Meclizine Chloridrate and Methyl- β -Cyclodextrin Associated with Monophosphoester Synthetic Phosphoethanolamine Modulating Proliferative Potential in Triple-Negative Breast Cancer Cells

Manuela Garcia Laveli da Silva, Luciana Bastianelli Knop and Durvanei Augusto Maria

Molecular Biology Laboratory, Butantan Institute, Sao Paulo, SP, Brazil

Abstract: Synthetic phosphoethanolamine (Pho-s) is a monophosphoester ester with anti-inflammatory and pro-apoptotic properties. Meclizine chloridrate (MC) is a histamine H1 receptor blocker that is also able to inhibit cellular respiration. However, MC does not inhibit cellular respiration in isolated mitochondria such as antimycin and rotenone. Methyl- β -cyclodextrin (M β CD) belongs to the β -cyclodextrin family, which is capable of removing cholesterol from the plasma membrane. The aim of this study was to evaluate the proliferative effects of meclizine chloridrate and methyl- β -cyclodextrin compounds associated with synthetic phosphoethanolamine in a triple-negative human breast tumor line, MDA-MB-231. Cell viability of the tumor line and normal cells FN1 was evaluated by MTT colorimetric test; the production of free radicals was determined by lipoperoxidation (LPO) test; and the percentage of cell cycle phases and proliferative index was evaluated by flow cytometry. Cell viability demonstrated a significant decrease with the treatments of M β CD, MC and Pho-s associated with MC. The production of free radicals decreases significantly in all treatments. In addition, a significant increase of DNA fragment and decrease in G0/G1 cell cycle phase were observed in cellular percentage with concentrations of 20 and 30 mM of Pho-s in association with MC and M β CD, respectively.

Key words: Human triple-negative breast cancer MDA-MB-231, synthetic phosphoethanolamine, meclizine chloridrate, methyl- β -cyclodextrin, cell cycle.

List of Abbreviations

MDA-MB-231: Triple-negative breast cancer cells line

TNBC: Triple-negative breast cancer

FN1: normal human fibroblast cells

Pho-s: Synthetic phosphoethanolamine

PLs: Phospholipids

ALPs: alkyl-lysophospholipid analogs

MC: Meclizine chloridrate

M β CD: Methyl- β -cyclodextrin

$\Delta\Psi_m$: Mitochondrial potential

1. Introduction

The clinical and molecular heterogeneity of breast

cancer is well known. The advancement and widespread application of technologies such as genomics, epigenomics, transcriptomics or proteomics provided several new insights into the molecular complexity of this disease [1]. Despite, clinical decisions still depend on the evaluation of three markers: the expression of endocrine receptors for estrogen and progesterone (ER and PGR, respectively) and HER2 expression. The definition of triple-negative breast cancer (TNBC) applies to all tumors that lack the expression of ER, PGR and HER2, which are molecular targets for therapeutic agents. However, chemotherapy remains the first treatment option for early-stage patients, and patients with advanced stage of TNBC [2]. TNBC patients have a worse clinical outcome when compared to results from patients with

Corresponding author: Durvanei Augusto Maria, Ph.D., research field: carcinogenesis. Email: durvanei@usp.br.

other breast cancer subtypes, due to a more aggressive clinical behavior and lack of known molecular targets for therapy. TNBC diagnosis depends on the precise assays of ER and PGR level expression proteins by immunohistochemistry (IHC), and HER2 by IHC and/or fluorescence *in situ* hybridization [3].

The alkyl-lysophospholipid analogs (ALPs) such as edelfosine, perefosine and miltefosinere present a novel class of phospholipids with antitumor activity. ALPs can be classified according to their molecular structure. Alkyl-lysophospholipids and alkylphosphocholines represent a new class of drugs, which differ from classical chemotherapeutic agents. ALPs do not interact directly in DNA, but act on cell membrane by lipid metabolism and signaling pathways [4, 5]. Phospholipids such as ALPs have been shown favorable responses in cancer treatments. ALPs are able to reduce the synthesis of phosphatidylcholine in tumor cells, which influence the turnover of phospholipids that induce apoptosis. Tumor progression and metabolic changes show high levels of phosphatidylcholine and phosphatidylethanolamine in breast cancers in comparison with normal breast tissue [6]. Phosphoethanolamine is a primary amine presented in various tissues at high intracellular concentrations. It has been implicated in several important cellular functions, such as osmoregulation, membrane stabilization and neuromodulation [4, 5].

