Nutrition and Cancer

ISSN: 0163-5581 (Print) 1532-7914 (Online) Journal homepage: http://www.tandfonline.com/loi/hnuc20

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To cite this article: Eliana Noelia Alonso, María Julia Ferronato, Norberto Ariel Gandini, María Eugenia Fermento, Diego Javier Obiol, Alejandro López Romero, Julián Arévalo, María Emilia Villegas, María Marta Facchinetti & Alejandro Carlos Curino (2017) Antitumoral Effects of D-Fraction from Grifola Frondosa (Maitake) Mushroom in Breast Cancer, Nutrition and Cancer, 69:1, 29-43, DOI: 10.1080/01635581.2017.1247891

To link to this article: http://dx.doi.org/10.1080/01635581.2017.1247891

Published online: 28 Nov 2016.

NUTRITION AND CANCER
2017, VOL. 69, NO. 1, 29–43
http://dx.doi.org/10.1080/01635581.2017.1247891

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Antitumoral Effects of D-Fraction from *Grifola Frondosa* (Maitake) Mushroom in Breast Cancer

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ABSTRACT

D-Fraction is protein-bound β-1,6 and β-1,3 glucans (proteoglucan) extracted from the edible and medicinal mushroom *Grifola frondosa* (Maitake). The antitumoral effect of D-Fraction has long been exclusively attributed to their immunostimulatory capacity. However, in recent years increasing evidence showed that D-Fraction directly affects the viability of canine and human tumor cells, independent of the immune system. Previously, we have reported that D-Fraction modulates the expression of genes associated with cell proliferation, cell death, migration, invasion, and metastasis in MCF7 human breast cancer cells. Therefore, the purpose of the current study is to investigate if this modulation of gene expression by Maitake D-Fraction really modulates tumor progression. In the present work, we demonstrate for the first time that Maitake D-Fraction is able to act directly on mammary tumor cells, modulating different cellular processes involved in the development and progression of cancer. We demonstrate that D-Fraction decreases cell viability, increases cell adhesion, and reduces the migration and invasion of mammary tumor cells, generating a less aggressive cell behavior. In concordance with these results, we also demonstrate that D-Fraction decreases tumor burden and the number of lung metastases in a murine model of breast cancer.

ARTICLE HISTORY
Received 26 November 2015
Accepted 4 August 2016

Introduction

The global burden of cancer increases every year largely because of population aging and growth as well as an adoption of cancer-associated lifestyle (1). Based on the GLOBOCAN 2012 estimates, about 14.1 million new cancer cases and 8.2 million cancer deaths are estimated to have occurred in 2012 worldwide. Breast cancer is, by far, the most frequently diagnosed malignant neoplasia and a leading cause of cancer death in females worldwide (2). Despite advances in early detection and the understanding of the molecular bases of breast cancer biology, the local and systemic recurrence of this disease remains a serious problem (3).

Today, conventional cancer therapies include surgical operation, radiotherapy, and chemotherapy, depending on the type of cancer and the stage of tumor progression. However, their side effects cause serious damage and suffering to patients (4,5). In this context, the role of immunotherapy as a supportive help to conventional therapy is gaining increasing prominence, because it improves the quality of life of cancer patients while helping to overcome cancer growth (6). Biologically active metabolites found in medicinal mushrooms, such as β-glucans, are potent immunomodulators with effects on both innate and adaptive immunity (7–10).

Grifola frondosa, commonly known as Maitake, is an edible and medicinal mushroom that has been used for centuries by the oriental medicine, especially in countries like China, India, Japan, and Korea (4,11). D-Fraction is protein-bound β-1,6 and β-1,3 glucans (proteoglucan) extracted from the fruit bodies of Maitake (12). The antitumor effect of D-Fraction has been attributed mainly to their immunostimulatory capacity. It was found that D-Fraction not only activates various immune effectors such as macrophages (13–17), natural killer (18), dendritic cells (19), and T-helper lymphocytes (14,20) that attack tumor cells but also potentiates the release of various mediators including lymphokines and interleukins.

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On the other hand, increasing evidence showed that D-Fraction has a direct antiproliferative or cytotoxic effect on human cancer cells including PC3 (prostate) (21), T24 (bladder), HepG2 (liver), U89 (brain), HL60 (leukemia) (22), MCF7 (breast) (22,23), and ACHN cells (kidney) (24,25). Furthermore, it was shown that Maitake D-Fraction also affects the viability of three canine cancer cells such as CF33 (mammary gland), CF21 (connective tissue), and CL1 cells (lymphoma) (22). In our previous work, we have demonstrated that Maitake D-Fraction modulates the switching on/off of genes associated with cell proliferation, cell death, migration, invasion, and metastasis in MCF7 human breast cancer cells (26). Hence, it is necessary to confirm these results evaluating the direct antitumoral actions of D-Fraction. Therefore, the purpose of the current study was to investigate the effects of Maitake D-Fraction on the cellular processes that govern the transformation of normal cells into malignant ones, including cellular proliferation, cell death, cell adhesion, cell migration, and cell invasion. In addition, we aimed at investigating the antitumoral effect of Maitake D-Fraction in an animal model of breast cancer.

Materials and methods

Bioactive Maitake D-Fraction

D-Fraction is a proteoglucan extracted from the medicinal mushroom *G. frondosa* (Maitake). In this work, we use Maitake D-Fraction® Pro 4X Liquid, which is made from D-Fraction extract, vegetable glycerin, and water by Mushroom Wisdom, Inc. (formerly Maitake Products, Inc.) New Jersey, USA. Maitake D-Fraction® Pro 4X Liquid does not contain alcohol, sugar, yeast, mold, corn, salt, wheat, artificial color, preservatives, or synthetic pesticides or fertilizers.

Cell culture

LM3 murine mammary adenocarcinoma cells (27) were a generous gift from E. Bal de Kier Joffé (Instituto de Oncología Ángel Roffo, Buenos Aires, Argentina). The LM3 cells lack estrogen receptor (ER) and progesterone receptor (PR) expression (28), but they express high levels of human epidermal growth factor receptor 2 (HER2) (29). Therefore, LM3 cells are classified as hormone-independent breast cancer cell lines. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 5% (v/v) fetal bovine serum (FBS, Gibco), L-glutamine (5 mM, Gibco), penicillin (Gibco, 100 U/mL), and streptomycin (Gibco, 100 μg/mL) at 37°C in a humidified 5% CO_2 air atmosphere.

Cell viability assays

The LM3 cells were plated at a density of 3,000 cells/well into 96-multiwell dishes in complete medium. They were treated with 30, 300, 750, 1500, 2250, and 3000 μg/mL of D-Fraction or vehicle for 24, 48, and 72 h. They were washed with phosphate-buffered saline (PBS), trypsinized, suspended in 100 μL of complete medium, and counted manually using a hemocytometer, as previously described (30). The experiment was repeated at least twice and performed in quadruplicate. Half-maximal inhibitory concentrations (IC_{50}) of D-Fraction were calculated using the sigmoidal dose-response equation employing GraphPad Prism 5.0 program. Additionally, cell viability was assessed by 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3 benzene disulfonate (WST-1) colorimetric assay (Roche). For this purpose, after treatment with D-Fraction or vehicle, the cells were incubated for 1 h at 37°C with the tetrazolium salt WST-1 and the absorbance of the formazan product was read at 440 nm. Vehicle treatment did not exert effects over cellular viability when compared to nontreated cells.

Cell cycle analysis

Cell cycle analysis was performed as previously described (31). In brief, LM3 cells were synchronized by deprivation of FBS for 20 h. Then, they were treated with D-Fraction (570.6 μg/mL) or vehicle for 24 h. After that, they were trypsinized, fixed with ice-cold 70% ethanol, stained with propidium iodide (PI, Roche), and analyzed for DNA content by FACScan Calibur Becton Dickinson. Data were analyzed by CellQuest software (Becton Dickinson). At least 100,000 cells were analyzed for each sample.

