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Clinical Oncology Translational Study Phase-1 with Antitumor Phosphorylethanolamine (2-AEH$_2$P) in Dogs with Neoplasms

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Abstract: The animal has the potential to serve as a clinical model for human disease, due to striking similarities and homologies in diseases. Early clinical development of a new drug may influence its final destination, and a careful and thoughtful approach to Phase-1 clinical trials is essential. Phosphorylethanolamine (2-AEH$_2$P) is a new cytostatic phospholipid agent that, unlike most chemotherapeutic drugs used today, does not target DNA or cytoskeleton, but act on the cell membrane. Studies carried out by our group have shown several effects of 2-AEH$_2$P controlling the progression of the cell cycle, in the alterations of the mitochondrial electrical potential capable of inducing apoptosis and autophagy in several types of human and animal tumor cells. The aim of this study is to establish the recommended safety dose of a 2-AEH$_2$P monophosphoester, in dogs with tumor following the Phase 1 study model proposed by Fibonacci. The Phase-1 study following the Fibonacci model, 2-AEH$_2$P was safe at all staggered doses up to 150 mg/Kg for 8 weeks. Intravenous administration in staggered doses according to the Fibonacci model, showed that the drug is safe, no advent of mortality during the study period or acute toxicity were observed. It is not a drug with hemolytic properties or that induces anemia. It does not lead to changes in liver and renal functions and was able to modulate leukocyte production. 2-AEH$_2$P is new compound with antitumor potential, being useful for future veterinary and human tumors, as a combination of chemotherapeutic agents.

Key words: Cancer, metabolism, phospholipids, monophosphoester, Fibonacci Model, clinical study.

1. Introduction

The Canine Comparative Oncology and Genomics Consortium (CCOGC), which integrates cancer research between human medicine and veterinary medicine, was established in 2004 to take full advantage of the assembly of domestic canine genomic sequences, which creates new opportunities to investigate canine cancers at the molecular level [1, 2]. The natural occurrence of tumors in companion animals is a fundamental strategy for the study of translational medicine drugs used in clinical trials for this species, and also in human patients.

The dog has been used as an experimental model for the development of new drugs for some time due to the physiological similarities in its anatomy and human systems, as well as its naturalness in the development of neoplastic diseases and its characteristics of progression. Malignant neoplasms in pet dogs are characterized by growth in an intact immune system, cellular tumor heterogeneity, development of recurrent or drug-resistant disease, host and tumor microenvironment, and capacity formation metastasis. Thus, canine cancers captures

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the "essence" of the human cancer problem in a way that other animal model systems cannot [2].

The risk of cancer in canine species and their large population of 52.2 million is an important factor in conducting clinical trials and may even lead to the acquisition of new drugs [2, 3]. Immunotherapy studies in the mid-1980s showed promise for the treatment of canine melanoma, and DNA vaccines were evaluated in the mid-2000s to assess their transformational potential in human patients [4, 5].

The early clinical development of a new drug may affect its final destination, so a cautious and thoughtful method to conduct Phase I clinical trials is essential. Each clinical trial has very specific qualifications, from the type and age of participants to life expectancy, weight, and, of course, cancer type. Phase I clinical trials in human and veterinary oncology are typically small, sequential studies involving patients with good hematology and metabolism who have progressed despite the possibility of conventional therapy. The aim of this study is to determine a new recommended safe dose for monophosphate, ethanolamine phosphate or 2-aminoethyl dihydrogen phosphate (2-AEH$_2$P) a lipid precursor.

The 2-AEH$_2$P is the basic structural component of phospholipids in cell membranes. They are essential molecules in play regulatory roles in cell division, signal transduction, activation, adhesion, autophagy and phagocytosis [6, 7].

Most 2-AEH$_2$P is distributed on the plasma membrane protoplasm surface together with phosphatidylserine, while choline containing phospholipids and sphingolipids are mainly distributed on the outer side of the membrane [8].

These anti-tumor phospholipids are easily inserted into the lipid bilayers of cell membranes. Thus, tumor cell proliferation is interfered with through complex mechanisms involving phospholipid and cholesterol metabolism. In addition to lipid dependent survival signaling pathways and autophagy changes. Although antitumor phospholipids also have antiparasitic, antibacterial, and antifungal effects, in this review, we provide a summary of the antileishmaniasis activity of these lipid analogues [9].

This study showed that 2-AEH$_2$P could induce cytotoxicity in tumor cells of murine B16-F10 melanoma and human SK-MEL-28, triple-negative breast cancer MDA-MB-231, human and murine 4T1, mammary carcinoma MCF-7, chronic myeloid leukemia cells K562 and K562 Lucena MDR+, but had no significant effect on normal cells, such as fibroblasts, lymphocytes, macrophages, endothelial cells and smooth muscle. It is effective in reducing the capacity of DNA synthesis, as well as inhibiting cell proliferation by arrested in the G2/M cell cycle phase. Changes related to pro-apoptotic effects reflect increased expression of active forms of caspase-3, 8, and 9 [10-21].

In this study, Fibonacci's model was used to treat tumors in dogs with 2-AEH$_2$P to evaluate the extent of clinical safety Phase-1 at a staggered dose. The design of Phase-1 trials is usually divided into rule-based dose escalation method and model-based dose escalation method. Escalation in rule-based methods is determined according to toxicity: the '3+3' design in which a cohort of three patients received an increased dose of the drug. For a given patient cohort, the dose increment increases until a predetermined endpoint is reached, usually the incidence of dose-limiting toxicities (DLTs).

2. Materials and Methods

2.1 Study Design

Phase I study was approved by the relevant Veterinary Medicine and Animal Science Faculty, Sao Paulo University, Ethical Committee (Process number CEUA 6260100718). All participating dog owners signed an informed consent document. This was a Phase I, single-center, open-label study designed to establish the maximally tolerated dose (MTD) and assess the overall safety of 2-AEH$_2$P that was administered on days 1, 8, 15 and 21. If treatment was

well tolerated, animals were allowed to receive repeated 28-day treatment cycles until the occurrence of progressive disease or toxicity.

The starting dose is the extrapolation of preclinical animal toxicity data, and the ascending dose was predetermined based on the Fibonacci sequence [22]. For this research, 12 dogs with only owners were used, namely patients from Estima Veterinary Hospital Granadeiro Guimarães, Taubaté, Sao Paulo and Luther King Veterinary Clinic, Sao Paulo (Table 1). The trial was conducted using a '3+3' design and treated at least three patients each dose level:

Cohort I dose: 30 mg/Kg weekly (4 weeks), n = 03
Cohort II dose: 60 mg/Kg weekly (4 weeks), n = 03
Cohort III dose: 100 mg/Kg weekly (4 weeks), n = 03
Cohort IV dose: 150 mg/Kg weekly (4 weeks), n = 03

2.2 Experimental Model Study (Figure 1)

2.3 Compound Preparation Monophosphoester 2-AEH$_2$P

The 2-AEH$_2$P was obtained from PhosphoVET®, the pure product was analyzed in plasma by inductive coupling and mass spectrometry. The 1M stock solution was diluted in water, pH 7.2. It was stored at 4 ℃ temperature and diluted in sterile phosphate saline and filtrated in sterile 0.22 μm membrane. Each dog of the different treatment groups received 2-AEH$_2$P in the recommended dose, intravenously, pH 7.2, diluted in a closed system bag with 100 ml of sterile 0.9% saline solution, following 20 min. These applications were performed with an interval of 7 days for a period of 4 weeks

2.4 Effect in vitro of Monophosphoester 2-AEH$_2$P in Dog Culture Cells

The primary canine osteosarcoma, adenocarcinoma mammary dogs and fibroblasts cell lines were routinely cultured in Modified Dulbecco's Medium or RPMI supplemented with 2 mM L-glutamine and 5% (v/v) FCS and maintained at 37 ℃ in 95% humidified atmosphere, containing 5% CO_2.Cells were plated in 96-well at a concentration of 1×10^4 cells per well.

Table 1 Description of selected patients diagnosed with malignancy.