Synthetic phosphoethanolamine (Pho-s) is a phosphoric ester known as amino ethyl ester phosphoric that was previously synthesized by our group. Studies have shown that treatment with Pho-s presents an antitumor effect on murine melanoma B16F10 cells [7], human breast adenocarcinoma MCF7 [8, 9], leukemic cells [10], without apparent effect on normal cells [8-12].

Meclizine chloridrate (MC) is the first H1 antihistamine piperazine class that has been used for decades against nausea and vertigo. Besides the anticholinergic activity, MC has shown to be a target for constituent androstane receptor (CAR) [13]. Unlike

classic respiratory inhibitors (antimycin and rotenone), meclizine does not inhibit respiration in isolated mitochondria, but in intact cells [14].

Cyclodextrins (CD) are macrocyclic oligomers of α -D-glucose with a hydrophilic exterior and a lipophilic core. CDs are named according to the number of glucose units presented in their structure: α , β or γ -CD if they contain six, seven or eight sucrose rings, respectively. β -CD, methyl- β -cyclodextrin (M β CD), and 2-hydroxypropyl- β -CD are mainly used in membranes' cholesterol manipulation studies (Chol). M β CDs are molecules that are widely used to remove and to carry cholesterol (Chol) from artificial and natural membranes [15].

The aim of this study was to evaluate the proliferative effects of meclizine chloridrate and methyl- β -cyclodextrin compounds associated with synthetic phosphoethanolamine in a triple-negative human breast tumor line (MDA-MB-231).

2. Materials and Methods

2.1 Cell Culture

MDA-MB-231 triple-negative breast cancer cell line was acquired from American Type Culture Collection (ATCC® HTB-26™, Rockville, MD, USA) and maintained in Leibovitz medium (Cultilab, Campinas, SP, Brazil). Medium was supplemented with 2 mM L-glutamine (Cultilab, Campinas, SP, Brazil), 24 mM sodium bicarbonate, 0.01% of antibiotics (Cultilab, Campinas, SP, Brazil), and 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil). Human normal fibroblasts FN1 were used as negative control group. Cells were cultivated in 5% CO₂ atmosphere at 37 °C as monolayers cultures. Cells viability was checked using Trypan Blue exclusion test.

2.2 Colorimetric Assay—MTT

MDA-MB-231 and FN1 cells viability were evaluated using MTT [3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyl tetrazolium bromide] (Sigma Chemical Co., St. Louis, MO, USA) colorimetric assay, which is

based on the reduction of formazan crystals by living cells [16]. Human normal fibroblast cells (FN1) were the control group and the drug action specificity. Tumor and normal cells were incubated in 96-well plates at a concentration 1×10^5 cells/mL and treated with groups described in Table 1. After treatment, the supernatant was collected and 100 μ L of MTT (Calbiochem-Darmstadt, Germany) at 5 mg/mL concentration was added in 96-well plates, and the cells were incubated for 3 h in the dark oven containing 5% CO₂ at 37 °C. Thus, the contents were removed and 100 μ L of methyl alcohol was added to dissolve formed and precipitated formazan crystals. The IC₅₀ value was calculated from the concentration-response curve. Morphological changes induced on cells were observed by inverted light microscopy (Carl Zeiss, Germany), and the images were captured by CCD-IRIS, color video camera (Sony Co.), Cyto Viewer Lite Program.

2.3 Determination of the Production of Free Radicals by Lipoperoxidation (LPO)

The quantification of lipid peroxidation is based on the formation of thiobarbituric acid reactive substances (TBARS), predominantly malondialdehyde (MDA), which occurs after cell membranes lipoperoxidation. These substances produce a characteristic coloration that is measured by spectrophotometer. Cell culture supernatants of MDA-MB-231 and FN1 cells used for MTT methods were collected prior to MTT cytotoxicity test and kept under-refrigerated at -20 °C. Samples were thawed at room temperature, 50 μ L of the sample was added to the wells and 250 μ L of 20% trichloroacetic acid (Sigma-Aldrich) was added in a microtube; 50 μ L of the same sample was added with 250 μ L of thiobarbituric acid 0.86% TBA

(Sigma-Aldrich, Cat.: T550-0). The microtubes were placed in a water bath at 100 °C for 20 min, followed by cooling at 0 °C for 20 min, and 8,000 rpm centrifugation for 4 min, and the supernatant was used for the quantification of TBARS. The reading was performed on the spectrophotometer (Thermo Plate) at wavelength of 535 nm.

