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Long-circulating and fusogenic liposomes loaded with a glucoevatromonoside derivative induce potent antitumor response

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ABSTRACT

Cancer is an important public health problem, being one of the leading causes of death worldwide. Most antineoplastic agents cause severe toxic effects and some types of cancer do not respond or are resistant to the existing pharmacotherapy, **necessitating** the research and development of new therapeutic strategies. Cardenolides have shown significant antitumor activity due to their ability to inhibit **the** Na^+K^+ATPase enzyme, and the expression of this enzyme is increased in tumor cells. Glucoevatromonoside containing peracetylated glucose hydroxyl groups (GEVPG) is a cardenolide derivative that has low solubility in aqueous media, which constitutes a barrier to its potential biological applications. In this context, the use of liposomes represents a promising strategy to deliver GEVPG, **thus** allowing its intravenous administration. In this study, long-circulating and fusogenic liposomes containing GEVPG (SpHL-GEVPG) were developed, and **their** chemical and physicochemical properties **were** evaluated. SpHL-GEVPG presented adequate properties, including a mean diameter of 182.2 ± 2.7 nm, a polydispersity index equal to 0.36 ± 0.03, a zeta potential of –2.37 ± 0.31 mV, and a GEVPG entrapment of 0.38 ± 0.04 mg/mL. Moreover, this formulation showed a good stability after having been stored for 30 days at 4 °C. The cytotoxic studies against breast (MDA-MB-231, MCF-7, and SKBR-3) and lung (A549) cancer cell lines demonstrated that SpHL-GEVPG treatment significantly reduced the cell viability. In addition, the SpHL-GEVPG formulation presented a good selectivity toward these cancer cells. The evaluation of the therapeutic efficacy of the treatment with SpHL-GEVPG showed a potent anticancer effect in **an** A549 human lung cancer xenograft model. SpHL-GEVPG administered at doses of 1.0 and 2.0 mg/kg (i.v.) induced antitumor effect comparable to paclitaxel given at dose of 10 mg/kg (i.v.) to mice. Therefore, the results of the present work indicate the potential applicability of SpHL-GEVPG as a new anticancer formulation.

1. Introduction

Cancer is an important public health problem, responsible for one in six deaths in the world. More than 14 million people develop cancer annually and by 2030 the annual rate is expected to rise to 21 million new cases [1]. Most of the anticancer drugs in clinical use are natural products or derivatives [2]. However, most antineoplastic agents cause severe toxic effects and some types of cancer do not respond or are resistant to the existing pharmacotherapy, thus demanding the search and development of new therapeutic strategies [3–5]. Epidemiologic evidence has led to the disclosure of cardenolides as potential antitumoral agents [6]. These compounds have been used as first-line agents for the therapy of congestive heart failure, since they are able to inhibit Na^+/K^+-ATPase, leading to a positive inotropic effect [7]. However, their use should be carefully monitored, since they have an extremely low therapeutic index [8]. Glucoevatromonoside containing peracetylated glucose hydroxyl groups (GEVPG) (Fig. 1) is a cardenolide, **which** can be obtained by semisynthesis **of** evatromonoside [9]. This compound exhibited a great cytotoxic activity against **the** PC3 human prostate cancer cell line as well as the glucoevatromonoside,

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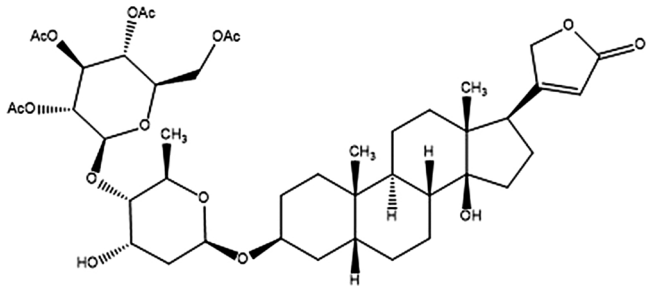

Fig. 1. Chemical structure of GEVPG.

presenting IC50 values equal to 38 nM and 37.2 nM, respectively [9]. GEVPG has low water solubility which limits its clinical application. In this context, the use of nanosystems as carrier, such as liposomes, represents a valid strategy to allow its intravenous administration and delivery. Liposomes are well-recognized drug delivery systems which can accommodate amphiphilic or lipophilic drugs in the bilayers, while hydrophilic compounds can be encapsulated within the aqueous inner compartment of the vesicles. A major drawback of conventional liposomes is the rapid uptake of these nanosystems *in vivo* by cells of the mononuclear phagocyte system (MPS). The coating of the liposome surface with inert biocompatible polymers, such as polyethylene glycol (PEG), can significantly prolong the circulation half-life of liposomes. Due to the increased circulation time of liposomes containing PEG-lipids and the leaky structure of the microvasculature in the solid tumor tissue, these liposomes have been shown to accumulate preferentially in tumor tissue. The capillary permeability of the endothelial barrier in newly vascularized tumors is significantly greater than that of normal tissues. This defective vascular architecture coupled with poor lymphatic drainage, induces an enhanced permeability and retention (EPR) effect [4]. Thus, these delivery systems are capable of targeting the tumor region, enhancing the therapeutic effect and minimizing systemic toxicity [10,11]. Furthermore, it is known that liposomes constituted by phosphatidylethanolamine derivatives, such as dioleoylphosphatidylethanolamine (DOPE), have a better fusogenic property with the cellular and endosomal membrane when compared to other liposomal formulations. This fusogenic property leads to the fusion of liposomes with membranes, and consequently, the release of drugs to the cytoplasm [12]. Therefore, the aim of the **present study was to encapsulate GEVPG in long-circulating and fusogenic liposomes (SpHL-GEVPG),** composed **of** DOPE, cholesteryl hemisuccinate (CHEMS), and distearoylphosphatidylethanolamine-polyethyleneglycol$_{2000}$ (DSPE-PEG$_{2000}$). The chemical and physicochemical properties of these liposomes were evaluated. The cytotoxicity effect of SpHL-GEVPG against breast cancer cell lines, such as MDA-MB-231, MCF-7, **and** SKBR-3; lung cancer cell line A549; **and** non-tumoral human fibroblasts (NTHF) were investigated. **In addition**, we conducted clonogenic and cumulative population doubling assays in order to determine the long-term effects after exposure of **the** A549 cell line to the SpHL-GEVPG treatment. Finally, the antitumor efficacy of SpHL-GEVPG was evaluated in Balb/C nude female mice bearing A549 lung tumor.

2. Materials and methods

2.1. Chemicals

Glucoevatromonoside containing peracetylated glucose hydroxyl groups (GEVPG) was synthetized as previously described by Munkert et al. [9]. The purity of GEVPG (> 98%) was previously assessed by chromatographic and spectroscopic data. DOPE and DSPE-PEG$_{2000}$ were supplied by Lipoid GmbH (Ludwigshafen, Germany). CHEMS, Sulforhodamine B (SRB), tris(hydroxymethyl)aminomethane (Tris base), phosphate saline buffer, sodium hydroxide 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), sodium bicarbonate, and trypsin were obtained from Sigma Aldrich (St. Louis, USA). Sodium chloride and acetonitrile were purchased from Merck (Frankfurt, Germany). All other chemicals used in this study were of analytical grade.

