Antineoplastic Activity of Products Derived From Cellulose-containing Materials: Study of Levoglucosenone and Structurally-related Analogous as New Alternatives for Breast Cancer Treatment

Damian Ignacio Delbart
Institute of Oncology Angel H Roffo: Instituto de Oncologia Angel H Roffo
German Francisco Giri
IQUIR: Instituto de Quimica Rosario
Agostina Cammarata
Institute of Oncology Angel H Roffo: Instituto de Oncologia Angel H Roffo
Lizeth Ariza Bareño
Institute of Oncology Angel H Roffo: Instituto de Oncologia Angel H Roffo
Natalia Loreley Amigo
Institute of Oncology Angel H Roffo: Instituto de Oncologia Angel H Roffo
Andres Bechis
Institute of Oncology Angel H Roffo: Instituto de Oncologia Angel H Roffo
Alejandra Graciela Suarez
IQUIR: Instituto de Quimica Rosario
Rolando Angel Spanevello
IQUIR: Instituto de Quimica Rosario
Laura Beatriz Todaro
Institute of Oncology Angel H Roffo: Instituto de Oncologia Angel H Roffo
Alejandro Urtreger (urtreger@yahoo.com.ar)
Institute of Oncology Angel H Roffo: Instituto de Oncologia Angel H Roffo https://orcid.org/0000-0002-2338-7821

Research Article

Keywords: levoglucosenone, breast cancer, soybean hulls, pyrolysis

Posted Date: August 6th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-758835/v1

Abstract

Purpose: Breast cancer is the leading cause of cancer death among women worldwide. For this reason, the development of new therapies is still essential. In this work we have analyzed the antitumor potential of levoglucosenone, a chiral building block derived from glucose, and three structurally related analogues obtained from soybean hulls pyrolysis.

Methods: Employing human and murine mammary cancer models, we have evaluated the effect of our compounds on cell viability through MTS assay, apoptosis induction by acridine orange / ethidium bromide staining and/or flow cytometry and the loss of mitochondrial potential by tetramethylrhodamine methyl ester staining. Autophagy and senescence induction were also evaluated by Western blot and β-galactosidase activity respectively. Secreted metalloproteases activity was determined by quantitative zymography. Migratory capacity was assessed by wound healing assays while invasive potential was analyzed using Matrigel-coated transwell chambers. *In vivo* studies were also performed to evaluate subcutaneous tumor growth and experimental lung colonization.

Results: Apoptosis was identified as the main mechanism responsible for the reduction of monolayer cell content induced by the compounds without detecting modulations of autophagy or senescence processes. Two of the four compounds were able to modulate *in vitro* events associated with tumor progression, such as migratory potential, invasiveness, and proteases secretion. Furthermore, tumor volume and metastatic spread were significantly reduced *in vivo* after treatment with the compounds.

Conclusion: We could obtain from soybean hulls, a material with almost no commercial value, a variety of chemical compounds useful for breast cancer treatment.

Introduction

Breast cancer is one of the most common malignancies and the leading cause of cancer death among women worldwide [1]. Statistics show that 1 in 8 women can develop this disease during their lifetime and only the 25% of the patients with metastatic disease survive after 5 years [2]. Although adjuvant and/or neoadjuvant treatments improve prognosis, conventional therapies (surgery, chemotherapy and radiotherapy) seem to have reached a plateau of therapeutic efficiency. For this reason, it is essential to look for new alternatives that limit tumor growth and/or prevent metastases development.

On the other hand, to address the environmental deterioration issue, there is a need to adopt actions to develop a sustainable bioeconomy in which obtain useful products from renewable sources and also in a lesser contaminant manner. In this way, biomass has received special attention since it represents the only source of renewable organic carbon. From a chemical point of view, the oxygenated nature, chemical diversity and chirality make biomass a suitable material to obtain a wide variety of highly useful compounds that could replace those obtained from petroleum [3].

A suitable and commonly employed way to process biomass is through a thermal treatment in the absence of oxygen known as pyrolysis. Using this technique, solid biomass can be converted to coal, liquid bio-oils and non-condensable gases [4]. The bio-oils find their main utility in the development of fuels, but hundreds of organic compounds are also present that could become valuable chemicals [5, 6]. An interesting and practically unexplored issue is the use of these bio-oils as a source of bioactive compounds [7–9].

In presence of a catalytic amount of acid, the cellulose backbone present in the vegetal material is degraded obtaining a bio-oil highly enriched in levoglucosenone (1,6-anhydro-3,4-dideoxy-*β*-D-glycero-hex-3-enopyranos-2-ulose) (compound **1**), a bicyclic compound with a 1,6-anhydrous bridge that fixes the conformation of this system and sterically hides the *β* (endo) side of the molecule [10, 11] (Fig. 1a). This compound has served as a building block for the synthesis of a wide array of chemicals making use of its functionalization and steric hindrance. Besides, the fact that it is a pure enantiomer also allows the possibility to obtain complex structures such as those found in natural products [3, 11]. The synthesis of Ras protein inhibitors through 1,3-dipolar additions to levoglucosenone's double bond [12], the generation of triazole [13] and iminosugars [14] derivatives of **1** are some of the strategies employed for the clinical use of levoglucosenone in cancer and other pathologies. Nevertheless, a drawback in all of these cases was the bioactive compounds required several complex synthetic steps in order to be prepared and tested.

Surprisingly, although levoglucosenone was obtained more than three decades ago, only one report showed that this molecule could inhibit *in vitro* hamster cells growth [15], and no further research about its activity has been described. We have recently demonstrated the obtaining of levoglucosenone from soybean hulls bio-oils, further determining that this molecule displays antibacterial activities

against *Salmonella* Typhimurium and other bacterial species [16]. In a further study, we developed several derivatives employing a Michael addition of aromatic thiols over the enone functionality. These derivatives along with its brominated analogous in C-3 (Fig. 1b) were effective in the induction of hepatocarcinoma cells death *in vitro* [17]. An important feature is that, in order to obtain these bioactive compounds, we needed only one synthetic step [17]. Therefore, our potential anticancer drugs can be easily synthesized at a low cost and from a renewable raw material.

In the present work we have analyzed the effect of levoglucosenone and the three structurally-related analogous previously reported, in mammary tumor progression. Employing different human and murine mammary cancer models, we could identify apoptosis as the main mechanism responsible of cell death induced by the compounds, without detecting a significant modulation of autophagy or senescence processes. Moreover, two of the four cell death-inducing compounds were able to modulate *in vitro* events associated with tumor progression such as migratory potential, invasiveness and proteases secretion. Finally, preclinical assays were conducted employing BALB/c mice. A clear impairment of both, tumor growth and metastasis dissemination were observed, allowing us to propose these compounds as an alternative for breast cancer treatment.

In this work, we faced a double challenge. On one hand, to transform waste material with almost no commercial value (soybean hulls) into a source of chemical compounds with productive uses and on the other, provide to the clinic with new tools that allow the effective treatment of breast cancer patients. We consider that both challenges were efficiently fulfilled with remarkable results.

Materials And Methods

Reagents and antibodies: Media for cell culture were from Gibco BRL Laboratories (Rockville, MD). Fetal bovine serum (FBS) was from GBO (Buenos Aires, Argentina). Transwell chambers were purchased to Corning Inc. (New York, NY) and Matrigel was obtained from BD Biosciences (Bedford, MA). Monoclonal anti-LC3II and anti-actin were purchased from BD Biosciences (San Diego, CA). Acrylamide, horseradish peroxidase conjugated anti-mouse antibodies, were obtained from Sigma (St. Louis, MO). Hybond-P membranes and chemiluminescence reagents (ECL) were from GE Healthcare Bio-Sciences (Little Chalfont, UK). Other reagents for polyacrylamide gel electrophoresis and zymography were obtained from Bio-Rad (Richmond, CA) and Annexin-V Kit was purchased from Thermo Fisher Scientific (Waltham, MA).

