CLINICS

BASIC RESEARCH

FTY720 induces apoptosis in B16F10-NEX2 murine melanoma cells, limits metastatic development *in vivo*, and modulates the immune system

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OBJECTIVE: Available chemotherapy presents poor control over the development of metastatic melanoma. FTY720 is a compound already approved by the Food and Drug Administration for the treatment of patients with multiple sclerosis. It has also been observed that FTY720 inhibits tumor growth *in vivo* (experimental models) and *in vitro* (animal and human tumor cells). The aim of this study was to evaluate the effects of FTY720 on a metastatic melanoma model and in tumor cell lines.

METHODS: We analyzed FTY720 efficacy *in vivo* in a syngeneic murine metastatic melanoma model, in which we injected tumor cells intravenously into C57BL/6 mice and then treated the mice orally with the compound for 7 days. We also treated mice and human tumor cell lines with FTY720 *in vitro*, and cell viability and death pathways were analyzed.

RESULTS: FTY720 treatment limited metastatic melanoma growth *in vivo* and promoted a dose-dependent decrease in the viability of murine and human tumor cells *in vitro*. Melanoma cells treated with FTY720 exhibited characteristics of programmed cell death, reactive oxygen species generation, and increased β-catenin expression. In addition, FTY720 treatment resulted in an immunomodulatory effect *in vivo* by decreasing the percentage of Foxp3+ cells, without interfering with CD8+ T cells or lymphocyte-producing interferon-gamma.

CONCLUSION: Further studies are needed using FTY720 as a monotherapy or in combined therapy, as different types of cancer cells would require a variety of signaling pathways to be extinguished.

KEYWORDS: FTY720; Murine Melanoma B16F10; Apoptosis; Metastasis; Reactive Oxygen Species; β-Catenin; Immunomodulation.

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INTRODUCTION

FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol; Fingolimod, Novartis) is a synthetic analogue of myoricin, originally isolated from the extract of the fungus *Isaria sinclairii*. The compound is a substrate for sphingosine kinases (SphK1 and SphK2); its phosphorylated form, FTY720-P, acts as an agonist of sphingosine 1-phosphate (S1P) receptors (S1P1, S1P3, S1P4, S1P5), binding to them with high affinity at sub-nano-molar concentrations (1). Several groups have shown that FTY720 increases allograft survival (2,3), promotes earlier recovery from ischemia and reperfusion injury (4), and attenuates autoimmune diseases (5,6). The mechanisms underlying the effects of FTY720 were first described as the sequestration of lymphocytes in the thymus and secondary lymphoid organs, thus preventing their migration to inflammatory sites. However, other mechanisms have been proposed for FTY720, such as immunomodulation. Using experimental models of transplantation and autoimmune disease, our group demonstrated that FTY720 prevented the increase in Th17 (T helper 17) cells, instead of promoting Treg (T regulatory) expansion (3).

Another important effect of FTY720 has been observed in tumor cells. The compound caused dose-dependent cell

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death in several human tumor cell lines, such as prostate cancer (DU145), bladder cancer (T24, UMUC3, HT1197), and hepatoma cells (HepG2, Huh-7, Hep3N) and a cisplatin-resistant renal lineage (ACHN) (7-10). Several mechanisms have been proposed to explain these antitumor effects, which depend on the cell line analyzed. Wallington-Beddoe et al. (11) reported that FTY720 induced autophagy in acute lymphoblastic leukemia cells; this effect was not mediated by the S1P1 receptor or by protein phosphatase 2a (PP2A) activation. Liao et al. (12) demonstrated autophagy-induced caspase 3-dependent apoptosis in U266 cells, a multiple myeloma cell line. Azuma et al. (13) showed that FTY720 induced apoptosis *in vitro* and metastasis reduction *in vivo* by inducing cytoskeletal changes and reduced integrin expression in a syngeneic breast cancer model. FTY720 interaction with the S1P1 receptor in activated B cell-like diffuse large B-cell lymphoma (ABC-DLBCL) blocked STAT3 (Signal transducer and activator of transcription 3) signaling and reduced lymphoma cell growth *in vitro* and *in vivo* (14).

For this reason, FTY720 has been proposed as an enhancer of the efficacy of anticancer therapies. Here, we show that FTY720 is effective *in vivo* against the B16F10-Nex2 model of syngeneic murine metastatic melanoma, and to better understand the pathways associated with the antitumor effects in our model, we evaluated the influence of the compound on human and murine melanoma cell lines *in vitro*.

MATERIALS AND METHODS

In vivo evaluation of pulmonary metastasis

Male C57BL/6 mice, 6-8 weeks old, were purchased from CEDEME-UNIFESP (Centro de Desenvolvimento de Modelos Experimentais - UNIFESP). The guidelines in "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, and all of the animal experiments were performed using protocols approved by the Ethics Committee for animal experimentation of Federal University of São Paulo, Brazil.

The animals were inoculated via the tail vein with 3×10^5 viable B16F10-Nex2 melanoma cells. They were then treated by gavage with 5 mg/kg/day of FTY720 (Novartis, Switzerland) or 0.2 mL of PBS for 7 days, starting on the same day of tumor cell inoculation. Eight days after the last dose of the drug, the mice were anesthetized, their lungs were harvested, and melanotic pulmonary nodules were counted using a stereomicroscope.

Cells and culture conditions

B16F10, a syngeneic murine melanoma cell line in C57BL/6 mice, was originally obtained from the Ludwig Institute for Cancer Research, São Paulo branch. B16F10-Nex2, a subline isolated at the Experimental Oncology Unit (Federal University of São Paulo (UNIFESP), Paulista School of Medicine, EPM-UNIFESP), retains the principal characteristics of the original tumor cell line, namely, low immunogenicity and moderate virulence *in vivo*. It forms lethal subcutaneous tumors without spontaneous metastasis, which can be obtained by endovenous injection. Human melanoma cell line A2058 was originally obtained from the Memorial Sloan Kettering Cancer Center, New York. Human cervical carcinoma (HeLa) cells were acquired from Dr. Hugo P. Monteiro, Federal University of São Paulo (UNIFESP), Paulista School of Medicine. The MCF-7 human breast carcinoma cell line was originally obtained from the Ludwig Institute for Cancer Research. The cells were cultivated in RPMI 1640 medium (pH 7.2; Gibco/Invitrogen, Minneapolis, MN) supplemented with 10 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid), 24 mM sodium bicarbonate, and 10% fetal calf serum, all from Gibco/Invitrogen, and 40 mg/mL of gentamycin (Hipolabor Farmacêutica, Sabará, MG, Brazil). The cells were maintained in culture flasks at 37℃ in a humidified atmosphere with 5% CO_2.

