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Article

Co-administration of paclitaxel and cisplatin liposomal improves efficacy and reduces toxicity of chemotherapy agents in murine breast cancer model

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Paclitaxel (PTX) and cisplatin (CDDP) are potent cytotoxic drugs that target distinct intracellular pathways employed together for breast cancer therapy. While studies indicate enhanced treatment efficacy with drug combinations, the concomitant rise in adverse effects remains a concern. This study assessed the potential of co-administration of these drugs encapsulated into pH-sensitive liposomes (SpHL) against a murine triple-negative breast cancer model. The cytotoxicity studies revealed a concentration-dependent relationship between drug concentration and cell viability for both drugs. The combination effect of free drugs at IC_{50} and IC_{50}x2 showed an additive effect, while co-treatment with SpHL-PTX:SpHL-CDDP at IC_{50}x4 (1:3 molar ratio) displayed strong synergism (CI = 0.52). Other combinations exhibited antagonism (CI > 2.0). *In vivo* studies were performed at PTX:CDDP 1:3 molar ratio in two regimen protocols: single or dual dose protocol, resulting in different cumulative doses. Tumor growth was significantly decreased when two doses of free or encapsulated drugs were used compared to single-dose administration. Notably, encapsulated dual-dose treatments demonstrated enhanced antitumor efficacy, diminished systemic toxicity, and zero mortality. In conclusion, our study underscores the promising potential of co-administering encapsulated drugs into SpHL, highlighting their superior efficacy and reduced toxicity in breast cancer treatment compared to free drugs.

Keywords: Synergism. cytotoxicity. Antitumor efficacy. Toxicity. Combined therapy.

INTRODUCTION

Paclitaxel (PTX) and cisplatin (CDDP) are conventional chemotherapeutic agents widely used in clinics due to their remarkable antitumor efficacy against various solid tumors. These drugs present different physicochemical and pharmacokinetic features as well as distinct mechanisms of action and toxicity profiles (Khosravi-Shahi, Cabezón-Gutiérrez, Custodio-Cabello, 2018; Sikov, 2015). CDDP is known to bind covalently to DNA and form DNA adducts, which triggers inhibition of cell cycle progression and induces apoptosis (Dasari, Tchounwou, 2014). On the other hand, PTX is a microtubule-stabilizing agent and causes abnormal mitotic spindle assembly, chromosome segregation, and cell cycle arrest (Zhu, Chen, 2019). The efficacy of these drugs in monotherapy regimens has a response rate of 25-35%, and the median overall survival of patients is less than 12 months (Isakoff *et*

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al., 2015).

It is well-known that the combination of two or more chemotherapeutic agents is a feasible approach for improving the effectiveness of antitumor treatment, reducing the side effects, and increasing the probability of achieving a patient's cure compared to monotherapy (Xu *et al*., 2015). The combination regimens can sensitize cancer response to drugs by synergetic effect and modulate different signaling pathways in cancer cells. This promising approach can result in lower doses of each drug and, consequently, reduced side effects, and offers more tolerable cancer treatments (Qin *et al*., 2018; Núñez *et al*., 2016). As CDDP and PTX exhibit distinct mechanisms of action, combined therapy with both drugs can synergistically reduce the therapeutic doses and chemoresistance to treatment. Studies have shown that the combined therapy of CDDP and PTX inhibits breast cancer growth and metastasis, hence providing a new therapeutic route to treating this disease (Wang *et al*., 2021; Sun *et al*., 2014). However, the toxicity profile is still a critical issue, and formulation development with an increased safe profile is required. Besides, a more effective combination strategy with the ability to coordinate the pharmacokinetics and biodistribution of multiple drug molecules is highly desirable to maximize combinatorial effects.