2.2. Cells

Breast cancer cell lines MDA-MB-231 (ATCC® HTB26™), MCF-7 (ATCC® HTB22™), and SKBR-3 (ATCC® HTB30™) were purchased from American Type Culture Collection (ATCC) (Manassas, USA) and grown in Dulbecco's Modified Eagle's Medium (DMEM), Eagle's Minimum Essential Medium (MEM) supplemented with 0.01 mg/mL human recombinant insulin, or McCoy's 5 A Modified Medium (McCoy), respectively. Human non-small cell lung cancer (NSCLC) A549 cells (ATCC® CCL185™) were grown in MEM. Non-tumoral human fibroblasts (NTHF) from primary gingival tissue culture were grown in DMEM and kindly supplied by Prof. Cláudia Maria Oliveira Simões (Laboratory of Virology, Universidade Federal de Santa Catarina, Florianópolis, Brazil) according to the Research Ethics Committee of the Universidade Federal de Santa Catarina, protocol number 021/2009. All cell lines were supplemented with 10% fetal bovine serum (FBS, Gibco) and maintained at 37 °C and 5% CO_2 in a humidified atmosphere. All cell lines were routinely tested for Mycoplasma contamination using Hoechst fluorescence staining.

2.3. Animals

Six- or seven-week-old female Balb/c nude mice (supplied by Faculty of Medicine, Universidade de São Paulo, São Paulo, Brazil) were maintained in appropriately isolated cages with free access to drinking tap water and food on a daily 12-h light/dark cycle. The experimental protocol was approved by the Committee on Care and Use of Experimental Animal Resources of the Universidade Federal de Minas Gerais (protocol number 303/2017) and followed the guidelines for the care and use of laboratory animals recommended by the Institute of Laboratory Animal Resources.

2.4. Preparation of SpHL-GEVPG

SpHL-GEVPG **was** prepared by the lipid hydration method as described by Bangham et al. [13]. Briefly, 1.2 mL of chloroform solution of DOPE 28.7 mM, 0.48 mL of chloroform solution of CHEMS 48 mM, and 0.3 mL of chloroform solution of DSPE-PEG$_{2000}$ 10 mM were transferred to a round bottom flask (total lipid concentration of 10 mM, molar ratio of 5.7 : 3.8 : 0.5, respectively). A lipid film was obtained by evaporating the chloroform under reduced pressure. Next, the lipid film was hydrated with 0.1 mL of NaOH 0.228 M to promote the complete ionization of CHEMS molecules. The lipid film containing NaOH solution was dissolved in 2 mL of chloroform and GEVPG chloroform solution equivalent to 0.5% (w/v) was added to generate a final lipid film after evaporation under reduced pressure. Then, the final lipid film was hydrated with 6 mL of NaCl solution 0.9% (w/v). The obtained mixture was subjected to vigorous shaking in a vortex, leading to the formation of multilamellar liposomes. The liposomes were downsized by sonication using a Cole Parmer® sonicator model CPX500 (Illinois, USA), for 5 min and amplitude of 21%. The unencapsulated GEVPG was removed from the liposome dispersion by centrifugation using a baby Thermo Scientific® centrifuge model 11210801, for 1 min and 5000 rpm. After centrifugation, the pellet and supernatant obtained consisted of unencapsulated GEVPG and purified SpHL-GEVPG, respectively. The blank liposomes were prepared under the same conditions without the addition of GEVPG.

2.5. SpHL-GEVPG characterization

The mean diameter and polydispersity index (PDI) of SpHL-GEVPG

Table 1
Acetonitrile/water gradient mixture used to analysis GEVPG by HPLC.

Time (min.)	Acetonitrile (%)	Water (%)
0	30	70
6	95	5
12	95	5
12.5	30	70
16	30	70

were determined by dynamic light scattering (DLS) at 25 °C and at an angle of 90°. The zeta potential was evaluated by DLS associated with electrophoretic mobility. The measurements were performed using the Zetasizer Nano ZS90 equipment (Malvern Instruments Ltd, Worcestershire, UK). The samples were diluted with NaCl solution 0.9% (w/v) solution. The amount of GEVPG in the liposomes was determined by HPLC before (non-purified SpHL-GEVPG) and after centrifugation (purified SpHL-GEVPG). The liposome samples were disrupted in isopropanol in a volume ratio of 1:5, respectively. Then, the samples were diluted in acetonitrile for HPLC analysis. The chromatographic apparatus consisted of a model G1311B pump, a model G1329B autoinjector, and a model G4212B variable wavelength UV detector (Agilent Technologies, Santa Clara, USA) controlled by Agilent OpenLab CDS EZChrom® software. Separations were performed using a 25 cm × 4.6 mm, 5 µm LiChrospher, RP-18 column (Merck SA, Germany). The eluent system consisted of an acetonitrile/water gradient mixture (Table 1), at a flow of 1.0 mL/minute. Samples (10 µL) were injected into the apparatus and the absorbance of the eluate was monitored at 220 nm. The HPLC method was validated and showed satisfactory specificity, linearity (2–10 µg/mL), precision, accuracy, and robustness (data not shown). The GEVPG entrapment was calculated by using the following equation:

$$GEVPG\ entrapment\ (\%) = \frac{GEVPG\ in\ purified\ liposomes}{GEVPG\ in\ non-purified\ liposomes} x\ 100$$

2.6. Transmission Electron Microscopy (TEM)

Morphological examination of SpHL-GEVPG was performed by means of TEM using a negative staining method. The liposomes were previously diluted 100 fold in NaCl solution 0.9% (w/v), and placed on a formvar and carbon coated copper grid and stained with a 2% (w/v) phosphotungstic acid solution containing 0.5% (w/v) bovine serum albumin and 0.5% (w/v) saccharose. The stained samples were characterized using a Tecnai G2 12 Spirit Biotwin FEI at 120 kV (Centro de Microscopia, UniversidadeFederal de Minas Gerais, Belo Horizonte, Brazil).

2.7. Stability assay

The determination of the storage stability of SpHL-GEVPG was performed **for** 30 days after preparation. This formulation was maintained at 4 °C in NaCl solution 0.9% (w/v). The parameters evaluated included mean diameter, PDI, zeta potential, and drug entrapment. The mean values of these parameters were compared with those obtained at time zero.

2.8. Release profile of SpHL-GEVPG

SpHL-GEVPG (370 µL corresponding to 185 µg GEVPG) was added to dialysis bags (10 kDa Sigma, USA) and immersed into 100 mL of HEPES buffer containing Tween 80 (1% w/v) at pH 7.4 or pH 5, maintaining the sink condition. The dialysis flasks were agitated using **an** incubator with orbital agitation model KS 4000i Control (IKA, Shangai, China), for 156 rpm, at 37 °C. The release profile of SpHL-GEVPG was performed at 1, 2, 4, 8, 12, and 24 h. The parameters evaluated included mean diameter, PDI, zeta potential, and drug release.

