

Synthetic Phosphoethanolamine Induces Apoptosis Through Caspase-3 Pathway by Decreasing Expression of Bax/Bad Protein and Changes Cell Cycle in Melanoma

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Summary

Phospholipids are potential antineoplastic agents that are abundant constituents of the cell membrane of eukaryotes and are supposed to be involved in specific intracellular signaling such as cell death. The aim of this study was to assess the *in vitro* and *in vivo* antitumor effects of synthetic phosphoethanolamine (PHO-S) on B16F10 murine melanoma cells and normal human fibroblasts. The cytotoxicity was evaluated by MTT assay and PHO-S was cytotoxic in melanoma cells but not in fibroblasts with IC₅₀% of 1.4 mg/ml to melanoma cells. *In vivo* antitumor activity was evaluated in a mice model subcutaneously injected with B16F10 melanoma cells. The mice treated with PHO-S in all concentrations showed a decrease of the tumor growth and metastasis. Cytometry analysis showed that the PHO-S blocked DNA synthesis, decreased number of melanoma cells in S phase and G₂/M, besides increasing number of apoptotic cells, inducing caspase-3 activity and decreasing Bad/Bax protein expression. Histologically, the dorsal tumors in the control group showed pigmented nodular masses with high vascularization and pleomorphic tumor cells. In the treated group, PHO-S reduction vascularization intratumoral with increased of collagen fibers and infiltrates neutrophils. The data indicate that PHO-S is a lipid compound potential with proapoptotic and antiproliferative effects but further work will be necessary to elucidate the antitumor mechanisms.

Keywords: Phosphoethanolamine; Melanoma; Apoptosis; Cell cycle; Caspase-3; Metastasis; Antiproliferative; Bax; Bad

Abbreviations: PHO-S: Synthetic Phosphoethanolamine; PC: Phosphatidylcholine; PE phosphatidylethanolamine

Introduction

The incidence of melanoma has been steadily increasing over the last three decades, and the discovery of better anti-cancer agents against melanoma is greatly needed. New anticancer agents need to be developed due to an inadequate biodistribution of therapeutic concentrations of chemotherapeutic agents against the tumor, as well as mutations suffered by the tumor cells resulting in molecular changes rendering cancer cells which are resistant to treatments [1,2]. These and many other factors such as reduction of therapeutic activity due to acidic microenvironment associated with hypoxia and the damage caused by the current chemotherapeutic therapies in normal cells are problems faced by patients during treatment, which drive the search for compounds, as well as therapeutic strategies for treating cancer [3].

Phospholipids are present in tissues and there is a special scientific interest in the elucidating of their role in the treatment of cancer. Tumor progression and accompanied adaptive metabolic changes as observed in breast cancers showed high levels of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in comparison with normal breast tissue [4]. The consistent ratios by which tumor cells alter the lipid metabolism are still unknown, but the metabolism of neoplastic cells has been the target for the development of new anticancer strategies. A class of potential synthetic antineoplastic phospholipids such as alkylphospholipids, collectively termed as “ether lipids” represents promising treatment for cancers. Ether lipids have been shown to be able to reduce the synthesis of PC in cancer cells interfering with turnover of phospholipids induced apoptosis. New lipid compounds and analogs to the ether lipids are currently being developed and tested clinical, one of which is Miltefosine (hexadecylphosphocholine), used for control of cutaneous metastases of human malignant melanoma [5,6]. In a

recent study, phospholipids exogenous PE affected HepG2 cell growth through apoptosis and down-regulation of Bcl-2, up regulation of Bax and increased caspase-3 levels [7,8].

In this study, we have evaluated the antitumor activity of synthetic phosphoethanolamine (PHO-S), an amino-ethyl phosphoric ester which was previously synthesized by our group. Therefore, the effects of PHO-S was evaluated using B16F10 melanoma cells as a model for cellular cytotoxicity and *in vivo* using animals induced with dorsal melanoma tumors.

Materials and Methods

Chemical

Amino-ethyl phosphoric ester was prepared according to [9] with purity over 99% analyzed by High-performance liquid chromatography (HPLC). The stock solution 1M was dissolved in water and stored at room temperature for *in vitro* and *in vivo* test.

Cell culture

B16F10 murine melanoma (CLR 6475) and human fibroblast (CCL-75) cell lines were obtained from the American Type Culture Collection (ATCC). B16F10 cells and fibroblasts were grown in RPMI-

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1640 medium, supplemented with 10% inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin and incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Cytotoxicity assay

B16F10 cells and fibroblasts were harvested in exponential phase and seeded in 96-well flat bottom tissue culture plates at a concentration of 1x10⁵ cells/100 µl/well. The cells were allowed to grow for 24 h, and treated with concentrations of PHO-S ranging from 0.005 to 6 mg/ml, in six replicates. The cells were then washed with PBS and allowed to grow in complete medium for 24 h. The cell viability was determined by the MTT (3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay. Briefly, 20 µl of MTT reagent (Sigma-Aldrich, St. Louis, USA) were added to each well at a final concentration of 5 mg/ml, incubated for 4 h at 37°C and centrifuged at 2000 rpm for 10 min. The medium was discarded and 100 µl of dimethyl sulfoxide were added to each well. The optical density was measured in a plate reader (Thermoplate TP Reader, Tokay Hit, Japan) at 540 nm. The 50% inhibitory concentration (IC₅₀) of PHO-S was determined from the dose-effect curve.

