

Antenor Pereira Bonfim Neto¹, Laertty Garcia de Sousa Cabral^{2,3}, Thalles Anthony Duarte Oliveira^{3,4}, Julio Carlstron Pacheco^{1,2}, Monique Gonçalves Alves^{1,3}, Mercedes Reyes Hernández¹, Rosa Andreia Nogueira Laiso¹ and Durvanei Augusto Maria^{1,2,3}

1. University of São Judas, Sao Paulo, SP 01246-000, Brazil

2. Faculty of Medicine, University of Sao Paulo (FMUSP), Sao Paulo, SP 01246-000, Brazil

3. Laboratory of Development and Innovation, Butantan Institute, Sao Paulo, SP 05503-900, Brazil

4. Faculty of Veterinary Medicine and Animal Science, University of São Paulo (FMZUSP), São Paulo, SP 01246-000, Brazil

Abstract: Antineoplastic phospholipids are a new class of antitumor agents. These molecules interact with the plasma membrane, changing numerous pathways that induce cell death, with high selectivity for cancer cells. A representative of this class of antineoplastic agents is 2-aminoethyl dihydrogen phosphate (2-AEH₂P). It is present in high intracellular concentrations in various tissues and organelles with antitumor, antiproliferative and pro-apoptotic action. Therefore, 4T1 triple-negative tumor cells were treated in different concentrations in order to assess the cytotoxic potential and its effects on the modulation of cell death pathways in association with the chemotherapeutic drug Paclitaxel. 2-AEH₂P promoted cytotoxicity in tumor cells and significant morphological changes, however, it did not cause these effects in normal cells. There was positive regulation of proteins involved in the intrinsic pathway of cell death by apoptosis and regulation of the phases of cell cycle progression. Furthermore, structural and distribution changes in mitochondria, as well as decreased cell density and regression of the cytoskeleton were observed. The 2-AEH₂P demonstrated a modulatory potential of apoptotic pathways inducing cell death, being a new compound with antitumor properties.

Key words: Breast cancer, 2-aminoethyl dihydrogen phosphate, apoptosis, cell cycle.

1. Introduction

Breast cancer occupies the second position in number of cases and, according to GLOBOCAN [1], for 2020, the global estimate is 2,179,457 and, for the year 2040, about 3,059,829 cases. In the female population, breast cancer is also commonly diagnosed (24.2%), being the most frequent in 154 countries and the leading cause of cancer death (15.0%) in 103 countries [2].

The most commonly diagnosed histological type is invasive ductal carcinoma (50-75%), followed by invasive lobular carcinoma (5-15%), and with mixed ductal/lobular carcinomas and other rarer histology constituting the remainder of the diagnoses [3]. The triple-negative subtype constitutes approximately 15% of all breast tumors and is characterized by the absence of expression of the estrogen receptor (ER), progesterone receptor (PR) or human epidermal growth factor receptor-type 2 (HER2) molecular targets [4]. Therefore, this type of tumor has a high recurrence rate between three to five years after diagnosis [5].

Primary tumor cells taken from spontaneous tumors, due to their similarity to breast tumor cells, can be used as an excellent study model [6]. Thus, the 4T1 murine breast carcinoma orthotopic model is ideal for studies of molecular, cellular and pathological bases,

Corresponding author: Durvanei Augusto Maria, Ph.D., research field: pharmacology. E-mail: durvanei@usp.br.

as well as studies of therapeutic strategies [7]. This tumor is highly tumorigenic and invasive, being able to spontaneously metastasize from the primary tumor to distant regions, as in lymph nodes, blood, liver, lung, brain and bone [8-10]. In addition, it has an easy implantation in the mammary gland and metastatic progression to lymph nodes and other organs similarly to triple negative human breast cancer, the human equivalent is MDA-MB-231 [11].

Breast cancer is known for its low response to traditional treatments adopted, such as chemotherapy and radiotherapy, therefore, the development of new therapeutic therapies is urgent. Thus, antineoplastic phospholipids and lipid precursors appear as a new class of antitumor agents. Without causing damage to DNA, these molecules interact with the plasma membrane, altering its update and inducing cell death, with high selectivity for cancer cells [12, 13]. The phospholipid 2-aminoethyl dihydrogen phosphate (2-AEH₂P), present in high intracellular concentrations in various tissues, is related to osmoregulation, neuromodulation and membrane stabilization [14, 15]. In addition, ethanolamine-derived phospholipids are present in the cell membrane as an important structural component, performing regulatory functions in cell division, signaling, autophagy and phagocytosis [16].

Studies carried out by our group on murine B16-F10 melanoma cells with 2-AEH₂P have demonstrated their antiproliferative effects and cell cycle block in S or G2/M phases [17]. The treatment caused antiproliferative effects reducing the Cyclin D1 mRNA as well as the transcription of the vascular endothelial growth factor receptor 1 (VEGFR1) gene and the expression of the VEGFR1 receptor [18]. In acute promyelocytic leukemia cell lines, after treatment with 2-AEH₂P, there was a decrease in the expression CD177⁺ and Gr-17+ in immature myeloid cells bone marrow, spleen and liver [19]. The action of 2-AEH₂P in the mitochondrial intrinsic pathway, promoted apoptotic effects in MCF-7 breast cancer cells, leading to an interruption followed by the

release of cytochrome c. The 2-AEH_2P induced apoptosis independent of Caspase-3 and stopped the cell cycle in phase G1 and decreased expression of cyclin D1 [20].