Patient	Cohort	Concentration (mg/kg)	Genre	Breed	Age (Years)	Weight (kg)	Histopathology
1	1	30	Female	Poodle	15	6.4	Tumor 1: Grade II solid type mixed breast tumor carcinoma; Tumor 2: Ulcerated extramedullary skin plasmacytoma Tumor 3: Mast cell lymphatic metastasis
2	1	30	Female	Shih-tzu	9	5.2	Grade II carcinoma
3	1	30	Female	MB	13	8.5	Grade I ductal breast carcinoma
4	2	60	Male	MB	11	18.7	transition cell carcinoma
5	2	60	Female	Poodle	13	11.4	transition cell carcinoma
6	2	60	Female	MB	11	33.3	Noninvasive papillary carcinoma
7	3	100	Female	MB	9	17.2	Grade III cribriform carcinoma
8	3	100	Male	Boxer	5	28.0	Grade II ulcerated squamous cell carcinoma
9	3	100	Female	Poodle	9	10.5	Tumor 1: Grade II solid type breast carcinoma Tumor 2: Grade I breast carcinoma
10	4	150	Female	MB	13	7.2	Tumor 1:Melanic melanoma Tumor 2: Lymphatic metastasis of melanoma
11	4	150	Female	Golden retriever	12	29.0	Tumor 1:Micropapillary carcinoma Tumor 2: Nodal metastasis from micropapillary carcinoma
12	4	150	Female	Pitbull	10	26.0	Tumor 1:Melanic melanoma Tumor 2: Mixed benign Tumor 3: Grade I tubulopapillary carcinoma Tumor 4: Grade I soft tissue sarcoma

MB = Mixed Breed

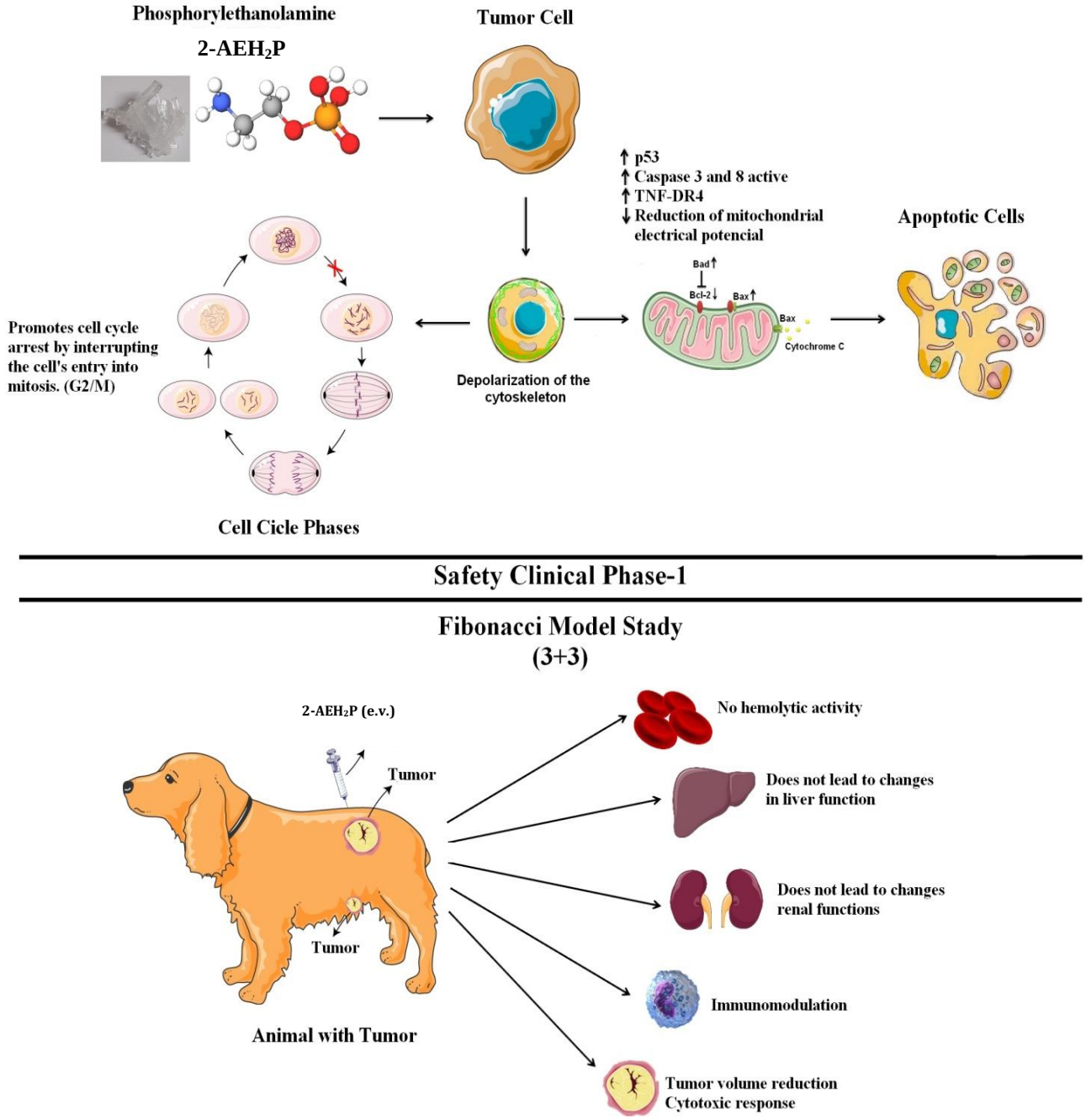

Fig. 1 Representative scheme of the experiment carried out.

The cells were allowed to grow for 24 h then treated with at 2-AEH$_2$P concentrations ranging from 5 to 100 mM, in six replicates. After 24 h of treatment, cell viability was determined by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Sigma, St Louis, MO, USA). Briefly, 20 µl of MTT solution were added to each well (final concentration, 0.5 mg. ml^{-1}) and the plate was incubated for an additional 4 h. Finally, 200 µl of dimethyl sulfoxide (DMSO) were added to each well and the absorbance was measured in a plate reader (Thermoplate TP Reader, Gendoji-cho, Fujinomiya-shi Shizuoka-ken, Japan) at 540 nm. The 50% inhibitory concentration (IC_{50}) values were calculated using a proper analysis (nonlinear regression-curve fit).

2.5 Sample Collection for Hematological and Biochemical Analysis

Blood samples from dogs from the different 2-AEH$_2$P treatment groups (Group-1 30 mg/kg; Group-2 60 mg/kg; Group-3 100 mg/kg and Group-4 150 mg/kg), were obtained by puncture of the jugular vein after local asepsis and 10 mL whole blood volume in the presence or absence of ethylene diamine tetraacetic acid (EDTA) anticoagulant, collected and separated plasma and serum fractions, stored at -20 ℃ for further processing. Hematological counts were performed on automated veterinary equipment from Hematology Analyzer IDEXX, ProCyteDx® and biochemical analyzes on a dry chemistry apparatus, Biochemical Analyzer Catalyst One®. Biochemical tests (urea, creatinine, alanine aminotransferase (ALT), asparate aminotransferase (AST), alkaline phosphatase (AF), calcium, albumin, globulin, total bilirubin, total protein, sodium, potassium, phosphorus, and glucose) were performed for all patients at the beginning of treatment and 1 week after the end of treatment.

2.6 Lipid Peroxidation (LPO)

The oxidative stress of membrane unsaturated lipids was assessed by measuring the content of malondialdehyde (MDA), an end product of fatty acid peroxidation that reacts with thiobarbituric acid (TBA) to form colored complexes. The thiobarbituric acid reaction substances (TBARS) were quantified by spectrophotometric determination (LPO method) [23]. The content of LPO in the supernatant of samples after and before irradiation was determined by MTT method.

3. Results

3.1 Evaluation of the Cytotoxic Effects of 2-AEH$_2$P in Tumor Cell Lines by MTT Assay

The results showed that the 2-AEH$_2$P had no effect on the activity of normal fibroblasts. However, after 24 h of treatment, 2-AEH$_2$P exhibited potent cytotoxic activity against all tumor cells used in this study, among which the killing effects on canine primary osteosarcoma and canine breast adenocarcinoma cells were the best, with IC_{50}% values of 7.93 mM and 8.01 mM, respectively (Fig 2).