Cell viability assay

B16F10-Nex2 or human tumor cells were seeded in a 96-well plate (10^3 cells/well/100 μL of complete medium) and were treated 12 h later with increasing concentrations of FTY720 (Novartis, Switzerland). Viable adherent cells were counted after 24 or 48 h using Trypan blue dye. For some of the experiments, the cells were previously incubated for 2 h with 10 mM N-acetyl-L-cysteine or 15 mM L-cysteine (both from Sigma-Aldrich, St. Louis, MO) and were then washed with PBS and treated with FTY720 for 12 h. Alternatively, B16F10-Nex2 cells were co-incubated with 12 μM FTY720 and 100 or 150 μM necrostatin-1 (Sigma-Aldrich) for 24 h.

Caspase-3 activity

The enzymatic activity of caspase-3 in B16F10-Nex2 cells induced by FTY720 treatment was assayed using the ApoTarget™ Caspase Colorimetric Protease Assay Kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Briefly, the cells were treated with 6 μM FTY720 for 6 h and were then harvested and incubated with chilled lysis buffer for 10 min in an ice bath. The lysate was centrifuged at 10,000 g for 1 min, and the protein concentration in the supernatant was measured using the Bradford method. The extract was diluted to 4 mg/mL of protein in lysis buffer, and 200 μg of the protein was incubated with 50 μL of DTT-containing reaction buffer and 200 μM enzyme substrate (DEVD-pNA) at 37℃ for 2 h in a 96-well plate. The reaction was read at 400 nm in a microplate reader (SpectraMax-M2 software).

Transmission electron microscopy

B16F10-Nex2 cells (5×10^4) were cultivated on plastic disks made from Aclar film, incubated with 12 mM FTY720 for 3 h, and then fixed in a mixture of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 20 h. After washing with sodium cacodylate buffer for 15 min, the cells were fixed with 1% osmium tetroxide in sodium cacodylate buffer for 2 h and were then incubated in an aqueous solution of 0.4% uranyl acetate for 30 min. After each treatment, the cells were washed with water for 10 min, and reactions were run at room temperature. All of the reagents were acquired from Sigma-Aldrich. The cells were dehydrated in graded ethanol solutions (70%, 95%, and 100%), treated with propylene oxide twice for 15 min, embedded in SPURR for 5 h at room temperature, and then embedded in SPURR for 72 h at 70℃, with suitable regions carefully selected for final trimming of the blocks. Ultra-thin sections from the selected regions were collected on grids and were stained in alcoholic 1% uranyl acetate and in lead citrate prior to examination using a Jeol 100 CX Electron Microscope (Tokyo, Japan).

TUNEL assay

DNA fragmentation was analyzed by TUNEL assay (In Situ Cell Death Detection kit, Roche Molecular Biochemicals, Mannheim, Germany). Briefly, B16F10-Nex2 cells (1×10^4) were cultivated on round glass coverslips for 24 h at 37℃. The cells were then treated with 6 μM FTY720 for 6 h, washed with PBS, and fixed for 30 min at room temperature with 2% formaldehyde (Sigma-Aldrich). After fixation, the cells were washed with PBS and were permeabilized with 0.1% Triton X-100 (Sigma Aldrich) for 30 min; then, they were washed again with PBS and incubated with TdT (terminal deoxynucleotidyltransferase) in reaction buffer with dUTP-fluorescein at 37℃ for 1 h. Finally, the cells were washed and stained with 10 μg/mL of DAPI (Invitrogen) for 10 minutes. As a positive control, cells were incubated with 1 μg/mL of actinomycin D (Sigma-Aldrich) for 2 h. Images were processed using ImageJ software (http://rsbweb.nih.gov/ij/). The cells were analyzed using an Olympus BX-51 fluorescent microscope (Olympus, Center Valley, PA, USA).

Chromatin condensation analysis

B16F10-Nex2 cells (1×10^4) were cultivated on round glass coverslips for 24 h at 37℃ and were then treated with 6 μM FTY720 for 4 h. The cells were washed with PBS and fixed for 30 min at room temperature with 2% formaldehyde (Sigma-Aldrich). After fixation, the cells were washed with PBS and then incubated with 2 μM Hoechst 33342 dye (Invitrogen) for 10 min. Images were processed with Image J software. Fluorescent cells were analyzed using an Olympus BX-51 microscope.

Superoxide anion production

The superoxide anion production was analyzed by dihydroethidium (DHE) assay (Invitrogen). Briefly, B16F10-Nex2 (1×10^4) cells were cultivated on round glass coverslips for 24 h and were treated with 6 μM FTY720 for 4 h. The cells were then incubated with 5 μM DHE at 37℃ for 30 min. As a positive control, cells were incubated with 5 mM H_2O_2 (Sigma-Aldrich) for 20 min. Images were processed with Image J software. Fluorescent cells were analyzed using an Olympus BX-51 microscope.