2.9. Sulforhodamine B assay

The viability of tumor**al** and non-tumoral cells was measured using Sulforhodamine B assay [14]. Briefly, MDA-MB-231, MCF-7, SKBR-3, A549, and NTHF cells were seeded into 96-well plates (1×10^4 cells/well). After 24 h of incubation at 37 °C and 5% CO_2, solutions of free GEVPG in DMSO [DMSO concentration in all treatments was inferior to 1% (v/v)] or SpHL-GEVPG were added to the wells (GEVPG concentration ranged from 0.001 µM to 5 µM in tumor cells, and from 0.4 µM to 50 µM in NTHF). Liposomes without GEVPG (blank liposomes) were diluted in the same way as SpHL-GEVPG. After 48 h of incubation, 10% trichloroacetic acid (TCA) was added to each well to fix cells for one hour. Plates were then washed with water to remove TCA, followed by staining with SRB for 30 min. Afterwards, the plate was washed with 1% (v/v) acetic acid to remove the unbound dye. **Then**, 10 mM Tris-Base solution (pH 10.5) was added to solubilize the protein-bound dye, and the optical density (OD) was read at 510 nm using a microplate spectrophotometer Spectra Max Plux 384 (Molecular Devices, Sunnyvale, USA). Paclitaxel (PTX) and doxorubicin (DOX) were used as positive controls against lung and breast cancer cell lines, respectively. The percentages of viable cells were plotted against each concentration, and the IC_{50} values were determined based on concentration-response curves using GraphPad Prism 6.0 (Graph Pad software, La Jolla, California, USA).

2.10. Selectivity index

The selectivity index (SI) of the free GEVPG in DMSO [DMSO concentration in all treatments was inferior to 1% (v/v)] or SpHL-GEVPG treatments against tumoral cells and non-tumoral cells was calculated as shown in the following equation:

$$SI = \frac{IC50\ NTHF}{IC50\ TUM}$$

Where $IC_{50\ NTHF}$ means the inhibitory concentration of 50% cell viability (IC_{50}) for NTHF and $IC_{50\ TUM}$ means the IC_{50} values for tumoral lines.

2.11. Clonogenic assay

The clonogenic assay was carried out as previously described with some modifications [15]. A549 cells were seeded in 12-well plates (10^5 cells/well). After 24 h of incubation at 37 °C and 5% CO_2, the cells were treated for 48 h with free GEVPG in DMSO [DMSO concentration in all treatments was inferior to 1% (v/v)] or SpHL-GEVPG at concentration equivalent to their respective IC_{50} values (44 and 27 nM, respectively). After 48 h of treatment, remaining cells were counted and seeded in 6-well plates (2×10^2 cells/well) in order to evaluate the ability of these cells to form colonies after 21 days without treatment. MEM supplemented with 10% FBS was replaced every three days. On day 21, colonies were fixed with ethanol 70°GL, followed by the staining with Giemsa. The colonies formed were counted using a microscope (Olympus, Tokyo, Japan) and photographed.

2.12. Determination of cumulative population doubling (CPD)

A549 cells were seeded in 12-well plates (10^5 cells/well) and incubated at 37 °C and 5% CO_2 for 24 h. The treatments were performed similarly as described above for clonogenic assay. After 48 h, remaining cells were counted and seeded in 12-well plates (2.5×10^4 cells/well) and followed by 21 days without treatment. A549 cells were trypsinized, counted, and re-seeded every three or four days, respecting a minimum of 20% and a maximum of 80% of cell confluence. Then, the

population doubling (CPD) values were determined and plotted in a graph *versus* days in culture. The CPD *versus* days graph was used to calculate CPD parameters, as described by Silva et al. [16].

2.13. Xenograft lung tumor model

An A549 lung cancer xenograft model was established as reported by Marostica et al. [17]. A549 cells were cultured in MEM supplemented with 10% FBS. They were grown to confluence and then trypsizined and counted. After centrifugation, A549 cells were resuspended with Matrigel™: MEM (1:1) and injected subcutaneously at the right lower flank (100 µL/animal, containing 10^6 A549 cells). Tumors were allowed to grow for 25 days.

2.13.1. Treatments

After growth of tumors, the animals were randomly divided into five experimental groups, **each** containing five or six animals. The negative control group was treated with blank liposomes. The animals of the positive control group received PTX treatment at dose of 10 mg/kg. The third group received SpHL-GEVPG treatment at dose of 0.5 mg/kg. The fourth group received SpHL-GEVPG at dose of 1.0 mg/kg. The fifth group received SpHL-GEVPG at dose of 2.0 mg/kg. The treatments were administered into the tail vein of A549 lung tumor-bearing mice every three days, **with** six administrations **being carried out**. The first day of treatment was considered day zero of this study.

2.13.2. Antitumor activity

The evaluation of the antitumor activity was based **on** the determination of the tumor volume (TV) calculated as previously described [18], where TV = 0.52 x (d1 x $d2^2$), **with** d1 and d2 **being** the largest and the smallest perpendicular diameters, respectively. These diameters were measured with a caliper MIP/E-103 (Mitutoyo, Suzano, SP, Brazil). Tumor growth was monitored every two days, from day zero to five days after the end of the treatment (day 20). Alterations of the TV were determined by considering the initial volume as 100% and calculating the percentages of increase or decrease according to this initial volume. The relative tumor volume (RTV) and the percentage of tumor growth inhibition (TGI) for each experimental group were calculated as shown in the following equations:

$$RTV = \frac{TV\ on\ day\ 20}{TV\ on\ day\ 0}$$

$$TGI = 1 - \frac{RTV\ from\ each\ treatment}{RTV\ of\ negative\ control\ group} x\ 100$$

2.13.3. Histological analysis

Five days after the end of the treatments (day 20), animals were anesthetized and euthanized. Kidney, liver, and spleen tissues were harvested and fixed in formalin (10% w/v in phosphate-buffered saline, PBS, pH 7.4) in order to investigate the toxicity. Then, these tissues were embedded in paraffin blocks, sectioned into a 5 µm thickness, placed onto glass slides, and stained with hematoxylin-eosin. Images of histological sections were captured using a digital camera (Spot Insight Color; SPOT Imaging Solutions, Sterling Heights, MI, USA) attached to a microscope Olympus BX-41 (Hamburgo, Germany).

2.14. Statistical analyses

The experiments were performed in triplicate and the data were evaluated with GraphPad Prism software (version 6.0, San Diego, USA). To confirm the normality and homoscedasticity of variance, D`Agostino and Bartlett tests were applied, respectively. Variables without normal distribution were transformed by the equation: y = log (y + 100). The differences between the experimental groups were tested by analysis of variance (one-way ANOVA followed by Tukey's test). For statistical analyses, the 95% confidence interval was used, and the differences were considered statistically significant when $p < 0.05$.

Table 2
Chemical and physicochemical properties of SpHL-GEVPG. Data expressed as mean ± standard deviation (SD) (n = 4).