3.2 Hematological Analysis

In this study, the analysis of the red series, included red blood cells (RBC) quantitation, hematocrit, hemoglobin dose, and blood volume index: (MCV-Mean Corpuscular Volume; MCH-Mean Corpuscular Hemoglobin; CMCH-Mean Corpuscular Hemoglobin Concentration, and red blood cell distribution width-RDW), microscopic examination of erythrocyte morphology. These together provide support for the diagnosis of the main causes of anemia or other myelodysplastic changes. Analysis of the total number of red blood cells over the 8 weeks of treatment in all animals treated with 30, 60, 100 and 150 mg/kg showed no significant differences, with compared initial treatment (Fig 3). The drug does not cause hemolysis.

The changes of the total number of leukocytes were observed 3 to 6 weeks after 2-AEH$_2$P administration (Fig 4). In group 2 receiving 30 mg/kg 2-AEH$_2$P, a decrease in leukocytes counts and significant changes in the absolute numbers of neutrophils, lymphocytes and eosinophils were observed at week 7 and 8 (Fig 5, 6 and 7). In Group 3 of animals receiving 100 mg/kg, an increase in leukocyte numbers was observed, with deviation in the amount of neutrophils, lymphocytes, and monocytes. No eosinophilia was observed after treatment. Animals receiving 150 mg/kg showed a decrease in the number of total leukocytes compared to the initial weeks of treatment, followed by an increase in the number of neutrophils and lymphocytes. In this group there was a significant percentage increase in eosinophils.

Tumor-induced platelet activation and coagulation does not only constitute a significant risk for thrombosis, but also contribute to tumor progression

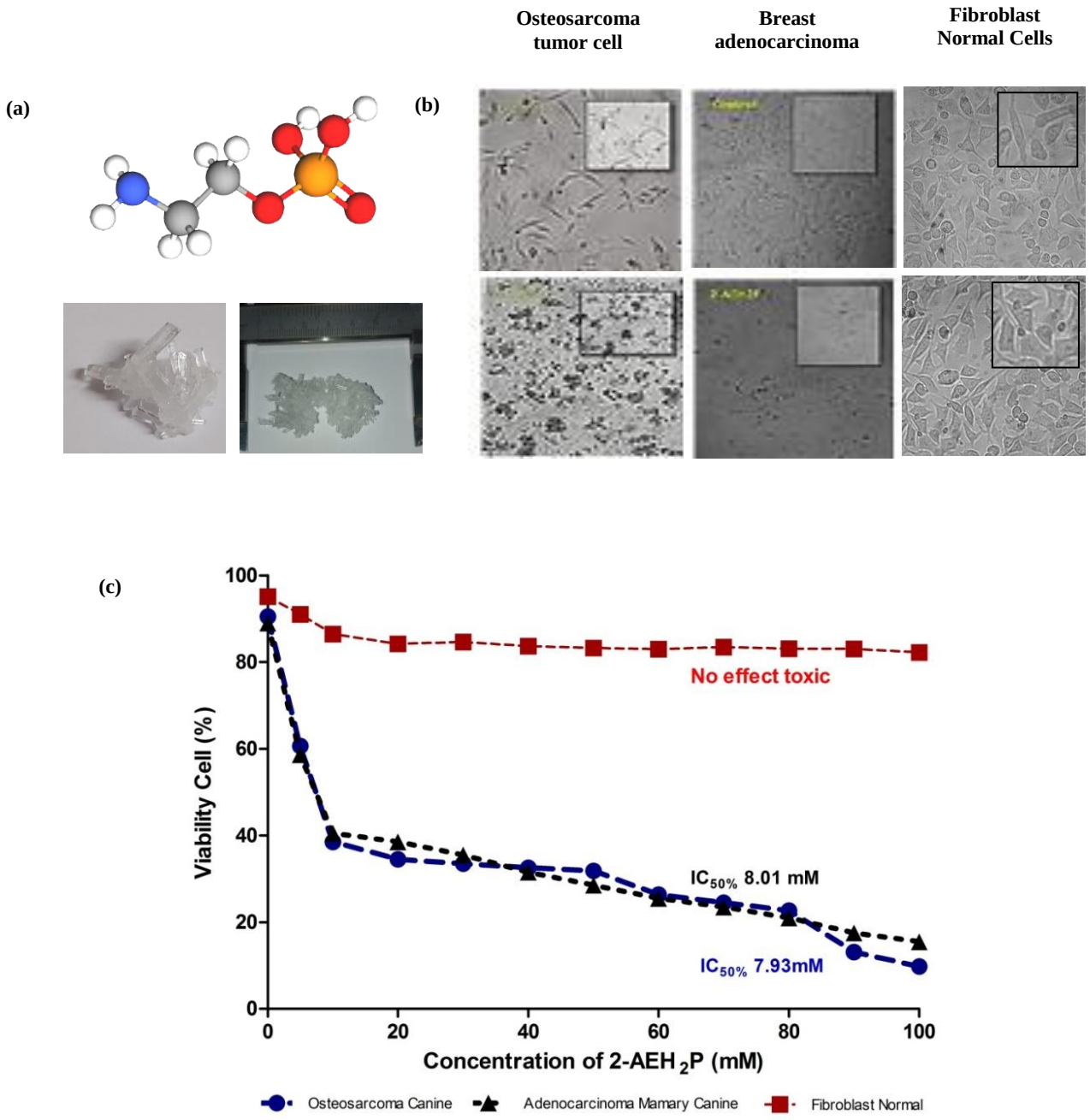

Fig. 2 Determination of cytotoxicity by the MTT colorimetric assay and cytological sends. The tumor and normal cells were treated with different concentrations of 2-AEH$_2$P. (a) 2-AEH$_2$P molecule and crystal formation after purification; (b) Photomicrographs of osteosarcoma cells, mammary adenocarcinoma and normal fibroblast; (c) Line graph shows the correlation of the cytotoxic effect expressed as mean value ± SD of three independent experiments.