Apoptosis analysis

Apoptosis was analyzed by flow cytometry using Annexin V-FITC (AV)/PI double staining as previously described (30). In brief, LM3 cells were treated with D-Fraction (570.6 μg/mL) or vehicle for 24 h. Then, they were washed with PBS and resuspended in binding buffer 1× at a concentration of 1×10^6 cells/mL. Then, 100 μL of suspension were taken for staining with 5 μL of AV and 10 μL of PI (50 μg/mL). The suspension was homogenized and incubated in the dark at room temperature for 15 min. Finally, 400 μL of binding buffer 1× was added to each sample. The cells were counted using FACScan Calibur Becton Dickinson and data were analyzed by CellQuest software (Becton Dickinson). We determined the percentage of cells with mark AV+/IP- (early apoptosis), AV+/IP+ (late apoptosis), AV-/IP+

(necrosis), and AV-/IP- (viable cells). At least 100,000 cells were analyzed for each sample.

Cell migration assay

Cell migration was studied by employing the "wound healing" assay as previously described (32). Briefly, LM3 cells were seeded in 35-mm Petri dishes and cultured until confluence. The cells were treated with D-Fraction (570.6 μg/mL) or vehicle. The cells were scraped with a 200-μL micropipette tip, denuding a strip of the monolayer. Then, they were observed and photographed every 4 h and for a shorter time than the doubling time (T_2) of the cell line (LM3 $T_2 = 23$ h). Images were captured with an inverted microscope (Nikon Eclipse TE2000-S) equipped with a digital camera (Nikon Coolpix S4). The uncovered wound area was measured and quantified at different intervals with Fiji (Fiji Is Just ImageJ).

Cell invasion assay

The invasion of LM3 cells was assessed in transwell chambers (Millipore) with Matrigel (BD Biosciences) as previously described (30). In brief, each transwell was coated with 100 μL of a 1:3 Matrigel in cold serum-free DMEM to form a thin continuous film on top of the filter. The lower chamber was filled with 600 μL of DMEM containing 5% (v/v) FBS. D-Fraction-treated cells (570.6 μg/mL) or vehicle-treated cells during 12 h (12,500 cells/well in 500 μL of complete DMEM medium) were transferred to each transwell in triplicate. After incubation for 12 h at 37°C, the cells on the upper side of the transwell membrane were removed by cotton swab and rinsed with PBS. Cells migrating to the lower side of the membrane were fixed in methanol 100% (v/v) for 10 min at room temperature and stained with 0.5% (w/v) crystal violet (Sigma) for 5 min. For each replicate, 10 randomly selected fields were photographed and the cells were counted with Fiji (Fiji Is Just ImageJ).

Cell adhesion assay

Cell adhesion was evaluated as previously described (33), with some modifications. Briefly, LM3 cells were treated with D-Fraction (570.6 μg/mL) or vehicle for 12 h. Then, cells were seeded into 96-well culture plates (10,000 cells/well, three wells for each condition) and were allowed to adhere. After 30 min, 1 h, 2 h, 5 h, 8 h, and 10 h, nonadherent cells were removed by gentle washing with PBS. Attached cells were fixed in 100% methanol for 10 min and stained with 0.1% (w/v) crystal violet (Sigma) for 20 min. Cells attached to the culture plate were observed with an inverted microscope (Nikon Eclipse TE2000-S), equipped with a digital camera (Nikon Coolpix S4). For each replicate, ten randomly selected fields were photographed and the cells were counted with Fiji (Fiji Is Just ImageJ).

Analysis of actin cytoskeleton

Immunofluorescence was performed as previously described (34). Briefly, LM3 cells were seeded on glass coverslips in 35-mm Petri dishes and cultured until 50% confluence. They were treated with D-Fraction (570.6 μg/mL) or vehicle for 12 h. After treatment, they were washed three times with PBS and fixed with paraformaldehyde 4% in PBS for 1 h. The cells were then permeabilized with 0.1% Triton in PBS and incubated with rhodamine-phalloidin (1:50) in PBS for 20 min. After that they were washed twice with PBS and finally stained with 4',6-diamidino-2-phenylindole (DAPI; 1:10,000 in PBS). Glass coverslips were mounted on glass microscope slides, and fluorescence images were acquired with an inverted microscope (Nikon Eclipse TE2000-S), equipped with a digital camera (Nikon Coolpix S4). Lamellipodia- and filopodia-containing cells located at the edge of the colonies were counted in 10 randomly chosen fields of each experimental sample (30).

Preparation of conditioned media

Secreted matrix metalloproteinase (MMP) activity was evaluated in conditioned media (CM). Briefly, the LM3 cells were plated at a density of 3,000 cells/well into 96-multiwell dishes and grown to 80% confluence in complete medium. The cells were treated with D-Fraction (570.6 μg/mL) or vehicle for 6 and 12 h. Then, they were incubated overnight at 37°C in a humidified 5% CO_2 air atmosphere with 4 μL of serum-free DMEM in the presence of D-Fraction (570.6 μg/mL) or vehicle. CM were individually collected, cold-centrifuged, aliquoted, and stored at −20°C. Finally, the cell number/well was determined by manual counting using a hemocytometer.

Zymography for MMP-2

MMP-2 activities in CM from LM3 cells were studied by measuring collagenolytic activity in substrate-impregnated gels, as previously described (35) with some modifications. Briefly, samples were collected and run on 10% SDS-polyacrylamide gels containing 3 mg/mL of gelatin under nonreducing conditions. After electrophoresis, gels were washed for 20 min using 2.5% Triton X-100 and subsequently incubated for 24 h at 37°C in agitation with buffer containing 50 mM Tris-HCl, 200 mM NaCl, 5 mM $CaCl_2$, and 0.02% Triton X-100 (pH 7.4). After incubation, gels were fixed and stained with 0.1% Coomassie Brilliant Blue G-250 in methanol/acetic acid/H_2O (30:10:60). Activity bands were visualized by negative staining. Gelatinolytic bands were visualized using a transilluminator and measured with Fiji

(Fiji Is Just ImageJ). Data were normalized to the amount of cells/well.

Western blotting

The LM3 cells were seeded in Petri dishes with complete medium and treated with D-Fraction (570.6 μg/mL) or vehicle for 15 min, 30 min, 2 h, 12 h, or 18 h. The treatment time varies according to the protein under study and, particularly for evaluating the expression of phospho-Akt (pAkt), cells were previously deprived of FBS for 18 h. Briefly, 80 μg or 140 μg of protein lysates was separated by SDS-PAGE on 12% and 15% gels, transferred onto nitrocellulose membrane, blocked with 5% nonfat 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-phospho-Akt (Ser 473) (Cell Signaling, #9271S), rabbit polyclonal anti-p53 (FL-393, Santa Cruz Biotechnology, sc-6243), rabbit polyclonal anti-pBad (Ser 136) (Cell Signaling, #9295), rabbit polyclonal anti-Bax (N-20) (Santa Cruz Biotechnology, sc-493), mouse monoclonal anti-Bcl-2 (C-2) (Santa Cruz Biotechnology, sc-7382), and rabbit polyclonal anti-E-cadherin (H-108) (Santa Cruz Biotechnology, sc-7870). Anti-β-actin (C-11) (polyclonal goat, Santa Cruz Biotechnology, sc-1615) was used as internal control for protein loading and analysis.

Murine model of syngeneic transplantation with LM3

In vivo studies were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Nine-week-old virgin female BALB/c mice, weighing around 20 g, were purchased from Facultad de Ciencias Veterinarias (La Plata, Argentina). Animals were given free access to water and food, and were housed in a climate-controlled room with a 12-h light/12-h dark cycle. Twelve animals were injected subcutaneously with LM3 cells (4×10^5 cells in 50 μL of serum-antibiotic-free DMEM) at level of the inguinal mammary glands. The cells were implanted out using 1-mL syringes with needles (BD) 0.80 × 25 mm (21Gx1).