Compounds: Preparation of levoglucosenone (compound **1**), and the synthetic derivatives: 3-bromolevoglucosenone (compound **2**), 4-(phenylthio)-dihydrolevoglucosenone (compound **3**) and 4-(*p*-methyl-phenylthio)-dihydrolevoglucosenone (compound **4**) has been described elsewhere [17].

Cell lines and culture conditions: LM3, MCF-7 and MDA-MB-231 cell lines were used in this study. The LM3 cell line was previously established in our laboratory from spontaneous BALB/c mammary adenocarcinoma with tumorigenic and metastatic capacities [18]. The human MDA-MB-231 (CRM-HTB-26) and MCF-7 (HTB-22) cell lines were obtained from ATCC. LM3 cells were grown in minimum essential medium (MEM) supplemented with 10% FBS and 80 µg/ml gentamycin. MDA-MB-231 and MCF-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM/F12) with the same supplement. All cell lines were cultured at 37°C in a humidified air atmosphere with 5% CO_2. Serial passages were performed treating the monolayers with 0.25% trypsin (Invitrogen, Carlsbad, CA) and 0.02% EDTA in Ca^{2+} free and Mg^{2+} free phosphate buffered saline (PBS) twice a week.

Cell viability assay: To assess the effect of the compounds on cell viability, cells were seeded onto 96 well plates in standard culture conditions. Twenty-four hours later cells were treated with different concentrations of the compounds (range 1–40 µM) during 48 h. Cell proliferation was evaluated using the MTS assay (Celltiter 96TM Non-Radioactive Proliferation Assay, Promega Madison, WI), as described by the manufacturer. Data were analyzed using the R package "ic50" [19] in order to determine the half maximal inhibitory concentration (IC_{50}) values for each compound.

Apoptosis detection: Cells growing on glass coverslips were treated with the IC_{50} value of the compounds or vehicle alone for 24 h and then stained with Acridine orange (10 µg/ml) and Ethidium bromide (10 µg/ml). Visualization was performed with a Nikon, Eclipse E400 epifluorescence microscope. Uniform green nuclei with organized structure were considered as live cells, orange and red nuclei were classified as early or late apoptotic cells respectively.

Apoptosis induction was further evaluated by Annexin-V staining and flow cytometry. Cells treated or not for 24 h with the IC_{50} obtained for the different compounds were collected and apoptosis was quantified as described by the manufacturer. Briefly, $1x10^6$

cells were washed and resuspended in 100 µl of 1X binding buffer. Then, cells were incubated 15 min in darkness at room temperature with 5 µl of Alexa488-conjugated Annexin-V. Cells were washed with 1X binding buffer and finally 5 µl of propidium iodide was added. Cells were mixed in darkness at room temperature for 10 min, then 300 µl of 1X binding buffer was added, and cells were mixed in an ice bath at dark. Cell suspension was examined under 488 nm excitation wavelength by flow cytometry using an Epics Elite ESP coulter cytometer (Beckman Coulter).

Loss of mitochondrial potential: Cells growing on glass coverslips were treated for 48 h with the IC_{50} of the compounds or vehicle alone as control. Then cells were washed with PBS and stained with 150 nM of tetramethylrhodamine methyl ester (TMRM) fluorescent dye (Image-iT™ TMRM Reagent, Molecular Probes, Thermo Fisher Scientific, Waltham, MA) in PBS. After 30 min incubation cells were washed again and mounted in PBS-Glycerol (1:1). Photographs were taken using a Nikon Eclipse E400 epifluorescence microscope equipped with a digital Coolpix Nikon camera (Tokyo, Japan).

Autophagy: Cells treated or not for 48 h with the different compounds IC_{50} were lysed and autophagy was assessed by determining LC3-I and LC3-II expression levels by Western blot as previously described [20].

Western blot: Semiconfluent monolayers treated for 48 h with the IC_{50} of the compounds, or vehicle as control, were washed twice with ice-cold PBS and then lysed with 1% Triton X-100 in PBS by scrapping with a Teflon scrapper. Samples were denatured by boiling in sample buffer with 5% β-mercaptoethanol and run in 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Fifty µg of protein was loaded in each lane and gels were blotted to Hybond-P membranes. Membranes were blocked by incubation during 1 h with PBS containing 5% skim-milk plus 0.1% Tween-20. Then membranes were incubated with the first antibody overnight at 4°C, and finally with a secondary antibody coupled to horseradish peroxidase. Detection was performed by chemiluminescence. Bands were digitalized with a Photo/Analyst Express System (Fotodyne Inc. Hartland, WI) and signal intensity was quantified with Gel-Pro Analyzer software.

Senescence: To detect senescence-associated β-galactosidase activity, a detection kit was used following the manufacturer's instructions (Biovision, Milpitas, CA, USA). Briefly, cells were seeded onto 12 well-plates and treated with different doses of the compounds up to 72 h. After the incubation, cells were rinsed with PBS and fixed with 2% formaldehyde and 0.2% glutaraldehyde for 15 min at room temperature. Next, 470 µl of staining solution, 5 µl supplement for staining and 25 µl of a 20 mg/ml X-gal solution were added to each well. After overnight incubation at 37°C, cells were rinsed again with PBS and mounted in PBS-Glycerol (1:1). Photographs were taken using a Nikon Eclipse E400 epifluorescence microscope equipped with a digital Coolpix Nikon camera (Tokyo, Japan).

Preparation of conditioned media (CM): Secreted metalloproteases (MMPs) activity was evaluated in CM. Briefly, subconfluent cell monolayers growing in 6-well plates were treated or not with the IC_{50} dose of the compounds for 24 h. Then, cells were extensively washed with PBS and subsequently incubated in FCS-free medium for another 24 h. Finally, CM were individually harvested, the remaining monolayers were lysed using a buffer containing 0.1 N NaOH and 2% Ca_2CO_3, and cell protein content was determined. CM samples were aliquoted, stored at -40ºC and used once after thawing.

Quantification MMPs secreted activity by zymography: MMPs enzymatic activity was determined by quantitative zymography [21]. Briefly, CM samples were run on a 9% SDS-PAGE gels co-polymerized with gelatin (1 mg/ml) under non-reducing conditions. After electrophoresis, gels were washed for 30 min with 2% Triton X-100 and subsequently incubated at 37°C for 48 h in a buffer containing 0.25 M Tris-HCl, 1 M NaCl, and 25 mM $CaCl_2$ (pH 7.4) for activity detection. Finally, gels were fixed and stained with Coomassie Brilliant Blue (0.1% Coomassie R-250 in 10% acetic acid and 30% methanol). Gelatinolytic bands were visualized as negative staining and quantified using the Gel Pro Analyzer program. Data were expressed in arbitrary units and was relativized to cell lysates protein content.

Migratory capacity: Wounds of approximately 400 µm width were performed in LM3 and MDA-MB-231 subconfluent monolayers, and cells were then allowed to migrate into the cell-free area for a period of 18 h in the presence or not of the IC_{50} dose of the compounds and low FCS concentration (1%). The same spot was photographed at different times using an inverted microscope (Eclipse TE2000-S, Nikon) equipped with a digital camera and the migratory area was determined using the Image-ProPlus 4.5 software. Cell migration was expressed as the percentage of the area occupied by the migratory cells in the original cell-free wounded area.

Invasion assay: The effect of the compounds on invasive capacity was analyzed employing transwell culture cameras (Corning, Lowell, MA). Filters (8 µm membrane pores) were coated with 0.1% gelatin on the lower side and with a thin layer (250 µg/ml) of Matrigel (Becton Dickinson Labware, Bedford, MA) on the upper side. The lower chamber contained 0.5 ml of culture media supplemented with human fibronectin (16 µg/ml) (Sigma, St. Louis, MO) as chemoattractant. Cells ($1x10^5$) pretreated or not for 24 h with the IC_{50} dose of compounds were seeded in the upper chamber in culture medium without FCS. Eighteen hours later, filters were removed, fixed with Carnoy and the upper surface was wiped with a cotton swab to remove non-invasive cells. Finally, the filters were stained with DAPI. Cells that invaded Matrigel, passed through the pores, and reattached the lower surface of the filters were considered as invasive and their fluorescent nuclei were counted using an epifluorescence microscope (Eclipse TE2000-S, Nikon). The number of cells per field was determined in 10 randomly selected fields.