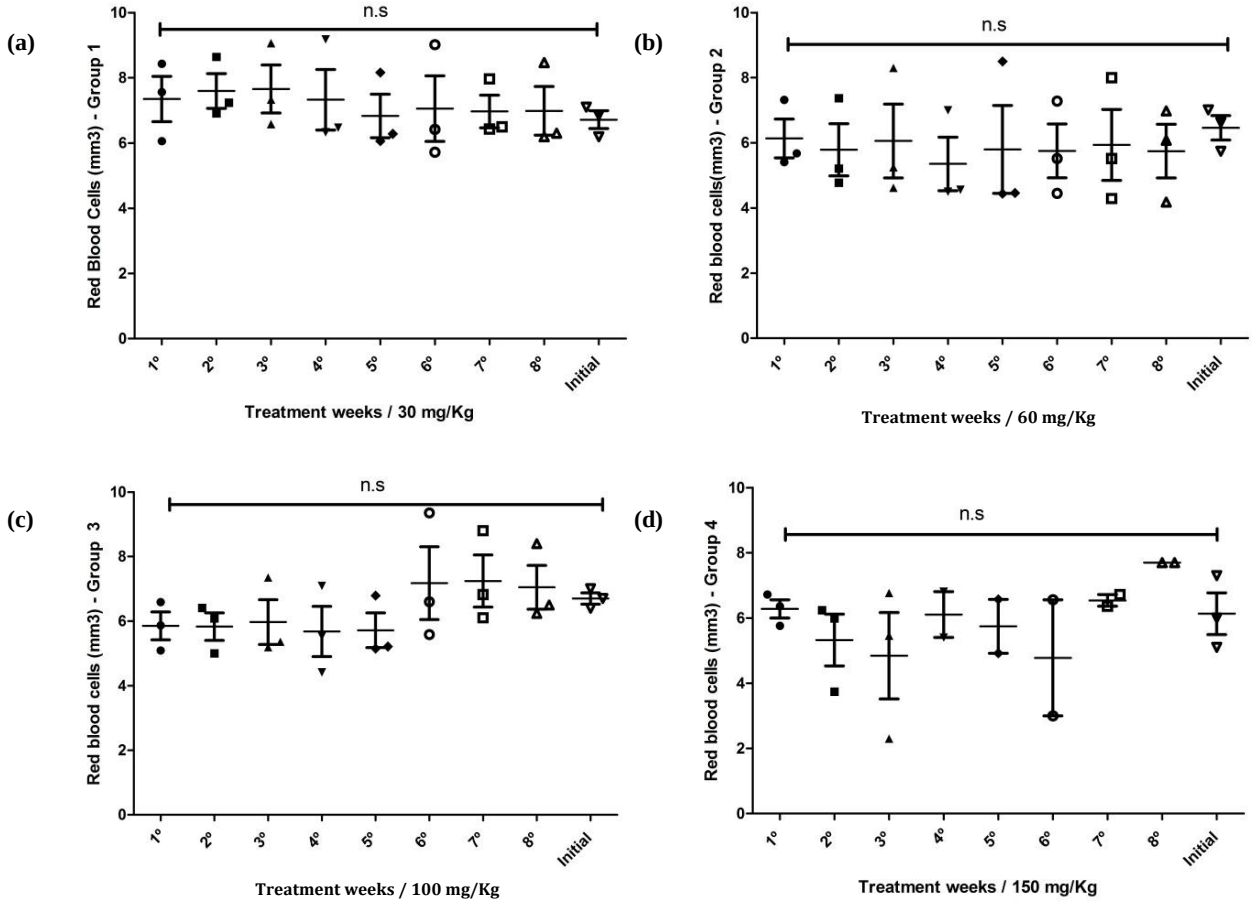

Fig. 3 Analysis of the total number red blood cells treated dose escalation from 30 to 150 mg/Kg for 8 weeks with injectable solution of 2-AEH$_2$P in dogs with neoplasms. (a) Mean value and ± SD of dogs treated 30 mg/Kg; (b) Mean value and ± SD of dogs treated 60 mg/Kg; (c) Mean value and ± SD of dogs treated 100 mg/Kg; (d) Mean value and ± SD of dogs treated 150 mg/Kg. The different mean value ± SD data were statistically compared by the test t Student and Anova Variance methods followed by the Tukey-Cremer multiple test. n.s = There was no significant statistical difference between different weeks and the initial study value.

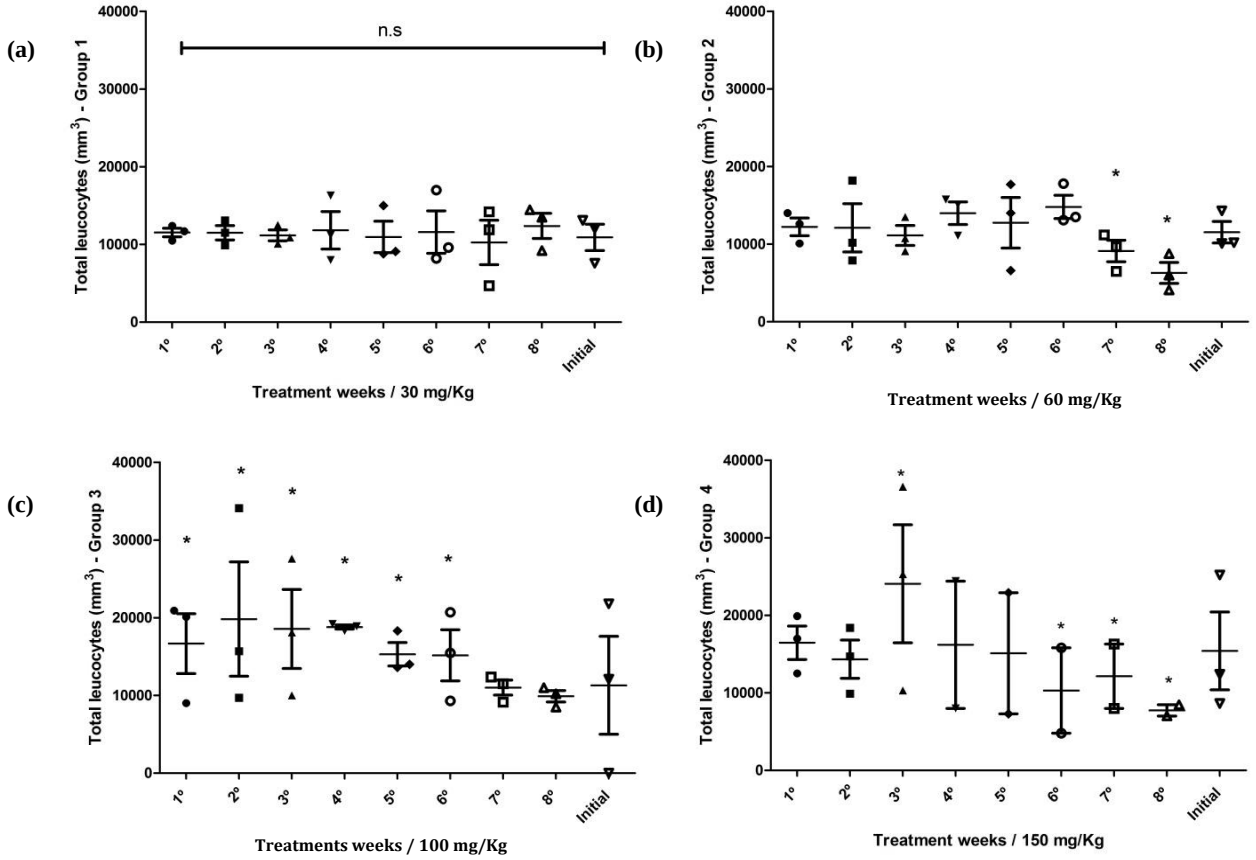

Fig. 4 Analysis of the total number leucocytes cells treated dose escalation from 30 to 150 mg/Kg for 8 weeks with injectable solution of 2-AEH$_2$P in dogs with neoplasms. (a) Mean value and ± SD of dogs treated 30 mg/Kg; (b) Mean value and ± SD of dogs treated 60 mg/Kg; (c) Mean value and ± SD of dogs treated 100 mg/Kg; (d) Mean value and ± SD of dogs treated 150 mg/Kg. The different mean value ± SD data were statistically compared by the test t Student and Anova Variance methods followed by the Tukey-Cremer multiple test. (n.s) No significant statistical differences and (*) Significance values p* < 0.05.