When tumors reached a width > 0.2 cm, LM3 tumor-bearing mice were randomly divided into two groups and were injected as indicated previously (36): 1) 5 mice with D-Fraction (15.63 mg/Kg in 50 μL of saline) and 2) 7 mice with vehicle of D-Fraction (glycerin-water in 50 μL of saline). Mice were injected subcutaneously in the tumor periphery five times a week for four weeks (total doses = 20). Tumor growth was measured each other day with digital calipers and tumor volume was calculated as $\pi/6 \times a \times b^2$, where a is the length in centimeters, and b is the width in centimeters. At the end-point animals were sacrificed by cervical dislocation. Tumors were then removed, weighed, measured, and put into liquid nitrogen or 10% formalin for further study. Tumor volume was calculated as $\pi/6 \times a \times b \times c$, where a, b, and c are the three tumor dimensions. Lungs were removed and fixed in Bouin's solution. The number of superficial lung metastases per mouse was counted by an investigator who was unaware of the sample assignment, with the aid of a Stereo Microscope (Nikon SMZ1500) coupled with High-Intensity Illuminator (Nikon NI-150) and a digital camera (Nikon DXM 1200F).

Tumors were excised, bisected along the longest axis, fixed for 24 h in 10% formalin in PBS, and processed into paraffin by standard procedures. In brief, after paraffin sections were dewaxed, they were rehydrated in a decreasing series of ethanol dilutions and either stained with Hematoxylin and Eosin (H&E) or used for immunohistochemical studies. Staining with H&E was used to observe histopathological characteristics. Mitotic index was calculated as the number of the mitotic figures observed in 10 fields at a magnification of 400× in the H&E-stained slides. The proliferation and apoptotic processes were studied by immunohistochemistry (IHC).

Immunohistochemistry

IHC was performed as previously described (37). IHC was carried out with the avidin-biotin complex immunoperoxidase technique. Five-μm sections of paraffin-embedded specimens were mounted on glass slides, deparaffinized with xylene, and rehydrated with graded alcohol. They were incubated for 15 min in 3% hydrogen peroxide in ethanol at 96°C to quench endogenous peroxidase. After washing in PBS, the sections were blocked for 30 min in 2% bovine serum albumin (in PBS) and then incubated overnight at 4°C with primary antibodies. After that, they were incubated with diluted biotinylated secondary antibody for 30 min and then incubated with VECTASTAIN ABC reagent (Vector Laboratories Inc.) for 30 min. Diaminobenzidine/H_2O_2 were used as substrates for the immunoperoxidase reaction. They were lightly counterstained with Harris Hematoxylin (Zymed Laboratories), dehydrated through graded ethanol and xylene, mounted with Permount (Fisher Scientific) for analysis by bright-field microscopy, and examined under an Olympus microscope (CX31). For negative controls, the slides were subjected to the same IHC process omitting the primary antibody. Primary antibodies used were goat polyclonal anti-Ki-67 (M-19, sc-7846) and rabbit polyclonal anti-Bax (N-20, sc-493) from Santa Cruz Biotechnology.

Evaluation of immunohistochemical staining

Immunostained sections were scored semiquantitatively based upon the proportion of tumor cells stained and the staining intensity, using the Immunoreactive Score (IRS) system (combining positive cell ratio and staining intensity) as suggested by Remmele and Stegner (38). Bax expression was evaluated as previously described (37). The IRS was calculated as the product of the staining intensity (graded as: 0 = no, 1 = weak, 2 = moderate, and 3 = strong staining) by the percentage of positively stained cells (0 = less than 10% of stained cells, 1 = 11–25% of stained cells, 2 = 26–75% of stained cells, and 3 = more than 75% of stained cells). The mean IRSs for Bax in 10 randomly chosen fields of the sample (400× magnification) were determined. Ki-67 expression was calculated by the percentage of positively stained cells (0 = less than 10% of stained cells, 1 = 11–25% of stained cells, 2 = 26–75% of stained cells, and 3 = more than 75% of stained cells).

Statistical analysis

The GraphPad Prism software package, version 5.00 was used for collection, processing, and statistical analysis of all data. Cell viability, adhesion, migration, cell cycle assays, and in vivo comparison of the tumor volume were analyzed with two-way Analysis of Variance (ANOVA) and Bonferroni post-tests. Cell invasion assay and effects on actin cytoskeleton were analyzed with Student's *t*-test and Chi-square test, respectively. Comparison of the tumor burden and number of lung metastases among different groups and IRS of IHC were analyzed by the nonparametrical Mann-Whitney U test. Statistical significance was determined at $P < 0.05$ level.

Results

Maitake D-Fraction decreases LM3 cell viability through induction of apoptosis

One of the most prominent characteristics of a cancer cell is its ability to proliferate constantly generating its own growth signals, being insensitive to antigrowth signals and evading apoptosis (39). So, to investigate the antitumor effects of D-Fraction in LM3, we first examined its effects on cell viability. LM3 cells were treated with different concentrations of D-Fraction (30, 300, 750, 1500, 2250, and 3000 μg/mL) and different treatment times (24, 48, and 72 h). Then, manual cell counting under Neubauer chamber and WST-1 assay were performed. As shown in Fig. 1A, a decrease in LM3 cell count was observed from 30 μg/mL of D-Fraction ($P < 0.05$) and 24 h onwards compared to vehicle-treated cells. This effect was dose- and time-dependent, and the IC_{50} values of D-Fraction at 24, 48, and 72 h were 570.6 μg/mL, 274.4 μg/mL, and 210.75 μg/mL, respectively. The colorimetric WST-1 assay confirmed the decrease of cell viability induced by D-Fraction in the murine mammary adenocarcinoma LM3 cells (data not shown).

In order to determine whether D-Fraction exerts a cytostatic or apoptotic effect on the tumor LM3 cell line, we performed PI staining followed by flow cytometry analysis. LM3 cells were treated with D-Fraction (570.6 μg/mL) or vehicle for 24 h. We found that D-Fraction decreases the number of LM3 cells in the G_0/G_1 phase, compared to vehicle (D-Fraction = 41.89% vs. vehicle = 58.65%, $P < 0.001$). This decrease in G_0/G_1 phase was accompanied by an increase in the number of cells in the $subG_0/G_1$ phase (D-Fraction = 23.91% vs. vehicle = 10.76%, $P < 0.001$) (Fig. 1B). In order to corroborate if this increase in the number of cells in the $subG_0/G_1$ phase was due to an induction of apoptosis, AV/PI double staining was performed and examined by flow cytometry after 24 h of D-Fraction (570.6 μg/mL) or vehicle treatment. As shown in Fig. 1C, the cell population in early apoptosis (AV+/PI−) increased from 4.38% in the vehicle-treated cells to 11.45% in the D-Fraction treated cells ($P < 0.05$). This shows that Maitake D-Fraction decreases LM3 cell viability through apoptosis, without affecting cell cycle progression.

The phosphoinositide-3-kinase (PI3K)/protein kinase B (Akt) pathway is frequently hyperactivated in many solid tumors such as breast cancer (40). This pathway regulates critical cellular functions including growth, proliferation, survival, and apoptosis, among others (41,42). Given the important role of PI3K-Akt signaling pathway in the cancer pathogenesis, we evaluated the effect of D-Fraction on Akt activation, which acts as the central mediator of the pathway, and some of their downstream targets. We found that Maitake D-Fraction treatment delays Akt phosphorylation at Ser 473 (pAkt-S473) in LM3 cells (Fig. 2A). In concordance with this, we found that the expressions of pBad and Bcl-2 were decreased after D-Fraction treatment, whereas the levels of p53 and Bax remained constant (Fig. 2B). Altogether these results suggest that D-Fraction could induce apoptosis through a decrease in PI3K-Akt pathway and a modulation of the Bax/Bcl-2 ratio.