In the global scenario, speaking of the incidence of breast cancer, as well as resistance and low responses to therapies and non-specific treatments, it is necessary to search for new therapeutic strategies. In the study, therefore, we sought to evaluate the cytotoxicity of $2\text{-}AEH_2P$ and its therapeutic effects, as well as the modulation of the pro-apoptotic pathway in murine 4T1 breast adenocarcinoma cells.

2. Material and Methods

2.1 Compound Preparation Monophosfoester 2-AEH₂P

The monophosphoester 2-aminoethyl dihydrogen phosphate (2-AEH₂P) was obtained from (PhosphoPure®), the pure product was analyzed in plasma by inductive coupling and mass spectrometry.

2.2 Cell Culture

The 4T1 line obtained from the American Type Culture Collection (ATCC® CRL-2539 [™], Rockville, MD, USA) and FN1 human fibroblast cells obtained from patients undergoing blepharoplasty normal dermatological surgery procedures, which were part of the FMUSP project 921/06, was grown in RPMI medium (LGC Biotecnologia, Cotia, SP, Brazil). The medium was supplemented with 2 mM l-glutamine (Cultilab, Campinas, SP, Brazil), 10 mM HEPES (Cultilab, Campinas, SP, Brazil), 24 mM sodium bicarbonate, 0.01% antibiotics and 10% of fetal bovine serum (Cultilab, Campinas, SP, Brazil). The cells were grown in 5% ambient CO₂ at 37 °C as monolayer cultures. The cells were checked for viability using the trypan blue exclusion test.

2.3 Cytotoxicity Assay by the MTT Method

Tumor cells $4T1 (10^5 \text{ cells/well})$ were seeded in 6-well plates and incubated for 24 and 48 h and were treated with concentrations of 2-AEH₂P ranging from

10 to 100 mM, Paclitaxel in concentrations from 0.5 to 8, 5 μ M and Paclitaxel in combination with 2-AEH₂P in concentration at 40 mM. The absorbance quantification was performed in an ELISA reader, and the inhibitory concentration of 50% (IC_{50%}) of 2-AEH₂P was determined from the dose-response curve.

2.4 Analysis of the Potential of Mitochondrial Membrane ($\Delta \mathbb{Z}m$)

The 4T1 tumor cells (10^5 cells/well) were seeded in 6-well plates and incubated for 24 h and treated with concentrations of 20, 40, 60 mM of 2-AEH₂P. After 24 h, the cells were harvested and washed once with phosphate-buffered saline (PBS) and stained with Rhodamine-123 (Rho 123) for 15 min at 37 °C. Then, the cells were washed once with PBS and maintained at 4 °C during measurements. The mitochondrial membrane potential ($\Delta \square$ m) was measured by flow cytometry with FACScalibur (Becton Dickinson). A total of 10,000 cells/samples were analyzed and the average fluorescence intensity recorded.

2.5 Evaluation of Morphological Changes in the Cytoskeleton by Laser Confocal Microscopy

The 4T1 tumor cells were plated in 24 wells (10^5) cells/well) at a concentration of 20, 40 and 60 mM of 2-AEH₂P, for 24 h. After the treatment period, the supernatant was removed and the cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Then, the cells were washed with PBS 3 times for 5 min each. The cell membrane was permeabilized with 0.1% Triton X-100 at room temperature for 15 min. The non-specific binding sites were blocked with PBS+1% BSA + 0.1% (100 µg/mL) at 37 °C for 40 min. After blocking, the cells were incubated with the combined FITC-Phalloidin (2 µg/mL) (Sigma Aldrich Code P5282) for 1 h. The cells were then washed with PBS 3 times for 5 minutes each and incubated with propidium iodide (PI) (20 µg/ml) for 5 min. Following the same cell culture above and the same concentrations, the cells were marked by rhodamine-123 (Molecular Probes, USA), for 15 min at 37 °C. After incubation, excess rhodamine-123 was removed and the cells washed in culture medium. After labeling the fluorochrome, the cells were washed 3 times with PBS for 5 min each under a confocal laser microscope (Carl Zeiss LSM 700; Leica, Mannheim, Germany).

2.6 Analysis of the Cell Cycle Phases

The 4T1 tumor cells were treated with 20, 40 and 60 mM of 2-AEH₂P at a density of 1 x 10⁶ per well for 24 h. The cultured cells were collected and fixed with 70% ethanol/20 mg/mL RNase (Sigma), and stored at -20 °C for 24 h. The samples were centrifuged and resuspended in 200 μ L of Fac's buffer, 20 μ L of Triton X-100 (Sigma-Aldrich), and 50 μ g/mL of propidium iodide (Sigma-Aldrich) maintained for 30 min at room temperature. The DNA content of each sample (10,000 events) was quantified for by flow cytometry, the data were analyzed by CellQuest software (Becton Dickinson) and the data were analyzed by ModFit program.