Properties	SpHL-GEVPG
Vesicle diameter (nm)	182.2 ± 2.7
PDI	0.36 ± 0.03
Zeta potential (mV)	−2.37 ± 0.31
GEVPG entrapment (%)	75.0 ± 7.7

3. Results

3.1. Characterization of SpHL-GEVPG

The chemical and physicochemical properties of SpHL-GEVPG, such as vesicle diameter, PDI, zeta potential, and the drug entrapment are summarized in Table 2. SpHL-GEVPG presented a mean diameter of 182.2 ± 2.7 nm and the mean PDI value of 0.36 ± 0.03, indicating that the vesicle population in the formulation **was** monodisperse. The formulation exhibited a zeta potential value near neutrality (-2.37 ± 0.31 mV), as expected for formulations containing PEG **in the** bilayer. The encapsulation percentage of GEVPG in liposomes was high (75.0%). This result should be attributed to the hydrophobic character of the GEVP molecule favoring its insertion into the lipid bilayer.

3.2. Transmission Electron Microscopy (TEM)

TEM was used to investigate the morphological characteristic of SpHL-GEVPG. As shown in Fig. 2, SpHL-GEVPG are spherical, without aggregation or fusion, monodisperse, and homogeneous. The images of SpHL-GEVPG observed by the TEM technique was of vesicles of varying diameters, predominantly of less than 200 nm, which were consistent with the results obtained from the particle sizes measured by DLS technique (shown in Table 2).

3.3. Stability assay

It is worth noting that SpHL-GEVPG showed good stability in terms of mean vesicle diameter, PDI (Fig. 3A), and GEVPG retention (Fig. 3B) over 30 days at 4 °C. No significant changes were observed in relation to time zero. In addition, the zeta potential values also kept constant over 30 days (data not shown).

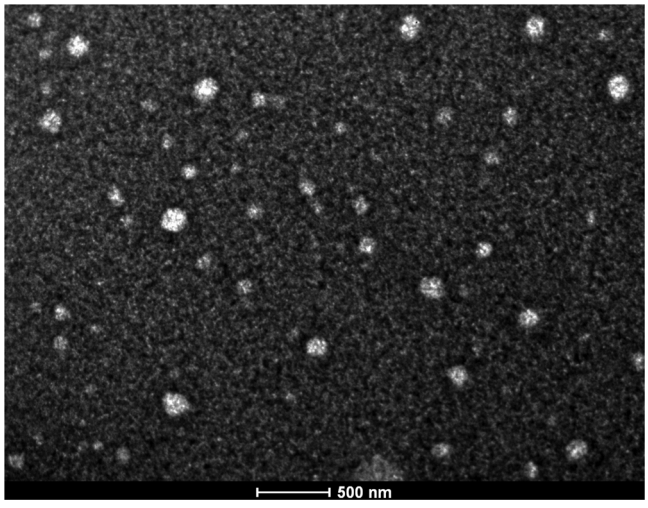

Fig. 2. TEM photomicrographs obtained for SpHL-GEVPG.

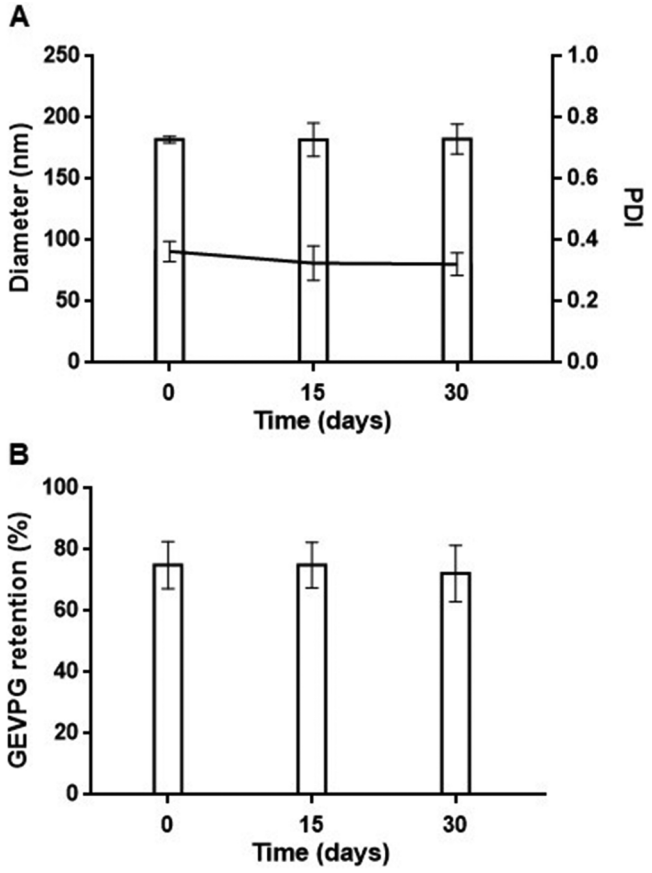

Fig. 3. Evaluation of the vesicle diameter and PDI (A), and GEVPG retention (B) from SpHL-GEVPG over 30 days of storage at 4 °C.

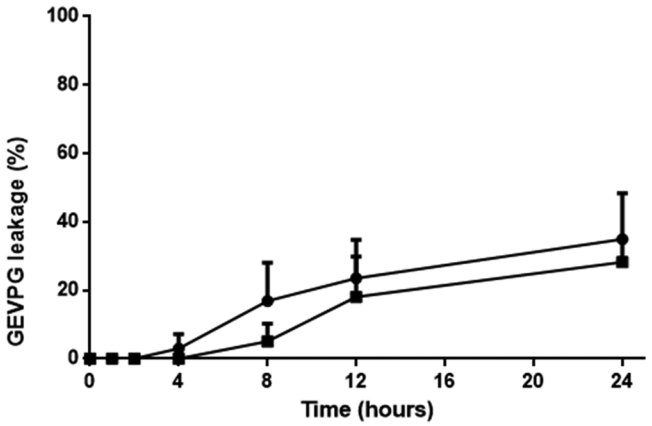

Fig. 4. Release profile of GEVPG from SpHL-GEVPG at pH 5.0 (circle) and pH 7.4 (square).

Table 4
Determination of SI of different treatments against breast and lung cancer cell lines and non-tumoral human fibroblasts (NTHF). *The SI value was determined by the ratio of IC_{50} of NTHF and IC_{50} of cancer cell lines (MDA-MB-231, SKBR3, MCF-7 or A549).

Cell line	SI ($IC_{50\ NTHF}/IC_{50\ TUM}$)*			
	GEVPG	SpHL-GEVPG	DOX	PTX
MDA-MB-231	283	24	> 60	_
SKBR3	353	25	> 185	_
MCF-7	138	12	> 100	_
A549	1092	149	_	> 500

3.4. Release profile of SpHL-GEVPG

After incubation of SpHL-GEVPG at different pH values, GEVPG leakage was evaluated as a parameter to determine the pH-sensitivity of the liposomal formulation, since the liposomes **containing** DOPE and CHEMS have sensitivity to acidic pH. The results are summarized in Fig. 4. In both evaluated pH values, the release of GEVPG from liposomes was low. At pH 7.4 after 24 h, the GEVPG leakage was 28.3 ± 6.8%, while at pH 5 it was 34.9 ± 13.4%. No significant change in the vesicle diameter, PDI, and zeta potential over 24 h could be observed (data not shown).