Among several strategies to reduce toxicity and increase the specificity of chemotherapeutic agents, drug delivery systems are the most promising since they act as modulators of pharmacokinetics and pharmacodynamics (Glassman, Muzykantov, 2019; van der Koog, Gandek, Nagelkerke, 2022). In the field of drug delivery, liposomes stand out with their diverse size range, permeability, fluidity, and composition, presenting a promising avenue in drug delivery for breast cancer. Their successful use in several FDA-approved medicines, with the translation of around 15 liposomal formulations into marketed products over the past three decades, underscores their potential (Bozzuto, Molinari, 2015). This system's ability to encapsulate lipophilic and hydrophilic drugs, such as PTX (aqueous solubility 5.5mg/L) and CDDP (aqueous solubility 2530mg/L), further enhances their appeal. Besides, liposomes might preferentially accumulate in tumors via the enhanced permeability and retention (EPR) effect, which results from more fenestrations in the vessels of newly vascularized tumors and defective lymphatic drainage in the tumor region (Ferreira *et al*., 2013; Pawar, Prabhu, 2019).

In the last years, our research group has studied the biological behavior of long-circulating and pH-sensitive liposomes carrying PTX or CDDP in different *in vitro* and *in vivo* experimental models, such as lung, breast, and pancreatic cancers. Overall, our data have shown that monotherapy regimens using CDDP or PTX liposomal were more effective in inhibiting cell proliferation and cancer progression compared to the drug in solution form (Franco *et al*., 2021; Monteiro *et al*., 2019; Carlesso *et al*., 2016; de Carvalho Maroni *et al*., 2012; Leite *et al*., 2012; Carvalho Júnior *et al*., 2007). The pH-sensitive liposomes are designed to remain stable at physiological pH and undergo structural destabilization at acidic pH, releasing the encapsulated drug at the specific tumor site. This is due to the significant pH difference between healthy tissues (pH 7.4) and the intracellular environment of tumors (pH 6.5), especially in the endosomes (pH of about 4.5–5.5). This difference in pH levels is a critical factor in the targeted drug delivery to the tumor site, allowing it to reach lethal concentrations in breast cancer cells (Ferreira *et al*., 2013; Franco *et al*., 2021; Monteiro *et al*., 2019).

To date, no evaluation of the combined effect of liposomal formulations has been conducted. Therefore, considering the previously reported benefits following the administration of PTX and CDDP-loaded liposomes individually, it becomes intriguing to explore the potential of co-administration of these long-circulating and pH-sensitive liposomes containing PTX and CDDP as an alternative approach for treating triple-negative breast tumors. To achieve this, liposomes were prepared and characterized, and a synergistic study was carried out to determine the best molar ratio for *in vivo* assays. Then, antitumor efficacy and preliminary toxicological

tests were performed in a 4T1-breast tumor model to validate our hypothesis.

MATERIAL AND METHODS

Material

PTX was purchased from Quiral Quimica do Brasil S/A (Juiz de Fora, Brazil). The lipids 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG2000) were purchased from Lipoid GmbH (Ludwigshafen, Germany). CDDP, cholesteryl hemisuccinate (CHEMS), and Cremophor® EL were acquired from Sigma Chemical Company (St. Louis, USA). Sodium chloride (NaCl), acetonitrile high-pressure liquid chromatography (HPLC), and dimethyl sulfoxide (DMSO) were obtained from Merck (Darmstadt, Alemanha). Water was purified using a Milli-Q apparatus (Millipore, Billerica, USA). All other chemicals and reagents used in this study were of analytical grade.

Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute Medium (RPMI 1640), sulforhodamine B, trypsin, ethylenediaminetetraacetic acid (EDTA) solution (0.5%), and trypan blue were supplied by Sigma-Aldrich (St Louis, EUA). Fetal bovine serum (FBS) was acquired from Gibco Life Technologies (Carlsbad, USA).