2.7 Apoptosis and Necrosis Detection Test

For the detection and evaluation of apoptosis, cells were seeded in 6-well culture plates and treated with 20, 40 and 60 mM 2-AEH₂P within 24 h. Cells were collected, washed and resuspended in 100 μ L PBS, followed by incubation. Then, the cells were stained with the apoptosis detection kit Anexina V-FITC/PI (BD Bioscience) and incubated in the dark at room temperature for 15 min. After incubation, 400 μ L binding buffer was added and the cells were analyzed by flow cytometry (FACScalibur, Becton Dickinson) using the CellQuest software to determine the percentage of apoptotic cells with at least 10,000 events per sample.

2.8 Expression of Markers by Flow Cytometry

The 4T1 tumor cells treated with 2-AEH₂P and the

control groups were incubated with 1 μ g of the specific antibodies involved in cell death for 1 h at 4 °C, using cell cycle progression regulators (cytocrome-c, Bax, Bad, Bcl-2, p53, Ki-67, caspase-3, caspase-8 and TNF-DR4). After this period, the cells were centrifuged at 1500 rpm and washed twice with ice-cold PBS and 0.2% BSA. The supernatant was discarded and the pellet was resuspended in 200 μ l of Facs buffer containing 0.1% paraformaldehyde. Read and analysis the expression of receptors on the surface of tumor cells was performed in a flow cytometer FACSCalibur (BD) at FL-1 intensity, and histograms were obtained and analyzed in Cell-Quest (BD). A least 10,000 events were purchased for each sample.

2.9 Synergy Finder 2.0 Analysis of Multiple Drug Combinations

The 4T1 adenocarcinoma cells were treated with phospholipid 2-AEH₂P and chemotherapy paclitaxel at 24 h and 48 h, respectively, to determine the synergistic effect between the drugs. SynergyFinder 2.0 software quantified the degree of synergy of a single drug's doubling effect as if they were acting independently (Bliss). The following higher-order formulations are used to quantify the multi-drug combination effect between 2 drugs:

$$S_{BLISS} = E_{A,B} - (E_A + E_B)$$

Synergy Score =
$$\frac{-\log(p)}{\log(0.05)} x \frac{t}{|t|}$$

3. Results

3.1 2-AEH₂P Has Cytotoxic Activity in 4T1 Triple-Negative Breast Tumor Cells

In order to investigate the cytotoxic effects of 2-aminoethyl dihydrogen phosphate (2-AEH₂P) in 4T1 tumor cells, a MTT colorimetric assay was performed in the periods of 24 and 48 h. In 4T1 tumor cells, 2-AEH₂P produces cytotoxic effects, with IC_{50%} values of 17.4 mM, 2.6 mM and 0.8 mM, in 24 h, 48 h and 72 h respectively. The cells when treated with Paclitaxel showed IC_{50%} values of 6.3 μ M in 24 h, 0.9 μ M in 48 h and 0.5 μ M in 72 h. The association of

Paclitaxel and 2-AEH₂P obtained a result of greater cell cytotoxicity at lower concentrations, with IC_{50%} of 2.4 μ M in the 24 h period and for the period of 48 and 72 h the IC_{50%} was 0.2 and 0.08 μ M respectively (Figure 1B, 1C and Table 1).

After 24 h treatment, 4T1 tumor cells showed significant morphological changes with the presence of cells in abnormal cell division containing aneuploidy. After the treatment of 2-AEH₂P associated with Paclitaxel, groupings of dead and detached cells from the substrate and spherical morphology were observed, as well as the formation of apoptotic bodies. Thus, the cytotoxic effect of 2-AEH₂P on 4T1 tumor cells has been proven and, in association with Paclitaxel, have a marked cytotoxic effect (Figure 1A).

3.2 Evaluation of Mitochondrial Electrical Potential and Cytoskeleton Rearrangement

The changes in the mitochondrial membrane potential were evaluated in the 4T1 tumor cells with the Rodamina 123 probe and showed a significant decrease in the mitochondrial electrical potential after the 24 h treatment with 2-AEH₂P. As well as integrity, the distribution of mitochondria has been altered, preferentially passing from the nucleus to the cytoplasm. In fact, the transfer of mitochondria from the nucleus to the cytoplasm is more accentuated in the higher concentrations of 2-AEH₂P (Figure 2A, 2C and 2D).

It is possible to observe by the staining with Faloidin and Propidium Iodide (PI) that the treatment with 2-AEH₂P promoted a reduction in cell confluence and some cells showed aneuploidy, aberrant cell division and cells showing increased cell volume. It was observed that the control group showed similar morphology, with spindle and mononucleated cells. The tumor cells in those treated with 60 mM became spherical due to themodification of the cytoskeleton, as well as the presence of nuclear fragmentation (Figure 2B).



Fig. 1 Determination of cytotoxicity and morphological changes in 4T1 murine triple-negative breast tumor cells. The cells were treated with different concentrations of $2-AEH_2P$ and the association $2-AEH_2P + Paclitaxel$. (a) Photomicrographs of the morphological analysis of the 4T1 tumor cells treated in the period 24 and 48 h; (b) Line graphs corresponding to cell viability after treatments at different concentrations in 24, 48 and 72 h; (c) The heatmap shows the correlation of the cytotoxic effect expressed in mean \pm SD of the treatments.