2.4 Cell Cycle Phases Analysis

MDA-MB-231 and FN1 cells were treated in groups I, II, III, IV, V, and VI for a period of 24 h. Treated and control cells were trypsinized and centrifuged at 1,400 rpm for 10 min. Then, the pellet was suspended in 70% alcohol solution, RNase alcohol, and stored in the freezer at -20 °C for 24 h. The samples were centrifuged at 3,000 rpm for 10 min and suspended in 200 μ L of Fac's buffer, 20 μ L of triton X-100 (Sigma-Aldrich), and 50 μ g/mL propidium iodide (Sigma-Aldrich), maintained by 30 min at room temperature, protected from light. Afterwards, the samples were transferred to cytometry tubes and had taken for analysis on FACScalibur (BD) flow cytometer at FL-1 fluorescence intensity; the histograms acquired were analyzed by the Cell-Quest-BD program.

3. Results

3.1 Cell Viability Evaluation

MDA-MB-231 and FN1 cells were incubated and treated with groups I, II, III, IV, V, and VI for 24 h. Tumor cells treated with Pho-s were observed under the inverted light microscope and showed morphological changes. It was observed with the appearance of lysis and formation of cell nuclei in the

Table 1 Experimental groups evaluated.

Group I	Negative control—medium culture
Group II	Synthetic phosphoethanolamine
Group III	Meclizine chloridrate
Group IV	Methyl- β -cyclodextrin
Group V	Synthetic phosphoethanolamine + Meclizine chloridrate
Group VI	Synthetic phosphoethanolamine + Methyl- β -cyclodextrin

supernatant from the concentration of 10 mM (IC₅₀% = 20.4mM). Treatment with MC showed a precipitate of the compound in the highest concentration, and morphological aspects of lysis and formation of cellular debris from the concentration of 0.39 mM, as well as a significant reduction in cell density (IC₅₀% = 6.0 mM). The treatment of Pho-s associated with MC showed compound's precipitation at the highest concentrations, morphological aspects of lysis, loss of cytoplasmic extensions and formation of cellular debris from the concentration of 0.39 mM (IC₅₀% = 0.6 mM). The compound M β CD caused decrease in cell density, loss of cytoplasmic extensions and morphological aspects (lysis) with IC₅₀% in value of 1.5 mM. Treatment with Pho-s and M β CD showed significant

cytotoxic effects, such as increasing in cell death and loss of cytoplasmic prolongations (IC₅₀% = 2.5 mM) (Figs. 1 and 2).

The experiment was conducted using human normal fibroblasts FN1 as the control group and as the specificity of drug action. In the treatment with Pho-s, it was observed morphological cellular lysis and debris formation from concentration of 70 mM to obtain IC₅₀% in the amount of 82 mM. Treatment with MC showed morphological aspects: lysis, loss of cytoplasmic extensions and pycnotic cells from the concentration of 20 mM (IC₅₀% = 27 mM). In the treatment using MC plus Pho-s, the aspects in cell morphology were similar to the treatment with Pho-s. However, the IC₅₀% was different in the two treatments,

Fig. 1 Cell viability of MDA-MB-231 tumor cells after 24 h (average values $\pm$ SD). (A) Pho-s; (B) Meclizine chloridrate and Meclizine chloridrate + Pho-s; (C) M β CD and M β CD + Pho-s (GraphPad Prism 5 Software) ($n = 3$, octuplicated).

Fig. 2 Cellular aspects of MDA-MB-231 tumor (control) and treated groups with Pho-s, Meclizine chloridrate, M β CD and their associations after 24 h of treatment (IC₅₀%). Inverted Microscope Light.

with the MC plus Pho-s with 31 mM. Treatments with M β CD compounds and M β CD plus Pho-s showed a precipitate at the highest concentrations (IC₅₀% = 70 and 80 mM, respectively) (Figs. 3 and 4).

3.2 Determination of Free Radicals Production by Lipoperoxidation (LPO)

Lipid peroxidation in cell membranes and the formation of free radicals were quantified by thiobarbituric acid reactive substances (TBARS) method. Malondialdehyde (MDA) is one of the final products of lipoperoxidation formed by the primary or secondary decomposition of intermediate products. MDA reacts with thiobarbituric acid, being widely used in the determination of oxidative stress. The treatment with Pho-s resulted in a significant difference in the formation of lipid peroxidic radicals of MDA-MB-231 breast tumor line. Whereas, the treatment with MC showed a significant decrease in the production of lipid peroxidic radicals, there was no statistical change in the lowest concentration. There was a significant decrease in the production of lipid peroxidic radicals at all concentrations in the treatment with MC plus Pho-s. Similarly, treatments with M β CD

and M β CD plus Pho-s also showed a significant decrease in the production of lipid peroxidic radicals in MDA-MB-231 tumor cells at all concentrations (Fig. 5).