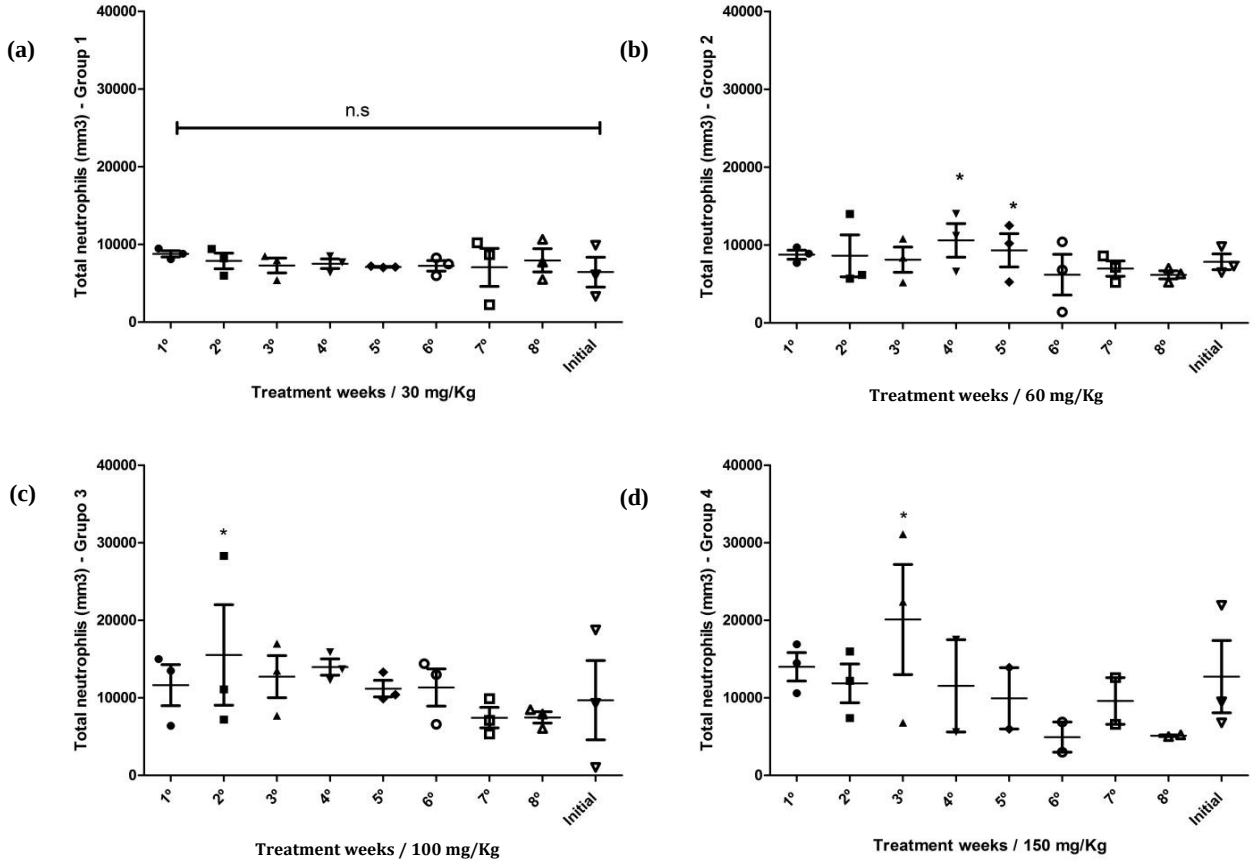

Fig. 5 Analysis of the total number neutrophils treated dose escalation from 30 to 150 mg/Kg for 8 weeks with injectable solution of 2-AEH$_2$P in dogs with neoplasms. (a) Mean value and ± SD of dogs treated 30 mg/Kg; (b) Mean value and ± SD of dogs treated 60 mg/Kg; (c) Mean value and ± SD of dogs treated 100 mg/Kg; (d) Mean value and ± SD of dogs treated 150 mg/Kg. The different mean value ± SD data were statistically compared by the test t Student and Anova Variance methods followed by the Tukey-Cremer multiple test. (n.s) No significant statistical differences and (*) Significance values p* < 0.05.

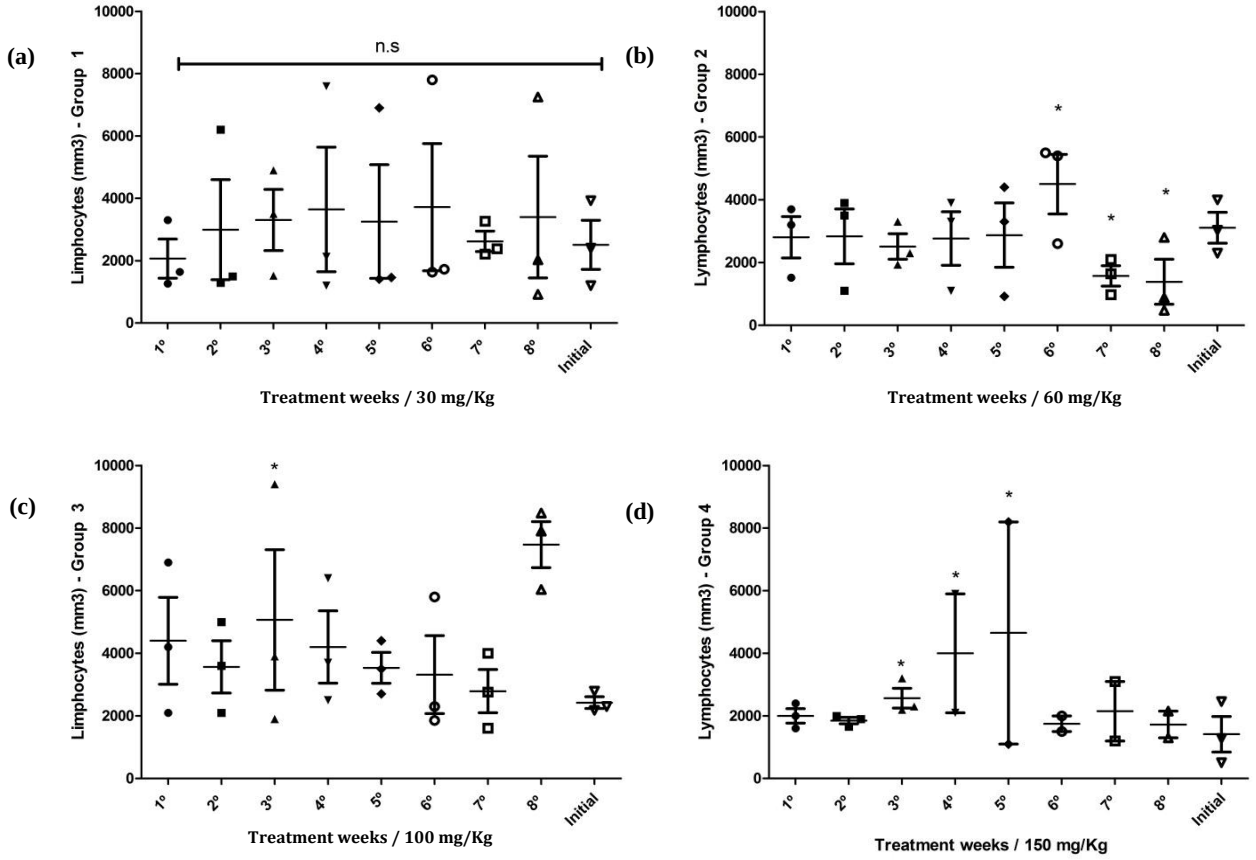

Fig. 6 Analysis of the total number lymphocytes cells treated dose escalation from 30 to 150 mg/Kg for 8 weeks with injectable solution of 2-AEH$_2$P in dogs with neoplasms. (a) Mean value and ± SD of dogs treated 30 mg/Kg; (b) Mean value and ± SD of dogs treated 60 mg/Kg; (c) Mean value and ± SD of dogs treated 100 mg/Kg; (d) Mean value and ± SD of dogs treated 150 mg/Kg. The different mean value ± SD data were statistically compared by the test t Student and Anova Variance methods followed by the Tukey-Cremer multiple test. (n.s) No significant statistical differences and (*) Significance values p* < 0.05.