Maitake D-Fraction decreases the migratory and invasive capability of LM3 cells in culture

Metastases are the cause of 90% of human cancer deaths (39). Tumor cells must acquire the capability to migrate and invade surrounding tissue to begin the physical translocation from the primary tumor to the site of spread (first phase of metastatic cascade) (43). So, to evaluate the effect of D-Fraction on migration of tumor cells, wound healing assays were performed. Confluent monolayers of LM3 cells were wounded. Wound closure was observed by optical microscopy during 20 h of treatment. Under treatment with

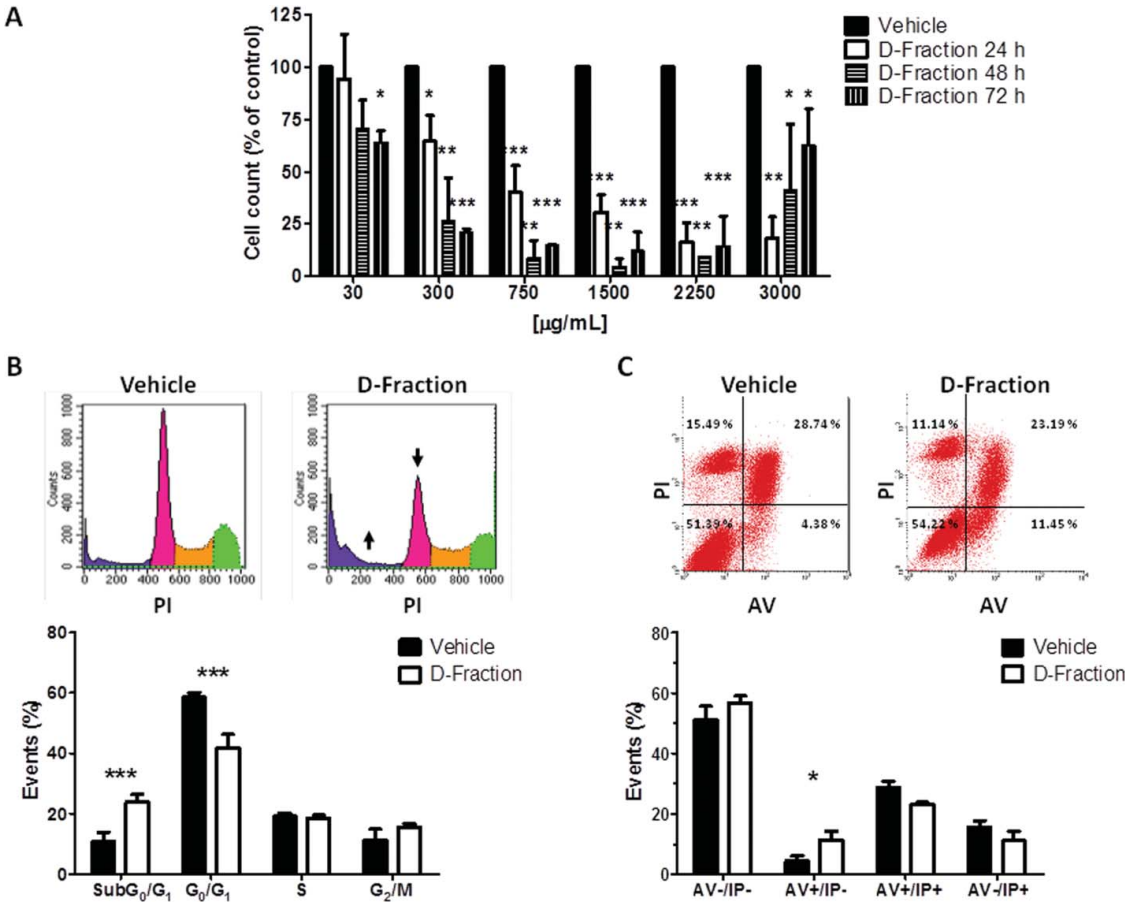

Figure 1. Maitake D-Fraction decreases LM3 cell viability through induction of apoptosis. A: Cell count was assessed in LM3 cells after 24, 48 or 72 h of treatment with different concentrations of D-Fraction or vehicle (glycerol-water). Data show the percentage of cells in relation to vehicle-treated cells. Dots: mean of at least two independent experiments performed in quadruplicate; bars: SEM. B: Cell cycle analysis of LM3 cells treated with D-Fraction (570.6 μg/mL) or vehicle for 24 h. Cells were stained with PI and analyzed for DNA content with FACScan Calibur Becton Dickinson. The increase of subG$_0$/G$_1$ and the decrease of G$_0$/G$_1$ cell populations are indicated by arrows and plotted in a graph (below). C: Apoptosis analysis of LM3 cells treated with D-Fraction (570.6 μg/mL) or vehicle for 24 h. Cells were stained with AV/PI and analyzed with FACScan Calibur Becton Dickinson. Representative images of AV/PI staining in LM3 cells following treatments are shown. Quantification of AV/PI stained cells is plotted in a graph (below). Two-way ANOVA and Bonferroni post tests. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

D-Fraction (570.6 μg/mL), LM3 cells decreased their migratory capability. Such effect was observed from 16 h of treatment onwards, when uncovered wound area in D-Fraction-treated cells was 46.94% vs. 23.20% in vehicle-treated cells ($P < 0.001$, Fig. 3).

Then, to study the invasive capacity of LM3 cells we used transwell chambers (Millipore) with Matrigel. Maitake D-Fraction treatment reduced the invasiveness of LM3 when compared with vehicle treatment (mean value of vehicle treatment = 65.67 vs. D-Fraction treatment = 58.67, $P < 0.05$) (Fig. 4).

Maitake D-Fraction increases LM3 cell adhesion

Adhesion is an important feature related to migration and invasion on metastasis development (44). To evaluate if Maitake D-Fraction is capable of modulating tumor cell adhesion, LM3 cells were treated with D-Fraction (570.6 μg/mL) or vehicle for 12 h. Then, LM3 cells were incubated for different times in 96-well plates. As shown in Fig. 5, after incubating cells for 2 h (vehicle = 4.42 vs. D-Fraction = 17.81) and 5 h (vehicle = 5.50 vs. D-Fraction = 14.39), D-Fraction significantly increased LM3 cell adhesion compared to vehicle ($P < 0.001$). Therefore, Maitake D-Fraction is capable of modulating the adhesive response of LM3 cells, further confirming that D-Fraction induces a less malignant phenotype.

Maitake D-Fraction decreases the number of filopodia and lamellipodia in LM3 cells

In most types of cell migration, the actin cytoskeleton is dynamically remodeled, resulting in the formation of protrusive structures and in the generation of intracellular forces that lead to cellular movement.

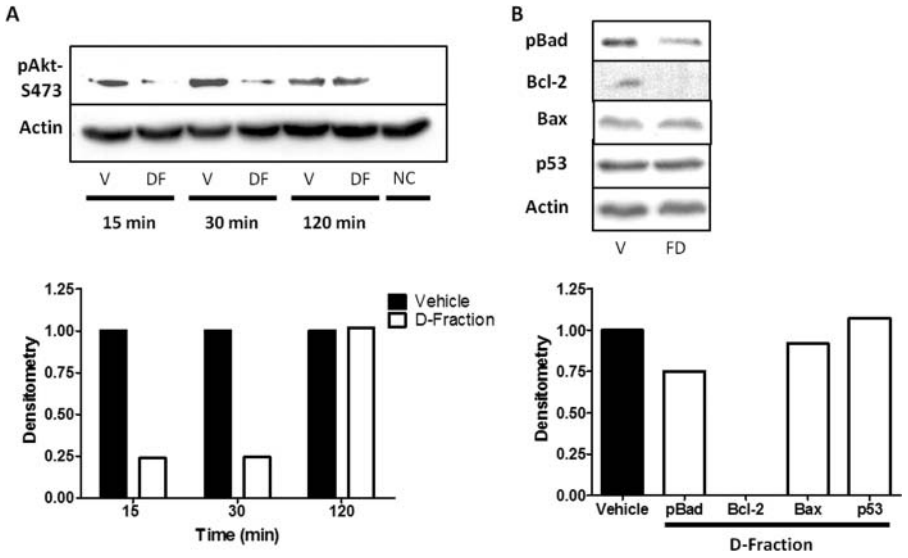

Figure 2. Maitake D-Fraction decreases antiapoptotic PI3K-Akt pathway and affect the balance Bax/Bcl-2 in LM3 cells. A: Following serum starvation, LM3 cells were subsequently treated with D-Fraction (570.6 μg/mL) or vehicle for 15, 30, or 120 min and cell lysates were subjected to WB analyses to detect pAkt-S473. B: LM3 cells were treated with D-Fraction (570.6 μg/mL) or vehicle for 18 h and cell lysates were subjected to WB analyses to detect pBad, Bcl-2, Bax, and p53. Protein loading was normalized with actin. The blots are representative of at least two independent experiments. The graph shows the densitometry of bands. V = vehicle, DF = D-Fraction, NC = negative control (LM3 cells deprived of FBS for 20 h).