3.5. Evaluation of the cytotoxicity

The IC_{50} and SI values were determined for each cell line and are summarized in Tables 3 and 4, respectively. The free GEVPG and SpHL-GEVPG treatments presented higher cytotoxicity against four human tumor cell lines than **against the** non-tumor cell**s** (NTHF). The encapsulation of GEVPG in liposomes did not alter its cytotoxicity against MDA-MB-231, SKBR3, MCF-7, and A549 cell lines ($p > 0.05$). It is worth noting that the treatments with GEVPG or SpHL-GEVPG for MDA-MB-231 and SKBR3 cells showed higher cytotoxicity than doxorubicin (positive control used in the clinic). In relation to cytotoxicity against A549 lung cancer cells, treatment with the liposomal formulation containing GEVPG showed to be more cytotoxic **than** the free GEVPG treatment and **had** a cytotoxicity profile similar to that obtained for paclitaxel treatment (positive control used in the clinic). The blank liposomes did not show any cytotoxicity effects when submitted under the same conditions.

It is interesting to note that the treatment of all tumor cell lines with GEVPG showed to be more selective than with the respective positive control (doxorubicin or paclitaxel). The encapsulation of GEVPG in liposomes led to a decrease **in** the SI values. This fact may be due to the mechanism of cellular entry of liposomes by endocytosis, contributing to the delivery of a higher dose of the cytotoxic agent inside **the** tumor cells.

3.6. Long-term cytotoxicity evaluation

Further experiments were conducted in order to determine if

Table 3
Determination of IC_{50} for cancer and non-cancer cell lines. Asterisks mean significant difference compared to other treatments on the same line (*$p < 0.05$ and ****$p < 0.0001$). Data expressed as mean ± SD (n = 3).

Cell line	IC_{50} (μM)			
	GEVPG	SpHL-GEVPG	DOX	PTX
MDA-MB-231	0.170 ± 0.007	0.168 ± 0.070	0.828 ± 0.148****	_
SKBR3	0.136 ± 0.002	0.158 ± 0.010	0.270 ± 0.019****	_
MCF-7	0.347 ± 0.032	0.343 ± 0.048	0.498 ± 0.134	_
A549	0.044 ± 0.008*	0.027 ± 0.005	_	0.020 ± 0.005
NTHF	48.05 ± 3.05	4.02 ± 0.07****	> 50	> 10

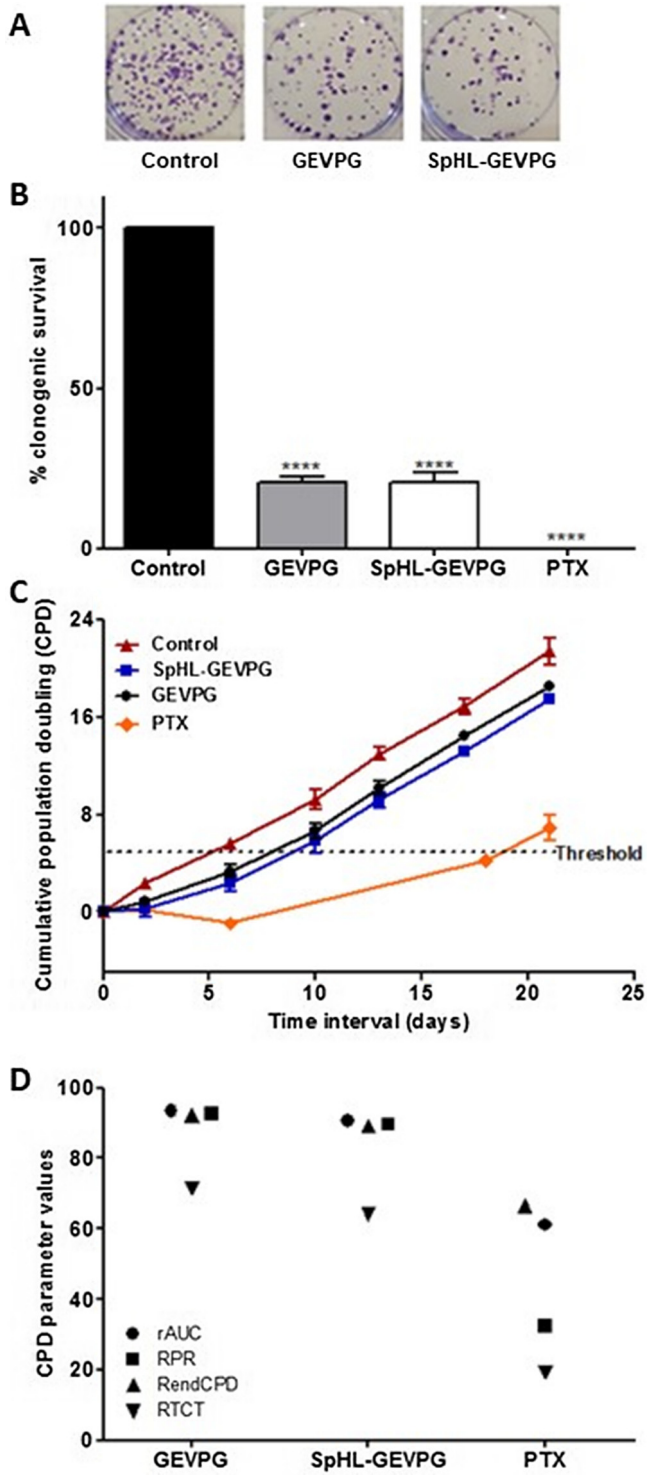

Fig. 5. Evaluation of long-term effects of the treatments of A549 cancer cell line with free GEVPG, SpHL-GEVPG or PTX. Photograph of plates containing colonies (A) and percentage of colony formation in relation to the control group for A549 lung cancer cell line when exposed to free GEVPG, SpHL-GEVPG or PTX (positive control) (B). Cumulative population doubling (C) and determination of their parameters (D) after treatment of A549 cells with GEVPG, SpHL-GEVPG or PTX. rAUC, RPR, RendCPD and RTCT signify area under the relative curve, relative proliferation rate, relative final CPD, time to reach the threshold, respectively.