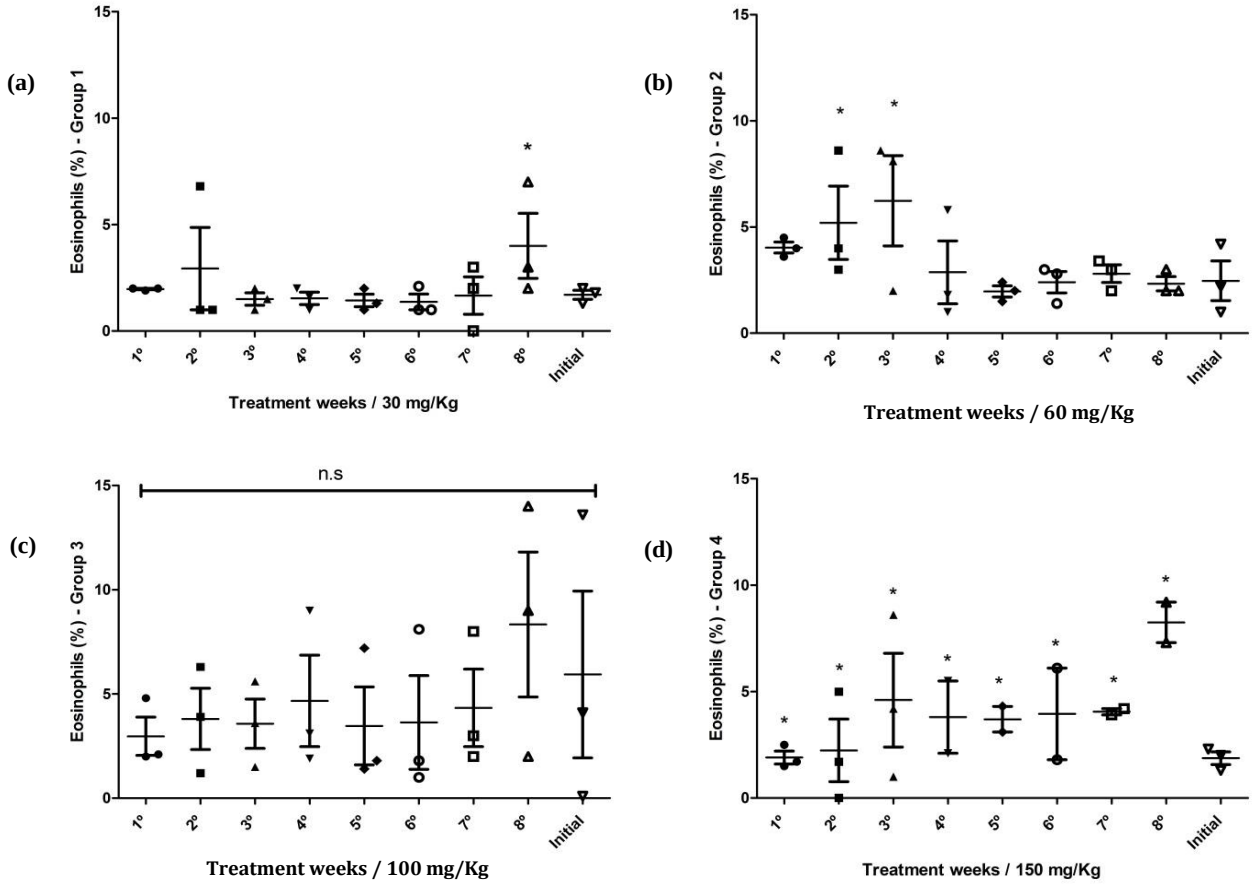

Fig. 7 Analysis of the percentual number eosinophils cells treated dose escalation from 30 to 150 mg/Kg for 8 weeks with injectable solution of 2-AEH$_2$P in dogs with neoplasms. (a) Mean value and ± SD of dogs treated 30 mg/Kg; (b) Mean value and ± SD of dogs treated 60 mg/Kg; (c) Mean value and ± SD of dogs treated 100 mg/Kg; (d) Mean value and ± SD of dogs treated 150 mg/Kg. The different mean value ± SD data were statistically compared by the test t Student and Anova Variance methods followed by the Tukey-Cremer multiple test. (n.s) No significant statistical differences and (*) Significance values p* < 0.05.

by promoting critical processes such as angiogenesis and metastasis. In some cases, these interactions suppress immune recognition and the elimination of tumor cells or promote arrest in the endothelium or embolization in the microvasculature and survival.

The number of platelets was modified at the different staggered doses of 2-AEH$_2$P. Quantitative changes significant occur in the groups of animals that received 60, 100 and 150 mg/Kg. There was a 60 mg /kg increase in dose and in the 100 and 150 mg/Kg dose there was a significant decrease (Fig 8).

3.3 Biochemical Analysis

The determination of pharmacological toxicity and safety was used as a qualitative and quantitative parameter for normal metabolic function of liver and renal parenchyma. Liver function was evaluated by measuring alanine in pyruvate and glutamate and alanine aminotransferase transaminase in which 2-ketoglutarate can reverse ammonia reaction. Non-significant data from biochemical analysis was not plotted.

Results obtained from animals in different 2-AEH$_2$P groups (Group 1 received 30 mg/Kg, Group 2 received 60 mg/Kg, Group 3 received 100 mg/Kg, and Group 4 received 150 mg/Kg) showed no significant difference in toxicity effects among any of the groups studied.

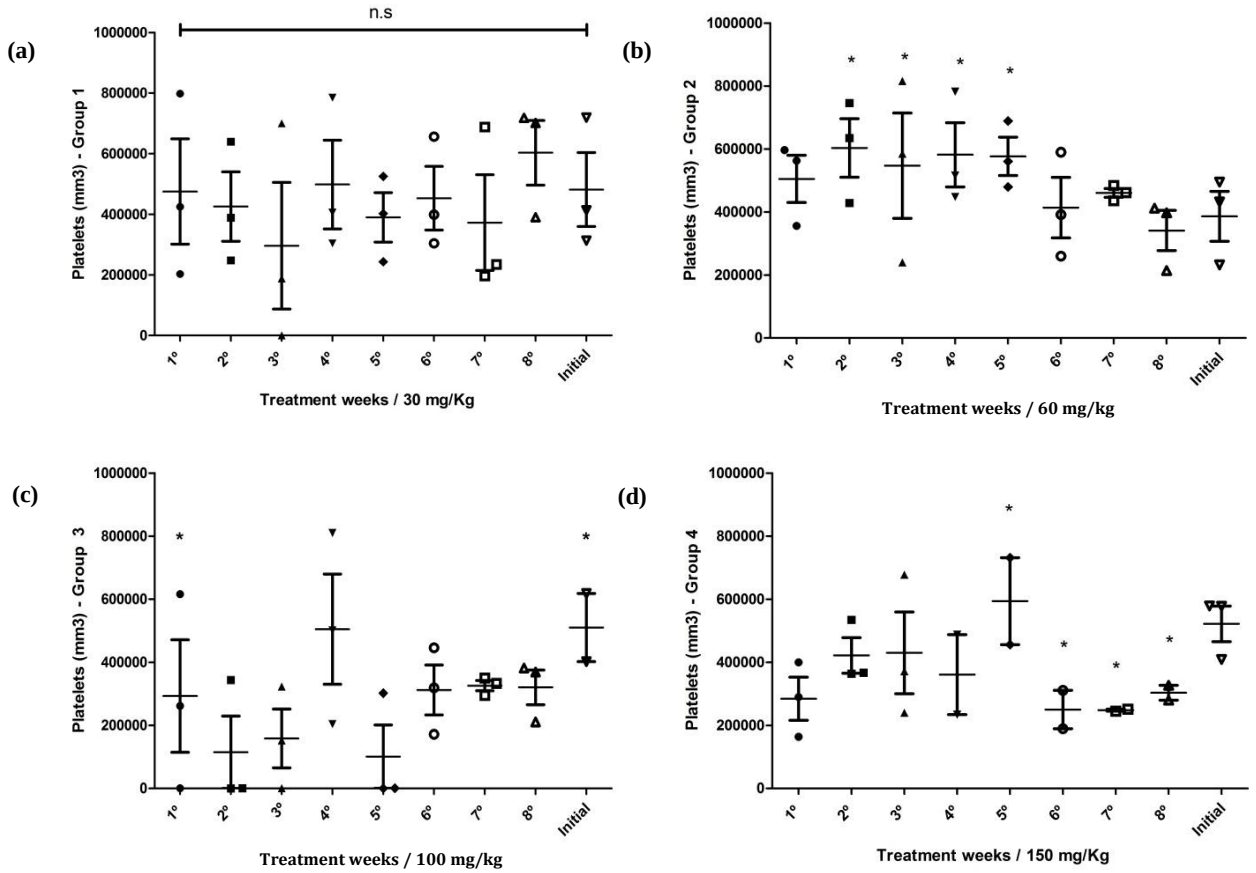

Fig. 8 Analysis of the total number platelets cells treated dose escalation from 30 to 150 mg/Kg for 8 weeks with injectable solution of 2-AEH$_2$P in dogs with neoplasms. (a) Mean value and ± SD of dogs treated 30 mg/Kg; (b) Mean value and ± SD of dogs treated 60 mg/Kg; (c) Mean value and ± SD of dogs treated 100 mg/Kg; (d) Mean value and ± SD of dogs treated 150 mg/Kg. The different mean value ± SD data were statistically compared by the test t Student and Anova Variance methods followed by the Tukey-Cremer multiple test. (n.s) No significant statistical differences and (*) Significance values p* < 0.05.

The evaluation of alkaline phosphatase activity was to monitor the function of the bile duct, an enzyme present in the bile duct lining membranes capable of removing the phosphate group from a large number of molecules, including proteins, nucleotides and alkaloids. In the different treatment groups with 2-AEH$_2$P, after the 8th week no significant changes in the alkaline phosphatase plasma concentration were observed.