In human normal fibroblast cells, a significant decrease in lipid peroxidic radical production was observed in the treatments with Pho-s, MC, and MC plus Pho-s at the highest concentrations. In the treatment with M β CD, and M β CD plus Pho-s, there was statistical difference at the concentration of 50 mM (Fig. 6).

3.3 Analysis of the Cell Cycle Phases by Flow Cytometry

Pho-s, MC and M β CD were tested for their ability to modify the distribution profile of the cell populations in the cell cycle phases. The concentrations evaluated to determine the populations at different stages of the cell cycle were defined from the calculations of inhibitory concentrations IC₅₀% obtained by cytotoxicity tests—MTT. All treatments showed significant increase of fragmented DNA and decrease of G₀/G₁ phase in tumor cells MDA-MB-231. However, the S phase was significantly different in the treatments with

Fig. 3 Cell viability of FN1 normal cells after 24 h (average values $\pm$ SD). (A) Pho-s; (B) Meclizine chloridrate and Meclizine chloridrate + Pho-s; (C) M β CD and M β CD + Pho-s (GraphPad Prism 5 Software) ($n = 3$, octuplicated).

Fig. 4 cellular aspects of FN1 normal cells (control) and the treated groups with Pho-s, Meclizine chloridrate, M β CD and their associations after 24 h (IC₅₀%). Obtained in Inverted Microscope Light.

Meclizine Chloridrate and Methyl- β -Cyclodextrin Associated with Monophosphoester Synthetic Phosphoethanolamine Modulating Proliferative Potential in Triple-Negative Breast Cancer Cells

Fig. 5 Production of free radicals by lipoperoxidation (LPO) in MDA-MB-231 tumor cells (Average values $\pm$ SD). (A) Phos-s; (B) Meclizine chloridrate; (C) M β CD; (D) Meclizine chloridrate + Phos-s; (E) M β CD + Phos-s. Significance values $p^* < 0.05$ and $p^{***} < 0.01$; ANOVA was obtained using variation of the test set for Turkey test ($n = 3$).

Fig. 6 Production of free radicals by lipoperoxidation (LPO) in FN1 normal cells (average values $\pm$ SD). (A) Phos-s; (B) Meclizine chloridrate; (C) M β CD; (D) Meclizine chloridrate + Phos-s; (E) M β CD + Phos-s. Significance values $p^* < 0.05$ and $p^{***} < 0.01$; ANOVA was obtained using variation of the test set for Turkey test ($n = 3$).

Fig. 7 Cell cycle phases of MDA-MB-231 tumor cells (control) and treated with Pho-s, Meclizine chloridrate, M β CD and their associations (mean $\pm$ SD). Significance values $p^* < 0.05$ and $p^{***} < 0.01$; ANOVA was obtained using variation of the test set for Turkey test ($n = 3$).

Fig. 8 Cell cycle phases of FN1 normal cells (control) and treated with Pho-s, Meclizine chloridrate, M β CD and their associations (mean $\pm$ SD). Significance values: $p^* < 0.05$ and $p^{***} < 0.01$; ANOVA was obtained using variation of the test set for Turkey test ($n = 3$).

the MC and the association MC plus Pho-s. The G2/M phase had no significant change in this treatment (Fig. 7).

Human normal fibroblast cells (FN1) treated with Pho-s at the lowest concentration did not show significant difference in the cell cycle phases distribution. However, it observed an increase of fragmented DNA and decrease of the G0/G1, S and G2/M phases in the treatment with the highest concentration of Pho-s. In treatments with Pho-s, MC, M β CD and their associations, fragmented DNA increased significantly and S and G2/M phases decreased in all concentrations. The G0/G1 phase decreased at the highest concentrations (Fig. 8).

4. Discussion

Cancer is one of the major problems in public health in the world. The number of individuals diagnosed with cancer increases all year with millions of deaths. Cancer is one of the most complicated and complex diseases that humans have already been encountered. Science has been advanced in this field in the last decades, but the pathology increases dramatically in number of cases and death year by year.