Western blotting

B16F10-Nex2 cells (3×10^7) were treated with 6 μM FTY720 for 1 h. Cytoplasmic and nuclear extracts were prepared using the Cellytic NuCLEAR Extraction Kit (Sigma-Aldrich), according to the manufacturer's instructions. Briefly, the cells were pelleted and swollen with lysis buffer for 15 min on ice. IGEPAL CA-630 was added to a final concentration of 0.6%, and the mixture was immediately centrifuged at 10,000×g for 30 sec. The supernatant at this stage constituted the cytoplasmic extract. The pellet was resuspended in extraction buffer and was then vortexed at medium speed for 30 min and centrifuged at 20,000×g for 5 min. The supernatant at this stage constituted the nuclear extract. The protein concentration was measured using the Bradford method, and 40 μg of protein from each extract was electrophoretically separated on a 10% SDS-PAGE gel and was then transferred to a nitrocellulose membrane (Millipore, Billerica, MA). The membranes were washed in washing buffer (10 mM Tris-HCl, pH 8.0, 150 nM NaCl, and 0.05% Tween 20, all from Sigma-Aldrich) and blocked with 5% skim milk (Molico; Nestle, São Paulo, Brazil) in washing buffer for 12 h at 4℃ with shaking. The membranes were then incubated for 16 h at 4℃ with mouse monoclonal antibodies (1:500) for the detection of murine β-catenin (Cell Signaling, MA, USA). After 1 h of incubation with 1:1000 rabbit anti-mouse peroxidase-conjugated antibody (Invitrogen), immunoreactive proteins were detected with enhanced chemiluminescence using an ECL detection system (GE Healthcare).

Flow cytometry

The spleens were removed from C57BL/6 mice, and cell suspensions were prepared by pressing the organs through a 400-μm sterile nylon mesh. Single-cell suspensions were placed in individual tubes and were incubated with lysis buffer for 1 min to cause hemolysis. For surface markers, 1×10^6 spleen cells were incubated for 20 min with rat anti-mouse antibody (BD Biosciences Pharmingen), CD4PerCP, CD3APC, and CD8PE. The cells were washed and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. For Foxp3 intracellular staining, 1×10^6 spleen cells were permeabilized with PBS containing 0.5 BSA and 0.1% saponin and were then incubated with anti-mouse Foxp3FITC (eBioscience – San Diego, CA) for 30 min at room temperature. The cells were washed with PBS containing BSA and 0.2% saponin, fixed with 4% paraformaldehyde, and analyzed using flow cytometry.

IFN-γ-producing cells were identified ex vivo using spleen cells (1×10^6) suspended in RPMI 1640 medium (Sigma-Aldrich Brasil Ltda), ionomycin (1 μg/mL), and the protein transport inhibitor brefeldin A (1 μg/ml) in a humidified incubator containing 5% of CO_2 at 37℃ for 4 h. The cells were washed twice in ice-cold PBS (Sigma-Aldrich Brasil LTDA), fixed overnight, and permeabilized with 0.2% Triton X-100 (6 min at 37℃). Then, the cells were washed twice in ice-cold PBS and were pelleted and stained (30 min at 4℃) for intracellular cytokines using anti-mouse IFN-γ Alexa-Fluor (eBioscience – San Diego, CA) flow cytometry analysis. The cells were analyzed in a FACSCalibur Cell Cytometer using Cell Quest Pro software (BD Biosciences). At least 10,000 events were evaluated.

Statistical analysis

Student's two-tailed t-test was used for statistical analysis, with p-values <0.05 considered statistically significant.

RESULTS

FTY720 reduces the development of metastatic melanoma *in vivo*

Male C57BL/6 mice were injected i.v. with 3×10^5 B16F10-Nex2 melanoma cells. The mice were treated by gavage with PBS (Control) or FTY720 (5 mg/kg/day) for 7 days, starting on the day of tumor cell injection, and the number of lung nodules was evaluated 8 days after the last dose of FTY720. The median number of lung nodules in the FTY720-treated group (125 nodules) decreased significantly when compared to the control C57BL/6 mice (more than 300 nodules) (Figure 1).

FTY720 has a cytotoxic effect on B16F10-Nex2 murine melanoma and human tumor cells *in vitro*

B16F10-Nex2 murine melanoma cells showed morphological alterations and a reduction in the number of adherent

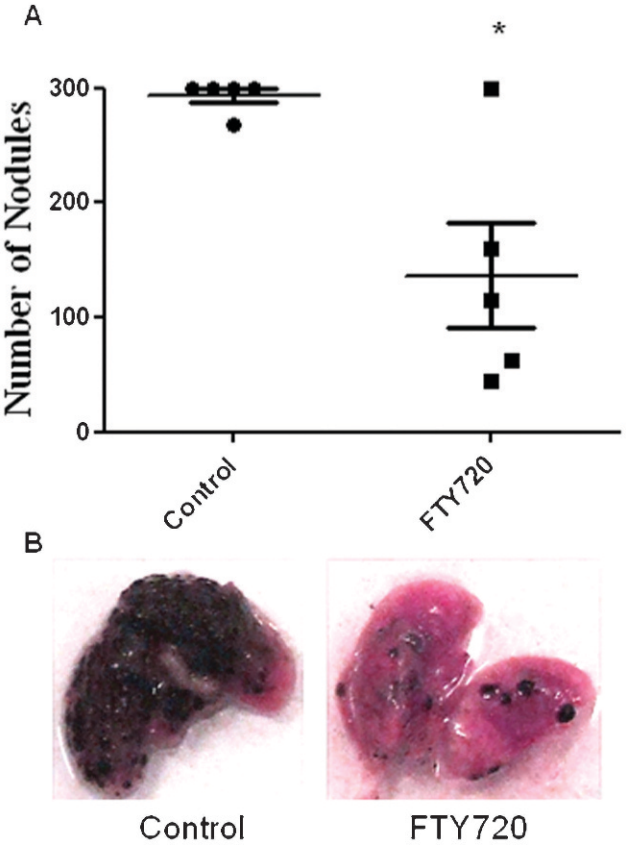

Figure 1 - *In vivo* evaluation of pulmonary melanoma metastasis after treatment with FTY720. **A)** C57BL/6 male mice were injected i.v. with 3×10^5 B16F10-Nex2 melanoma cells. The mice were treated by gavage with PBS (Control) or with FTY720 (5 mg/kg/day) for 7 days starting on the day of tumor cell inoculation, and the number of metastatic nodules in the lungs was evaluated 8 days after the last dose of the compound (n = 5 animals per group). *$p<0.04$. **B)** Representative images of C57BL/6 mouse lungs 15 days after melanoma cell inoculation.

cells after treatment with FTY720, as shown by light microscopy in Figure 2A. When these cells were collected with trypsin and were counted using a vital stain, a time- and dose-dependent cytotoxic effect was observed on B16F10-Nex2 cells (Figure 2B); additionally dose-dependent cytotoxic effects were observed on A2058 human melanoma, HeLa human uterine cervix carcinoma and MCF-7 human breast carcinoma cells (Figure 2C) incubated with FTY720 for 24 h. A2058 melanoma was the most resistant cell line to FTY720 treatment, followed by breast carcinoma MCF-7. All of the tumor cells evaluated, except for A2058 melanoma, showed IC_{50} values less than 10 μM.