Animals: For *in vivo* assays, randomized inbred female BALB/c mice, 2–4 months old, obtained from the Animal Care Division of the Institute of Oncology "Angel H. Roffo" were employed. Mice were housed 6 per cage, kept under an automatic 12 h light/12 h darkness schedule, and provided with sterile pellets (Cooperacion, SENASA N° 04-288/A) and tap water *ad libitum*. For housing, rectangular polycarbonate cages (20 x 29 x 14 cm) with irradiated pine wood (15 KGy) as bed were employed. All animal studies were conducted in accordance with the standards of animal care as outlined in the NIH and ARRIVE Guide for the Care and Use of Laboratory Animals. Besides, protocols have the approval of the Committee for the Use and Care of laboratory Animals (CICUAL) of the Institute of Oncology "A. H. Roffo", University of Buenos Aires. Animals were euthanized by CO_2 inhalation at the experimental endpoint. We established the human endpoint when mice show one of the following signs: Loss of > 20% of the initial weight, lethargy, bristling coat and/or hemorrhagic diarrhea.

Lung colonization assay: To study the effect of the compounds on lung colonization, an experimental metastasis assay was performed as previously described [22]. Briefly, $2x10^5$ LM3 cells pretreated or not with compounds for 24 h were injected in the lateral tail vein of syngeneic BALB/c mice using a 27-gauge needle. Cell viability was higher than 95% as determined by trypan blue exclusion test, and the order of injection of different groups was randomized to eliminate any difference that may bias the outcome. Mice were monitored daily and sacrificed 21 days later. Lungs were removed, fixed in Bouin's solution and the number of superficial lung nodules was counted under a dissecting microscope.

Tumor growth: Exponentially growing LM3 cell monolayers were harvested with trypsin-EDTA, washed with MEM and resuspended in the same medium at a final concentration of $1x10^6$ cells/ml. Then a 0.2 ml cell suspension was subcutaneously injected in mice left flank. Cell viability was higher than 95% as determined by trypan blue exclusion test. Animals were randomly divided into three groups and once the tumor was detected, they were treated twice a week with an intraperitoneal injection of 60 µg/kg of the compounds **1** or **2** or with vehicle in physiological solution as control. Mice were monitored daily and tumor-perpendicular diameters were measured with a sliding caliper twice a week. Tumor volume was calculated using the following formula V= ¾ (π x L x 2 W), where L is the longest and W is the shortest diameter. Animals were necropsied 21 days after cell inoculation.

Statistical Analysis: All assays were performed in triplicate, and independent experiments repeated at least twice. Statistical differences between groups were calculated by applying ANOVA, Chi Square or Kruskal-Wallis tests, as indicated. A value of $p < 0.05$ was considered to be significant.

Results

Effect of levoglucosenone and its derivatives on in vitro cell proliferation

In order to investigate whether levoglucosenone (compound **1**, Fig. 1a) and structurally-related derivatives (compounds **2** to **4**, Fig. 1b) alter the proliferative potential of breast cancer cells, we employed three tumor derived cell lines: LM3 (murine); MCF7 and MDA-MB-231 (human). The main difference between these breast cancer models relies in the expression of the hormonal and Her2 receptors. The LM3 cell line is hormone-independent and overexpresses the Her2 receptor. MCF7 expresses estrogen and progesterone receptors but does not overexpress Her2 and MDA-MB-231 is considered triple negative, since it does not express any of these receptors. The selection of these cell models aims to simulate a variety of patient-clinical conditions.

We found that levoglucosenone and the structurally-related derivatives strongly impair the *in vitro* proliferation of all the cell lines analyzed. The obtained IC_{50} values were in a range of low micromolar concentrations, with the best results obtained for the LM3 and

MDA-MB-231 cells (Table 1). Interestingly, a comparative analysis of sensitivity showed that our derivatives were as good as other drugs approved for the treatment of mammary cancer or even better (Table 1).

Table 1
Comparison of IC_{50} values between compounds and antitumor drugs currently employed for breast cancer treatment

Compound	Cell line IC_{50} (μM)		
	MCF7	MDA-MB-231	LM3
1	52.54 ± 2.07	12.60 ± 0.89	14.42 ± 0.54
2	53.14 ± 3.02	11.12 ± 1.44	11.76 ± 2.14
3	50.50 ± 0.70	13.35 ± 0.76	14.53 ± 0.35
4	51,50 ± 2.12	15.80 ± 0.77	12.32 ± 1.40
5-FU[a]	419.46	122.85	-
Olaparib[a]	285.38	62.54	-
Cyclophosphamide[a]	231.77	195.56	-
Palbociclib[a]	43.30	14.57	-
Fulvestrant[a]	1.03	25.98	-
Doxorubicin[a]	0.01	1.26	-

[a] Data were collected from www.cancerrxgene.org. No information is available regarding LM3 cells.

Mechanism involved in the in vitro proliferation impairment

In view that levoglucosenone and its analogous affect the *in vitro* proliferation of mammary cell lines, we decided to investigate which mechanisms are involved in this process. First, we assessed apoptosis induction through acridine-orange/ethidium bromide staining. All compounds induced the appearance of orange/red nuclei in LM3 and MCF-7 cell lines (Fig. 2a). Since this staining is consistent with apoptotic cells, this process was further quantified by flow cytometry employing Annexin-V-FITC/propidium iodide staining (Fig. 2b). Levoglucosenone and its derivatives proved to be strong apoptosis inductors, increasing cell death between ~ 50 to ~ 80 % in the LM3 line.

As a general rule, chemotherapy agents generate a cytoplasmic stress that activates apoptosis via the intrinsic pathway. The hallmark of this process is mitochondrial outer membrane permeabilization mediated by members of the Bcl-2 family [23]. To test this event, we employed the tetramethylrhodamine methyl ester (TMRM) fluorescent dye which accumulates in healthy mitochondria with intact membrane potential, showing an intense red fluorescence. As shown in Fig. 2c, almost no staining could be detected after all treatments, indicating that the compounds trigger mitochondrial membrane depolarization, confirming the induction of apoptosis via the intrinsic pathway.

In order to determine the existence of other mechanisms responsible for the reduction in monolayers cell content, we tested the induction of autophagy and senescence processes. Autophagy was assayed through Western blot, analyzing the expression of LC3 protein. The conversion of LC3I into LC3II, a protein tightly bound to the autophagosome membrane, highly correlates with autophagic activity in mammalian cells [24]. As shown in Fig. 2d and e, no significant differences in LC3II/LC3I ratio could be detected in LM3 and MCF-7 treated cells, relative to the control condition. This result allows us to confirm that autophagy is not induced by treatment with the compounds.

Despite being a mechanism of cell aging and not of cell death, we proceeded with the study of the senescence process, since the lack of replicative potential could finally affect monolayers cell content. As shown in Fig. 2f, none of the compounds induced the presence of blue or light-blue cells in β-galactosidase assays ruling out senescence as a mechanism induced by the compounds. Altogether,

our results support apoptosis as the main mechanism involved in the antiproliferative effect of levoglucosenone and the corresponding analogous.