The total plasma albumin concentration of animals treated with 2-AEH$_2$P at baseline and after 8 weeks of the experimental treatment protocol proposed by Fibonacci model was determined. The liver is the only organ capable of albumin. About 12% to 20% of hepatic synthesis capacity is available for the synthesis of this protein, not being influenced by serum levels per se, but depends on a complex interaction between colloid osmotic pressures in hepatic extracellular fluid. It is an important protein reserve, as well as a carrier of free fatty acids, amino acids, metals, calcium, hormones and bilirubin, besides participating in blood pH regulation, acting as an anion. Serum levels of hormones known to stimulate this synthesis (corticosteroids, anabolic steroids and thyroxine), presence of proinflammatory cytokines that inhibit this synthesis, and nutritional status, including the availability of energy, protein and micronutrients. Mean values obtained from plasma albumin concentration in the different groups treatments were not changed over the weeks of treatment.

The assessment of renal function is of utmost importance in clinical practice, both for the diagnosis, prognosis and monitoring of kidney disease. Urea is the major nitrogen metabolite derived from protein breakdown by the body, with 90% of this analytic excreted by the kidneys and the remainder eliminated by the gastrointestinal tract and skin. Although freely filtered by the glomerulus, not actively resorbed or secreted, urea is a poor predictor of glomerular filtration, as 40%-70% returns to plasma through a tubular passive diffusion process that is dependent on urinary flow.

We results showed initial values for urea showed no significant differences between the different treatment groups, but Group 3 (100 mg/Kg) had higher levels of urea than the other groups. After the 8 weeks of treatment, no significant differences were found between the different groups, although Group 4 receiving 150 mg/Kg had a higher creatinine level when compared to the other groups.

The sodium, potassium and phosphorus electrolytes showed no quantitative changes between treatment groups or even over the 8 weeks.

The dosage of total proteins and glycemic levels initially show significant differences between animals of all groups animals after 8 weeks.

The calcium in the blood is made up of three fractions: ionized calcium, serum protein-bound calcium and anion-complex calcium. Ionized calcium is the most important fraction from a biological point of view, representing about 50% of total calcium, as it acts as a regulatory ion in many metabolic processes. Maintaining calcium concentrations in extracellular fluids is of utmost importance as calcium is involved in numerous functions such as cell division, muscle contraction, hormone secretion and blood clotting.

Calcium levels initially obtained in the different groups showed significant differences. Regarding the administered concentrations, a significant decrease occurred in groups 3 and 4. After 8 weeks, calcium levels normalized, with a significant increase in group 4 values (Fig 9).

3.4 Lipid Peroxidation

Lipid peroxidation was estimated in terms of thiobarbituric acid reactive species (TBARS). The

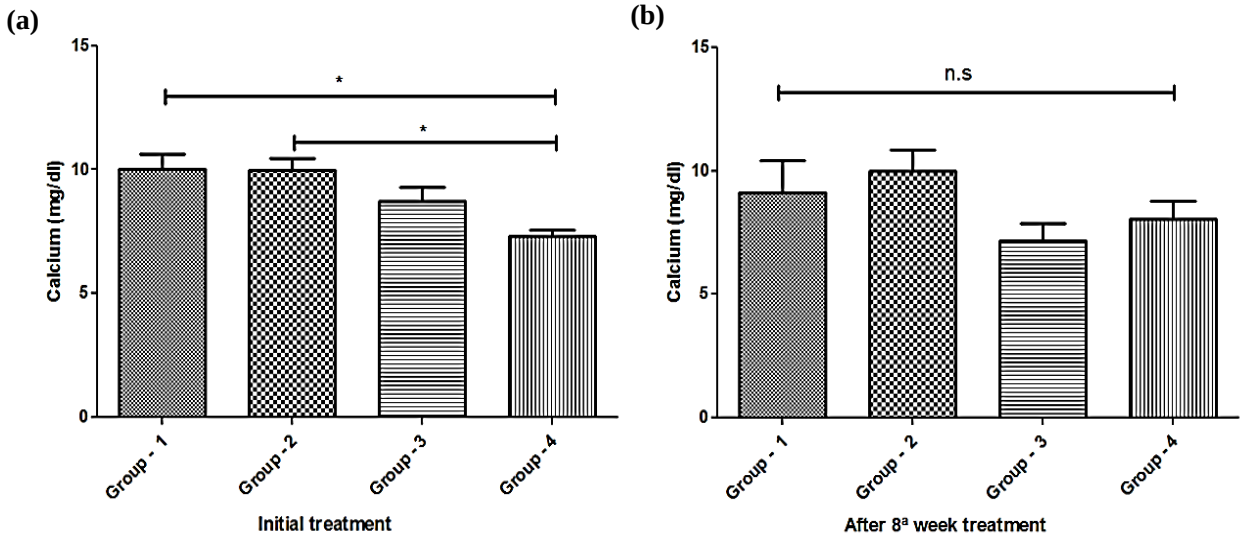

Fig. 9 Biochemical determination of total calcium levels in tumor dogs treated with 2-AEH$_2$P injection at an ascending dose from 30 to 150 mg/Kg for 8 weeks. (a) Mean value and ± SD of initial treatment protocol and (b) Mean value and ± SD of 8 weeks treatment protocol. The different mean value ± SD data were statistically compared by the test t Student and Anova Variance methods followed by the Tukey-Cremer multiple test. (n.s) No significant statistical differences and (*) Significance values p* < 0.05.

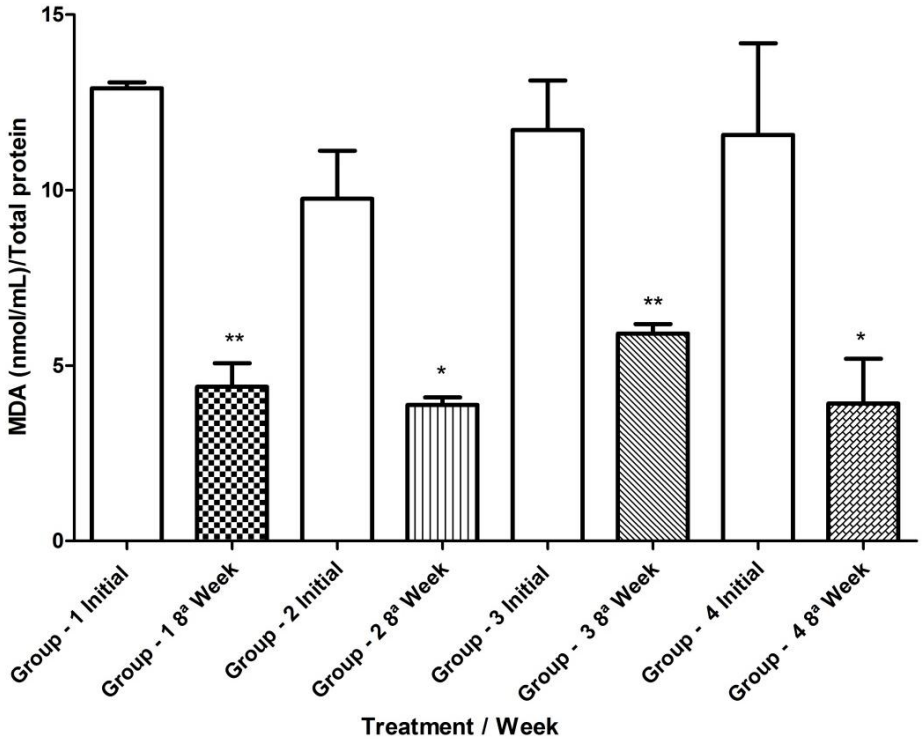

Fig. 10 Determination of lipidic peroxidation radical formation in plasmas of dogs with neoplasias, from the quantification and production of malondialdehyde (MDA) in relation to the amount of total protein. Mean value and ± SD of 8th weeks treatment protocol. The different mean value ± SD data were statistically compared by the test t Student and Anova Variance methods followed by the Tukey-Cremer multiple test. (*) Significance values p* < 0.05 and p < 0.01.**

quantification of the formed MDA values was corrected by the ratio of optical density to the amount of total proteins. Lipoperoxide formation in the plasmas of animals of all treatment groups decreased significantly compared to baseline and after 8 weeks of treatment (Fig 10).