FTY720 induces apoptosis in tumor cells

Next, we evaluated the cell death pathway induced by FTY720 in B16F10-Nex2 murine melanoma cells.

After incubation with 6 μM FTY720 for 4 h, a significant increase in tumor cells showing chromatin condensation was observed after cell staining with Hoechst 33342. Forty percent of FTY720-treated cells, but only 7% of control cells, showed evident chromatin condensation (Figure 3A). By transmission electron microscopy, important nuclear alterations were also observed after the compound treatment, mainly early chromatin aggregation (after 3 h) along with complete nuclear membrane disruption (Figure 3B).

FTY720 treatment also induced strong DNA degradation in B16F10-Nex2 cells detected by TUNEL assay. While 87% of treated cells showed DNA degradation, only 2.5% of control cells were positive on TUNEL assay (Figure 3C). Strong activation of caspase-3, an important hallmark of apoptosis, was observed in FTY720-treated cells (3D). The addition of necrostatin, a necroptosis inhibitor (15), to the cell culture before FTY720 treatment did not prevent FTY720-induced death, indicating that necrosis is not involved in the B16F10-Nex2 cell death pathway that is induced by FTY720 (Figure 3E).

Accumulation of reactive oxygen species and nuclear/cytoplasmic β-catenin in B16F10-Nex2 cells after FTY720 treatment

Reactive oxygen species (ROS) are important regulators of apoptosis (16). To evaluate the participation of ROS in FTY720-induced cell death, intracellular anion superoxide production was detected using DHE after short (4 h) incubation with a low dose (6 μM) of FTY720. The fluorescence intensity of cells treated with FTY720 was as high as that of the positive control treated with 5 mM of H_2O_2, with the majority of cells being positive in both treatments, while untreated cells showed a few positive cells with low fluorescence (Figure 4A). The role of ROS in FTY720-induced apoptosis was confirmed by measuring cell viability after pre-incubation of B16F10-Nex2 cells with the ROS scavenger N-acetyl-l-cysteine (NAC) or L-cysteine, following treatment with the compound. It was observed that both antioxidants partially inhibited cell death in the presence of FTY720 at the doses used (10 mM NAC and 15 mM L-cysteine) (Figures 4B-C). This result suggests that ROS are among the apoptosis effector molecules induced by FTY720.

Decreased expression of β-catenin has been reported in advanced and metastatic prostate cancer, and Chua et al. (17) reported that FTY720 restored β-catenin expression and suppressed the growth of human prostate cancer xenografts in nude mice. In agreement with this observation, B16F10-Nex2 cells exhibited significantly increased expression of β-catenin in their nuclei and cytoplasms after FTY720 treatment for 2 h compared to untreated cells, as observed by immunoblotting (Figure 4D).

Immunomodulation by FTY720

To assess other possible roles played by FTY720 in the melanoma model, we removed splenocytes from C57BL/6 mice 15 days after B16F10-Nex2 injections and investigated ex vivo the percentages of T lymphocytes (CD4+ and CD8+), Treg cells (CD4+Foxp3+), and IFN-producing lymphocytes by flow cytometry. Mice treated with FTY720 evidenced a significant decrease in the percentage of CD4+T cells, whereas no significant change was observed for CD8+ T cells (Figure 5A and 5C). Additionally, Treg cells decreased in mice treated with FTY720, but no change was observed in lymphocytes producing IFN-γ (Figure 5B and 5D).

DISCUSSION

Patients with advanced melanoma have a poor prognosis, with 1-year survival rates as low as 33% and a median overall survival (OS) of approximately 9 months (18).