Effect on proteases secretion, migratory and invasive potential

Enhancement of matrix metalloproteinases (MMPs) secretion and/or activation in cancer tissues represents one of the critical steps in tumor invasion, angiogenesis and metastatic spread due to their involvement in the degradation of extracellular matrix and cell adhesion proteins [25, 26]. As shown in Fig. 3a and b, employing direct zymography assays, we could confirm that both LM3 and MDA-MB-231 cells secrete detectable amounts of MMP-9 to the CM. Moreover, MMP-9 enzymatic activity was significantly reduced after treatment with compounds **1** and **2**. On the other hand, compounds **3** and **4** showed a slight non-significant reduction in MMP-9 secreted activity.

Next, we evaluated whether the modulation of tumor secreted proteases correlates with changes in migratory or invasive processes. Migratory capacity was evaluated through wound migration assays as described in material and methods section. As shown in Fig. 3c, d, e and f, compounds **1** and **2** caused a ~70–80% decrease in cell motility in both cell lines, while compounds **3** and **4** also induced a significant reduction in migratory potential but in a lesser degree (~30%).

Using Matrigel-coated transwells we studied the effect of the compounds in cell invasion. As shown in Fig. 3g and h, and in agreement with the previously described results, a significant decrease in the invasive capacity was observed after treating LM3 cells with the compounds **1** and **2**. These compounds reduced ~50% *in vitro* invasiveness after 20 h treatment. No effect could be detected after treating LM3 cells with compounds **3** and **4**.

In vivo studies: modulation of metastatic dissemination and tumor growth

In order to propose the use of the compounds in clinical settings, we investigated their *in vivo* effect employing the LM3 cell line. This tumorigenic and metastatic cell model was developed in our laboratory [18] and has the advantage of being syngeneic in BALB/c mice, making the *in vivo* experimentation more reliable.

First, we evaluated the effect on experimental lung colonization. For this purpose, pretreated LM3 cells were injected in the lateral tail vein of BALB/c mice, and 21 days later mice were sacrificed, and lung nodes were counted. As shown in Fig. 4a and Table 2, compounds **1** and **2** significantly reduced both incidence and number of macroscopic lung lesions as compared to the control group. Confirming the results obtained *in vitro*, compounds **3** and **4** were unable to affect experimental lung colonization and therefore no clinical benefits are expected from their employment.

Table 2
Experimental lung colonization parameters

	Control	Compound 1	Compound 2	Compound 3	Compound 4
Incidence (%) (Mean ± S.E.)	100 ± 0	77 ± 5.7*	83 ± 7.1*	100 ± 0	100 ± 0
Number Median (range)	52 (32–89)	2.5 (0–10)#	7 (0–8)#	88 (52–99)	58 (9–79)

Each group consisted of at least 10 female mice. Values are the average of three independent experiments. *$p < 0.05$ vs. control, Chi Square Test, #$p < 0.05$ vs. control, Kruskall-Wallis test.

The slight effect on cell migration and proteases secretion together with the inability to alter invasive and metastatic processes, displayed by compounds **3** and **4**, prompt us to perform the *in vivo* systemic studies employing only levoglucosenone and its brominated variant (compounds **1** y **2**).

Toxicity curves and survival analysis were performed inoculating animals with three different doses of the compounds **1** and **2** (up to 120 μg/kg) for four weeks, administering them twice a week. No changes in weight and/or in animal behavior could be detected. Furthermore, no liver toxicity or alterations in hematological and laboratory parameters were observed at the end of the treatment (data not shown) allowing the study of the compounds on tumor growth.

Animals were subcutaneously inoculated with LM3 cells and when tumors were detectable, mice received an intraperitoneal dose of 60 µg/kg of the compounds or vehicle in physiological solution as control twice a week for 3 weeks. As shown in Fig. 4b, compound **2** induced a strong and significant inhibition of local tumor growth. The compound **1** showed a milder effect nevertheless, it became significant after 15 days treatment. In both cases, the delay in tumor growth sustained over time. Altogether, our results strongly suggest that levoglucosenone and its brominated variant could become a promissory and useful tool for anticancer therapy.

Discussion

Breast cancer is the most frequently diagnosed cancer in women worldwide and is also the leading cause of women cancer death in more than 100 countries [1]. Targeted therapy has changed the course of breast cancer treatment, however, these therapies often block a single pathway and may become ineffective, due to the existence of redundant and/or alternative pathways on transformed cells [27]. Therefore, it is imperative to develop new and more efficient therapies, in order to generate a major impact on the evolution of this disease. In this sense, the objective of the present work was to demonstrate the potential use of a renewable, abundant and low-cost starting material, such as soybean hulls, to obtain relevant compounds for breast cancer treatment.

MCF-7 cells proved to be the most cell-death resistant model, displaying the higher IC_{50} value as compared with the other cell lines. Despite the differences in hormonal and Her2 receptors expression, it is important to note that unlike LM3 and MDA-MB-231 lines, MCF-7 cells are unable to express caspase-3, the final effector of the apoptosis cascade [28]. This caspase-3 deficiency, results in atypical apoptotic properties, and may probably be responsible for the higher resistance observed.

Interestingly, a comparative sensitivity evaluation showed that our compounds were as good as some drugs employed nowadays for breast cancer treatment. Moreover, in some cases they showed even better results (www.cancerrxgene.org). The major differences were observed when comparing in MDA-MB-231 cell line, compounds 1–4 with 5-fluorouracil, showing almost 10-fold higher effectiveness. Similar relations were obtained for the alkylating agent cyclophosphamide and the PARP1/2 inhibitor Olaparib. Moreover, the compounds are as potent as Palbociclib, a drug approved in recent years to treat breast cancer and the first CDK4/6 inhibitor to be accepted as a cancer therapy [29].

To elucidate mechanisms underlying the reduced proliferative capacity observed *in vitro*, first we examined the effect of each agent on apoptosis induction. A clear induction of this process was evidenced for all the compounds in LM3, MDA-MB-231 and MCF-7 cell lines upon staining with ethidium bromide/acridine orange and by Annexin-V flow cytometry. Moreover, the compounds also triggered mitochondrial stress which could be responsible of apoptotic induction through the intrinsic pathway [23].

It is generally accepted that autophagy blocks the induction of apoptosis, and caspase associated apoptosis shuts off the autophagic process [30, 31]. Nevertheless, autophagy and apoptosis could occur sequentially in a way in which autophagy precedes apoptosis [32]. Then, the rationale behind the study of the autophagic process relies in the fact that the regulation of this process in cancer cells is complex since it can favor tumor cells survival under stress conditions, but can also induce cell death [24]. None of the compounds induced the autophagic process and replicative senescence was also not visualized in any of the analyzed cell lines.

The ability to secrete proteolytic enzymes able to degrade one or more components of the extracellular matrix defines in part the invasive potential of tumor cells. We have analyzed the secretion of MMP-9, a member of the gelatinase family, whose activity is increased in a wide variety of tumors [25, 33]. An important inhibition of this protease activity, associated to compounds **1** and **2** treatment, could be detected. Proteases have two main functions related to extracellular matrix degradation. On one hand, the facilitation of invasive processes and on the other the modulation of cell migration, two key factors in malignant progression and metastasis development [34, 35]. Both migration and invasion capabilities were impaired when cells were treated with the compounds, showing levoglucosenone and its brominated derivative the most dramatic effects. All these findings led us to investigate the *in vivo* effects of the compounds, with special attention to compounds **1** and **2**, since *in vitro* results clearly indicate that these compounds could interfere with biological processes associated with tumor growth and metastatic spread. In fact, the treatment with these compounds not only affected the metastatic potential but also produced an important impairment in tumor growth. It is important to note that in all cases, the mice remained healthy throughout the entire experiment, a highly desired situation in a chemotherapy treatment. Compounds **3** and **4** were also assayed for their experimental metastatic ability. The rationale behind this assay rely in the fact that the proteolytic activity and invasive potential may not be considered essential in this process since cells are inoculated directly into the bloodstream and therefore the first steps of the metastatic cascade are omitted. However, as

already observed for *in vitro* events associated with tumor progression, these compounds were unable to alter the metastatic potential of LM3 cells.