Animal studies and treatment regimen

Thirty female Balb-c mice, six to 8 weeks old, weighting approximately 25 g, were maintained in Butantan Institute of animal care, and given water and feed ration *ad libitum*. The animals were divided into four treatment groups: 7 mg/Kg PHO-S, 14 mg/Kg PHO-S, 7mg/Kg Taxol and saline control, and were followed for 50 days. All animal experiments were conducted under a protocol in agreement with ethical principles in animal research, and approved by the ethics committee for animal research of Butantan Institute (process number 566/09).

Inoculation of mice with B16F10 melanoma cells

The B16F10 variant of B16 murine melanoma cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate and 100 U/ml penicillin and 100 µg/ml streptomycin. Adherent cells were detached from plates with 0.1% trypsin and 0.2% EDTA. After trypsin inactivation with 10% FBS, viable cells were counted based on trypan blue dye exclusion method. For the *in vivo* experiments 5x10⁴ cells suspended in 100 µl of PBS were injected subcutaneous (s.c) in the flanks of mice. Ten days after inoculation, the tumors became macroscopically apparent.

Antitumor activity

Mice were inoculated with B16F10 melanoma cells as described above. Treatments were started on day 10, when the tumor implanted in the animals reached a volume of 60–100 mm³. The mice were randomly allocated to groups of ten animals, each group was injected intraperitoneally (i.p.) with one of the following preparations: PHO-S 7 mg/Kg, PHO-S 14 mg/Kg and Taxol 7 mg/Kg and saline for the control group. The tumor sizes were measured three times a week using a caliper-like instrument during the experiment, and converted to tumor weight by the equation: tumor weight = (length² × width)/2. The experiment was ended on day 50, but the survival of the animals in each experimental group was monitored over 180 days.

Determination of tumor growth in mice

Animals were s.c inoculated with 5x10⁴ B16F10 cells (day zero). The tumor volume was estimated on alternate days, from two-dimensional tumor measurements performed with a slide caliper following the above-mentioned formula. Tumor volume of the treated group was compared with that of the control group and the results were expressed

as T/C, where T/C % = (tumor mean in treated group/tumor mean in control group) X 100.

Flow cytometric DNA analysis

B16F10 tumor cells were treated with 1.6 mg/ml mg and 3.3 mg/ml PHO-S and were harvested, washed with PBS and resuspended in 300 µl 0.03 g/l trypsin, 10 mM Tris (pH 8.0). After 15 min incubation at room temperature, 100ul of the neutralization solution (0.5 g/l trypsin inhibitor, 0.1 g/l RNase A and 1.2 g/l spermine) was added and incubation continued for 15 min. After centrifugation cells were resuspended in 300 µl PBS and fixed by the addition of ice-cold ethanol 70%. Prior to analysis, cells were incubated with 1.8µg/ml propidium iodide solution (Sigma, St. Louis, MO) and incubated in the dark for 30 min. Flow cytometric analysis was performed using a FACScan flow cytometry system (Becton Dickinson, San Jose, CA). The DNA content in the cell cycle phases (G₀/G₁, S and G₂/M) was analyzed by the Cell-Quest software and by the ModFit LT software (Becton, Dickinson, NJ, USA).

Obtaining the tumors cells of mice with B16F10 dorsal melanoma

Tumors B16F10 treated with PHO-S and control groups were aseptically removed and placed in cold Hanks' balanced salt solution with 50 units/ml heparin, necrotic tissues were excised and remaining tumor was minced by using a scalpel. The explant was washed in phosphate buffer saline, dissociation of 0.5 g minced tissue was performed in a cold enzyme cocktail of 5 mg collagenase type II, 5 mg collagenase type IV, 10 mg DNase, 5 ml RPMI-1640 for 45 min of constant mixing. The tumor cells suspension was passed through gauze, Hanks' balanced salt solution washed, then washed with PBS without Ca⁺⁺ or Mg⁺⁺. Tumor cells were suspended in FACS buffer (BD) at 10⁵ cells/ml, and labeled with a 1:100 dilution monoclonal antibody to mice CD31 (is expressed constitutively on the surface of adult and embryonic endothelial cells and is weakly expressed on many peripheral leukocytes and platelets), CD34 (expressed on the surface of lymphohematopoietic stem and progenitor cells, small-vessel endothelial cells, embryonic fibroblasts and some cells in fetal and adult nervous tissue), and anti-CD3 (molecules known as the T cell receptor and generate an activation signal in T lymphocytes), were utilized negative control to characterization cell tumor population in flow cytometric analysis. B16F10 tumor cells used were negative for markers CD31, CD34 and anti-CD3.

Assessment of apoptosis by caspase-3

Caspase-3 activity was determined by flow cytometry in cells obtained from dorsal tumors of the groups treated with 7 and 14 mg/Kg PHO-S and control using NucView assay. NucView™ 488 Caspase-3 substrate (Biotium Inc., USA) is a cell membrane-permeable caspase substrate designed for detecting caspase-3 activity within live cells. The substrate is cleaved by caspase-3 in the cytoplasm of apoptotic cells, releasing a fluorescent DNA dye that is able to enter the nucleus and stain the DNA. Briefly, 200 µl of cell suspension were transferred to a flow cytometry tube. Two microliters of NucView™ 488 caspase-3 substrate were added to the cells with or without Z-VAD-FMK, nonspecific fluorescent control (isotype) for pancaspase inhibitors followed by incubation at room temperature in the dark for 15 min. The cells were analyzed using flow cytometry FACScan flow cytometer (Becton Dickinson and Co.) and from dot plots of events recorded in the FL2 channel.