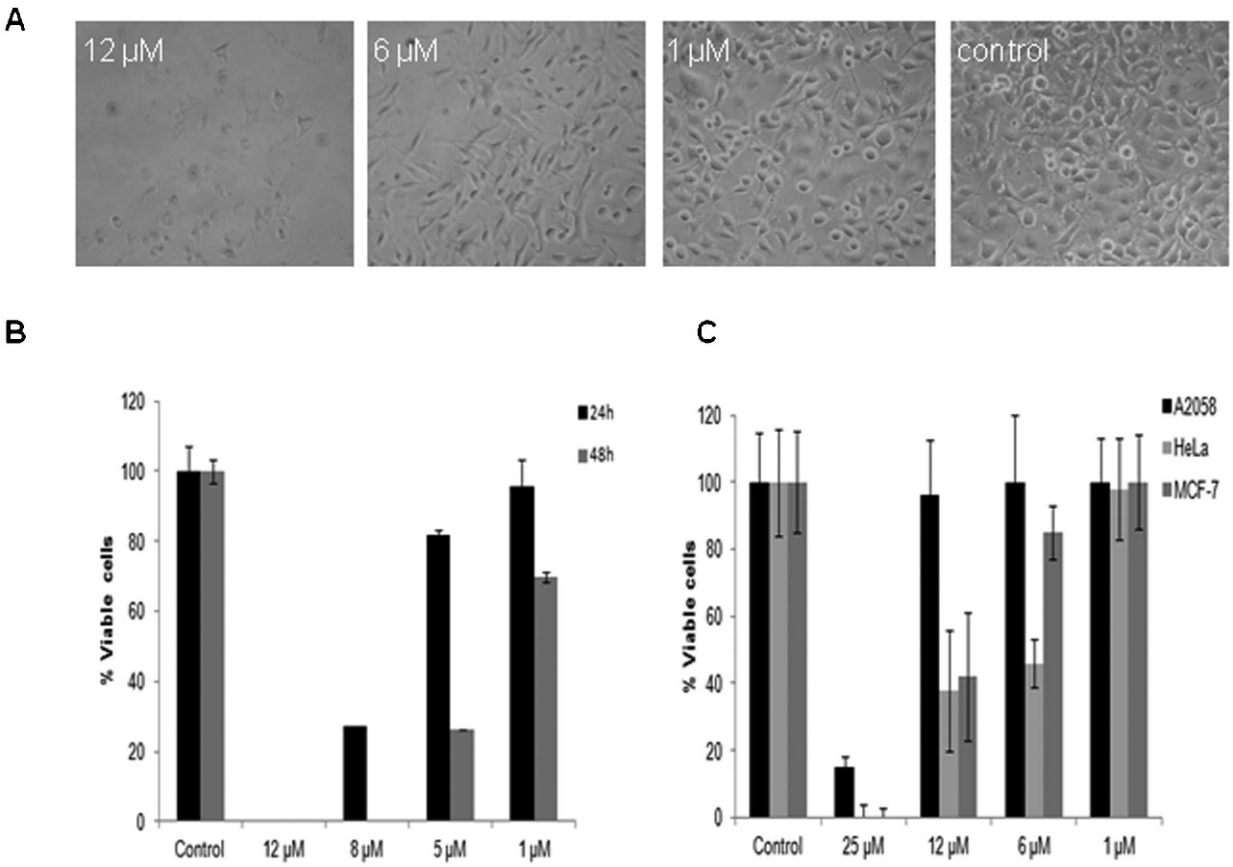

Figure 2 - *In vitro* effects of FTY720 on murine melanoma and human tumor cell lines. **A)** B16F10-Nex2 morphological alterations, after treatment with 1, 6, and 12 µM FTY720, were analyzed by light microscopy after 24 h. Control, untreated cells. Magnification, 40X. **B)** Viable B16F10-Nex2 cells were counted after 24 or 48 h of treatment with increasing doses of FTY720. **C)** The A2058, HeLa, and MCF-7 human tumor cell lines were incubated with increasing doses of FTY720, and cell viability was evaluated after 24 h. Each experiment was performed at least twice. The bars represent means and SDs.

Systemic treatments are ineffective in general because metastatic melanoma is largely refractory to the available therapies. Dacarbazine (DTIC) remains the most commonly used systemic agent, and when used alone or in combination with other agents, the response rates are less than 20%, with an OS of less than 9 months (19). Interleukin-2 trials reported durable complete responses in less than 10% of patients, with significant toxicity (20).

FTY720 has been approved by the FDA for the treatment of patients with refractory multiple sclerosis (21). Additionally, this compound has been shown to exert anti-cancer functions against chronic myelogenous leukemia (22) and gastrointestinal tumors via activation of PP2A (23) by unknown mechanisms.

Our results showed that FTY720 could limit B16F10-Nex2 metastatic melanoma development *in vivo*. Mice treated with 5 mg/kg/day of FTY720 for 7 days, starting on the same day as B16F10-Nex2 melanoma cell inoculation evidenced a significant reduction in the number of metastatic lung nodules when evaluated 15 days later. In agreement with this finding, in a model of lung cancer induction by a chemical carcinogen (urethane), it was recently shown that FTY720 decreases tumor growth, which is associated with reduced cell proliferation and VEGF (endothelial growth factor) expression, in addition to increased expression of activated caspase-3 (24).

LaMontagne et al. (25) reported a reduction in tumor cell growth *in vivo* after the inoculation of B16/BL6 melanoma cells in mouse ears and treatment with 3 mg/kg/day of FTY720 for 14 days. Both the volumes and weights of primary and cervical lymph node metastases were significantly reduced after FTY720 treatment. *In vitro*, the compound presented no cytotoxic or antiproliferative effects on melanoma cells at a dose of 1000 nM (1 µM), and the *in vivo* effects were attributed to the inhibition of tumor angiogenesis. In agreement with this observation, we found that 1 µM FTY720 had no effects on tumor cell apoptosis after 24 h of culture. In contrast, 5 µM FTY720 caused a 20% decrease in cell viability, whereas 8 µM FTY720 caused 70% cell death in 24 h and complete extermination of cells in 48 h. A dose of 12 µM was able to eliminate all viable cells after 24 h. Additionally, LaMontagne used B16/BL6 cells possessing an aggressive metastatic capability, whereas we evaluated B16F10-Nex2 cells, which presented moderate virulence. In LaMontagne's research, experimental cells could not even be subjected to prolonged incubation (>48 h) due to their rapid proliferative rate. Our results showed that FTY720 inhibition of cancer development is dose-dependent. For human therapy, FTY720 should be used in higher doses or in combination with classic chemotherapy, as human tumor cells likely possess more aggressive metastatic capabilities.