Phospholipids are present in many tissues, and a special interesting in the role of them in the cure of cancer is present in current days [17]. Phosphoethanolamine is a precursor of phosphatidylcholine and phosphatidylethanolamine involved in the turnover of cell membrane phospholipids. It takes part in lipid signaling pathways, act as ligands or generate intermediate substrates [18, 19]. The phospholipids such as alkylphospholipids (ALPs) have been shown good results in the treatment of cancers. ALPs are able to reduce the synthesis of phosphatidylcholine in cancer cells, which influences the turnover of phospholipids to induce apoptosis. The tumor progression and metabolic changes as observed in breast cancers showed high levels of phosphatidylcholine and phosphatidylethanolamine in comparison with normal breast tissue [20].

Treatments with Pho-s, MC, M β CD and their associations have been shown to be effective in decreasing the viability of treated human breast tumor cells MDA-MB-231 at different concentrations. The cell viability evaluated after 24 h of treatment showed the reduction of the cellular viability time-concentration dependent. Treatment with Pho-s in human normal fibroblast cells showed cytotoxicity only at the highest concentrations. Several studies have been published showing that the antineoplastic phospholipids act on tumor cell membranes, interfering with turnover of phospholipids in contrast of conventional chemotherapeutic agents. This process occurs due to their stability, in which their linkages are not metabolized and can interfere with signaling lipids, causing apoptosis in malignant tumor cells [21].

Pho-s was cytotoxic to all tumor cell lines studied by our research group: EAT (Ehrlich ascites tumor); B16F10 cells (murine melanoma); MCF7 cells (human breast adenocarcinoma); H292 cells (lung cancer); SKMEL-28 and MEWO cells (human melanoma). Besides that, treatment with Pho-s was not cytotoxic to normal cells such as fibroblasts and endothelial cells [7-12]. The results of cytotoxicity assays indicate that Pho-s promotes their anti-tumor effects through a mechanism that appears to be common to all strains without promoting significant cytotoxic effects on normal cells [10, 11].

MC is a first generation piperazine class of H1-antihistamine that has been in use for decades for prophylaxis against nausea and vertigo. Like many H1-antihistamines, meclizine has anticholinergic activity, and it has also been shown to target constitutive androstane receptors. The androstane receptor is a key regulator of xenobiotic and endobiotic metabolism [22].

MC mechanism was evaluated using overall metabolic profile of meclizine in cells to detect changes in the intracellular metabolites of intermediate metabolism. The metabolic profile revealed a marked increase in the intracellular levels of

phosphoethanolamine (PETN), which is an intermediate in the CDP-ethanolamine (Etn) of the phosphatidylethanolamine (PE) biosynthesis of the Kennedy pathway. Biochemical experiments confirmed the direct inhibition of CTP: cytidyl transferase phosphoethanolamine (PCYT2), a rate-limiting enzyme of the CD β pathway of the Kennedy pathway. Inhibition of PCYT2 results in the accumulation of its substrate, PETN, which directly inhibits mitochondrial respiration. This study identified a novel molecular target of meclizine that binds the CDP-Etn of the Kennedy pathway with mitochondrial respiration [13, 14]. Meclizine inhibits phosphate cytidyltransferase 2 (PCYT2) and causes an increase in cytosolic phosphoethanolamine, an ethanolamine derivative that is a central precursor in the biosynthesis of phospholipids' membrane [13]. Treatment with MC + Pho-s potentiated the antitumor activity of MC compound, decreasing the IC₅₀% concentration by 10-fold in triple-negative breast cells (MDA-MB-231). However, this effect was not observed in human normal fibroblasts cells (FN1).

The cholesterol content of tumor cells is usually increased through up-regulation of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, a regulatory enzyme in cholesterol synthesis, which leads to a coalescence of membrane rafts, and can stimulate cancerogenic pathways [23]. Omega-3 polyunsaturated fatty acids suppress HMG-CoA reductase and have anticancerogenic properties through the induction of cell necrosis or apoptosis [24, 25]. Other well known anticancerogenic lipids that interfere with membrane-raft functions through cholesterol homeostasis are the alkylphospholipids, such as edelfosine [26]. George and Wu [27] suggested that the significance of the membrane-raft composition and integrity for cell viability and proliferation is cell-type specific, due to fine tuning of the signaling pathways that can lead to cell death or cell survival. Therefore, cholesterol-enriched membrane domains are targets for raft-disturbing agents to affect cell

proliferation and viability. Cytotoxic effects of such agents can lead to three forms of cell death at least: apoptosis, autophagic cell death, and necrosis. Cholesterol depletion with methyl- β -cyclodextrin (M β CD), which withdraws plasma-membrane cholesterol, renders melanoma cells susceptible to apoptosis and triggers apoptosis in breast and prostates cancer cell lines, which have abundant membrane rafts [28].