Throughout this work, we have evaluated several biological aspects of levoglucosenone and related analogous, successfully demonstrating their potential utility for breast cancer treatment. Although more research is needed to deeply understand the underlying events, signals, and molecular targets, we could demonstrate for the first time the safety and effective use of our compounds. It is important to note that levoglucosenone and the related analogous have the great advantage of being easily synthesized and at low cost also using renewable raw materials with practically no commercial value. Altogether, these features make the compounds an interesting and economically affordable alternative for breast cancer treatment.

Declarations

Funding

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and the Universidad Nacional de Rosario.

Competing interests

Authors declare no competing interests.

Availability of data and materials

All the data generated during this study are included in this article.

Code availability

Not applicable.

Authors' contributions

DID, GFG and AC carried out all experiments, prepared figures and drafted the manuscript. LAB, NLA and AB participated in data analysis and results interpretation. AGS and RAS performed a critical revision of the manuscript. LBT and AJU conceived the study and participated in data analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal studies were conducted in accordance with the standards of animal care as outlined in the NIH and ARRIVE Guidelines for the Care and Use of Laboratory Animals. Protocols have the approval of the Committee for the Use and Care of laboratory Animals (CICUAL) of the Institute of Oncology "A. H. Roffo", University of Buenos Aires.

Consent for publication

Not applicable.

References

1. BrayF,FerlayJ,SoerjomataramI,SiegelRL,TorreLA,JemalA(2018)Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries.CA Cancer J Clin68:394–424.https://doi.org/10.3322/caac.21492
2. HowladerN,NooneAM,KrapchoM,MillerD,BrestA,YuM,RuhlJ,TatalovichZ,MariottoA,LewisDR,ChenHS,FeuerEJ,CroninKA(2021)SEER Cancer Statistics Review,1975–2018.https://seer.cancer.gov/csr/1975_2018/
3. CombaMB,TsaiYH,SarottiAM,MangioneMI,SuarezAG,SpanevelloRA(2018)Levoglucosenone and Its New Applications: Valorization of Cellulose Residues.Eur J Org Chem2018:590–604.https://doi.org/10.1002/ejoc.201701227
4. LiuC,WangH,KarimAM,SunJ,WangY(2014)Catalytic fast pyrolysis of lignocellulosic biomass.Chem Soc Rev43:7594–7623.https://doi.org/10.1039/c3cs60414d

5. WangH,MaleJ,WangY(2013)Recent Advances in Hydrotreating of Pyrolysis Bio-Oil and Its Oxygen-Containing Model Compounds.ACS Catal3:1047–1070.https://doi.org/10.1021/cs400069z
6. AdhikariS,SrinivasanV,FasinaO(2014)Catalytic Pyrolysis of Raw and Thermally Treated Lignin Using Different Acidic Zeolites.Energy & Fuels28:4532–4538.https://doi.org/10.1021/ef500902x
7. Mohan D, Shi J, Nicholas DD, Pittman CU Jr, Steele PH, Cooper JE (2008) Fungicidal values of bio-oils and their lignin-rich fractions obtained from wood/bark fast pyrolysis. Chemosphere 71:456–465. https://doi.org/10.1016/j.chemosphere.2007.10.049
8. Booker CJ, Bedmutha R, Vogel T, Gloor A, Xu R, Yeung KK, Scott IM, Conn KL, Berruti F, Briens C (2010) Experimental Investigations into the Insecticidal, Fungicidal, and Bactericidal Properties of Pyrolysis Bio-oil from Tobacco Leaves Using a Fluidized Bed Pilot Plant. Ind Eng Chem Res 49:10074–10079. https://doi.org/10.1021/ie100329z
9. FriedmanM(2013)Rice brans, rice bran oils, and rice hulls: composition, food and industrial uses, and bioactivities in humans, animals, and cells.J Agric Food Chem61:10626–10641.https://doi.org/10.1021/jf403635v
10. ShafizadehF,FurneauxRH,StevensonTT(1979)Some reactions of levoglucosenone.Carbohydrate Research71:169–191.https://doi.org/10.1016/S0008-6215(00)86069-3
11. Corne V, Botta MC, Giordano EDV, Giri GF, Llompart DF, Biava HD, Sarotti AM, Mangione MI, Mata EG, Suarez AG et al (2013) Cellulose recycling as a source of raw chirality. Pure Appl Chem 85:1683–1692. https://doi.org/10.1351/PAC-CON-12-11-10
12. MullerC,Gomez-Zurita FrauMA,BallinariD,ColomboS,BittoA,MarteganiE,AiroldiC,vanNeurenAS,SteinM,WeiserJ,etal(2009)Design, synthesis, and biological evaluation of levoglucosenone-derived ras activation inhibitors.ChemMedChem4:524–528.https://doi.org/10.1002/cmdc.200800416
13. TsaiYH,Borini EtichettiCM,DiBenedettoC,GirardiniJE,MartinsFT,SpanevelloRA,SuarezAG,SarottiAM(2018)Synthesis of Triazole Derivatives of Levoglucosenone As Promising Anticancer Agents: Effective Exploration of the Chemical Space through retro-aza-Michael//aza-Michael Isomerizations.The Journal of organic chemistry83:3516–3528.https://doi.org/10.1021/acs.joc.7b03141
14. PratesiD,MatassiniC,GotiA,AngeliA,CartaF,SupuranCT,SpanevelloRA,CardonaF(2020)The glycomimetic based approach towards selective carbonic anhydrase inhibitors.ACS Medicinal Chemistry Letters.https://doi.org/10.1021/acsmedchemlett.9b00590
15. NishiY,MiyakawaY,KatoK(1989)Chromosome aberrations induced by pyrolysates of carbohydrates in Chinese hamster V79 cells.Mutat Res227:117–123.https://doi.org/10.1016/0165-7992(89)90007-9
16. Giri GF, Viarengo G, Furlán RLE, Suarez AG, Véscovi EG, Spanevello RA (2017) Soybean hulls, an alternative source of bioactive compounds: Combining pyrolysis with bioguided fractionation. Ind Crop Prod 105:113–123. https://doi.org/10.1016/j.indcrop.2017.05.005
17. Giri GF, Danielli M, Marinelli RA, Spanevello RA (2016) Cytotoxic effect of levoglucosenone and related derivatives against human hepatocarcinoma cell lines. Bioorg Med Chem Lett 26:3955–3957. https://doi.org/10.1016/j.bmcl.2016.07.007
18. UrtregerA,LadedaV,PuricelliL,RivelliA,VidalM,DelustigE,JoffeE(1997)Modulation of fibronectin expression and proteolytic activity associated with the invasive and metastatic phenotype in two new murine mammary tumor cell lines.Int J Oncol11:489–496.https://doi.org/10.3892/ijo.11.3.489
19. FrommoltP,ThomasRK(2008)Standardized high-throughput evaluation of cell-based compound screens.BMC Bioinformatics9:475.https://doi.org/10.1186/1471-2105-9-475
20. BerardiDE,FlumianC,RodriguezCE,BessoneMI,CiriglianoSM,JoffeED,FiszmanGL,UrtregerAJ,TodaroLB(2016)PKCdelta Inhibition Impairs Mammary Cancer Proliferative Capacity But Selects Cancer Stem Cells, Involving Autophagy.J Cell Biochem117:730–740.https://doi.org/10.1002/jcb.25358.
21. Berardi DE, Ariza Bareno L, Amigo N, Canonero L, Pelagatti MLN, Motter AN, Taruselli MA, Diaz Bessone MI, Cirigliano SM, Edelstein A et al (2021) All-trans retinoic acid and protein kinase C alpha/beta1 inhibitor combined treatment targets cancer stem cells and impairs breast tumor progression. Sci Rep 11:6044. https://doi.org/10.1038/s41598-021-85344-w
22. Bessone MID, Berardi DE, Cirigliano SM, Delbart DI, Peters MG, Todaro LB, Urtreger AJ (2020) Protein Kinase C Alpha (PKCalpha) overexpression leads to a better response to retinoid acid therapy through Retinoic Acid Receptor Beta (RARbeta) activation in mammary cancer cells. J Cancer Res Clin Oncol 146:3241–3253. https://doi.org/10.1007/s00432-020-03368-7
23. GalluzziL,VitaleI,AbramsJM,AlnemriES,BaehreckeEH,BlagosklonnyMV,DawsonTM,DawsonVL,El-DeiryWS,FuldaS,etal(2012)Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012.Cell Death Differ19:107–120.https://doi.org/10.1038/cdd.2011.96