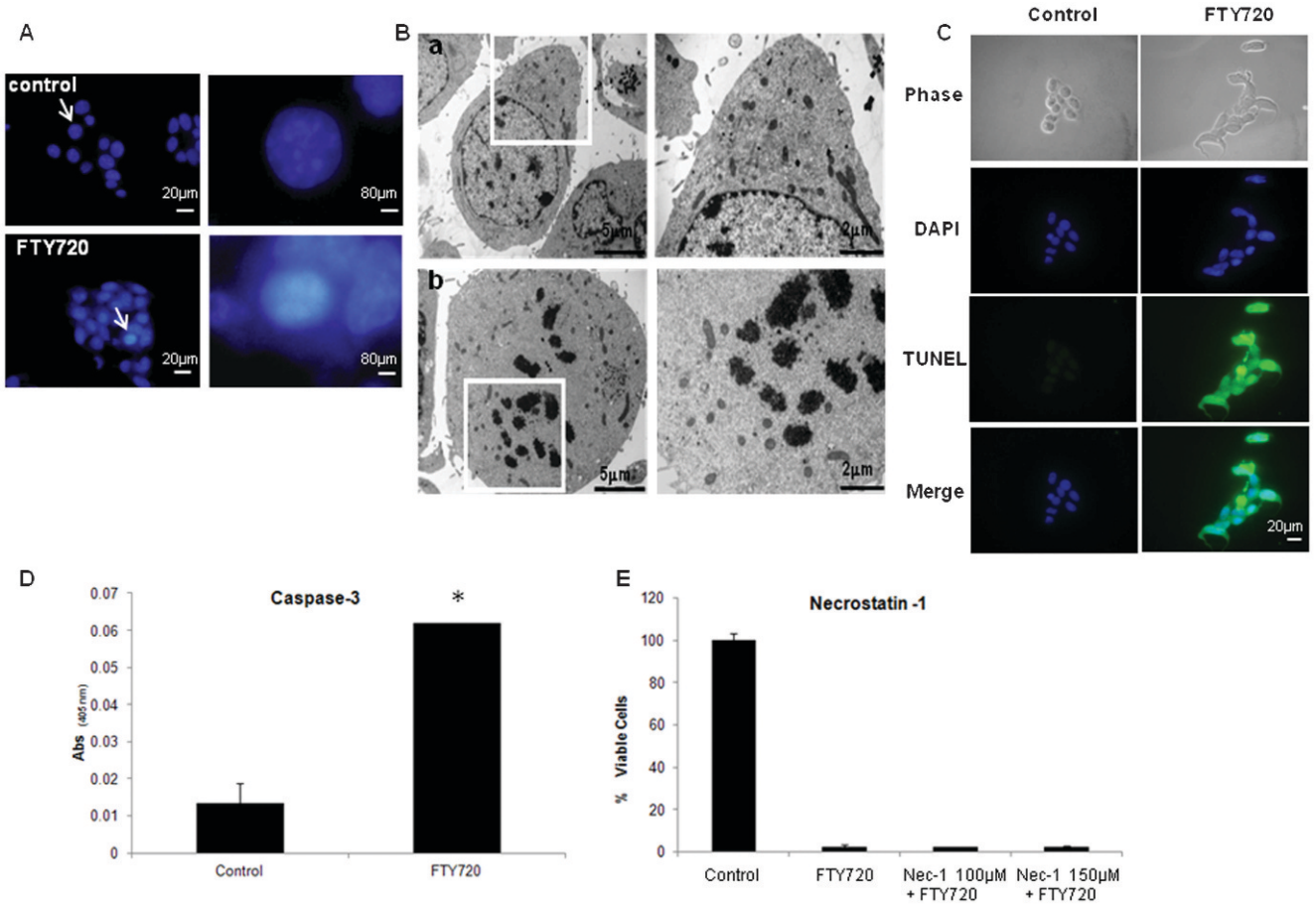

Figure 3 - FTY720 induces apoptosis in murine melanoma B16F10-Nex2. **A)** Murine melanoma cells were treated with 6 µM FTY720 for 4 h, and chromatin condensation was analyzed by fluorescence microscopy after DNA staining with Hoechst 33342. Arrows indicate cells, shown in the images on the right side, with nuclear condensation apparent in FTY720-treated cells. **B)** Transmission electron microscopy of B16F10-Nex2 cells after treatment with 12 µM FTY720 for 3 h. (a) Control and (b) FTY720. Squares indicate regions shown in images on the right side. **C)** Melanoma cells were treated with 6 µM FTY720 for 6 h, and DNA degradation was observed with TUNEL assay by fluorescent microscopy. Images of treated (right) and control (left) cells in phase contrast and after DAPI nuclear staining, TUNEL staining, and merging of both images. **D)** In the same conditions described in (C), activation of caspase-3 by FTY720 in B16F10-Nex2 cells was determined by colorimetric assay. **E)** B16F10-Nex2 cells were treated with 12 µM FTY720 for 24 h in the presence or absence of 100 or 150 µM necrostatin-1. *$p<0.04$. Experiments A and C were performed at least 3 times, and the experiments represented in B, D, and E were repeated twice. The bars represent means and SDs. Control, untreated cells.

FTY720 also reduced the viability of a human melanoma cell line (A2058) isolated from a metastatic lesion. After 24 h, a dose of 25 µM eliminated 90% of viable cells. However, when compared to other human tumor lineages (HeLa and MCF-7), the melanoma cell line was the most resistant to the compound.

Next, we investigated the mechanism involved in FTY720-mediated reduction of B16F10-Nex2 cell viability *in vitro*.

Several mechanisms have already been described for FTY720 death induction in tumor cells, and this effect is dependent on the tumor cell type.

Liao et al. (12) showed that FTY720 induced caspase-3-dependent apoptosis in dose- and time-dependent manners in U266 multiple myeloma cells by down-regulating the antiapoptotic proteins Mcl-1, bcl-2, and survivin and the cleavage of Bid. In these cells, apoptosis was preceded by autophagy, as measured by the conversion of LC3-I into LC-3II, a marker for autophagosome degradation.

In a lung tumor model, Saddoughi et al. (26) showed that FTY720 interacts with the inhibitor (I2PP2A/SET) of the tumor-suppressor enzyme PP2A, inducing RIPK1 (receptor-interacting serine/threonine-protein kinase 1)-mediated programmed necrosis (necroptosis) inhibited by necrostatin, but apoptosis and autophagy were not involved in the cell death process.

Our light microscopy results suggested that B16F10-Nex2 melanoma cells were not immediately lysed by FTY720 (Figure 2A), and electron microscopy did not reveal the formation of cytoplasmic vacuoles after treatment (Figure 3B). Together with caspase-3 activation, chromatin condensation and DNA degradation, these results strongly suggest that FTY720 treatment induced B16F10-Nex2 apoptosis and that autophagy was not involved in the induction of apoptosis. The addition of a necroptosis inhibitor (necrostatin-1) to the cell culture before FTY720 treatment did not prevent the death caused by the compound, indicating that necrosis is not involved in the B16F10-Nex2 cell death pathway that is induced by FTY720.