Detection of apoptosis by Annexin V/PI double-staining in flow cytometry

The Annexin V-FITC/PI apoptosis detection kit (BD Bioscience) was used to detect the effects of PHO-S after treatment of the animal and

control. B16F10 cells were collected from dorsal tumor as previously described and centrifuged for 15 min. The pellet was suspended with $1 \times$ binding buffer (100 μ l). The cells were stained with Annexin V (5 μ l) and PI (5 μ l), and incubated for 15 min at room temperature in the dark. After incubation, cells received 400 μ l $1 \times$ binding buffer and then were analyzed by flow cytometry FACScan (Becton Dickinson and Co.) using CellQuest Software, which can determine the percentage of apoptotic cells. PI was excited at 488 nm, and fluorescence was analyzed at 620 nm.

Measurement of Bcl-2, Bax and Bad expression by flow cytometry

The expression of the anti-apoptotic protein Bcl-2 and pro-apoptotic proteins Bad/Bax was measured in the cells obtained from dorsal tumors of the groups treated with PHO-S and control. Briefly, 10^6 cells were incubated with 100 μ l PBS 0.1% triton X-100 on ice for 30 min. Primary antibody anti-Bcl-2, anti-Bad/Bax (Abcam, UK) was then added, and the cells were incubated on ice for 30 min. The cells were washed twice with PBS, and incubated on ice with secondary antibody Alexa Fluor[®] 647 (Invitrogen CA, USA) for 30 min. The cells were centrifuged, washed and the cells were resuspended in 300 μ l PBS. The flow cytometry analysis was performed using a FACScan system (Becton-Dickinson, San Jose, CA).

Histological and histochemical analysis

Tissue specimens were fixed in 10% buffered formalin and routinely processed for paraffin embedding. Sections of 5 μ m were obtained and stained with: Hematoxylin-eosin to assess tissue integrity; Van Gieson to differentiate between collagen and smooth muscle and to demonstrate the increase of collagen; and Picosirius red under polarized light to detect differences in overall connective tissue.

Statistical analysis

Unpaired Student's T test was used to evaluate cell survival and cell growth. Analysis of Variance (ANOVA) was used to assess the flow cytometry results and the growth tumor inhibition curve. All values were expressed as mean \pm SD. In all analysis, $P < 0.05$ was considered statistically significant. The survival time plot (Kaplan-Meier test) and survival comparison between groups were carried out using the Graph Pad Prism statistical software.

Results

Cytotoxic effects of PHO-S on B16F10 melanoma

The results showed that PHO-S was cytotoxic against B16F10 cells at concentrations 1.5 to 6 mg/ml (IC₅₀ 1.4 mg/ml). Normal fibroblasts were not affected (Figure 1). The B16F10 cells showed morphological changes under PHO-S treatment with "blebs" formation, spindle-shape and cell detachment even at non-toxic concentrations as compared to the control cells (Figure 2).

PHO-S inhibits metastasis, increases survival rate and decreases tumor volume

Taxol reduced 60% the tumor volume compared with control, but the reduction of tumor volume in the mice treated with PHO-S was significantly higher in approximately 86% as compared with the control and treated with Taxol. The survival rates were significantly increased in the treated groups and no significant weight loss was observed (Figure 3a and Figure 3b). Untreated mice showed increased number of metastasis 98.5 ± 1.2 in the lung, kidneys, liver and new blood vessels. Treatment with PHO-S 7 mg/Kg and 14mg/Kg resulted in inhibition respectively 5.4 ± 1.7 and 13 ± 1.8 showing a greater efficacy in

reducing the number of metastases compared to Taxol 34.2 ± 1.7 (Figure 4a and Figure 4b).

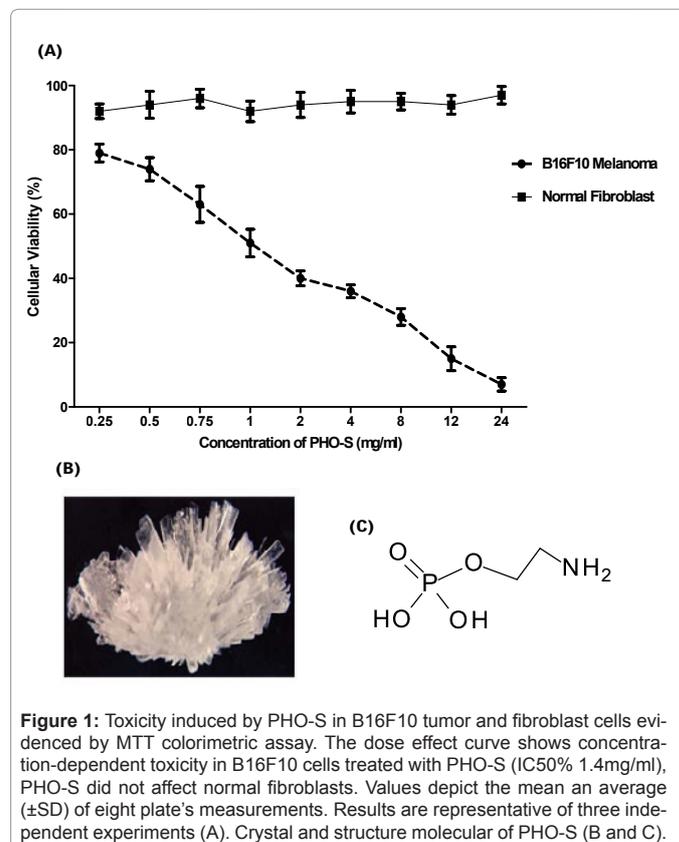
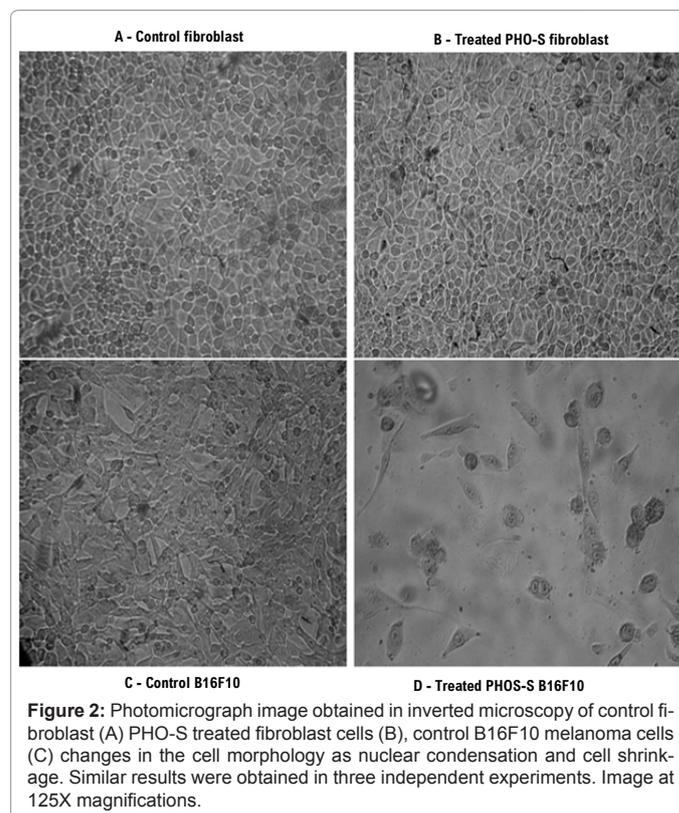