Treatments with M β CD and their association with Pho-s showed similar cytotoxic effect, such as decreased viability and cell density in MDA-MB-231 tumor cells, while there was no significant cytotoxicity in FN1 normal cells.

The lipid peroxidation induced by free radicals is the most widely investigated process. Cellular membrane is one of the components most affected by the high concentration of polyunsaturated fatty acids susceptible to peroxidation [29]. The increase in the formation of reactive oxygen species (ROS) induces to a DNA damage, promoting mutagenic modifications in nucleotides, protein oxidation, and lipid peroxidation, playing an important role in tumorigenesis. ROSs are constantly produced in cells as byproducts of cellular metabolism, performing important functions as mediators of protection mechanisms, such as apoptosis, phagocytosis, and detoxification reactions [30].

There was a decrease in the production of free radicals peroxidic in all treatments with MDA-MB-231 tumor cells. These data corroborate the MTT colorimetric experiment, which shows a decrease in dose-dependent cell viability. However, there was statistical difference in treatments with Pho-s, MC and MC + their associations in normal FN1 cells.

The eukaryotic cell cycle is controlled by a regulatory network, general characteristic that is conserved since yeast to humans. It proceeds through regulated transitions ensuring that specific events are kept in an orderly fashion. The discovery of cyclin-dependent cyclins and kinases (CDKs), elucidation of the underlying mechanisms of

transcriptional control, checkpoint signaling, and the characterization of ubiquitin ligase regulatory pathways have revealed the regulatory principles of the general cell cycle that are shared between eukaryotes [31].

Pho-s has been shown to be effective in reducing DNA synthesis rate and in cell proliferation, which induces cell cycle arrest in the G2/M phase, causing a decrease in cyclin D1 mRNA during the transcription of VEGFR1 gene and in expression of receptor VEGFR1 [7, 10]. A significant decrease in percentage of cells in the G0/G1 phase was observed for all treatments compared to the control group [32]. These data corroborate our results, in which we observed a reduction of cell populations in the G0/G1 phase of cell cycle. Results for the ability to modify the distribution profile of cell populations in cell cycle phases for treatment with meclizine chloridrate were not found in the literature.

5. Conclusions

The results of our study demonstrated that the MC associated with Pho-s treatment showed specificity for triple negative human breast adenocarcinoma cells (MDA-MB-231) when compared to the cytotoxicity obtained in human normal fibroblast cells (FN1) (IC50% ~30 times). A significant decrease in lipoperoxide radical production was observed in the treatments for human normal fibroblast cells using Pho-s, MC and MC plus Pho-s at the highest concentrations. There was a significant reduction in the production of lipoperoxide radicals in MDA-MB-231 tumor cells at all concentrations for treatments with the combination of M β CD and M β CD plus Pho-s. MDA-MB-231 tumor cells showed significant increase of fragmented DNA and decrease of the G0/G1 phase in all treatments on the ability to alter cell populations in the cell cycle phases. Pho-s treated human normal fibroblasts cells did not show significant difference in the cell cycle phases at the lowest concentration. In the treatments with MC, M β CD and their with Pho-s, the fragmented

DNA increased significantly and the S and G2/M phases decreased at all concentrations. The treatments with the associations of MC plus Pho-s showed to be a powerful antiproliferative in tumor cells.

Funding

Fundação de Amparo a Pesquisa do Estado de São Paulo—FAPESP (Process number 2016/15596-4) and Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq (Process number 306124/2015-7).

Conflict of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

MGLS had substantial experimental contributions to conception and designing the study, acquisition, analysis, and interpretation of data; also participated in the draft of paper. LBK contributed in designing, interpreting the data, and in drafting the paper. DAM was the responsible researcher and had substantial experimental contributions to conception, acquisition, analysis and data interpretation, as well as in drafting the paper.

All authors read and approved the final manuscript.

Acknowledgements

Gilberto Chierice for synthesizing the compound used in this study.