24. Berardi DE, Campodonico PB, Diaz Bessone MI, Urtreger AJ, Todaro LB (2011) Autophagy: friend or foe in breast cancer development, progression, and treatment. Int J Breast Cancer 2011:595092. https://doi.org/10.4061/2011/595092
25. HuangH(2018)Matrix Metalloproteinase-9 (MMP-9) as a Cancer Biomarker and MMP-9 Biosensors: Recent Advances.Sensors18:3249.https://doi.org/10.3390/s18103249
26. MondalS,AdhikariN,BanerjeeS,AminSA,JhaT(2020)Matrix metalloproteinase-9 (MMP-9) and its inhibitors in cancer: A minireview.Eur J Med Chem194:112260.https://doi.org/10.1016/j.ejmech.2020.112260
27. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144:646–674. https://doi.org/10.1016/j.cell.2011.02.013
28. WangS,HeM,LiL,LiangZ,ZouZ,TaoA(2016)Cell-in-Cell Death Is Not Restricted by Caspase-3 Deficiency in MCF-7 Cells.J Breast Cancer19:231–241.https://doi.org/10.4048/jbc.2016.19.3.231
29. Finn RS, Dering J, Conklin D, Kalous O, Cohen DJ, Desai AJ, Ginther C, Atefi M, Chen I, Fowst C et al (2009) PD 0332991, a selective cyclin D kinase 4/6 inhibitor, preferentially inhibits proliferation of luminal estrogen receptor-positive human breast cancer cell lines in vitro. Breast Cancer Res 11:R77. https://doi.org/10.1186/bcr2419
30. MaiuriMC,ZalckvarE,KimchiA,KroemerG(2007)Self-eating and self-killing: crosstalk between autophagy and apoptosis.Nat Rev Mol Cell Biol8:741–752.https://doi.org/10.1038/nrm2239
31. Tilija Pun N, Jang WJ, Jeong CH (2020) Role of autophagy in regulation of cancer cell death/apoptosis during anti-cancer therapy: focus on autophagy flux blockade. Arch Pharm Res 43:475–488. https://doi.org/10.1007/s12272-020-01239-w
32. MarinoG,Niso-SantanoM,BaehreckeEH,KroemerG(2014)Self-consumption: the interplay of autophagy and apoptosis.Nat Rev Mol Cell Biol15:81–94.https://doi.org/10.1038/nrm3735
33. Yao Z, Yuan T, Wang H, Yao S, Zhao Y, Liu Y, Jin S, Chu J, Xu Y, Zhou W et al (2017) MMP-2 together with MMP-9 overexpression correlated with lymph node metastasis and poor prognosis in early gastric carcinoma. Tumour Biol 39:1010428317700411. https://doi.org/10.1177/1010428317700411
34. Shay G, Lynch CC, Fingleton B (2015) Moving targets: Emerging roles for MMPs in cancer progression and metastasis. Matrix Biol 44–46:200–206. https://doi.org/10.1016/j.matbio.2015.01.019
35. QuesnelA,KaragiannisGS,FilippouPS(2020)Extracellular proteolysis in glioblastoma progression and therapeutics.Biochim Biophys Acta Rev Cancer187:188428.https://doi.org/10.1016/j.bbcan.2020.188428

Tables

Table 1: Comparison of IC_{50} values between compounds and antitumor drugs currently employed for breast cancer treatment

Compound	Cell line IC_{50} (mM)		
	MCF7	MDA-MB-231	LM3
1	52.54 ± 2.07	12.60 ± 0.89	14.42 ± 0.54
2	53.14 ± 3.02	11.12 ± 1.44	11.76 ± 2.14
3	50.50 ± 0.70	13.35 ± 0.76	14.53 ± 0.35
4	51,50 ± 2.12	15.80 ± 0.77	12.32 ± 1.40
5-FU[a]	419.46	122.85	-
Olaparib[a]	285.38	62.54	-
Cyclophosphamide[a]	231.77	195.56	-
Palbociclib[a]	43.30	14.57	-
Fulvestrant[a]	1.03	25.98	-
Doxorubicin[a]	0.01	1.26	-

[a] Data were collected from www.cancerrxgene.org. No information is available regarding LM3 cells.

Table 2: Experimental lung colonization parameters

	Control	Compound 1	Compound 2	Compound 3	Compound 4
Incidence (%) (Mean ± S.E.)	100 ± 0	77 ± 5.7*	83 ± 7.1*	100 ± 0	100 ± 0
Number Median (range)	52 (32-89)	2.5 (0-10)#	7 (0-8)#	88 (52-99)	58 (9-79)

Each group consisted of at least 10 female mice. Values are the average of three independent experiments. *p<0.05 vs. control, Chi Square Test, #p<0.05 vs. control, Kruskall-Wallis test.