Figure 1: Toxicity induced by PHO-S in B16F10 tumor and fibroblast cells evidenced by MTT colorimetric assay. The dose effect curve shows concentration-dependent toxicity in B16F10 cells treated with PHO-S (IC₅₀ 1.4mg/ml), PHO-S did not affect normal fibroblasts. Values depict the mean an average (\pm SD) of eight plate's measurements. Results are representative of three independent experiments (A). Crystal and structure molecular of PHO-S (B and C).



PHO-S inhibits melanoma cells proliferation and decreases the number of cells in the G2/M phase

Tumor cells treated with PHO-S 3.3 mg/ml reduced their proliferative activity ($P < 0.001$) in G0/G1, S and G2/M phases when compared with the control group. The treatment induced apoptosis as detected in a prominent sub-G1 apoptotic peak. The mice group treated with 1.6 mg/ml PHO-S presented a significant increase ($P < 0.001$) in apoptotic cells and a decrease in cell cycle progression (G0/G1 and G2/M). Quiescent cells significantly decreased ($P < 0.001$) in both concentrations of the treatment (Figure 5a-5c).

The expression of apoptotic and anti-apoptotic proteins were analyzed in tumor cells treated with PHO-S

The relationship of Bax/Bcl-2 binding was measured to determine whether Bax signaling was a possible mechanism for the PHO-S to induce apoptosis. The treatment with both concentrations unaffected the Bcl-2 expression in the control and PHOS-treated groups. PHO-S 7 mg/Kg and 14 mg/Kg induced downregulation of Bax and Bad protein. Since Bax-Bax dimerization is a prerequisite for mitochondrial translocation of apoptotic proteins such as cytochrome c, followed by apoptosis. Suggesting that PHO-S induces apoptosis independent of blockade of Bcl-2 (Figure 6).