Tuble I	Tuble ((Infi 1830%) values for TTT marm	e anpie negative bi	east cancer tanior cens.		
Cell	Treatment	IC _{50%} Value			
		24 h	48 h	72 h	
4T1	Paclitaxel	6.3 µM	1.2 μM	0.5 μΜ	
	2-AEH ₂ P	17.4 mM	2.6 mM	0.8 mM	
	Paclitaxel+2-AEH ₂ P	2.4 µM	0.2 µM	0.08 µM	

Table 1 Table with IC_{50%} values for 4T1 murine triple-negative breast cancer tumor cells.



Fig. 2 Photomicrographs of 4T1 murine triple-negative breast tumor cells and analysis of the mitochondrial electrical potential ($\Delta \Psi m$). (a) Photomicrograph of 4T1 tumor cells with mitochondria marked in green; (b) Photomicrograph of 4T1 tumor cells with nuclei marked in red and cytoskeleton marked in green, analyzed by laser confocal microscopy; (c) Analysis of mitochondrial electrical potential ($\Delta \Psi m$) in murine 4T1 triple-negative breast cancer tumor cells analyzed by flow cytometry; (d) Bar graph showing the $\Delta \Psi m$ expressed as mean \pm SD of three independent experiments analyzed by the ImageJ software. The statistical differences were obtained by the multiple comparison tests ANOVA and Tukey-Kramer. * p < 0.05, ** p < 0.01 and ***p < 0.001.

3.3 The Interference of 2-AEH₂P in the Cell Cycle in 4T1 Triple-negative Breast Tumor Cells

It was possible to observe significant changes in the phases of the cell cycle, when compared to the control group, in 12 h and 24 h, after the treatment of $2-AEH_2P$. In the 12 h treatment period, the lowest concentration caused a reduction in the cell population in all phases of the cell cycle and, in 24 h, an abrupt decrease in the G2/M phase, in the highest

concentration. There was also a notable increase in DNA fragmentation after treatment with 2-AEH_2P , which was potentiated in the treatment of 40 mM, which indicates a higher concentration of dead cells, due to necrosis and apoptosis (Figure 3A and 3B).

3.4 2-AEH₂P Induced 4T1 Triple-negative Breast Tumor Cells to Cell Death by Apoptosis

The 2-AEH₂P monophosphoester induces a

significant reduction in the number of viable cells in all treatment groups compared to control groups. After treatment, the cell population in both initial and late apoptosis increased significantly, at all concentrations. Treatment with 2-AEH₂P increased the population of cells in late apoptosis and, in the concentration above 40 mM, there was a decrease in cell density,and increase in necrosis cells when compared to the control group (Figure 4).



Fig. 3 Analysis of cell cycle phases in 4T1 murine triple-negative breast tumor cells. The cells were treated with 2-AEH₂P at a concentration of 20 and 40 mM, in the period of 12 and 24 h. (a) Distribution profile of 4T1 tumor cells in the cell cycle phases in the period 12 h; (b) Distribution profile of 4T1 tumor cells in the cell cycle phases in 24 h. The bar graphs show the correlation of the effect of the different correlations on the cell cycle expressed as mean \pm SD. The statistical differences were obtained by ANOVA and the Tukey-Kramer multiple comparison test. * p < 0.05, ** p < 0.01, *** p < 0.001 and no significant (ns).



Fig. 4 Cytometric analyzes of the determination of necrosis and initial and late in 4T1 murine triple-negative breast tumor cells. The cells were treated at 20, 40 and 60 mM, in the period 24 h. The statistical differences were obtained by ANOVA and the Tukey-Kramer multiple comparison test. * p < 0.05, ** p < 0.01, *** p < 0.001 and no significant (ns).

3.5 The Reduction of Cell Proliferation by Modulation of Pro-apoptotic Proteins due to the Effects Caused by 2-AEH₂P

We investigated whether caspase 3-8 and the death receptor (DR4) mav be involved in monophosphoester-mediated apoptosis 2-AEH₂P. Thus, it was possible to observe a significant increase in these proteins in 4T1 cells treated with 2-AEH₂P and, mainly, in the concentration of 40 mM (Figure 5A). The pro-apoptotic proteins of the Bcl-2, Bax and Bad family, which are also involved in the permeability of the mitochondrial membrane, were also evaluated. The pro-apoptotic caspases 3 and 8 had a significant increase, while the anti-apoptotic protein (Bcl-2) was suppressed, when compared to the control group, and even more accentuated in the concentration of 40 mM (Figure 5B).

The expression of cytochrome c was also analyzed and it was evident that 2-AEH_2P induced the release of cytochrome c, after 24 h of treatment, a mechanism that occurs during cell death by apoptosis by the intrinsic route. The expression of p53 was also analyzed, which showed an increase mainly in the 40 mM concentration of 2-AEH_2P . When analyzing KI-67, it was evident that associative treatment decreased the proliferation of tumor cells (Figure 5C).