Some chemopreventive cancer agents induce apoptosis in part through ROS generation and the disruption of redox

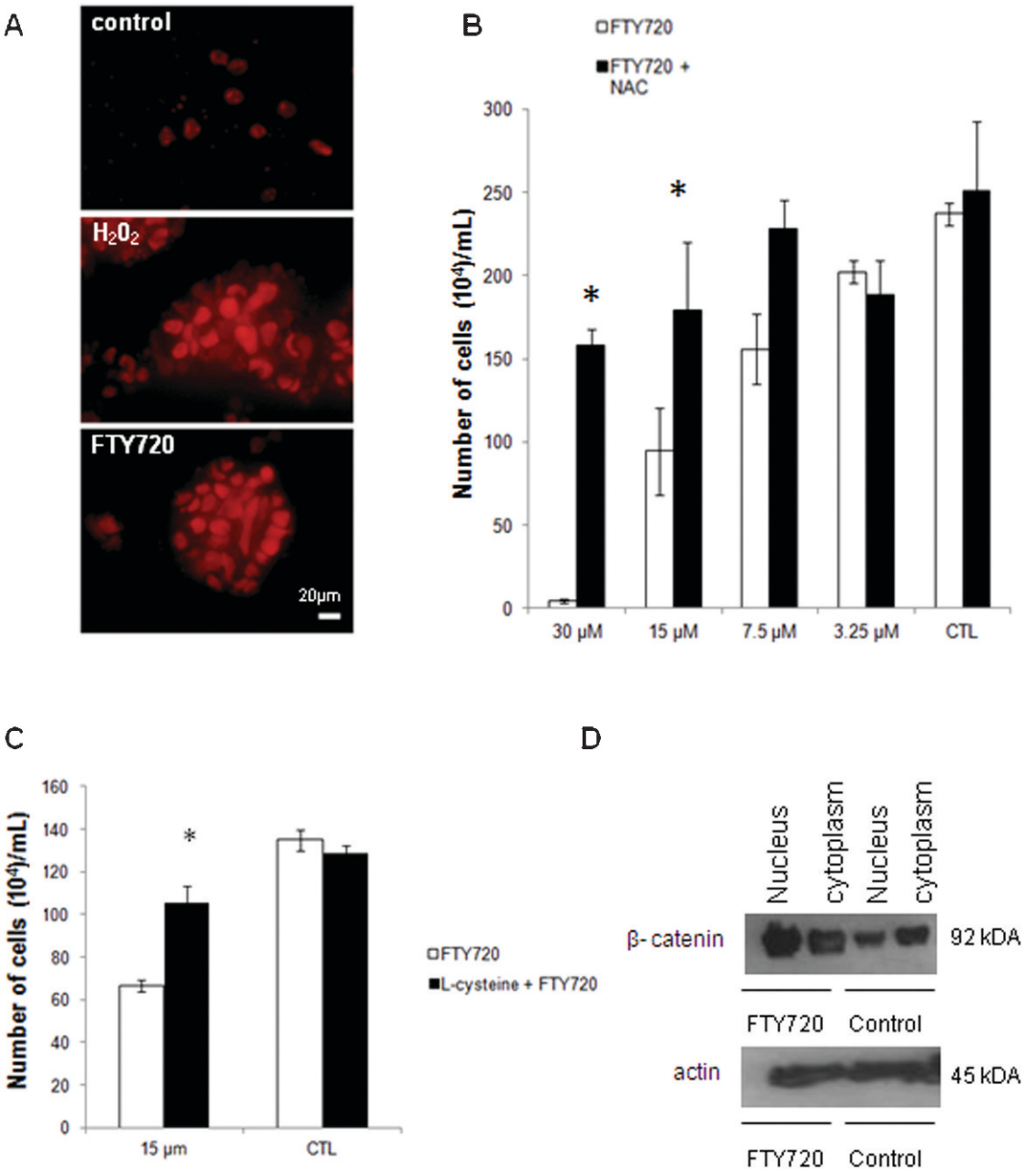

Figure 4 - ROS production and β-catenin expression by B16F10-Nex2 cells after FTY720 treatment. **A)** B16F10Nex-2 cells were incubated or not with 6 μM FTY720 for 4 h, and ROS production was determined by DHE assay and fluorescence microscopy. H_2O_2, positive control treated with 5 mM hydrogen peroxide. **B)** B16F10Nex-2 cells were treated with 3.25-30 μM FTY720 after 2 h of pre-incubation with 10 mM NAC or **C)** 15 μM FTY720 and pre-incubation with 15 mM L-cysteine, and cell viability was evaluated after 12 h. **D)** Expression of β-catenin was analyzed by western blot of nuclear and cytoplasmic extracts of B16F10Nex-2 cells incubated with 6 μM FTY720 for 1 h. *$p<0.04$.

homeostasis (27). In addition to byproducts of mitochondrial respiration, ROS are also key signaling molecules that regulate mitochondrial dysfunction and apoptotic events (28). As secondary messengers, ROS can trigger the release of pro-apoptotic proteins from the mitochondrial intermembrane space into the cytosol, such as cytochrome c, caspase-9, apoptosis-inducing factor, and Smac/DIABLO (second mitochondria-derived activator of caspases) (29), elevating intracellular Ca^{2+} concentrations and activating the caspase cascade (30). In acute lymphoblastic leukemia cells, the inhibition of ROS production by 10 μM NAC treatment prevented caspase-independent cell death (11).

Our results indicated that ROS are implicated in FTY720-induced cell death. B16F10-Nex2 melanoma cells treated with a low dose (6 μM) of FTY720 greatly increased their production of superoxide anions, as analyzed by DHE staining. In addition, the presence of the antioxidants NAC and L-cysteine partially inhibited the cell death induced by FTY720, suggesting that there are other regulating factors, in addition to ROS, in the FTY720-induced cell death of B16F10-Nex2 cells.