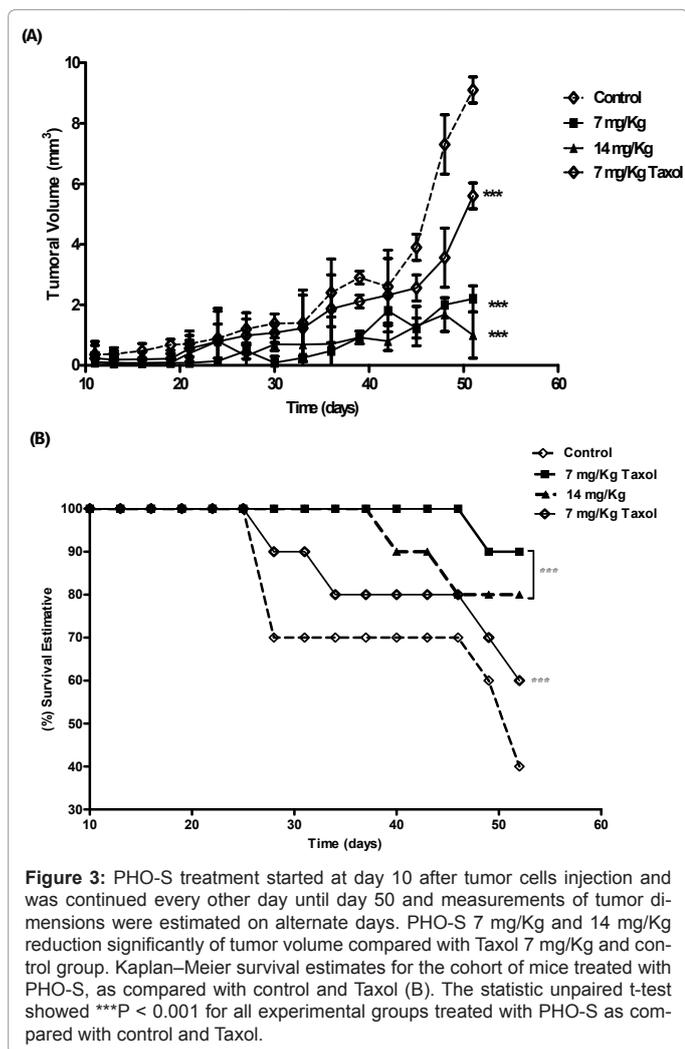


Figure 3: PHO-S treatment started at day 10 after tumor cells injection and was continued every other day until day 50 and measurements of tumor dimensions were estimated on alternate days. PHO-S 7 mg/Kg and 14 mg/Kg reduction significantly of tumor volume compared with Taxol 7 mg/Kg and control group. Kaplan–Meier survival estimates for the cohort of mice treated with PHO-S, as compared with control and Taxol (B). The statistic unpaired t-test showed $***P < 0.001$ for all experimental groups treated with PHO-S as compared with control and Taxol.