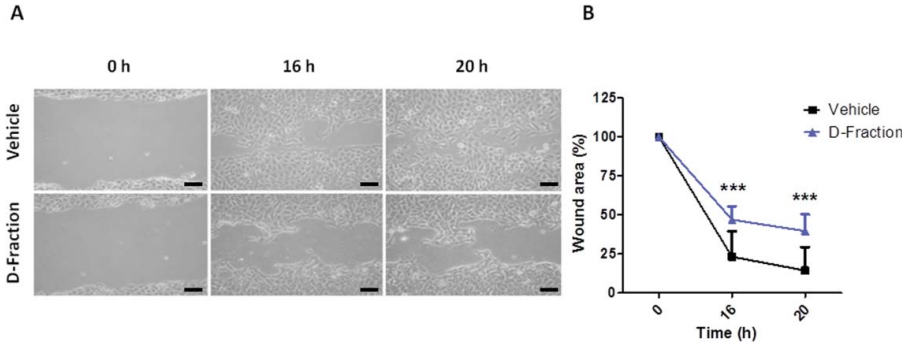

Figure 3. Maitake D-Fraction decreases migration of LM3 cells. A: Representative phase-contrast pictures of the wound healing assay in LM3 cells. A linear scratch wound was made along the culture plate, and cells were treated with D-Fraction (570.6 μg/mL) or vehicle. Wound closure was monitored during 20 h and photographed at 0, 16, and 20 h. Magnification: 200x, scale bars represent 125 μm. B: The graph represents the mean percentage (±SD) of uncovered wound area taking the value at 0 h as 100% of one representative experiment. Two-way ANOVA and Bonferroni post tests. $^{***}p < 0.001$.

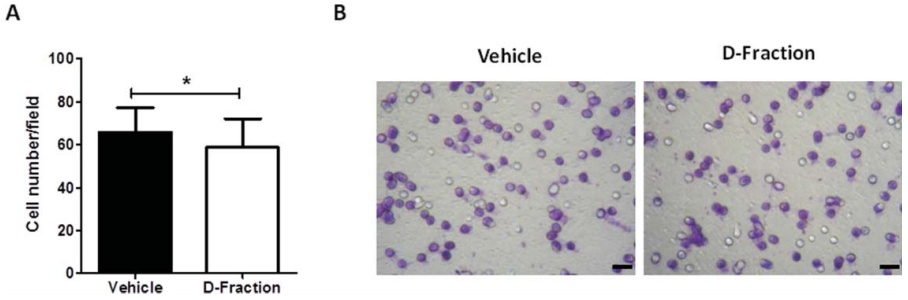

Figure 4. Maitake D-Fraction decreases invasion of LM3 cells. LM3 cells were treated with D-Fraction (570.6 μg/mL) or vehicle for 12 h and cell invasion was analyzed using Matrigel-coated transwell inserts. A: The graph represents the mean ± SEM number of invasive cells after treatment with D-Fraction or vehicle. Each assay was performed in triplicate and ten fields from each insert were counted. Student's t test; $^{*}p < 0.05$. B: Representative pictures of cell invasion assays in LM3. Magnification: 400x, scale bars represent 50 μm.

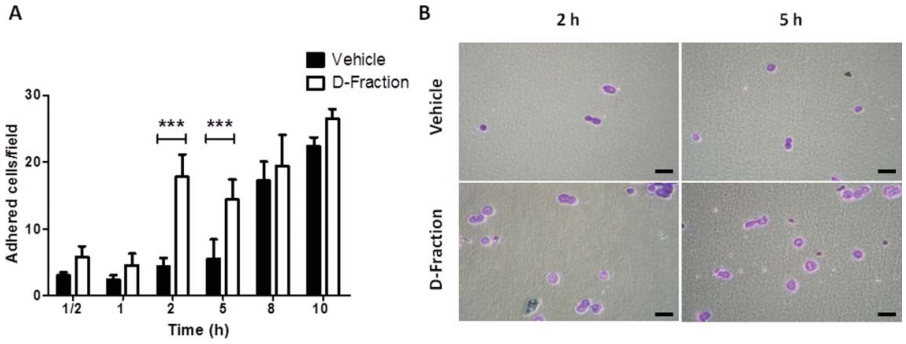

Figure 5. Maitake D-Fraction increases LM3 cell adhesion. LM3 cells were treated with D-Fraction (570.6 μg/mL) or vehicle for 12 h. Then, they were seeded into 96-well culture plates and were allowed to adhere for different times. A: The graph represents the number of adhered cells after treatment with D-Fraction or vehicle. Each assay was performed in triplicate and ten fields from each well were counted. Data show mean $\pm$ SD of one representative experiment. Two-way ANOVA and Bonferroni post tests. $^{***}p < 0.001$. B: Representative pictures of cell adhesion assay in LM3. Magnification: 400x, scale bars represent 50 μm.

Lamellipodia and filopodia are actin-rich structures that typically are associated with cell migration (45,46). Having seen the effect of Maitake D-Fraction on the cellular migration of LM3 cell line, we analyzed if this affected the actin cytoskeleton by staining F-actin with rhodamine-conjugated phalloidin. As observed in Fig. 6, we found that the number of LM3 cells with cytoplasmic protrusions (lamellipodia and filopodia) were reduced from 47.86 $\pm$ 5.31 to 29.55 $\pm$ 1.09 ($P < 0.05$) after 12 h of Maitake D-Fraction treatment (570.6 μg/mL). Thus, Maitake D-Fraction reorganizes the actin cytoskeleton in LM3 cells and decreases the cellular motility. This result is in agreement with the decreased migration of D-Fraction-treated LM3 cells observed in the wound healing assay.

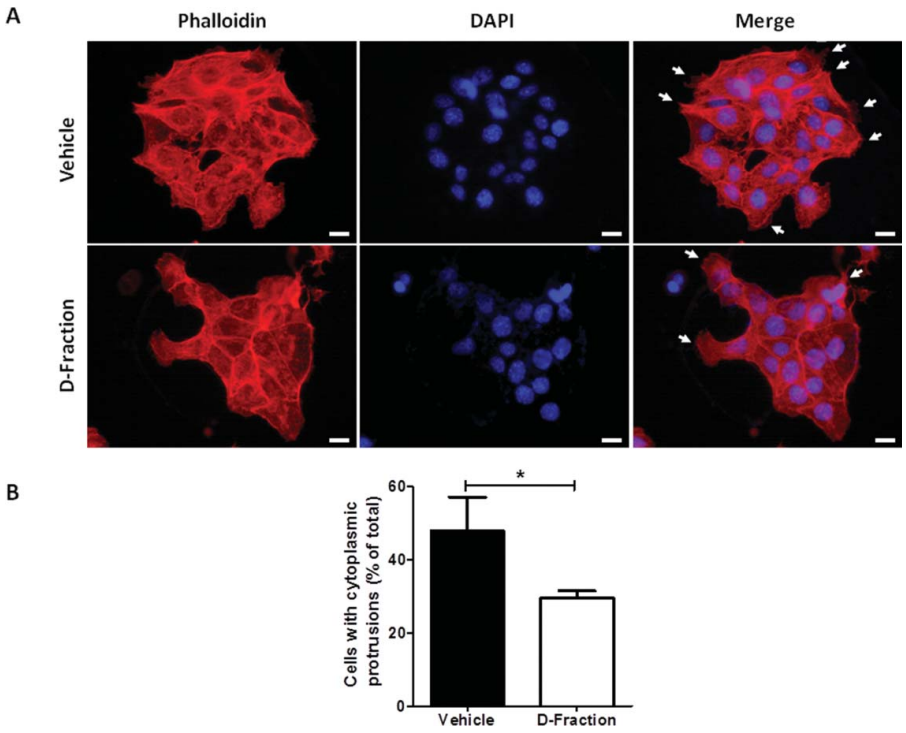

Figure 6. Maitake D-Fraction decreases the number of filopodia and lamellipodia in LM3 cells. LM3 cells were treated with D-Fraction (570.6 μg/mL) or vehicle for 12 h. Cells were fixed, stained with rhodamine-conjugated phalloidin (F-actin) and DAPI (nuclei). The images were acquired with a light fluorescence microscope. A: Representative fluorescence images. Arrows indicate cytoplasmic protrusions (lamellipodia or filopodia). Magnification: 400x, scale bars represent 20 μm. B: Cells were scored for the presence of cytoplasmic protrusions (lamellipodia or filopodia) and these were counted in 10 randomly chosen fields. The assay was performed in triplicate. Values are the mean $\pm$ SD of three independent experiments. Chi-square test was applied. $^{*}p < 0.05$.