References

- [1] Curtis, C., et al. 2012. "The Genomic and Transcriptomic Architecture of 2,000 Breast Tumours Reveals Novel Subgroups." *Nature* 486: 346-52.
- [2] Bianchini, G., Balko, J. M., Mayer, I. A., Sanders, M. E., and Gianni, L. 2016. "Triple-Negative Breast Cancer: Challenges and Opportunities of a Heterogeneous Disease." *Nature Reviews*.
- [3] Keegan, T. H. M., DeRouen, M. C., Press, D. J., Kurian, A. W., and Clarke, C. A. 2012. "Occurrence of Breast Cancer Subtypes in Adolescent and Young Adult Women." *Breast Cancer Research* 14: 55.

- [4] Turner, O., Phoenix, J., and Wray, S. 1994. "Developmental and Gestational Changes of Phosphoethanolamine and Taurine in Rat Brain, Striated and Smooth Muscle." *Experiamcnial Physioology* 79: 681-9.
- [5] Bierhanzl, V. M., Riesová, M., Taraba, L., Cabala, R., and Seydlová, G. 2015. "Analysis of Phosphate and Phosphate Containing Headgroups Enzymatically Cleaved from Phospholipids of *Bacillus subtilis* by Capillary Electrophoresis." *Anal Bioanal Chem.* 407: 7215.
- [6] Morse, D. L., et al. 2009. "Characterization of Breast Cancers and Therapy Response by MRS and Quantitative Gene Expression Profiling in the Choline Pathway." *NMR Biomed.* 22: 114-27.
- [7] Ferreira, A. K., Meneguelo, R., Marques, F. L. N., Randin, A., Mendonça, O., Neto, S. C., Chierice, G. O., and Maria, D. A. 2012. "Synthetic Phosphoethanolamine: A Precursor of Membrane Phospholipids Reduce Tumor Growth in Mice Bearing Melanoma B16-F10 and *in Vitro* Induce Apoptosis and Arrest in G2/M Phase." *Biomedicine & Pharmacotherapy* 66 (7): 541-8.
- [8] Ferreira, A. K., Meneguelo, R., Pereira, A., Mendonça, O., Chierice, G. O., and Maria, D. A. 2013. "Synthetic Phosphoethanolamine Induces Cell Cycle Arrest and Apoptosis in Human Breast Cancer MCF-7 Cells through the Mitochondrial Pathway." *Biomedicine & Pharmacotherapy* 67: 481-7.
- [9] Laveli-Silva, M. G. 2017. "Antitumor Evaluation of Synthetic Phosphoethanolamine and Liposomal Formulation DODAC/Synthetic Phosphoethanolamine in Human Breast Tumor Cells." Master's thesis, University of Sao Paulo. Available from: <http://www.teses.usp.br/teses/disponiveis/5/5160/tde-11052017-142120/pt-br.php>.
- [10] Ferreira, A. K., Santana-Lemos, B. A. A., Rego, E. M., Filho, O. M. R., Chierice, G. O., and Maria, D. A. 2013. "Synthetic Phosphoethanolamine Has *in Vitro* and *in Vivo* Anti-leukemia Effects." *British Journal of Cancer* 109: 2819-28.
- [11] Ferreira, A. K., Meneguelo, R., Pereira, A., Filho, O. M. R., Chierice, G. O., and Maria, D. A. 2012. "Anticancer Effects of Synthetic Phosphoethanolamine on Ehrlich Ascites Tumor: An Experimental Study." *Anticancer Research* 32: 95-104.
- [12] Ferreira, A. K., Freitas, V. M., Levy, D., Ruiz, J. L., Bydlowski, S. P., Rici, R. E., Filho, O. M., Chierice, G. O., and Maria, D. A. 2013. "Anti-angiogenic and Anti-metastatic Activity of Synthetic Phosphoethanolamine." *PLoS One* 8: 57937.
- [13] Gohil, V. M., Zhu, L., Baker, C. D., Cracan, V., Yaseen, A., Jain, M., Clish, C. B., Brookes, P. B., Bakovic, M., and Mootha, V. K. 2013. "Meclizine Inhibits Mitochondrial Respiration through Direct Targeting of Cytosolic Phosphoethanolamine Metabolism." *The Journal of Biological Chemistry* 288: 35387-95.
- [14] Gohil, V. M., Sheth, S. A., Nilsson, R., Wojtovich, A. P., Lee, J. H., Perocchi, F., Chen, W., Clish, C. B., Ayata, C., Brookes, P. S., and Mootha, V. K. 2010. "Nutrient-Sensitized Screening for Drugs That Shift Energy Metabolism from Mitochondrial Respiration to Glycolysis." *Nat. Biotechnol.* 28: 249-55.
- [15] Rudrangi, S., R., S., Bhomia, R., Trivedi, V., Vine, G. J., Mitchell, J. C., and Alexander, B. C. 2015. "Stephen Richard Wicks Influence of the Preparation Method on the Physicochemical Properties of Indomethacin and Methyl- β -Cyclodextrin Complexes." *International Journal of Pharmaceutics* 479: 381-90.
- [16] Mosmann, T. 1983. "Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays." *Journal of Immunological Methods* 65: 55-63.
- [17] Siegel, R. L., et al. 2015. "Cancer Statistics." *Ca Cancer J Clin.*
- [18] Rosanna Cazzolli, et al. 2006. "Phospholipid Signalling through Phospholipase D and Phosphatidic Acid." *IUBMB Life* 58: 457-61.
- [19] Eyster, K. M. 2007. "The Membrane and Lipids as Integral Participants in Signal Transduction: Lipid Signal Transduction for the Non-lipid Biochemist." *AdvPhysiol Educ.* 31: 5-16.
- [20] Lorenzo, V. P., et al. "Theoretical Study of Phosphoethanolamine: A Synthetic Anticancer Agent with Broad Antitumor Activity."
- [21] VanBlitterswijk, W. J., and Verheij, M. 2008. "Anticancer Alkylphospholipids: Mechanisms of Action, Cellular Sensitivity and Resistance, and Clinical Prospects." *Curr Pharm Des.* 14: 2061-74.
- [22] Huang, W., Zhang, J., Wei, P., Schrader, W. T., and Moore, D. D. 2018. "Meclizine Is an Agonist Ligand for Mouse Constitutive Androstane Receptor (CAR) and an Inverse Agonist for Human CAR." *Mol. Endocrinol.* 18: 2402-8.
- [23] Li, Y. C., Park, M. J., Ye, S. K., Kim, C. W., and Kim, Y. N. 2006. "Elevated Levels of Cholesterol-Rich Lipid Rafts in Cancer Cells Are Correlated with Apoptosis Sensitivity Induced by Cholesterol-Depleting Agents." *Am J Pathol.* 168: 1107-18.
- [24] Funahashi, H., Satake, M., Hasan, S., Sawai, H., Newman, R. A., Reber H. A., et al. 2008. "Opposing Effects of n-6 and n-3 Polyunsaturated Fatty Acids on Pancreatic Cancer Growth." *Pancreas* 36: 353-62.
- [25] Sauer, L. A., Blask, D. E., and Dauchy, R. T. 2007. "Dietary Factors and Growth and Metabolism in Experimental Tumors." *J Nutr Biochem.* 18: 637-49.