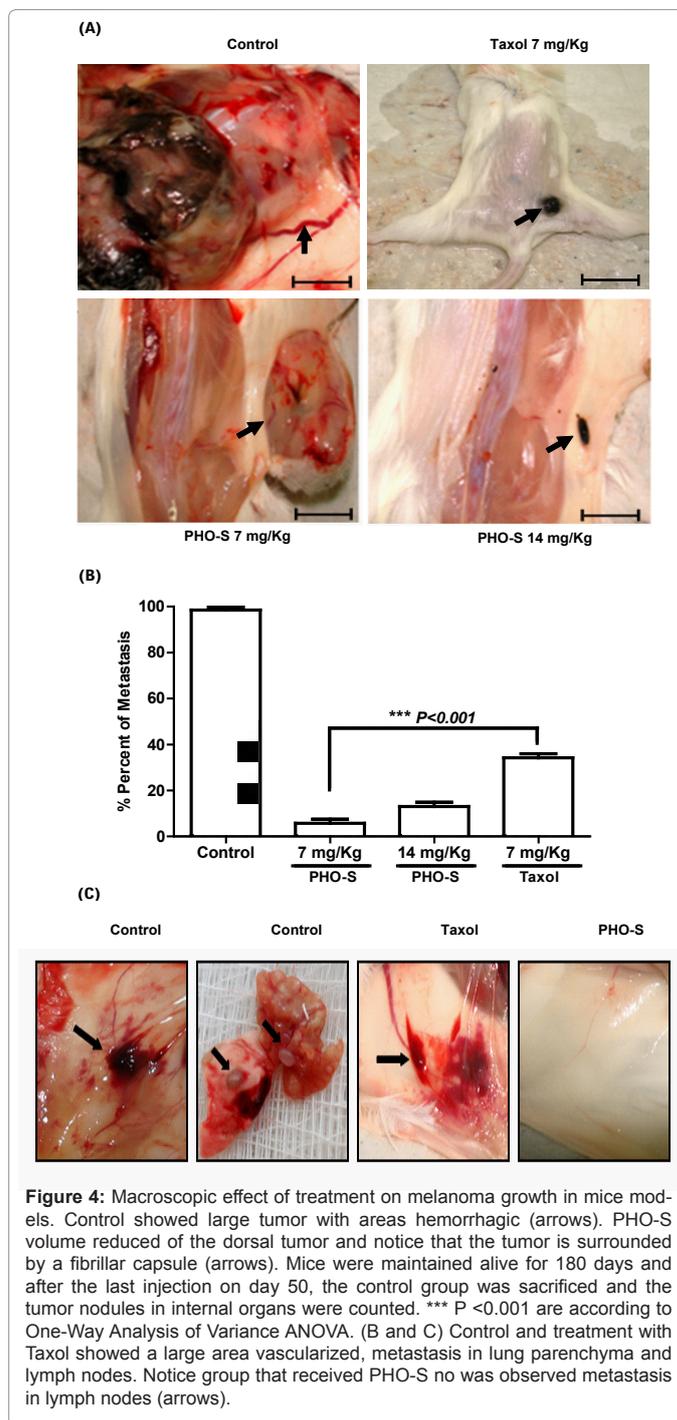
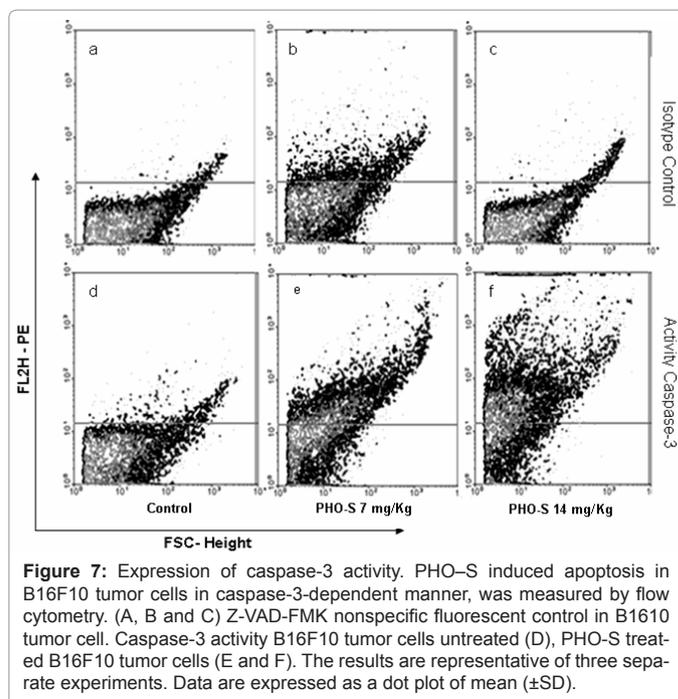
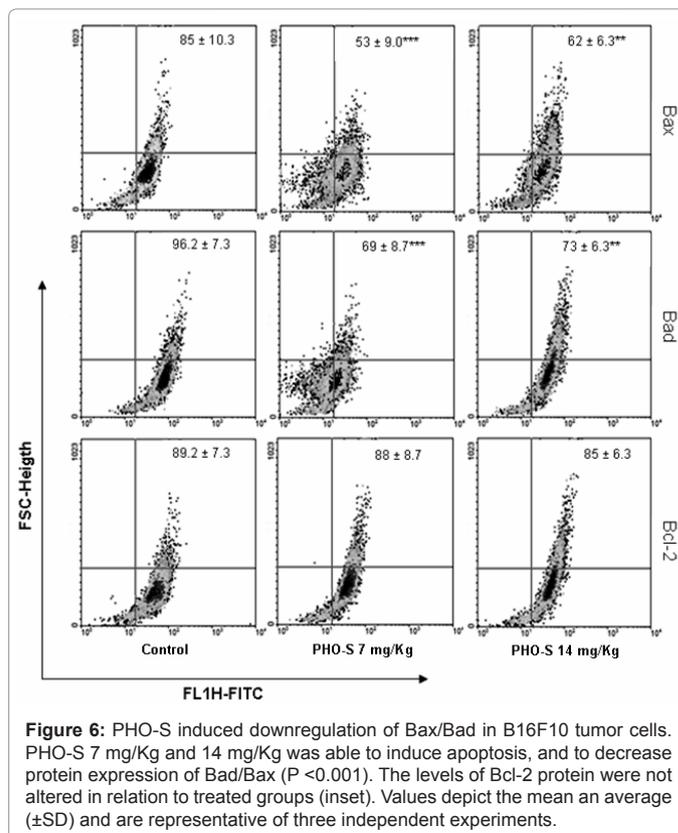
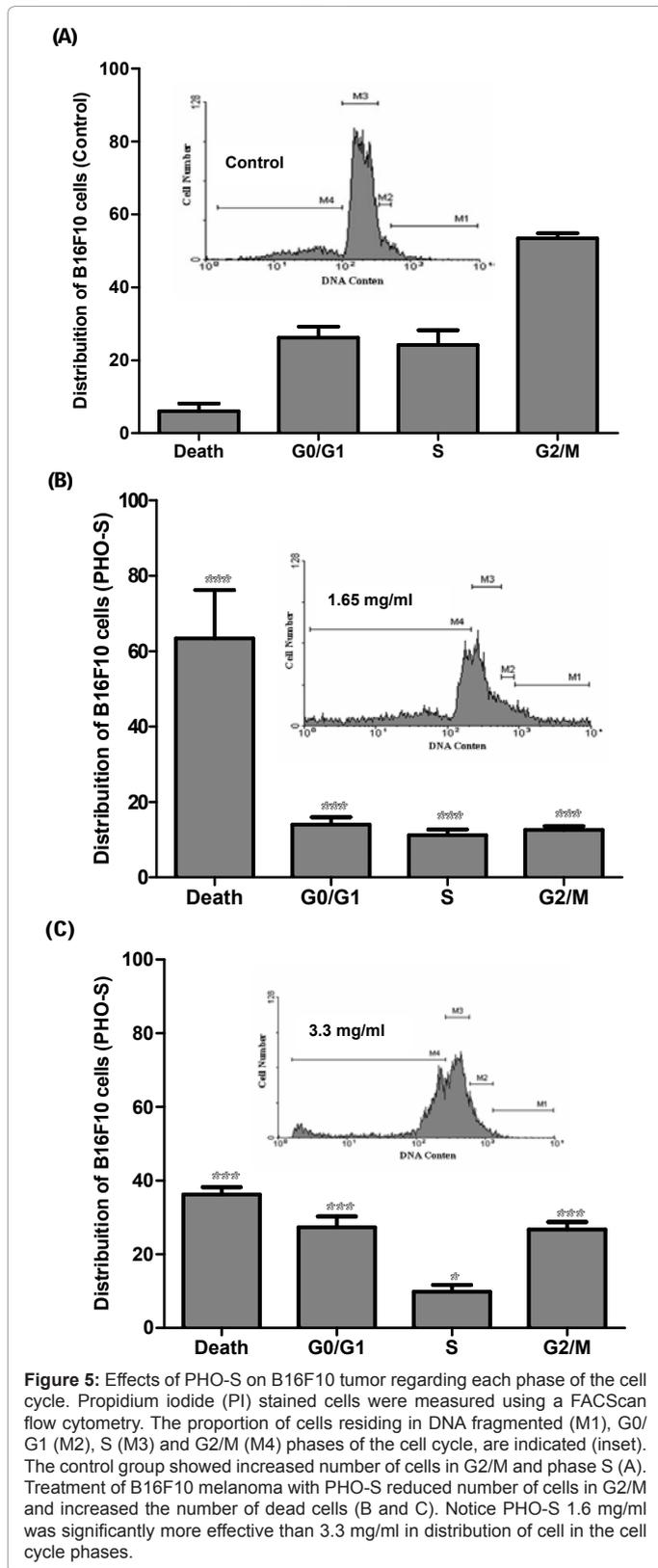


Figure 4: Macroscopic effect of treatment on melanoma growth in mice models. Control showed large tumor with areas hemorrhagic (arrows). PHO-S volume reduced of the dorsal tumor and notice that the tumor is surrounded by a fibrillar capsule (arrows). Mice were maintained alive for 180 days and after the last injection on day 50, the control group was sacrificed and the tumor nodules in internal organs were counted. $*** P < 0.001$ are according to One-Way Analysis of Variance ANOVA. (B and C) Control and treatment with Taxol showed a large area vascularized, metastasis in lung parenchyma and lymph nodes. Notice group that received PHO-S no was observed metastasis in lymph nodes (arrows).