Maitake D-Fraction decreases activity of MMP-2 secreted by LM3 cells and increases E-cadherin expression

It is known that degradation of the extracellular matrix (ECM) is a key event for invasion and metastasis of tumor cells (47,48). MMPs are a family of endopeptidases capable of degrading essentially all components of the ECM (49) and, hence, their high activity has been associated with tumor progression of many cancer types (50,51). To explore the mechanism by which D-Fraction decreases the invasiveness of LM3 cells, we evaluated their effect on MMP-2 activity by zymography. As shown in Fig. 7A, we found that Maitake D-Fraction decreased secreted MMP-2 activity by LM3 cells after 18 h and 24 h of treatment, compared to vehicle. These results suggest that D-Fraction reduces the invasion of LM3 cells, at least in part, through the decreased activity of MMP-2.

In addition, we evaluated if Maitake D-Fraction affects the expression of E-cadherin in LM3 cells. E-cadherin is one of the main cell adhesion molecules between epithelial cells (52,53) and has been shown to play an important role in epithelial-mesenchymal transition (EMT), a process that promotes the development of cancer metastasis (54, 55). As shown in Fig. 7B, we found that D-Fraction (570.6 μg/mL) treatment increases the expression of E-cadherin in LM3 cells compared to vehicle treatment. This result suggests that D-Fraction promotes the epithelial phenotype of tumor cells, thus decreasing their invasive potential.

Maitake D-Fraction reduces tumor burden and lung metastases in a LM3 syngeneic mammary carcinoma murine model

Given the potent effects of Maitake D-Fraction on LM3 cells observed in culture, we decided to investigate if similar results could be observed in vivo. To this end, we used a murine model of subcutaneous syngeneic LM3 cellular transplantation. Treatment of animals with D-Fraction or vehicle was performed as described in Materials and methods. First, we evaluated the effect of D-Fraction on the primary tumor volume and weight. As shown in Fig. 8A, Maitake D-Fraction retarded tumor growth when compared to the vehicle-treated mice. After 18 doses, D-Fraction-treated mice had significantly smaller tumors than vehicle-treated mice (D-Fraction = 0.66 cm^3 vs. vehicle = 1.39 cm^3, $P < 0.01$). Furthermore, the ex vivo tumor volume (Fig. 8B) and weight (Fig. 8C) were significantly decreased by Maitake D-Fraction treatment, compared to vehicle treatment (0.99 cm^3 vs. 1.81 cm^3, $P < 0.05$ and 0.94 g vs. 1.69 g, $P < 0.05$).

The LM3 syngeneic murine model of breast cancer produces lung metastases (27), and therefore it is

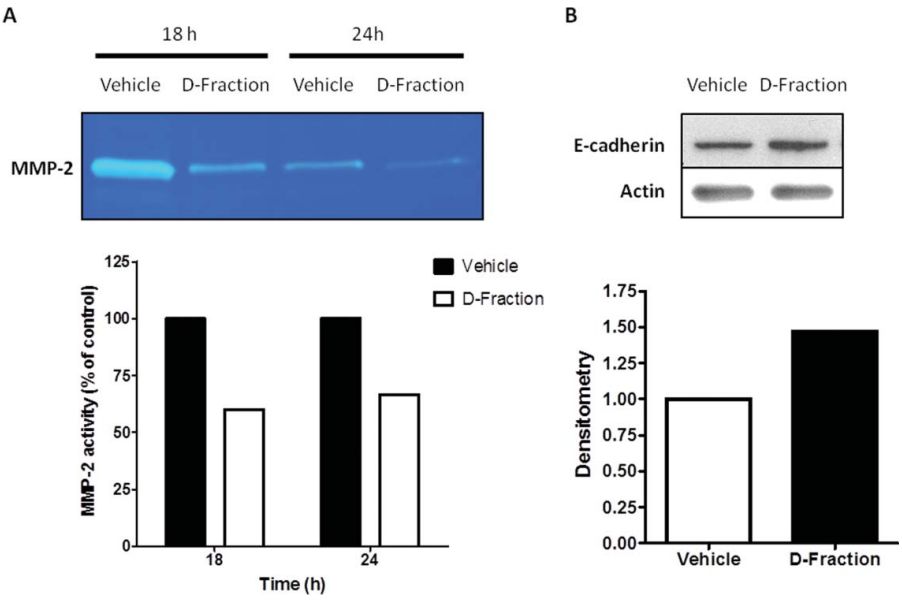

Figure 7. Maitake D-Fraction decreases the activity of MMP-2 secreted by LM3 cells and increases E-cadherin expression. A: LM3 cells were pretreatment with D-Fraction (570.6 μg/mL) or vehicle for 6 h or 12 h and then they were incubated in serum-free medium for 12 h in presence of D-Fraction or vehicle. Conditioned media were collected. Gelatinolytic bands were visualized using a transilluminator and measured with Fiji (Fiji Is Just ImageJ). Data were normalized to the amount of cells/well. Data are representative of at least two independent experiments. B: LM3 cells were treated with D-Fraction (570.6 μg/mL) or vehicle for 12 h and cell lysates were subjected to WB analyses to detect E-cadherin. Protein loading was normalized with actin. The blots are representative of at least two independent experiments. The graph shows the densitometry of bands.