Liposome preparation

PTX-loaded liposomes (SpHL-PTX) were prepared using the thin film hydration method as previously described (Monteiro *et al*., 2019). Briefly, liposomes composed of DOPE, CHEMS, and DSPE-PEG2000 at a molar ratio of 5.7:3.8:0.5, respectively, were prepared at a final lipid concentration of 40 mmol/L. Chloroform aliquots of the lipids and PTX (2.0 mg/mL) were transferred to a round-bottom flask, and the organic solvent was removed under reduced pressure. NaOH (0.456 mol/L) solution was added to the resulting film in equimolar ratio to CHEMS to guarantee its complete ionization. Following, the film was hydrated by NaCl 0.9% w/v (final volume equal to 10 mL). The obtained vesicles were sequentially submitted to the high-intensity probe sonication (20% amplitude) for 5 min in an ice bath using a high-intensity ultrasonic processor (R2D091109 model; Unique Instruments, Indaiatuba, Brazil). Free PTX was removed by centrifugation (HeraeusMultifage X1R Centrifuge, Germany) at 3000 rpm at 4 °C for 10 min.

The protocol described above was followed for CDDP liposomes (SpHL-CDDP) preparation except for adding the drug. In this case, the lipid film was hydrated with a CDDP (2 mg/mL) solution in NaCl 0.9% w/v (final volume 10 mL). The mixture was stirred in a vortex until liposome vesicle formation. The liposomes were then sequentially filtrated in 0.22 μm. Nonentrapped CDDP was removed by ultracentrifugation (Optima L-80XP; Beckman Coulter, Brea, California, USA) at 10 °C for 90 min. The empty liposomes (without drug, SpHL) were prepared as described for SpHL-PTX.

Preparation of free drugs

For *in vitro* assays, a solution of free PTX was freshly prepared by dissolving 8.5 mg of the drug in 2.0 mL of DMSO. Immediately before cell treatment, the solution was diluted at different concentrations in a cell culture medium. The highest DMSO concentration used was 0.4% (v/v). For the *in vivo* study, PTX was prepared in a micellar dispersion by solubilizing 6.0 mg of the drug in 1.0 mL of a mixture of Cremophor® EL: dehydrated ethanol (1:1 v/v) under vigorous stirring. Before intravenous injection, this dispersion was diluted in NaCl 0.9 % (w/v) solution at a 1.0 mg/mL concentration.

Concerning free CDDP, for *in vitro* e *in vivo* tests, 10.0 mg of the drug were dissolved in 5.0 mL of NaCl 0.9 % (w/v) at 37 °C and subsequently diluted either in cell culture medium or NaCl 0.9 % (w/v) according to the adopted protocol.

Physicochemical Characterization

SpHL-CDDP and SpHL-PTX were characterized by average diameter, zeta potential, polydispersity index (PDI), encapsulation efficiency (EE), and fixed aqueous layer thickness (FALT). The average diameter and PDI were determined by dynamic light scattering (DLS) at 25°C and a 90° angle by unimodal analysis. The zeta potential was evaluated by determining DLS associated with the electrophoretic mobility at an angle of 90° and a temperature of 25°C. All samples were diluted (30-folds) in a 0.9% NaCl (w/v) solution previously filtered in 0.45μm, and the measurements were performed in at least three batches in triplicate using a Zetasizer Nano ZS90 (Malvern Instruments Ltd, Malvern, England).

The EE of CDDP in liposomes was determined by atomic absorption spectroscopy using a Varian SpectrA–Zeeman (Varian, Freemantle, Australia) as previously described (Vieira *et al*., 2013). For the EE of PTX, high-performance liquid chromatography was used according to a validated method (Barbosa *et al*., 2015a). For both drugs, the EE was determined according to eq. (1):

EE= (Drug in purified SpHL / drug in non-purified SpHL) x 100 (1)

FALT was evaluated by the variation of the zeta potential measured in different ionic strengths. Aliquots of liposomal formulations (SpHL, SpHL-CDDP, and SpHL-PTX) were diluted in aqueous NaCl solution at 5, 10, 20, 150, and 500 mmol/L. Zeta potential was measured, and then FALT was estimated from a plot ln (Zeta potential) versus k. The Debye Huckel parameter (k) corresponds to $3.3\sqrt{C}$, where C represents the electrolyte concentration of the solution. The slope of the obtained plots indicates the thickness of the fixed aqueous layer in nm.