4. Discussion

In this study, we used the Fibonacci method for staggered dose. 2-AEH$_2$P is monophosphoester selective toxicity in canine primary tumor cell, no changes modified proliferative normal cells. Phase I dose-finding trials aim to acquire an optimal recommended dose for a new compound for additional testing in phase II trials. For cytotoxic drugs, this dose corresponds to the highest dose associated with an acceptable level of toxicity, called the maximum tolerated dose (MTD). In this study, several successive cohorts of dogs bearing tumor treated with increasing doses of compound 2-AEH$_2$P administered intravenously at concentrations of 30, 60, 100 and 150 mg/Kg were performed.

The 2-AEH$_2$P administered in this project was a lipid monophosphoester. 2-AEH$_2$P is a monoester is converted in the liver to the three mitochondrial inner membrane phospholipids: phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine. These three phospholipids are directly implicated in the mitochondrial inner membrane permeability and directly interfere with the transmembrane potential [24].

In preclinical models, our group demonstrated that 2-AEH$_2$P has selective toxicity to tumor cells without deleterious effects on normal cells. Animals with murine melanoma tumors treated with 2-AEH$_2$P showed significant reduction in tumor mass, as well as an increase in survival rate. The treated animals had a reduction in the spread of tumor cells and metastasis and a reduction and/or absence of neovascularization.

The evaluation of the cell cycle phases of tumors treated with 2-AEH$_2$P showed that the treatment-induced stop in the quiescent and non-proliferative G0/G1 phases, with an increase in the population of cells with fragmented DNA content, and an increase in active caspase-3, 8 and 9 [10-21].

Hematological analysis revealed that the compound showed no myelosuppression and was useful to stimulate red blood cell production. The histology of dorsal tumors treated with 2-AEH$_2$P showed a modulating effect by stimulating the synthesis of fibrillar components, promoting the replacement of tumor cell density by area of intra-tumoral fibrosis. Histochemical analyzes of the fibrillar matrix components of collagen type I and pro-collagen showed that 2-AEH$_2$P induces "new" synthesis of the extracellular matrix, possibly by replacing tumor cell density, as well as by increasing the areas of fibrosis found in the treated groups. In animals treated with 2-AEH$_2$P that by radio-labeled scintillation [^{99m}Tc(V)-DMSA2] during the experimental period, tumor regression was demonstrated by noninvasive methods by specific labeling of solid tumors, and there were significant differences between animals treated with different concentrations of 2-AEH$_2$P.

Our data initially show that the concentrations used and the proposed scheme for the Phase-1 study by the Fibonacci model. 2-AEH$_2$P is selective for *in vitro* tumor cells, is not toxic when administered at staggered doses, since in none of the study groups (30 to 150 mg) did not cause the death of any animal, so it is safe to be administered at other stages of the clinical study. Typically, this class of antitumor phospholipids causes several serious side effects, such as anemia, hemolytic, upper digestive tract bleeding, and consumption of clotting factors. New compounds of the same chemical class as 2-AEH$_2$P in clinical use, such as edelfosine and miltefosine, which cause various side effects, especially on red blood cells, with 2-AEH$_2$P these effects were not found [25-28].

No significant differences in hematimetric indices were found in all 2-AEH$_2$P treatment groups. Therefore, no qualitative and quantitative changes in red blood cells were found, with any differences in mean corpuscular volume, mean corpuscular hemoglobin, which reveal the size of red blood cells and hemoglobin content, important data for the diagnosis or monitoring of anemia or polycythemia.

The total leucocytosis counts changed in the groups receiving 100 and 150 mg/Kg. The leukocytosis found was accompanied by an increase in the number of lymphocytes and monocytes, in particular in the group of animals receiving 150 mg/Kg eosinophilia also occurred. In the analysis of leukocyte morphology, polymorphonuclear leukocytes with variations such as the presence of rods, metamielocytes, myelocytes and promyelocytes, toxic granulations, cytoplasmic vacuoles and abnormal inclusions were not observed.

The quantitative changes of platelets in this study reflected the difference in the role of 2-AEH$_2$P in microenvironment tumor.

The relationship between cancer and thrombosis has been known since 1865, when Armand Trousseau first described that localized cancer can induce venous thrombus formation. Tumor-associated thrombosis is one of the most common clinical manifestations in cancer patients and is associated with poor prognosis and disease-free survival. The main reason for the high thrombotic risk in cancer patients is that tumor cells can activate platelets and stimulate aggregation through direct and indirect mechanisms [29]. The 2-AEH$_2$P is modulated positivity production platelets.

Biochemical characteristics of liver function were determined by the quantitative evaluation of transaminases, alkaline phosphatase, albumin, globulin and total bilirubin in the serum of animals treated with 2-AEH$_2$P from baseline to 8 weeks later in groups 1 to 4. No significant changes were observed in these determinations, demonstrating that the compound was not hepatotoxic or choledochal toxic [30].

Ferreira et al (2012) demonstrated the modulation of the levels of various biochemical markers in a

model of murine breast carcinomatosis. Biochemical analysis of the parameters of untreated mice revealed elevated serum AST, ALT and lactate dehydrogenase (LDH) levels, as the tumor growth. Animals treated with 2-AEH$_2$P at a concentration of 70 mg/Kg showed no significant changes in AST, ALT, and LDH compared to untreated mice (Figure 7A and B). The authors suggest that inhibition of tumor ascites by 2-AEH$_2$P is reasonable to avoid liver failure [18].

Renal function in this study was assessed by quantitative determinations of markers, urea and creatinine, and 2-AEH$_2$P showed no significant differences between studied periods and administration doses [31].

The levels of potassium, sodium and phosphorus electrolyte do not show significant changes during treatment. Measurements of ionizable calcium successively showed a significant increase in plasma levels. This increase reflects the modulation effect of 2-AEH$_2$P, which is caused by its electrochemical properties [32]. The measured serum calcium is affected by the level of albumin and needs to be calibrated against the serum albumin [33].

There were no significant differences in total protein levels and blood glucose levels at different therapeutic doses. However, it should be noted that reprogramming of energy metabolism is considered to be one of the characteristics of cancer, which actively promotes the development of cancer. Oncogenic events lead to dysregulation of metabolic pathways, which in turn provide a selective advantage to cancer cells that enable to proliferate and survive in an adverse environment. In addition, rebound metabolism actively contributes to tumorigenesis through metabolite production, interaction with signaling pathways, and metabolite-dependent epigenetic regulation. The altered metabolic program of cancer cells also affects other cells residing in the tumor microenvironment and contributes to regulate processes deeply involved in cancer development, such as angiogenesis, inflammation and tumor immunity [34-37].

Malondialdehyde (MDA) is one of the end products of lipoperoxidation formed by the primary or secondary decomposition of intermediate products, and reacts with thiobarbituric acid, being widely used in the determination of oxidative stress. Plasma lipoperoxide formation was measured at baseline and after 8 weeks in the different treatment groups and showed significant reductions in all dosing doses. The excessive increase of reactive oxygen species (ROS) formation induces DNA damage, promoting mutagenic nucleotide modifications, protein oxidation and lipid peroxidation, playing an important role in tumorigenesis [38].

Oxidants generally produce protein modifications leading to loss of function and increasing the degradation rate of oxidized proteins, promoting changes in their structure and permeability, which leads to inhibition of cell growth and death. Lipid peroxidation can trigger the apoptosis process, activating the intrinsic apoptosis pathway present in all cells, whether or not dependent on caspase pathway phosphorylation. These data corroborate the safety potential and possibly the efficacy of 2-AEH$_2$P in controlling tumor growth or even modulating immunological effects [39].

5. Conclusions

The 2-AEH$_2$P is a new phosphomonoester with anti-tumor, antiproliferative and anti-inflammatory properties. In a Phase-1 study following the Fibonacci model, 2-AEH$_2$P was safe at all doses of 150 mg/Kg for 8 weeks. It is not a drug that has hemolytic properties or induces anemia. 2-AEH$_2$P maintained the leucocytes production independent tumor progression. It does not lead to changes in liver transaminases and renal depuration functions, was able to modulate leukocyte production and modulated free lipoperoxide formation. It is a new compound with antitumor potential, being useful for oncology veterinary.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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