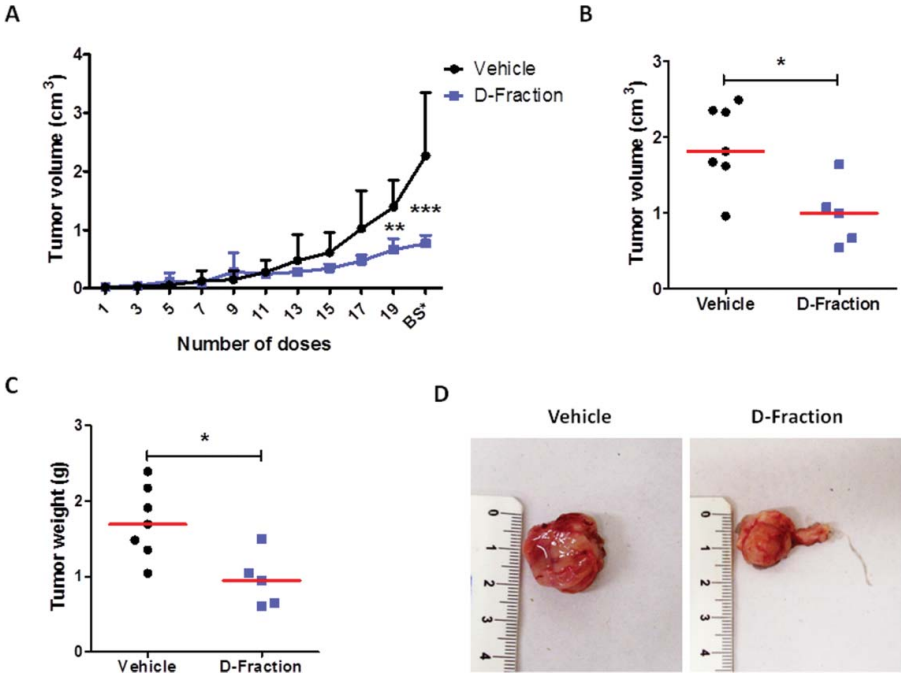

Figure 8. Maitake D-Fraction reduces tumor burden in LM3 syngeneic mammary carcinoma murine model. Tumor-bearing BALB/c mice were treated with D-Fraction (15.63 mg × Kg^{-1} × $día^{-1}$; n = 5) or vehicle (n = 7). A: Graph showing the kinetic of tumor growth. The tumor volume was calculated as $\pi/6 \times a \times b^2$, where a is the length and b is the width of the tumor. Shown is the mean ± SD. Two-way ANOVA and Bonferroni post-test. BS* means before the sacrifice. B: Graph showing the tumor volume. Ex vivo tumor volume was calculated as $\pi/6 \times a \times b \times c$, where a, b, and c are the three tumor dimensions. Each point represents the volume of each tumor and the lines represent the median value. C: Graph showing the tumor weight. Each point represents the weight of each tumor and the lines represent the median value. D: Representative pictures of the tumors from mice treated with D-Fraction or vehicle. Mann-Whitney U test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

possible to study the effect of D-Fraction on this process. As shown in Fig. 9 and in concordance with the previously observed effect on LM3 cell migration and invasion, we found a lower number of lung metastases per animal in D-Fraction-treated mice (median = 0, range 0–1) than in the vehicle-treated mice (median = 2, range 0–12) ($P < 0.05$).

Since a reduction in tumor burden was observed with Maitake D-Fraction treatment, we aimed at studying whether apoptosis and/or proliferation processes were affected in animal primary tumors. To study cellular proliferation we quantified the number of mitotic figures in H&E-stained tumor slides and Ki-67 expression by IHC and to analyze apoptosis we evaluated Bax expression by IHC. The number of mitotic figures (D-Fraction median = 50 vs. vehicle = 70) and the expression of Ki-67 (D-Fraction median = 0.125 vs. vehicle = 0.2) in tumors did not change significantly in Maitake D-Fraction-treated mice compared to vehicle-treated animals (Fig. 10). On the contrary, the expression of Bax in tumors increased in D-Fraction-treated mice compared to vehicle-treated mice (median IRS = 1.9 vs. 0.4, respectively; $P < 0.05$, Fig. 10). These results suggest that Maitake D-Fraction in vivo impairs tumor growth by inducing apoptosis.

Discussion

Medicinal mushrooms have been shown to exert therapeutic action against the development of cancer, primarily because they contain a number of biologically active compounds (4). These include high-molecular-weight compounds (HMW) such as polysaccharides (β-glucans), glycoproteins, proteins, proteoglycans, as well as low-molecular-weight compounds (LMW) such as quinones, isoflavones, catechols, triacylglycerols, sesquiterpenes, and steroids (56). For a long time, each of these groups of mushroom metabolites was linked to an exclusive antitumor action. So, while HMW exerted their antitumor action by activation of the immune response of the host organism, LMW acted directly on the tumor cell interfering with particular cellular signal transduction pathways linked to cancer development and progression (5,56–59). For example, it has been shown that LMW are able to inhibit molecular targets, such as Nuclear Factor kappa B, mitogen-activated protein kinases, aromatase and sulfatase, matrix metalloproteinases, cyclooxygenase, DNA topoisomerases, and DNA polymerase (59). However, increasing evidence shows that some polysaccharides (HMW) also have direct action on the tumor cells (10). Such is the case of Maitake D-Fraction.

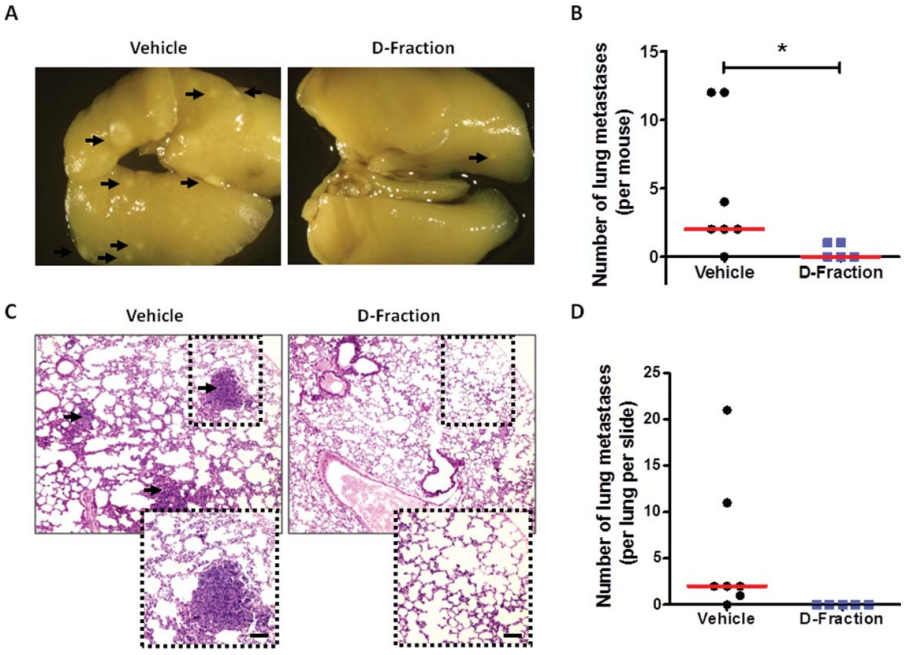

Figure 9. Maitake D-Fraction reduces lung metastases in LM3 syngeneic mammary carcinoma murine model. A: Representative pictures of the metastases macroscopically observed in the lungs from mice treated with vehicle and D-Fraction. B: Graph showing the quantification of the number of lung metastases per mouse. The lines represent the median value. C: Representative pictures of the metastases observed in H&E-stained lung slides from mice treated with vehicle and D-Fraction. Magnification: 40x and 100x, scale bars represent 320 μm for the inserts. D: Graph showing the quantification of the number of lung metastases. One slice per lung and per animal was performed and the number of metastases was quantified. The lines represent the median value. Mann-Whitney U test. $^{*}p < 0.05$.

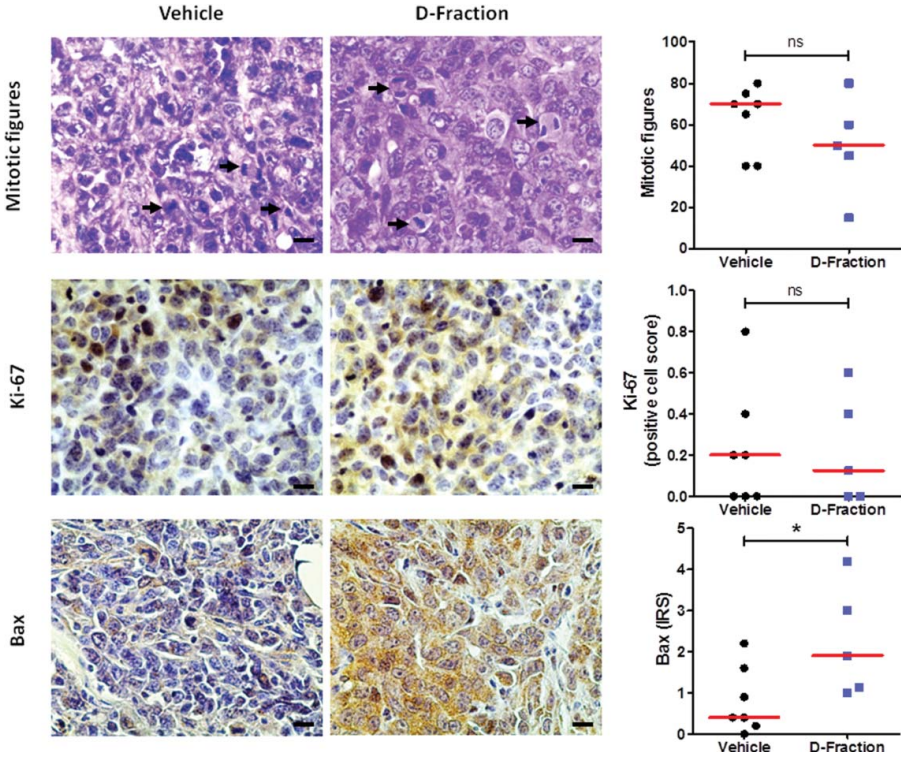

Figure 10. Maitake D-Fraction does not affect the mitotic index or Ki-67 expression but increases Bax expression in LM3 syngeneic mammary carcinoma murine model. Representative micrographs of H&E-stained tumor tissues showing mitotic figures and of immunohistochemical staining of tumor tissues showing Bax and Ki-67 expression and their respective quantifications. The quantifications were performed in 10 randomly chosen fields. Lines indicate the median value. Mann-Whitney U test. $^{*}p < 0.05$. Magnification: 400x, scale bars represent 60 μm.