PHO-S induces caspase-dependent apoptosis in B16F10 melanoma

The control B16F10 cells (2.7%) showed nonspecific fluorescent, while 17% were labeled for caspase-3. Cells treated with 14 mg/Kg PHO-S-induced increasing activity of caspase-3 with 45% fluorescent specific showed a statistically significant with 4.7% fluorescent nonspecific. Flow cytometric analysis revealed caspase-3 in the tumor-bearing animals group treated with 7 mg/Kg PHO-S with 4.9% of the tumor cells population with fluorescent nonspecific and 62% with specific fluorescent to caspase-3 active (Figure 7). The cells were stained with two

cell markers, annexin V to measure phosphatidylserine translocation to the extracellular leaflets (early apoptosis) and PI to measure the loss of phospholipid membrane integrity (late apoptosis/necrosis). Treatment



of the B16F10 tumor cells with PHOS-S in all concentrations resulted in 41% ± 2.1 increase of early apoptotic cells and 8.2% ± 0.8 increase of late apoptotic/necrotic cells, compared with controls (10% ± 1.2 apoptotic cells, 21% ± 2.6 necrosis, 7.9% ± 0.9 early apoptotic cells). These data suggest that PHO-S is highly effective in controlling tumor progression by apoptosis (Figure 8).

Histological examination

The control group showed a higher number of metastasis, enhanced cellular pleomorphism with intracellular and extracellular melanin deposition. Dorsal tumors showed increased mitotic figures, newly formed blood vessels, hemorrhagic areas, and rare intratumoral necrosis without inflammatory infiltration. Treatment with PHO-S at either concentration tested decreased irrigation without metastatic and increased necrotic, fibrotic areas with extensive intra-tumor collagen type I and increased number of infiltration neutrophils (Figure 9).

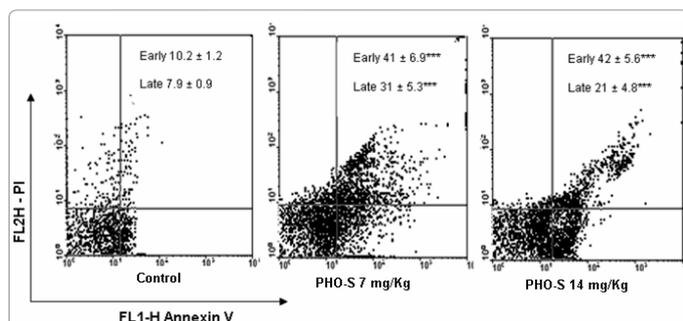


Figure 8: Detection of the apoptotic cells by flow cytometry. Tumors cells from treated animals were stained with annexin V (apoptotic cells) and PI (necrosis). PHO-S induced an increase in the population of apoptotic cell in both concentrations. The results are representative of three separate experiments. Data are expressed as a dot plot of mean (\pm SD).

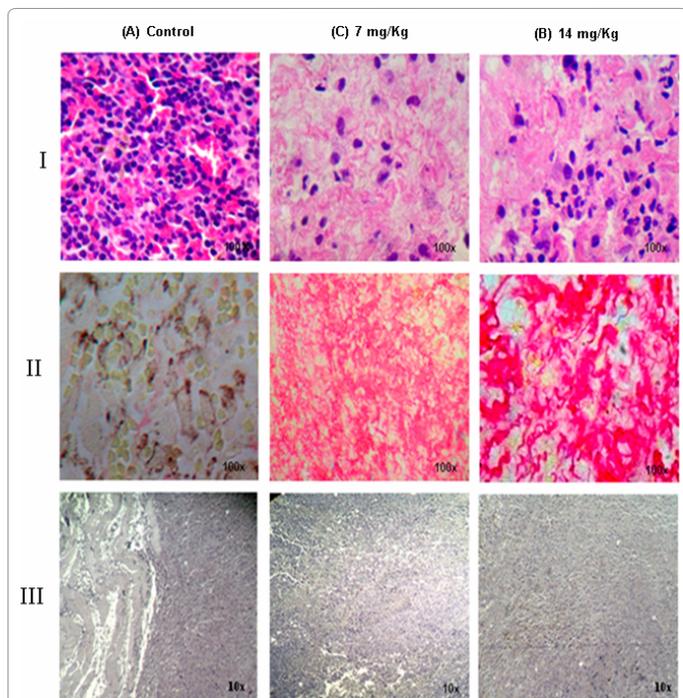


Figure 9: PHO-S inhibition of the melanoma growth in treated mice. Representative photomicrographs of tumor sections stained with hematoxylin eosin. (I) A, B and C PHO-S decreased density of tumor cells the amount of inflammatory cells, the cellular degeneration and fibrosis. The control shows large hemorrhagic areas with numerous tumor cells. (II) A, B and C. Photomicrographs of Sirius Red-stained melanoma tumor sections from treated mice showing a large area of intratumor collagen deposition, not observed in the control. (III) A, B and C Verhoeff-van Gieson stain showed that significant decrease of elastic fibers around endothelial cells was observed in the PHO-S treated groups.