3.6 The Association of 2-AEH₂P with Paclitaxel Potentiated the Antiproliferative Effects

In order to evaluate the associative effects between 2-AEH₂P and the chemotherapeutic Paclitaxel, in 4T1 tumor cells, we used the SynergyFinder2.0 software with the Bliss analysis model. The data obtained by the association demonstrate that Paclitaxel had an additive effect, with a synergy score of 4.486 in 24 h of treatment and 4.931 in treatment after 48 h (Figure 6 and Table 2). This pharmacological association promoted independent cytotoxic effects, with breakdown of the cellular cytoskeleton and alteration of cell structures, such as mitochondria. In morphological analysis, the cells showed spherical conformation and marked cytoplasmic retraction, as well as loss of cell adhesion and decreased cell density (Figure 1A).



Fig. 5 Analysis of marker expression in 4T1 murine triple-negative breast tumor cells. The expression of the markers was quantified by flow cytometry, after 24 h of treatment with the monophospester 2-AEH₂P. (a) Expression of caspase 3, caspase 8 and TNF-DR4; (b) Expression Bcl-2, BAD and BAX; (c) Expression of P53, cytochrome C and Ki-67 depending on the treatments. Bar graphs demonstrate the protein expression level as mean \pm SD of three independent experiments. The statistical differences were obtained by ANOVA and the Tukey-Kramer multiple comparison test. * p < 0.05, ** p < 0.01 and *** p < 0.001.



Fig. 6 Determination of the additive effect of the combination of 2-AEH₂P and Paclitaxelin 4T1 murine triple-negative breast tumor cells. The bar graphs show additive or synergistic additivity of the varied concentrations, in 24 and 48 h, of 2-AEH₂P and Paclitaxelin 4T1 tumor cells. The antagonistic effect is observed in the colored space between white and green (≤ 0), the additive effect and synergism is observed in the colored space between white and red (> 0 and < 10 additive; > 10 synergistic). Color saturation is proportional to the magnitude of the difference between these values.

Table 2	Table showing the combination	ion of drugs and the	synergy score for that	t combination in 24 h and48 h
---------	-------------------------------	----------------------	------------------------	-------------------------------

Tumor Cell	Drug combination	Synergy score	Effect	Method
<i>A</i> T1	Paclitaxel(µM) - 2-AEH ₂ P (mM) 24 h	4.486	Additive	Bliss
411	Paclitaxel(μ M) - 2-AEH ₂ P (mM) 48 h	4.931	Additive	Bliss

4. Discussion

Antineoplastic phospholipids emerge as a new antitumor strategy that acts directly on the plasma membrane, altering its renewal and, consequently, inducing cell death [12, 13]. Therefore, the monophosphoester 2-AEH₂P was shown to be effective in the ability to inhibit the proliferation of 4T1 tumor cells, after 24 and 48 h of treatment. The data obtained corroborate studies published by our group, which 2-AEH₂P showed cytotoxic potential in human breast adenocarcinoma cells MCF-7 and in human chronic myeloid leukemia cells K562 and K562-Lucena MDR+ (Multi drug resistance related to P-Glycoprotein) [19, 20].

Due to the rapid cell division and high proliferation rate, tumor cells have a greater energy requirement than normal cells [21]. Thus, the tumor cells alter their metabolism and these changes try to supply the constant need for nutrients and energy [22]. Therefore, since mitochondria serve as the "potency" of cells, they can be successful targets for tumor cell therapies [22]. These organelles are dynamic and play a central role in the apoptotic process, therefore, the decrease in mitochondrial potential ($\Delta \square m$) is known as a trigger for cell death due to apoptosis and this mechanism may be associated with the intrinsic apoptotic signaling pathway [23]. Analyzing the $\Delta \mathbb{Z}m$ of Ehrlich tumor cells, it showed that the effects of 2-AEH₂P are attributed to its ability to induce apoptosis by reducing $\Delta \square m$ [24]. These data corroborate the results obtained in this work, since 2-AEH₂P induced a similar mechanism in 4T1 tumor cells, reducing $\Delta \square$ m, which may be a key event to trigger signals that will result in cell death due to apoptosis.

The reduction in mitochondrial electrical potential and the increase in pro-apoptotic proteins, linked to membrane permeability are related to the release of cytochrome c by mitochondria [25]. It was possible to observe the increased expression of cytochrome c in 4T1, as well as in other tumor lines after treatment with 2-AEH₂P, reinforcing its role in modulating the intrinsic pathway of apoptosis [19, 20, 26-28].

Most of these apoptotic events are modulated by caspases, which are divided into initiators (8, 9 and 12) and executors (3, 6 and 7), the latter being responsible for morphological changes in the nucleus and cytoskeleton [29]. The 2-AEH₂P increased the expression of caspases 3 and 8 in 4T tumor cells. The activation of different caspases can initiate or amplify the apoptosis processes, depending on the mitochondrial pathway or independent caspases [30]. Results also obtained in other studies corroborate that 2-AEH₂P modulates the intrinsic pathway of apoptosis in response to the activation of caspase 8 [17, 31].

The Bcl-2 protein exhibits an anti-apoptotic effect and Bax and Bad work in the opposite way, being able to induce cell death, therefore being pro-apoptotic proteins [25]. Our results show an increase in Bad and Bax expression and a reduction in Bcl-2 in 4T1 tumor cells after treatment with 2-AEH₂P. These data obtained are convergent to those reported in the literature [32] where 2-AEH₂P increased the expression of Bax and Bad and had a negative regulation of Bcl-2 in human hepatocarcinoma [33] and human breast adenocarcinoma MCF-7 [27].