GEVPG and SpHL-GEVPG treatments affect the long-term survival of A549 lung cancer cells. The clonogenic assay showed that the treatments of A549 lung cancer cells with GEVPG or SpHL-GEVPG at dose equal to 44 nM and 27 nM, respectively, were able to inhibit approximately 75% of formation of colonies compared to **the** control group. The treatment of these cells with paclitaxel at dose of 20 nM **totally** inhibited the formation of colonies (positive control) (Figs. 5A and B). These findings suggest that SpHL-GEVPG treatment may be an interesting alternative for decreasing tumor recurrence by resistance of tumor cells to the treatment with antineoplastics. These results were similar to those obtained either for treatment of **the** A549 cell line with convalotoxin at 10 nM [19] or evatromonoside at 100 nM [20]. The evaluation of the reacquired proliferative capacity by means of the determination of cumulative population doubling was carried out and the results are presented in Figs. 5C and D. The cells treated with GEVPG (44 nM) or SpHL-GEVPG (27 nM) reacquired the proliferation capacity over the 21 days; however, they showed a significant difference in relation to the control group with lower growth of the cells (Fig. 5C). This difference may be related to the ability of cardenolides, such as GEVPG, to inhibit DNA replication from topoisomerase II binding, paralyzing the cell division cycle [21]. It can be seen that surviving cells after treatment with PTX at the dose of 20 nM (positive control) proliferated slowly over the 21 days. This was expected because PTX is a microtubule stabilizing agent that interferes with the process of the mitotic spindle of dividing cells, leading to arrest in metaphase [22].

The **following** CPD parameters were calculated as proposed by Silva et al. [16] to better quantify the effect of the treatments on A549 cells: relative final CPD (RendCPD), which compares the CPD value obtained at the end of 21 days for the treated groups in relation to the control group; area under the relative curve (rAUC), which determines the overall effect of the treatment from the area under the curve of each treated group relative to the control group; time to reach the threshold (RTCT), which evaluates the delay in the proliferation rate of each treated group relative to the control group; and the relative proliferation rate (RPR), which determines the relative growth rate of the surviving cells in each group in relation to control group. A549 cells treated with GEVPG or SpHL-GEVPP presented values of RendCPD, RPR, and rAUC equal to 90% compared to **the** control group. The RTCT values in both groups of treatment were about 60% in comparison to **the** control group (Fig. 5D). All parameters decreased when A549 cells were treated with free GEVPG or SpHL-GEVPG, indicating that long-term treatments affect A549 cell proliferation. It should be noted that the treatment of the A549 cells with the blank liposomes presented a similar profile to the control group both in the clonogenicity assay as well as in the cumulative population doubling.

3.7. Antitumor activity evaluation

The antitumor efficacy of SpHL-GEVPG was evaluated in A549 lung tumor bearing-Balb/C nude female mice, by assessing the tumor volume variation over time. As shown in Fig. 6, the tumor volume in the blank liposomes and SpHL-GEVPG (dose of 0.5 mg/kg) treatment groups increased rapidly over time. By contrast, the tumor volume was significantly lower in mice treated with SpHL-GEVPG at doses of 1.0 and 2.0 mg/kg, or PTX at dose of 10 mg/kg, than in mice from the two other treatment groups. In addition, the treatment of mice with SpHL-GEVPG at doses of 1.0 and 2.0 mg/kg **significantly** reduced the RTV compared to those animals treated with blank liposomes (Table 5). SpHL-GEVPG treatment at 0.5 mg/kg did not significantly decrease the tumor growth compared to blank liposome-treated xenograft mice. Besides, the tumor growth inhibition was higher with the SpHL-GEVPG treatment at dose of 1.0 and 2.0 mg/kg (67.6%) compared to others. It is worth noting that the tumor volume variation of PTX-treated mice (dose of 10 mg/kg) was similar to that observed for SpHL-GEVPG-treated mice (dose of 1.0 mg/kg). These findings suggest that the SpHL-GEVPG treatment **is more potent than the PTX treatment, which requires the use of a dose 10 times greater to approach a similar therapeutic effect.**

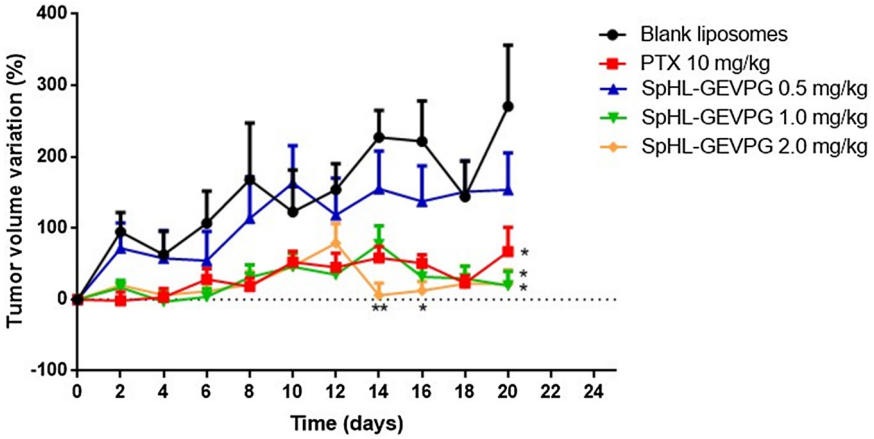

Fig. 6. Variation of xenograft lung tumor volume after treatment with SpHL-GEVPG, PTX and blank liposomes. Animals received intravenously blank liposomes (negative control), PTX 10 mg/kg (positive control), SpHL-GEVPG 0.5 mg/kg, SpHL-GEVPG 1.0 mg/kg or SpHL-GEVPG 2.0 mg/kg.

Table 5
Relative tumor volume and tumor growth inhibition after administration of SpHL-GEVPG, PTX or blank liposomes by intravenous route. Asterisks mean significant difference compared to blank liposomes (*$p < 0.05$ and **$p < 0.01$). Data represent the mean ± standard error (SE). The number of animals was equal to six, except for animals treated with blank liposomes ($n = 5$).

Treatment	RTV	TGI (%)
Blank liposomes	3.7 ± 1,9	–
PTX 10 mg/kg	1.7 ± 0,8 *	54.1
SpHL-GEVPG 0.5 mg/kg	2.5 ± 1,3	32.4
SpHL-GEVPG 1.0 mg/kg	1.2 ± 0,5 **	67.6
SpHL-GEVPG 2.0 mg/kg	1.2 ± 0,5 **	67.6

3.8. Preliminary toxicity analyses

The body weight variation was evaluated during and five days after the end of the treatments, as an indicator of toxicity. No significant body weight changes were detected for all treated groups and no death was recorded (data not shown). In addition, histological analyses of the tumor and different organs were performed at the end of the treatment period, and this evaluation revealed no evidence of toxicity in the kidney, liver, and spleen of the animals of all treatment groups. Besides that, for all the treatment groups analyzed, the neoplasia presented well delimited growth consisting of epithelial cells in a predominantly solid arrangement and sometimes presenting cystic cavities with eosinophilic secretion. The cells presented moderate nuclear pleomorphism, with evident nucleoli and broad and granular cytoplasm. The stroma is sparse and delicate and the areas of necrosis in the center of the tumor mass are sometimes extensive (Fig. 7).

