol induction of E-cadherin levels. Thus, it has been demonstrated that calcitriol promoted differentiation of MDA-MB-231 cells by inducing de novo E-cadherin expression [33] and that it reduced migration and invasion through up-regulation of E- cadherin in squamous cell carcinoma [42].

Calcitriol action is limited by its catabolism, occurring mainly by the CYP24A1 resulting in 1,24,25-$(OH)_3$ D_3, a metabolite with substantially lower affinity for the VDR. Enhanced mammary cell catabolism of calcitriol would be predicted to limit the action of the hormone. Indeed, amplification of the CYP24A1 gene was reported in human breast tumors [45] and analysis of the datasets from The Cancer Genome Atlas confirms that a subset of human breast cancers exhibit alterations in the CYP24 gene, with the most frequent changes being amplifications and up-regulation at the mRNA level [10]. Therefore, the antitumor activities of calcitriol or many vitamin D analogues may be limited by their mammary catabolism. In this regard, EM1 presents limitations in its metabolization through 24-hydroxylation due to the presence of a triple bond between the carbons 23 and 24, so its metabolic transformation might be reduced [20] and its antitumoral effects might be prolonged over time. However, further pharmacokinetic studies should be conducted aimed at investigating whether there is any metabolic transformation of the compound.

In conclusion, EM1 vitamin D analogue is highly effective in reducing lung metastasis of a hormone-independent breast cancer animal model, demonstrating also lack of hypercalcemic and other toxic effects on sub-acute administration of the compound.

Conflict of interest

The authors declare no conflicts of interest.

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