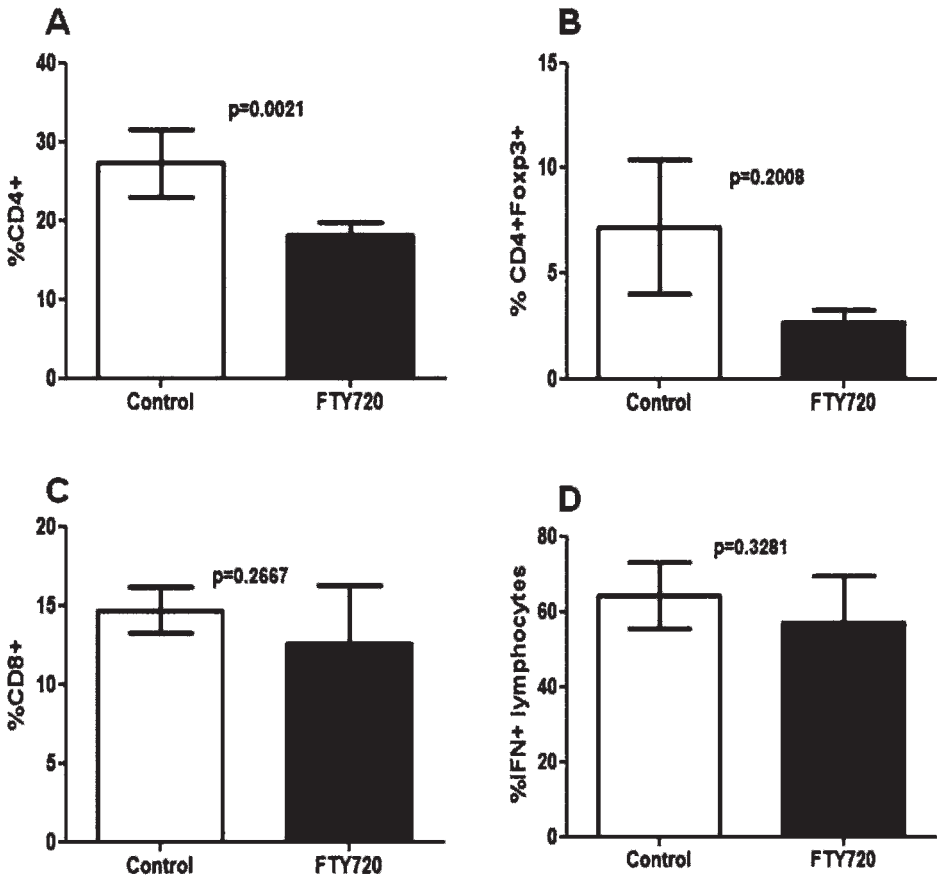

Figure 5 - Ex vivo evaluation of C57BL/6 mouse spleen cells by flow cytometry, showing the percentages of CD4+T cells, CD4+Foxp3+ cells, CD8+ T cells, and lymphocytes producing IFN-γ. Mice injected with B16F10Nex-2 cells and treated (FTY720) or not (Control) had their spleens harvested 15 days later for the evaluation of **A)** CD4+ T cells, $*p = 0.0021$; **B)** CD4+Foxp3+ cells; **C)** CD8+ T cells; **D)** IFN-γ-producing lymphocytes.

β-catenin is a key component of the Wnt/β-catenin signaling pathway and a mediator of the Ras/phosphatidylinositol 3-kinase (PI3K) pathways (31). Active β-catenin interacts with transcription factors, such as T cell factor/lymphoid enhancer (TCF/LEF), CBP and p300, resulting in target gene transcription, and it also binds to cadherins in the cell membrane to provide structural support for adhesion (32,33). The β-catenin pathway is well known as an enhancer of proliferation and survival in tumor cells. However, its overexpression or accumulation has also been reported to induce apoptosis in several tumor cell lines. Raab et al. (34) demonstrated that inhibition of PKC (protein kinase C) resulted in the accumulation of active β-catenin, which contributes to enzastaurin-induced cell death in multiple myeloma cells. Overexpression of a stable form of β-catenin or inhibition of endogenous β-catenin degradation has been reported to lead to G2 cell cycle arrest and apoptosis in epidermal keratinocytes (35). In malignant prostate cancer, reduced expression of nuclear β-catenin was associated with poorer prognosis (36), and restored functions of β-catenin and E-cadherin were associated with decreased microvessel density and VEGF expression in a mouse model of prostate cancer (17). It has recently been shown that the activation of Wnt/β-catenin signaling *in vitro*, *in vivo* (murine melanoma model), or in patient tumors results in increased levels of nuclear β-catenin, and this finding has been correlated with a beneficial prognosis (37,38).

In our study, there was increased expression of β-catenin in the nuclei and cytoplasms of B16F10-Nex2 cells treated with FTY720, suggesting that this molecule could be involved in the FTY720-induced cell death observed in these murine melanoma cells.

In addition to the reduced development of metastatic melanoma *in vivo* and increased melanoma cell death *in vitro* caused by FTY720, our results ex vivo showed a decrease in CD4+Foxp3+ cells in mice injected with B16F10-Nex2 melanoma cells and treated with FTY720. Treg cells have been shown to facilitate tumor development (39); thus, FTY720 could act as an immunomodulator by decreasing the numbers of these cells. Additionally, the capability of lymphocytes to produce IFN-γ was not changed by FTY720 treatment.

This study demonstrated that a low dose of orally administered FTY720 was effective in limiting murine metastatic melanoma development *in vivo* and that the compound induced apoptosis in these tumor cells *in vitro*, without indications of autophagy or necroptosis. Apoptotic cell death was regulated by ROS and by increased expression of β-catenin. FTY720 efficacy was obtained only in higher doses when added *in vitro* to A2058 human melanoma cells isolated from a metastatic lesion. In addition to the cancer cell death induced by FTY720, we also observed immunomodulation in the host immune system

caused by the compound, with a significant reduction in Treg cell numbers.

It has already been shown that FTY720, administered in association with chemotherapy, improves anticancer effects mainly by inducing cell death (40). Further studies are needed using FTY720 as a monotherapy or in combined therapy, as different types of cancer cells could require the involvement of a variety of signaling pathways to be eliminated completely.

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AUTHOR CONTRIBUTIONS

Pereira FV cell viability assays, *in vivo* experiment, participation in the manuscript design, statistical analysis, and helped to draft the manuscript. Arruda DC analysis of superoxide anion, chromatin condensation, transmission electron microscopy analysis and TUNEL assay. Figueiredo CR caspase-3 activation. Massaoka MH β-catenin detection. Matsuo AL cell viability assays. Bueno V *in vivo* treatment with FTY720, FACS analysis, participation in the manuscript design, and draft of the manuscript. Rodrigues EG conceived of the study, coordination and manuscript design, draft of the manuscript. All authors read and approved the final version of the manuscript.

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