4. Discussion

It is known that the mean diameter of vesicle **and the** diameter distribution profile are important characterization parameters to ensure the safety of the parenteral administration of liposomes [23,24]. SpHL-GEVPG presented the appropriate vesicle mean diameter, enabling their intravenous administration and the accumulation of these particles in the tumor area due to the known enhanced permeability and retention (EPR) effect [4]. It is well known that the determination of zeta potential allows predicting the stability of the liposomes. Vesicles that present high negative or positive zeta potential values have lower tendency to aggregation, due to electrostatic repulsion [23]. The zeta potential value of the SpHL-GEVPG near neutrality is due to the presence of PEG molecules coupled to the DSPE-PEG_{2000}, which reduce the electrophoretic mobility of particles [23,25]. However, the presence of PEG forms a steric barrier that avoids the vesicle proximity, increasing the stability of liposomes [26]. Thus, the good storage stability of SpHL-GEVPG in terms of mean vesicle diameter can be attributed to this fact. Concerning GEVPG entrapment percentage, the high value obtained might be explained by the formation of “pockets” in the lipid bilayer, where probably the GEVPG hydrophobic molecules were inserted, as occurs with other hydrophobic molecules such as paclitaxel [23,24]. **That the** GEVPG entrapment **remained** stable for 30 days **could be** due to the GEVPG lipophilic character that favors a stronger interaction with **the** lipid bilayer [27,28]. The slow release of the GEVPG from liposomes might once again indicate that the compound was strongly incorporated in **the** lipid bilayer, contributing to the stability of SpHL-GEVPG. Moreover, the strong interactions between GEVPG molecules and **the** lipid bilayer might be responsible **for** the similar release profile at both evaluated pH (7.4 and 5.0), leading to a decrease in sensitivity to acidic pH medium. SAXS studies of liposomes of the same lipidic constitution containing hydrophobic molecules, such as ursolic acid or paclitaxel, were carried out by our research group and showed the occurrence of changes in lipid self-assembly which may explain this reduction of pH-sensitivity [29,30]. Despite this, it is known that liposomes composed of DOPE:CHEMS are capable of releasing the encapsulated material into the cytoplasm due to their fusogenic character, which allows them to internalize in the cells more easily than conventional liposomes [4,31]. This fusogenic character can be explained by the fact that DOPE molecules provide a more hydrophobic bilayer surface due to the low hydration of their polar headgroup, allowing energetically more favorable interactions with the cell membrane. On the other hand, DOPE molecules may also be directly involved in the merging process, since they form the H_{II} phase, which is relevant as an intermediary stage in membrane fusion. During the fusion process between the cell and liposomal membranes the stalk mechanism may be involved. Briefly, a stalk is a semi-toroidal structure that forms between two closely apposed membranes and that makes the facing monolayers of the two membranes continuous. A transient H_{II} phase is formed during the stalk formation, which can explain the high fusogenic capacity of liposomes composed of DOPE [12,32]. Despite this, it is known that liposomes composed of DOPE:CHEMS are capable of releasing the encapsulated material into the cytoplasm due to their fusogenic character, which allows them to internalize in the cells more easily than conventional liposomes [4,31]. Herein we analyzed the cytotoxic effects of SpHL-GEVPG and free GEVPG against three human breast cancer cell lines (MDA-MB-231, MCF-7, and SKBR3) and in one non-small cell lung cancer cell line (A549). Breast cancer cell lines with different patterns of gene expression were used, according to the presence or absence of estrogen (ER), progesterone (PR), and human epidermal growth factor type 2 (HER-2) receptors. Breast cancer cell

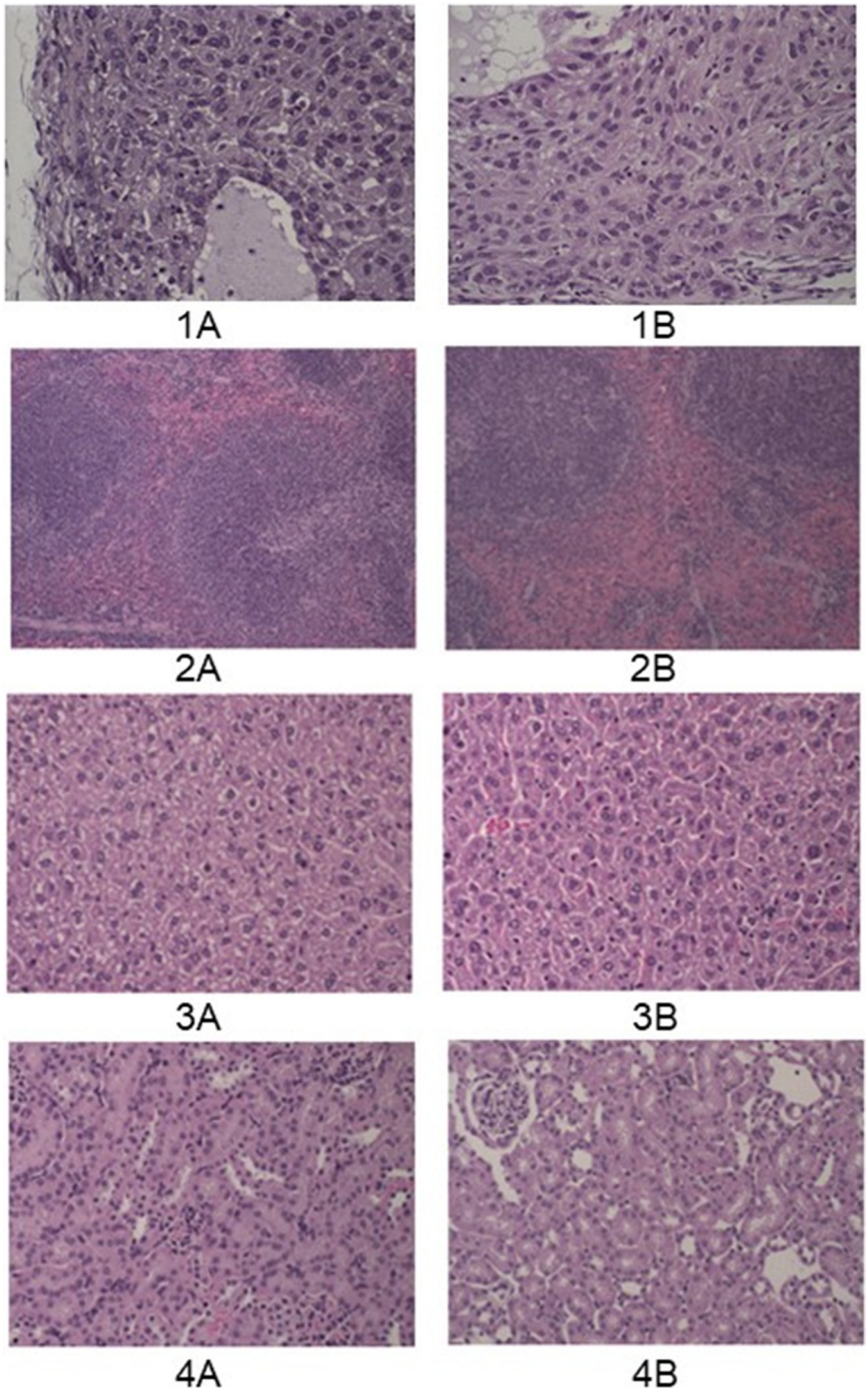

Fig. 7. Photomicrographs representative of histological preparations stained with hematoxylin-eosin solution to tumor (1), spleen (2), liver (3) and kidney (4) of Balb/C nude mice treated with blank liposomes (A) and SpHL-GEVPG 2.0 mg/kg (B).