The p53 protein regulates the S transition to the G2/M phase of the cell cycle, which involves the regulation of cell division cycle 2 (Cdc2 kinase), essential for cells to enter mitosis [32]. Thus, our results showed an increase in p53 expression in the treatment with 2-AEH₂P and similar data were found in squamous cell tumor lines of the oral cavity squamous cell carcinoma (SCC9 and SCC25) [26] and in human breast tumor lines [27] when treated with 2-AEH₂P.

We analyzed the percentage of distribution of tumor populations in the phases of the cell cycle after treatment with 2-AEH₂P and the data show an increase in fragmented DNA and a significant decrease in all other phases of the cycle. The increase in the population of cells in the G2/M phase was also

observed, when treated with 2-AEH_2P , in murine melanoma cells B16-F10 and in breast cancer MCF-7 [17, 20].

The 2-AEH₂P induced cell death by apoptosis and not by necrosis, which was confirmed by the Annexin/PI assay. In this study, 2-AEH₂P increased the percentage of cells in late apoptosis compared to the treated control group. This same compound was tested in other tumor cell lines and obtained results similar to those observed in this work [17, 19, 28]. Therefore, the data indicate that 2-AEH₂P modulates the cell cycle of 4T1 tumor cells by altering the cell proliferation process that is so intense in these tumor cell types.

The cytotoxic effect of 2-AEH₂P associated with the chemotherapy drug Paclitaxel was evidenced, an increase in the antiproliferative potential. The effect of this association was clearly evident in changes in cell morphology, which showed a marked cytoplasmic retraction and regression of the cytoskeleton. Paclitaxel inhibits cell growth and stabilizes the polymerization of microtubules, preventing the cell from replicating and remaining in the G2 phase of the cell cycle [34, 35].

The association with Paclitaxel was considered a potent antineoplastic agent used in several types of tumors, such as pancreas, breast, stomach, ovary and lung cancer [36, 37]. This chemotherapy promotes the formation of exceptionally stable microtubules, inhibiting the normal dynamics of the reorganization of the network formed by microtubules necessary for mitosis and cell proliferation [38]. In addition, Paclitaxel induces the accumulation of cells in the G2/M phases of the cell cycle and leads to apoptosis in sensitive cells [39, 40]. It was observed, in MCF-7 breast cancer cells, treated with this drug, the presence of apoptotic bodies in 24 h of treatment and peaks in 48-72 h [41, 42].

5. Conclusions

The 4T1 triple-negative breast tumor cell, after treatment with the 2-aminoethyl dihydrogen

phosphate monophosphoester, showed selective cytotoxicity and significant morphological changes in cell death due to apoptosis. The association with the chemotherapy drug Paclitaxel, then, which had additive effects, may favor antitumor therapy and mitigate possible adverse effects. In addition, when the cells were treated with 2-AEH₂P, there was positive regulation of proteins involved with the intrinsic pathway of cell death by apoptosis. There was an increase in populations of cells in late apoptosis, as well as structural and distribution changes in mitochondria and a decrease in cell density. In addition, 2-AEH₂P decreased the proteins that regulate cell proliferation, increased fragmented DNA and altered the distribution of the cell cycle phases. Thus, the 2-AEH₂P monophosphoester has been shown to have specificity in tumor cells and plays a modulatory role in apoptotic pathways, thus being a promising antitumor and antiproliferative agent.

References

- International Agency for Research on Cancer, World Health Organization. Cancer Tomorrow. https://gco.iarc.fr/tomorrow/home. Published 2018. Accessed September 26, 2020.
- [2] Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., Jemal, A. 2018. "Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries." *CA Cancer J Clin* 68 (6): 394-424.
- [3] Dillon, D., Guidi, A. J., dan Schnitt, S. J. 2014. 'Chapter 25: Pathology of Invasive Breast Cancer' in Harris, J. R., Lippman, M. E., Morrow, M., Osborne C. K. *Diseases of the Breast*, 5th ed, Wolters Kluwer Health, Philadelphia.
- [4] Denkert, C., Liedtke, C., Tutt, A., von Minckwitz, G. 2017. "Molecular Alterations in Triple-Negative Breast Cancer-the Road to New Treatment Strategies." *Lancet* 389 (10087): 2430-2442.
- [5] Foulkes, W. D., Smith, I. E., Reis-Filho, J. S. 2010. "Triple-Negative Breast Cancer." N Engl J Med 363 (20): 1938-1948.
- [6] Heppner, G. H., Miller, F. R., Shekhar, P. M. 2000.
 "Nontransgenic Models of Breast Cancer." *Breast Cancer Res* 2 (5): 331-4.
- [7] Evangelista, G. C. M., Salvador, P. A., Soares, S. M. A., Barros, L. R. C., da Cunha Xavier, F. H., Abdo, L. M., & Gameiro, J. 2019. "4T1 Mammary Carcinoma

Colonization of Metastatic Niches Is Accelerated by Obesity." *Front Oncol* 9: 685.