It has been shown that Maitake D-Fraction has antiproliferative or cytotoxic activity on canine and human cancer cells, implying that D-Fraction is capable of crossing the species barrier (21–25). Furthermore, we have previously reported (23,26) that Maitake D-Fraction modulates the expression of genes associated with cell proliferation, cell death, migration, invasion, and metastasis among others, in human breast cancer cells (MCF7). Of all capabilities that enable tumor growth and metastatic dissemination (the hallmarks of cancer) (60), Maitake D-Fraction has only been studied in relation to cellular viability. Hence, it is now necessary to investigate the direct effects of Maitake D-Fraction on the cellular processes that govern the transformation of normal cells into malignant ones, including cellular proliferation, cell death, cell adhesion, cell migration, and cell invasion.

In this study, we demonstrated for the first time that Maitake D-Fraction is able to act directly on murine mammary tumor cells, modulating different cellular capabilities involved in the development and progression of cancer.

The present data show that Maitake D-Fraction decreases the viability of LM3 hormone-independent breast cancer cells. We also demonstrate that D-Fraction induces apoptosis in these cells. In our previous work (23), we have demonstrated that Maitake D-Fraction also decreases the viability of human mammary adenocarcinoma MCF7 cells. Unlike LM3, MCF7 cells are hormone-dependent. They express ER and PR (61,62) but lack expression (62) or only express very low levels (29) of HER2. These results suggest that Maitake D-Fraction affects breast cancer cell viability regardless of hormonal receptor status. In this regard, it has been proposed that β-glucans could affect the viability of breast cancer cells by regulating expression of genes associated with endocrine resistance (63).

PI3K-Akt pathway is perhaps the most commonly activated signaling pathway in human cancer. Akt is a downstream multifunctional kinase, which acts as the central mediator of the PI3K-Akt pathway (42). It is well known that the full activation of Akt requires its phosphorylation at serine 473 (pAkt-S473) (64). Upon activation, pAkt phosphorylates a large number of downstream substrates. Particularly, pAkt promotes cellular survival through direct inactivation by phosphorylation of multiple proapoptotic proteins including Bad (42,65). Bad can form a heterodimer with survival factors: Bcl-2 and Bcl-xL. However, the phosphorylation of Bad by pAkt-S473 prevents this interaction, inactivating its proapoptotic function and restoring antiapoptotic function of Bcl-2 or Bcl-xL (64,66). Here, we demonstrated that Maitake D-Fraction delays Akt phosphorylation at Ser 473 and therefore decreases Akt activity (67). In concordance with PI3K-Akt pathway, we also demonstrated that D-Fraction decreases Bad phosphorylation. Therefore, these results show that, at least in part, the apoptotic effect of D-Fraction is mediated by attenuation of the PI3K/Akt/Bad pathway. Furthermore, *in culture* we detected that D-Fraction markedly decreased Bcl-2 expression without affecting the expression of Bax. Thus, these results suggest that D-Fraction induce apoptosis through modulation of proapoptotic and antiapoptotic proteins.

Although much progress in both diagnostic and therapeutic approaches has been made in the treatment of breast cancer, metastases remain the leading cause of death of this disease. The metastatic process involves detachment from the primary tumor, invasion into the surrounding tissue, intravasation, extravasation, and colonization of the target organ (43). In this work, we demonstrate that Maitake D-Fraction increases LM3 breast cancer cell adhesion, thereby decreasing the metastatic ability of these cancer cells. It has been shown that expression of fibronectin (FN) in the LMM3 cells (which originated from a more metastatic tumor than LM3) increases their adhesiveness and reduces their migratory capacity (68). Hence, it is possible that Maitake D-Fraction could increase the expression of FN in LM3 cells, leading to the increased cell adhesion that we demonstrated. In agreement with this, we also demonstrated that D-Fraction decreases the migratory and invasive capability of LM3 *in culture*.

It is well known that many of the steps involved in the metastatic process require cell motility (69). Reorganization of the actin cytoskeleton is the primary mechanism of cell motility and is essential for most types of cell migration (46). Two cellular features are typically associated with motile cell migration: lamellipodia and filopodia, which are actin-rich protrusive structures (45). Here, we demonstrated that Maitake D-Fraction decreases the number of lamellipodia and filopodia in LM3 cells thereby decreasing their motility.

Furthermore, MMPs have been widely associated with the metastatic process. Particularly, it has been suggested that the modulation of expression, secretion, and/or activation of MMP-2 may provide new strategies for breast cancer treatment (70,71). Moreover, it has been shown that low levels of MMP-2 are associated with a favorable prognosis in patients with hormone-receptor-negative breast tumors (72). In this work, Maitake D-Fraction was shown to decrease the activity of MMP-2 secreted by LM3 cells. This explains, at least in part, the mechanism by which D-Fraction reduces the invasive capability of LM3.

Finally, the antitumoral effects of Maitake D-Fraction that we demonstrated in culture were corroborated in vivo. In a murine model of subcutaneous syngeneic LM3 cell transplantation, we found that Maitake D-Fraction treatment decreases tumor burden. The observed

increase in Bax protein in the animal primary tumor tissues suggest that this decrease in tumor growth could be produced through apoptosis induction. Furthermore, we found that Maitake D-Fraction treatment decreases the number of lung metastases in the LM3-BALB/c animal model. It is likely that this in vivo antimetastatic effect of D-Fraction is the result of increased adhesion and decreased motility, migration, and invasion produced by Maitake D-Fraction on LM3 cells.

Having established the direct antitumoral effect of the proteoglucan D-Fraction, further investigation is warranted addressing if these antitumoral effects are due to the D-Fraction components acting cooperatively or if they are exerted by an individual component of the extract.

Conclusions

We provided evidence that Maitake D-Fraction, proteoglucan obtained from edible and medicinal mushroom *G. frondosa*, has a direct antitumoral action on LM3 murine mammary adenocarcinoma cells. We demonstrate that Maitake D-Fraction decreases cell viability, increases cell adhesion, and reduces the migration and invasion of LM3 cells, generating a less aggressive cell behavior. In concordance with these results, we also demonstrate that Maitake D-Fraction decreases tumor burden and the number of lung metastases in a murine model of syngeneic transplantation of LM3 cells.

Acknowledgments

We would like to thank Dr. Gabriela A. Balogh (Laboratorio de Biología Celular y Molecular, BIOMED UCA-CONICET) for the provision of Maitake D-Fraction® Pro 4X Liquid, which was produced by Mushroom Wisdom, Inc. (formerly Maitake Products, Inc.) New Jersey, USA. We are grateful to Dr. Elisa Bal de Kier Joffe (Instituto de Oncología Ángel Roffo, Buenos Aires, Argentina) for providing the LM3 cells.

Funding

This work was supported by the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICTs 2012-1595 and 2012-0966) and by Universidad Nacional del Sur, Bahía Blanca, Buenos Aires, Argentina (PGI 24/B172).

Declaration of interest

All authors declare that there are no conflicts of interest.

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