subtypes **overexpressing** hormone receptors can be treated with receptor-specific therapy; however, when hormone receptors do not express, the cells **become** resistant and thus do not respond to specific therapy [33]. It can be observed that IC_{50} values obtained after SpHL-GEVPG treatment against MDA-MB-231 (triple negative), MCF-7 (ER^+), and SKBR3 ($HER2^+$) were similar to those obtained after free GEVPG treatment (Table 3). These results indicate that the incorporation of GEVPG into liposomes did not provoke any impairment in the cytotoxic effect of GEVPG against breast cancer cells. Concerning A549 lung cancer cell**s**, the IC_{50} value obtained after SpHL-GEVPG treatment was significantly lower than **the** IC_{50} value obtained after free GEVPG treatment. This result suggests that liposomes may facilitate GEVPG uptake by A549 cells and thus enhance cytotoxicity. Similar results of cytotoxicity were obtained by our research group with free glucoevatromonoside against A549 cells [34], showing that peracetylated glucose hydroxyl groups do not affect the cytotoxic effect. Similarly, we have also demonstrated that these long-circulating and fusogenic liposomes containing paclitaxel or ursolic acid were able to induce cytotoxic response against breast and prostate cancer cell lines [27,29]. In addition, it is important to mention that the SpHL-GEVPG treatment against breast or lung cancer cell lines used in this study was comparable or even better than PTX and DOX treatments, which are currently used in the clinic. The higher cytotoxic effect of free GEVPG and SpHL-GEVPG compared to DOX, against triple-negative breast cancer MDA-

MB-231, is a promising result since this type of breast cancer has the worst prognosis and **a** higher chance of recurrence, and therefore requires the research **on** new therapeutic strategies [33]. Furthermore, a promising agent anticancer, at the same time, must present maximum activity against cancer cells and minimal toxicity toward healthy cells [34]. One way to assess drug selectivity is by determining the SI. In our studies, free GEVPG was up to 1000-fold more selective toward MDA-MB-231, SKBR3, MCF-7, and A549 cells. SpHL-GEVPG treatment was also selective toward the same tumor cell lines, but the selectivity demonstrated by GEVPG treatment encapsulated in liposomes was lower than that of the free GEVPG treatment **(**Table 4**)**. The difference of SI values between free GEVPG and SpHL-GEVPG treatments can be explained by the internalization of the liposomes in the cells, with release of the GEVPG from the endosomes, increasing the cytotoxicity of the compound [29,35,36]. Other studies have shown a selective cytotoxic effect of cardenolides against tumor cells; **for example**, evatromonoside and glucoevatromonoside were **shown to be** approximately 4 and 8.5 times more cytotoxic, respectively, against A549 cells than against MRC-5 normal fetal lung fibroblast cells [20,34]. It is known that cardenolides do not present high cytotoxicity against healthy cells, because of the different expression of subunits of Na^+/K^+-ATPase. Cancer cells overexpress α subunit, which is responsible for binding of cardenolides [7,37,38]. These findings suggest that SpHL-GEVPG treatment could be a potential strategy for cancer therapy. As previously discussed, the EPR effect allows the passive targeting of liposomes to the tumor region [4]. This characteristic, together with the selectivity of the SpHL-GEVPG treatment to cancer cells, may increase antitumor efficacy and decrease systemic toxicity. Long-term *in vitro* cytotoxicity experiments are important since the resistance of tumor cells to the treatment is a major cause of tumor recurrence [16]. The clonogenic assay showed that SpHL-GEVPG is able to reduce significantly the number of colonies formed by A549 survival cells after treatment. In relation to CPD parameters, the RendCPD, RPR, and rAUC values were 10% less than the values of the control group. The RTCT values indicate that the proliferation rate of cells without treatment is about 0.4-fold greater than that of surviving cells after treatment with GEVPG or SpHL-GEVPG. These results indicate that SpHL-GEVPG might be a promising alternative for decreasing tumor recurrence [16]. Similar results of clonogenic assay and CPD were obtained for A549 survival cells after long-term treatments with 10 nM convallotoxin and 10–100 nM evatromonoside [19,20]. Finally, the antitumor efficacy of the SpHL-GEVPG treatment was evaluated in A549 lung tumor-bearing Balb/C nude female mice. Blank liposomes did not induce toxicity or antitumor activity in the experimental animal model, being then chosen as negative control of our experiments [39]. From the obtained results (Table 5 and Fig. 6), it can be seen that the liposomal formulation containing GEVPG (doses equal to 1.0 or 2.0 mg/kg) was 5 to 10 times more potent than PTX treatment at dose equal to 10 mg/kg (positive control). In addition, SpHL-GEVPG treatment at dose of 1.0 mg/kg demonstrated an antitumor effect similar to that observed for SpHL-GEVPG treatment at dose of 2.0 mg/kg, indicating that the increase of the dose to more than 1.0 mg/kg **does** not improve the antitumoral effect. The doses used in this study were based **on** previous studies which determined the maximum tolerated dose of the cardenolides ouabain and digitoxin, 5 mg/kg and 10 mg/kg, respectively, after their intraperitoneal administration route in healthy mice [40]. Besides that, twelve administrations of the cardenolide UNBS1450 at doses of 10 and 20 mg/kg significantly inhibited the tumor growth in the non-small cell lung cancer xenograft model [41,42]. Therefore, the dose equal to 1.0 and 2.0 mg / kg of the SpHL-GEVPG treatment appears to be safe and the preliminary toxicity study corroborates this behavior. Another important point to note is SpHL-GEVPG's potent antitumor activity which led to significant inhibition of tumor growth with the use of an extremely low dose (1.0 mg/kg). Considering these preliminary experiments, the doses of SpHL-GEVPG (0.5, 1.0, and 2.0 mg/kg) were selected with safety. From the obtained results (Table 5 and Fig. 6), it can be seen that the liposomal formulation was 5 to 10 times more potent than positive control (PTX 10 mg/kg).

5. Conclusion

In conclusion, the results of the present study demonstrated that SpHL-GEVPG was successfully developed and demonstrated a good stability after having been stored for 30 days. The cytotoxic studies against breast and lung cancer cell lines demonstrated that SpHL-GEVPG treatment significantly reduced the cancer cell viability. In addition, the SpHL-GEVPG formulation was selective for these tumor cells in comparison to human non-tumor cells. Long-term *in vitro* studies confirmed that SpHL-GEVPG treatment decrease**s** the growth capacity of surviving tumor cells and the ability of these cells to form colonies. The evaluation of antitumoral effect of the treatment with SpHL-GEVPG in a human lung cancer xenograft model showed that SpHL-GEVPG inhibited tumor growth. Therefore, the results from this study suggest the potential applicability of SpHL-GEVPG as a new and promising anticancer formulation.

Conflicts of interest

None.

Acknowledgments

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