- [8] Pulaski, B. A., and Ostrand-Rosenberg, S. 1998. "Reduction of Established Spontaneous Mammary Carcinoma Metastases Following Immunotherapy with Major Histocompatibility Complex Class II and B7.1 Cell-based Tumor Vaccines." *Cancer Res* 58 (7): 1486-93.
- [9] Lelekakis, M., Moseley, J., Martin, T., Hards, D., Williams, E., Ho, P., Lowen, D., Javni, J., Miller, F., Slavin, J., and Anderson, R. 1999. "A Novel Orthotopic Model of Breast Cancer Metastasis to Bone." *Clin Exp Metastasis* 17 (2): 163-70.
- [10] Bazm, M. A., Naseri, L., & Khazaei, M. 2018. "Methods of Inducing Breast Cancer in Animal Models: A Systematic Review." World Cancer Research Journal 5 (4): e1182.
- [11] Pulaski, B. A., Terman, D., Khan, S., Meuller, E., and Ostrand-Rosenberg, S. 2000. "Cooperativity of *Staphylococcal aureus* Enterotoxin B Superantigen, Major Histocompatibility Complex Class II, and CD80 for Immunotherapy of Advanced Spontaneous Metastases in a Clinically Relevant Postoperative Mouse Breast Cancer Model." *Cancer Res* 60 (10): 2710-5.
- [12] Ríos-Marco, P., Marco, C., Gálvez, X., Jiménez-López, J. M., Carrasco, M. P. 2017. "Alkylphospholipids: An Update on Molecular Mechanisms and Clinical Relevance." *Biochim Biophys Acta* 1859 (9 Pt B): 1657-67.
- [13] Ríos-Marco, P., Ríos, A., Jiménez-López, J. M., Carrasco, M. P., Marco, C. 2015. "Cholesterol Homeostasis and Autophagic Flux in Perifosine-Treated Human Hepatoblastoma HepG2 and Glioblastoma U-87 MG Cell Lines." *Biochem Pharmacol* 96 (1): 10-9.
- [14] Turner, O., Phoenix, J., and Wray, S. 1994.
 "Developmental and Gestational Changes of Phosphoethanolamine and Taurine in Rat Brain, Striated and Smooth Muscle." *Exp Physiol* 79 (5): 681-9.
- [15] Lee, S., Kim, J., Shim, G., Kim, S., Han, S. E., Kim, K. et al. 2012. "Tetraiodothyroacetic Acid-Tagged Liposomes for Enhanced Delivery of Anticancer Drug to Tumor Tissue via Integrin Receptor." *Journal of Controlled Release* 164 (2): 213-20.
- [16] Bakovic, M., Fullerton, M. D., and Michel, V. 2007.
 "Metabolic and Molecular Aspects of Ethanolamine Phospholipid Biosynthensis: The Role of CTP: Phosphoethanolamine Cytidyltransferase (Pcyt2)." *Biochem Cell Biol* 85 (3): 283-300.
- [17] Ferreira, A. K., Meneguelo, R., Marques, F. L., Radin, A., Filho, O. M., Neto, S. C., Chierice, G. O., 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." *Biomed Pharmacother* 66 (7): 541-8.

- [18] Ferreira, A. K., Freitas, V. M., Levy, D., Ruiz, J. L., Bydlowski, S. P., Rici, R. E., Filho, O. M., Chierice, G. O., Maria, D. A. 2013. "Anti-angiogenic and Anti-metastatic Activity of Synthetic Phosphoethanolamine." *PloS One* 8 (3): e57937.
- [19] Ferreira, A. K., Santana-Lemos, B. A., Rego, E. M., Filho, O. M., Chierice, G. O., Maria, D. A. 2013. "Synthetic Phosphoethanolamine Has *in vitro* and *in vivo* Anti-leukemia Effects." *Br J Cancer* 109 (11): 2819-28.
- [20] Ferreira, A. K., Meneguelo, R., Pereira, A., Filho, O. M., Chierice, G. O., Maria, D. A. 2013. "Synthetic Phosphoethanolamine Induces Cell Cycle Arrest and Apoptosis in Human Breast Cancer MCF-7 Cells through the Mitochondrial Pathway." *Biomed Pharmacother* 67 (6): 481-7.
- [21] Fadaka, A., Ajiboye, B., Ojo, O., Adewale, O., Olayide, I., & Emuowhochere, R. 2017. "Biology of Glucose Metabolization in Cancer Cells." *Journal of Oncological Sciences* 3 (2): 45-51.
- [22] DeBerardinis, R. J, & Chandel, N. S. 2016.
 "Fundamentals of Cancer Metabolism." *Sci Adv* 2 (5): e1600200.
- [23] Haider, T., Tiwari, R., Vyas, S. P., & Soni, V. 2019.
 "Molecular Determinants as Therapeutic Targets in Cancer Chemotherapy: An Update." *Pharmacol Ther* 200: 85-109.
- [24] Mosmann, T. 1983. "Rapid colorimetric assay for celular growth and survival: Application to Proliferation and Cytotoxicity Assays." *J Immunol Methods* 65 (1-2): 55-63.
- [25] Ferreira, A. K., Meneguelo, R., Pereira, A., Filho, O. M. R., Chierice, G. O., Maria, D. A. 2012. "Anticancer Effects of Synthetic Phosphoethanolamine on Ehrlich Ascites Tumor: An Experimental Study." *Anticancer Research* 32 (1): 95-104.
- [26] Kandelous, H. M., Salimi, M., Khori, V., Rastkari, N., Amanzadeh, A., & Salimi, M. 2016. "Mitochondrial Apoptosis Induced by *Chamaemelum Nobile* Extract in Breast Cancer Cells." *Iran J Pharm Res* 15 (Suppl): 197-204.
- [27] Carvalho, L. K., Higashi, D. 2017. "Evaluation of Pro Apoptotic Effects of Synthetic Phosphoethanolamine and DODAC/FOS Liposomal Formulation in Oral Cavity Squamous Cell Carcinoma Cells." Faculdade de Medicina, University of Sao Paulo. [citado 2020-10-25]. doi: 10.11606/D.5.2017.tde-31072017-132005.
- [28] Laveli-Silva, M. G. 2017. "Antitumor Evaluation of Synthetic Phosphoethanolamine and Liposomal Formulation DODAC/synthetic Phosphoethanolamine in