- [26] Marco, C., Rios-Marco, P., Jimenez-Lopez, J. M., Segovia, J. L., and Carrasco, M. P. 2014. "Antitumoral Alkylphospholipids Alter Cell Lipid Metabolism." In *Anti-cancer Agents in Medicinal Chemistry*.
- [27] George, K. S., and Wu, S. 2012. "Lipid Raft: A Floating Island of Death or Survival." *Toxicology and Applied Pharmacology* 259: 311-9.
- [28] Resnik, N., Repnik, U., Kreft, M. E., Sepčić, K., Maček, P., Turk, B., et al. 2015. "Highly Selective Anti-cancer Activity of Cholesterol-Interacting Agents Methyl- β -Cyclodextrin and Ostreolysin A/Pleurotolysin B Protein Complex on Urothelial Cancer Cells." *PLoS ONE*.
- [29] Sodergren, M. H., et al. 2005. "Carcinosarcoma of the Biliary Tract: Two Case Reports and a Review of the Literature." *Eur J Gastroenterol Hepatol*. 17: 683-5.
- [30] Li, Q., et al. 2006. "A Possible Cooperation of SOD1 and Cytochrome c in Mitochondria-Dependent Apoptosis." *Free Radic Biol Med*. 40: 173-81.
- [31] Bertoli, C., Skotheim J. M., and Bruin, R. A. M. 2013. "Controle da transcrição do ciclo celular durante as fases G1 e S." *Nature Reviews Molecular Cell Biology* 14: 518-28.
- [32] Luna, A. C. L., et al. 2016. "Potential Antitumor Activity of Novel DODAC/PHO-S Liposomes." *International Journal of Nanomedicine* 11: 1577-91.