Figures

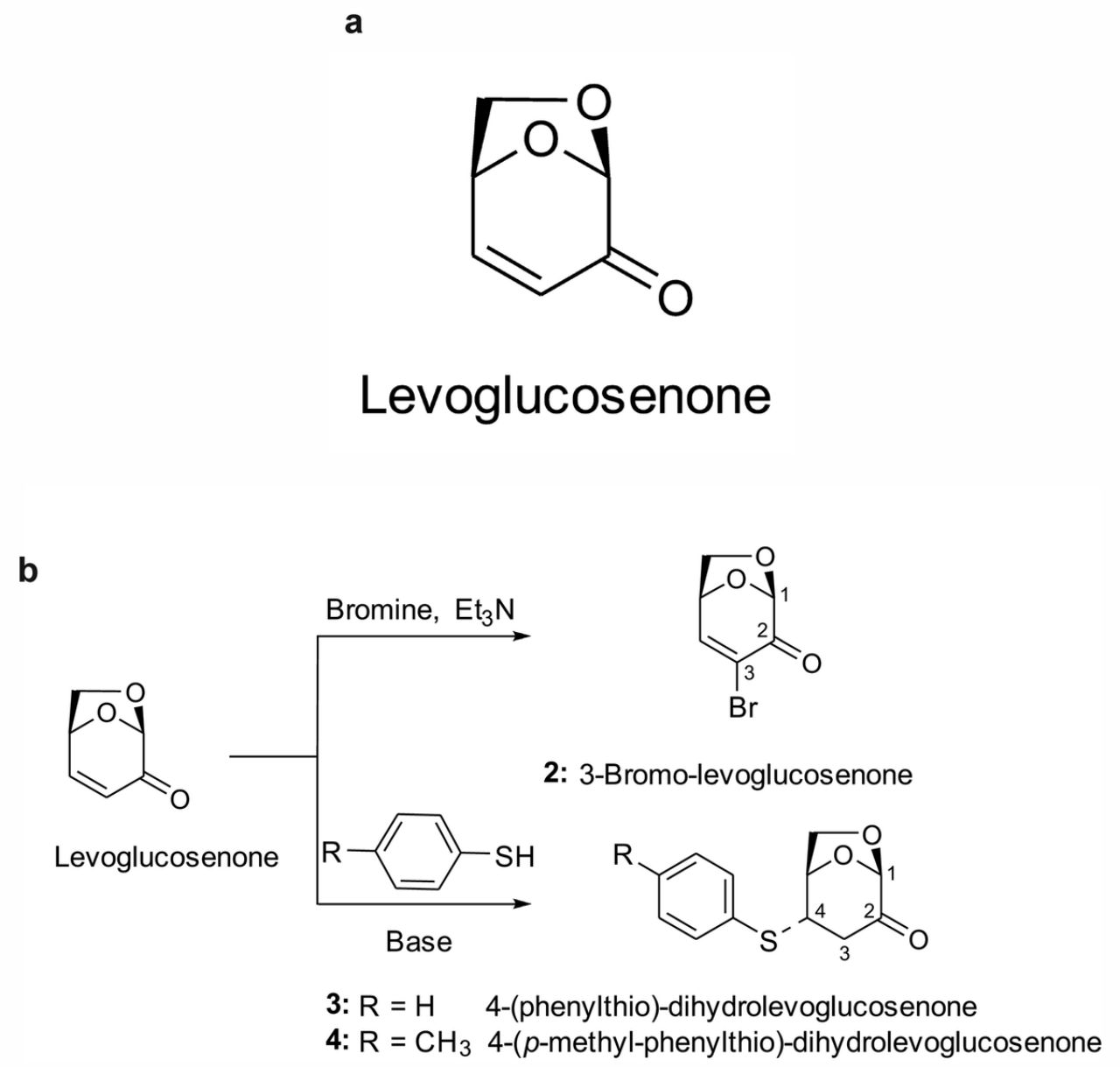

Figure 1

Chemical structure of the compounds Panel a: Levoglucosenone (1). Panel b: Obtaining of the different Levoglucosenone derivatives levo 3-bromolevoglucosenone (2), 4-(phenylthio)-dihydrolevoglucosenone (3) and 4-(p-methyl-phenylthio)-dihydrolevoglucosenone (4)

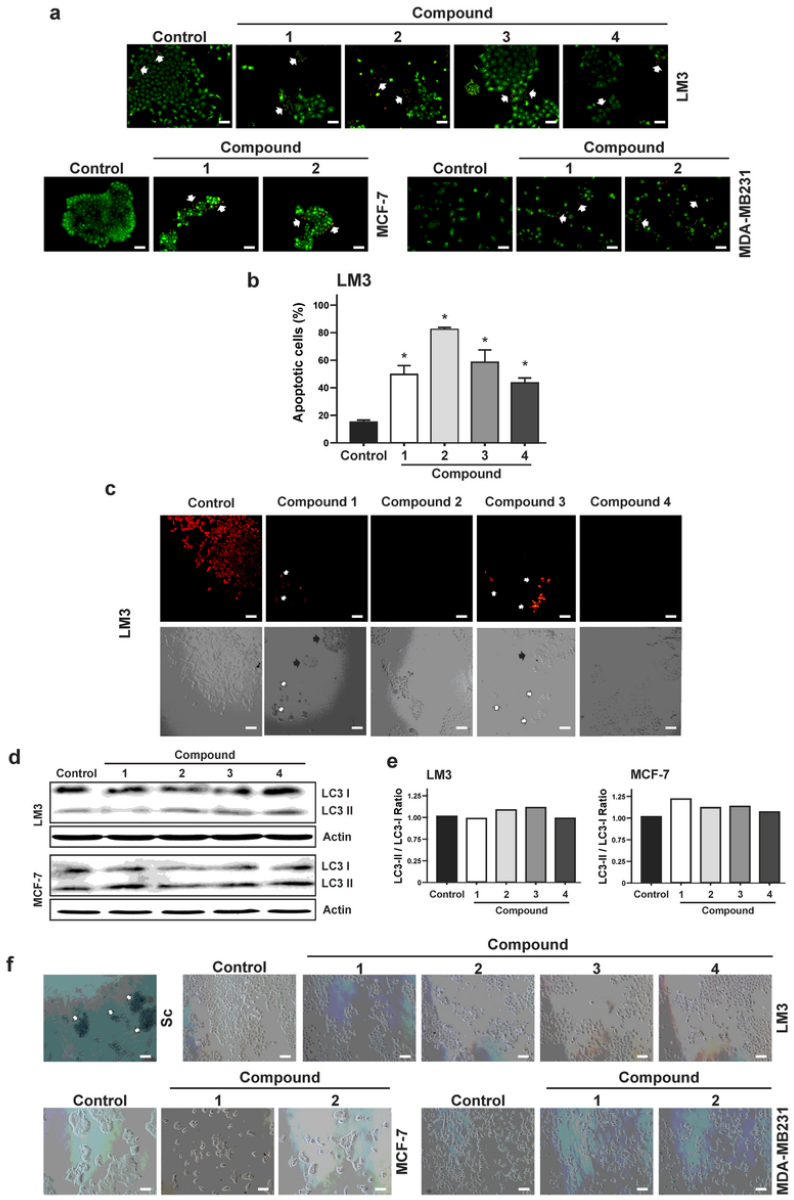

Figure 2

Modulation of apoptosis, autophagy and senescence processes Panel a: Cells growing on glass coverslips were treated with the IC50 dose of the compounds or vehicle as control. Unfixed cells were stained with acridine orange/ethidium bromide and apoptosis was evaluated by fluorescence microscopy. White arrows indicate the presence of apoptotic cells or cell groups. Two independent experiments were performed with similar results (Scale bar: 50 μm). Panel b: Quantification of Annexin-V staining by flow cytometry. LM3 cells were treated with the IC50 dose of the compounds or vehicle as control for 24 h and apoptotic cells were quantified. Data represent the mean ± S.D. of triplicate determinations, *p<0.05 vs. control, ANOVA test. Three independent experiments were performed with similar results. Panel c: Cells pretreated with the compounds were incubated with the TMRM fluorescent dye and further evaluated by fluorescence microscopy. White arrows indicate the presence of cells with functional mitochondria and black arrows indicate the presence of apoptosis-committed cells. Two independent assays were performed with similar results (Scale bar: 50 μm). Panel d: Immunoblot assays for LC3I and LC3II detection. Whole cell lysates prepared from LM3 and MCF-7 pretreated cells were resolved on 10 % SDS-PAGE and blotted against LC3 protein (50 μg/lane). Actin immunoblotting was used as a loading control. A representative experiment is shown. Similar results were observed in two independent experiments. Panel e: Densitometric analysis of panel D. Signal intensity was quantified and LC3II/LC3I ratio was determined. Panel f: Cells treated with the compounds were stained with a specific buffer for the detection of senescence-associated β-galactosidase activity. Sc melanoma cells treated with all-

trans retinoic acid were used as technique control. White arrows indicate the presence of blue-stained senescent cells. Two independent experiments were performed with similar results (Scale bar: 50 μm)