Human Breast Tumor Cells." Masters' these. University of Sao Paulo. Available from: http://www.teses.usp.br/teses/disponiveis/5/5160/tde-110 52017-142120/pt-br.php.

- [29] de Oliveira Conceição, T., da Silva, M. G. L., Maria, D. A. 2019. "Phosphomonoester Phosphoethanolamine Induces Apoptosis in Human Chronic Myeloid Leukemia Cells." *Journal of Pharmacy and Pharmacology* 7 (7): 434-450.
- [30] Bogliolo, L., Murrone, O., Piccinini, M., Ariu, F., Ledda, S., Tilocca, S., & Albertini, D. F. 2015. "Evaluation of the Impact of Vitrification on the Actin Cytoskeleton of *in vitro* Matured Ovine Oocytes by Means of Raman Microspectroscopy." *J Assist Reprod Genet* 32 (2): 185-93.
- [31] Van Der Luit, A. H., Vink, S. R., Klarenbeek, J. B., Perrissoud, D., Solary, E., and Verheiji, M. 2007. "A New Class of Anticancer Alkylphospholipids Uses Lipid Rafts as Membrane Gateways to Induce Apoptosis in Lymphoma Cells." *Mol Cancer Ther* 6 (8): 2337-45.
- [32] de Lima Luna, A. C., Saraiva, G. K. V., Ribeiro Filho, O. M., Chierice, G. O., Neto, S. C., Cuccovia, I. M., Maria, D. A. 2016. "Potential Antitumor Activity of Novel DODAC/PHO-S Liposomes." *Int J Nanomedicine* 11: 1577-91.
- [33] Mcllawain, D. R., Berger, T., Mak, T. W. 2013. "Caspase Functions in Cell Death and Disease." *Cold Spring Harb Perspect Biol* 5 (4): a008656.
- [34] Ruan, S., Jia, F., Li, J. 2017. "Potential Antitumor Effect of Harmine in the Treatment of Thyroid Cancer." *Evid*

Based Complement Alternat Med doi: 10.1155/2017/9402615.

- [35] Galluzzi, L., Kroemer, G. 2011. "Necroptosis Turns TNF Lethal." *Immunity* 35 (6): 849-51.
- [36] Zhang, S., Han, Z., Kong, Q., Wang, J., Sun, B., Wang, G., ... & Li, H. 2010. "Malignant Transformation of Rat Bone Marrow-derived Mesenchymal Stem Cells Treated with 4-nitroquinoline 1-oxide." *Chem Biol Interact* 188 (1): 119-26.
- [37] Rowinsky, E. K., Donehower, R. C. 1993. "The Clinical Pharmacology of Paclitaxel (Taxol)." *Seminars in Oncology* 20 (4 Suppl 3): 16-25.
- [38] Yamazaki, S., Sekine, I., Saijo, N. 1998. "Paclitaxel (Taxol): A Review of Its Antitumor Activity and Toxicity in Clinical Studies." *Gan To Kagaku Ryoho* 25 (4): 605-15.
- [39] Al-Hajeili, M., Azmi, A. S., Choi, M. 2014.
 "Nab-Paclitaxel: Potential for the Treatment of Advanced Pancreatic Cancer." *Onco Targets Ther* 7: 187-92.
- [40] Kumar, A., Hoskins, P. J., Tinker, A. V. 2015. "Dose-Dense Paclitaxel in Advanced Ovarian Cancer." *Clin Oncol (R Coll Radiol)* 27 (1): 40-7.
- [41] Buzdar, A. U., Singletary, S. E., Valero, V., Booser, D. J., Ibrahim, N. K., Rahman, Z., Holmes, F. A. 2002. "Evaluation of Paclitaxel in Adjuvant Chemotherapy for Patients with Operable Breast Cancer: Preliminary Data of a Prospective Randomized Trial." *Clin Cancer Res* 8 (5): 1073-9.
- [42] Rowinsky, E. K., Donehower, R. C. 1995. "Paclitaxel (Taxol)." N Engl J Med 332 (15): 1004-14.