Discussion

Cancer is basically a cell disease, characterized by a loss in the mechanisms which drive the proliferation and cellular differentiation. Melanoma is a kind of cancer which represents about 1% of all tumors and is responsible for 75% of skin cancer deaths. The standard treatment for metastatic melanoma and Dacarbazine is currently the only FDA-approved chemotherapy drug for metastatic stage IV melanoma, but its therapeutic response is poor and only 15-20% of patients with favorable prognosis have increased survival rate [10-12].

The present results showed that PHO-S is cytotoxic and inhibits B16F10 cells growth in a concentration-dependent manner ($IC_{50}=1.4$ mg/ml), and revealed no induced cytotoxicity and morphologic changes in normal human fibroblasts (CCL-75) indicating that compound a potential anti-cancer agent which may produce less toxicity to normal cells.

Solid tumors that present relatively slow cellular divisions, such as lung, colon and breast carcinomas, constitute more than 90% of all the types of human cancer and, for this reason, they are in urgent need for new drugs. In recently released a new guideline for the treatment of metastatic melanoma and Taxol was considered by National Comprehensive Cancer Network (v.1.2009 ME-D) as first-line treatment for melanoma, and is considered highly effective therapy when used in combination with Dacarbazine e IL-2. Based on these new guidelines and the results obtained in this study, the PHO-S was highly effective when compared to Taxol in reducing tumor volume and increased survival rate [13]. Ally the need for developing new drugs for the treatment of malignant tumors such as melanoma the PHO-S is considered as a future agent to combat the malignant tumors. The characteristics of malignant tumors which are the most influent on the development of targets for prognosis of malignant melanoma are aggressiveness multidrug resistance and metastasis remains that an obstacle in cancer therapy [14-16]. Melanoma found to be less than or equal to 1.0 mm are at a lower risk of nodal involvement. In patients with thin melanomas (depth of less than 1 mm), the rate of nodal involvement seemed so low that an elective lymph node dissection was not warranted. For melanomas with depths greater than 4mm, the risk of systemic metastasis seemed to outweigh the potential benefit of elective lymph node dissection [17,18].

In the in vivo study, B16F10 cells of the control group showed spreading nodules in several organs and macroscopic areas of intense neovascularization. These metastases were preferentially distributed in the lung, liver parenchyma and satellite lymph nodes. The reports on the molecular mechanisms involved in the metastatic process led many investigators to look for experimental therapies directed towards inhibiting or regulating one or subsequent steps of the metastatic cascade [19]. In the present study, PHO-S 7 and 14mg/Kg significantly inhibited the number of metastasis compared to Taxol 7mg/Kg. These results suggest that the compound has antimetastatic activities, but complementary studies are required to determine its action mechanisms.

Many chemotherapeutic agents cause cell-cycle arrest but not apoptosis. Different concentrations of PHO-S significantly increased apoptotic cells, suggesting that it is a selective and specific compound in inducing cell death. Flow cytometry analysis indicates that PHO-S induces an increase in quiescent cell phase proportion, a decrease in DNA synthesis phase and in G2/M cells. Membrane phospholipid degradation is rapid when the cells re-enter G1 phase after quiescence. During G1 phase, both the choline pathway and phospholipase-mediated PC hydrolysis are, therefore, at their maximum activity and

are maintained in S phase [20,21]. The increased number of cells in G1 suggests that inhibition of cell proliferation and of G1-S phase transition in PHO-S treated B16F10 melanoma is a result of the activation of caspases, the key effector molecule in apoptosis that are potential targets for pharmacological modulation of cell death.

PHO-S treatment of tumor-bearing mice showed a significant increase in the proportion of cells in apoptosis the caspase-3 pathway, and a decrease in G2/M, indicating that the outcome is apoptotic cell death. Considering the general resistance of melanoma cells to apoptosis, these results emphasize the potential of PHO-S as a new agent for the treatment of cancer.

Imbalance of the Bax/Bcl-2 ratio would render tumor cells which are more resistant to a wide variety of cell death stimuli, including all chemotherapeutic drugs [22-24]. In this study we show that potent PHO-S induced apoptosis via caspase-3 independent the downregulation of Bcl-2 protein or presence of once Bax is released from Bcl-2 inducing Bax-Bax oligomerization protein complex follows and inserts in the outer mitochondrial membrane, causing mitochondrial permeabilization and release of the proapoptotic factors AIF, Smac/Diablo, and cytochrome c.

Histological analysis of B16F10 melanoma tumors in the control group showed intense spreading of the tumor, which is typical of its aggressiveness [25,26]. The tumors of mice treated with PHO-S had predominant infiltration as neutrophils being that entrapped melanoma cells produced and secreted high levels of a cytokine interleukin-8, attracting neutrophils and increasing tethering β 2 integrin expression cellular adhesion molecule-1 on melanoma cells and β 2 integrin on neutrophils interacted, promoting anchoring to endothelium. Transendothelial migration and lung metastasis development decrease in melanoma cells have the potential to decrease metastasis development by disrupting interaction with neutrophils [27]. Histochemical analysis of control group tumors revealed a low density of fibrillar collagen and increase in fenestrated newly formed vessels. In the PHO-S-treated groups, there was intense deposition of collagen type I and pro-collagen fibers around the tumor mass and a decrease in newly formed blood vessels.

In conclusion, PHO-S demonstrated high selectivity and specificity against tumor cell proliferation and dissemination triggered through increased apoptosis caspase-3 pathway and downregulation of Bad/Bax. This study adds to a better understanding of PHO-S action mechanisms and provides a potential tool for melanoma treatment.

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