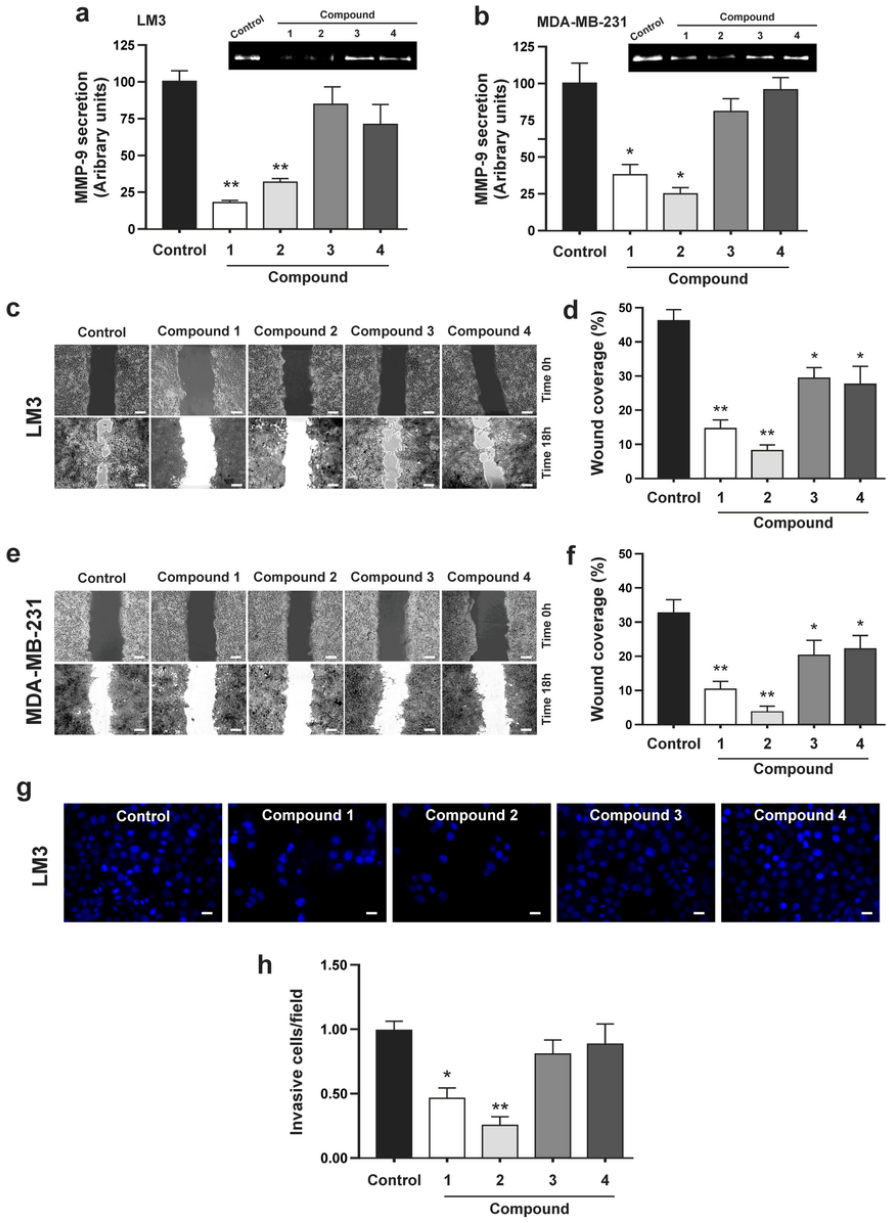

Figure 3

Modulation of soluble MMP-9 activity, migratory and invasive potential Panel a and b: MMP-9 activity was analyzed by zymography in CM of LM3 and MDA-MB-231 monolayers treated with the IC50 dose of the compounds or vehicle as control. Data were expressed in arbitrary units as the mean ± SD of triplicate determinations, *p<0.05 vs. control, **p<0.01 vs. control, ANOVA test. Results are representative of three independent experiments. Inset: a representative MMP-9 gelatin zymography is shown. Panel c and e: Monolayers were wounded at time zero and cells were allowed to migrate into the cell free area for 18 h in the presence of the compounds or vehicle as control. Representative photographs of the same spot are shown (Scale bar: 50 μm). Panel d and f: Densitometric analysis of panel C. Cell migration was quantified calculating the percentage of the area occupied by cells that migrated into the original cell-free wounded area. Data are expressed as the mean ± S.D. of triplicate determinations. *p<0.05 vs. control, **p<0.01 vs. control, ANOVA test. At least 3 independent experiments were performed with similar results. Panel g: The invasive capacity of cells pretreated with the IC50 dose of the compounds or vehicle as control was determined employing transwell devices coated with Matrigel. Three independent experiments were performed with similar results (Scale bar: 50 μm). Panel h: Quantification of panel E. Invasive cells were quantified by counting 10 randomly selected fields of each condition. Data are expressed as the mean ± S.D. of triplicate determinations. *p<0.05 vs. control, **p<0.01 vs. control, ANOVA test. Three independent experiments were performed with similar results

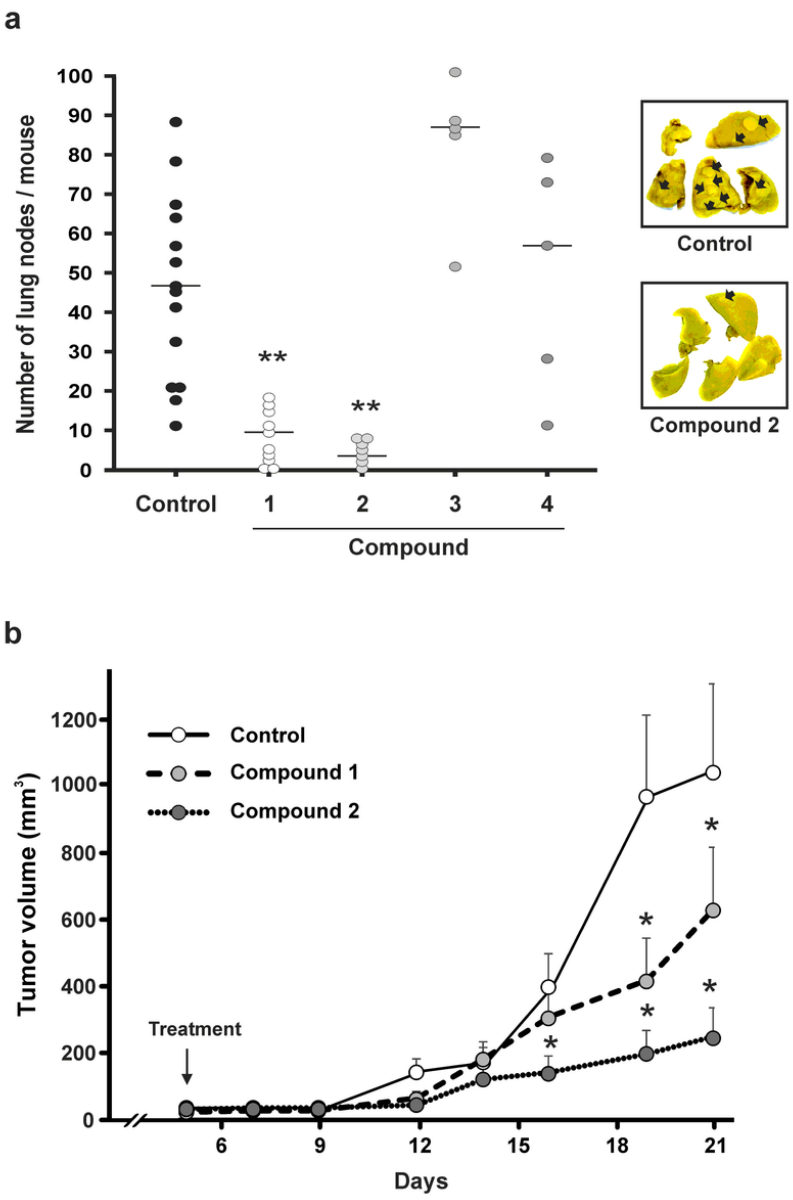

Figure 4

Evaluation of metastatic dissemination and tumor growth Panel a: LM3 cells pretreated or not with compounds were injected in the lateral tail vein of BALB/c mice. Twenty-one days later mice were sacrificed and the number of surface lung nodes was determined under a dissecting microscope. Data are expressed as median and range of lung nodules per animal. *p<0.05 vs. control, Kruskall-Wallis test. Three independent experiments were performed with similar results. Inset: Representative lungs of control and compound 2 treated cells are shown. Panel b: LM3 cells were harvested from subconfluent monolayers and subcutaneously inoculated into BALB/c mice. Animals were treated twice a week with an intraperitoneal injection of 60 µg/kg of the compounds 1 and 2 or with vehicle in physiological solution as control. Size of the two perpendicular diameters was recorded and used to calculate tumor volume. Each data point represents mean ± S.D. (n=5). *p<0.05 vs. control, ANOVA test. Two independent experiments were performed with similar results.