In vitro studies

Cell Culture

4T1 (murine breast cancer ATCC® CRL-2539™) cell lineage was obtained from the American Type Culture Collection (ATCC, Manassas, USA) and grown in RPMI-1640 supplemented with 10% FBS (v/v). Cells were maintained at 37°C in 5% CO2 and 95% humidity until confluency was reached. Then, they were harvested by trypsinization, centrifuged (5 min at 330 x g), and resuspended in 1.0 mL of cell medium. A viable cell count was performed in a Neubauer hemocytometer using trypan blue (1:1) dye exclusion method.

Cytotoxicity assays

The cytotoxicity was measured using sulforhodamine B (SRB) assay (Vichai, Kirtikara, 2006). Briefly, 4T1 cells were seeded (5×10^4/well in 96 well-plates), and after 24 h at 37°C in a 5% CO2 humidified atmosphere, cells were exposed to free CDDP or SpHL-CDDP (0.065 to 100 μM) and free PTX or SpHL-PTX (0.01 to 10 μM) and SpHL (without drug). Subsequently, the cells were incubated for 48 h, and then 100 μL of 10% trichloroacetic acid (TCA) was added to each well to fix the cells for 1 hour. The plates were washed with Milli-Q water and stained with SRB for 30 min. Finally, the plates were washed with 1% v/v acetic acid, and 100 μL of Tris-Base solution (10 mM, pH 10.5) was added to solubilize the protein-bound dye. The cell density was determined by measuring the optical absorbance at 510 nm with a Spectra Max Plus 384 microplate spectrophotometer (Molecular Devices, Sunnyvale, USA). Cell viability was determined by the percentage of absorbance compared to the untreated cells used as a control. Half maximal inhibitory concentration (IC50) was calculated using GraphPad Prism software.

Synergism analyses

4T1 cells were treated with each drug alone or in combination at fixed ratios equivalent to the respective IC50 values (i.e., at IC_{50} x 0.25, x 0.5, x 1, x 2, and x 4) for 48 h. Cell viability was determined by SRB assay, as described above. A combination at a molar ratio of

PTX: CDDP 1:3 was adopted from the IC_{50} value of the PTX liposome. This ratio was based on the doses used elsewhere (Leite *et al*., 2012; Monteiro *et al*., 2019). Considering the IC50 value equal to 0.4 μM, the PTX concentrations used were (0.1, 0.2, 0.4, 0.8, and 1.6 μM) and CDDP concentrations were (0.3, 0.6, 1.2, 2.4 and 4.8 μM). The degree of interaction between PTX and CDDP was calculated through the combination index (CI) equation, based on the median-effect principle of the mass-action law, using the software Calcusyn (Biosoft, Cambridge, U.K). According to the CI theorem, CI values lower than 0.9, between 0.9 and 1.45, and higher than 1.45 indicate synergism, additive effect, and antagonism, respectively, as described by Chou (2006).

Assessment of Antitumor Efficacy and Treatment-Related Toxicity

Female Balb/c mice (8 weeks old, 20 ± 2.0g) were obtained from the Bioterism Center of the Federal University of Minas Gerais (CEBIO-UFMG). The mice were housed in cages in a controlled environment with a temperature set at 25 ± 2 °C, a 30–70 % humidity range, a 12 h light-dark cycle, and free access to food and water. In vivo studies were conducted under the approval of the local Ethics Committee on Animal Use (CEUA/UFMG, protocol #75/2014) following the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Aliquots of 100 μL containing 2.5 × 10^6 4T1 cells in RPMI were injected subcutaneously into the right flank of female BALB/c mice. Tumor cells were allowed to grow for seven days; then, animals were randomly divided into five experimental groups (seven animals per group) that received: (1) SpHL; (2) free CDDP:PTX-one dose; (3) free CDDP:PTX-two doses; (4) SpHL- CDDP: SpHL-PTX-one dose or (5) SpHL-CDDP:SpHL-PTX-two doses. Each treated animal received 8.0 mg/kg and 7.5 mg/kg in one dose or 12.0 and 11.5 mg/kg split into two administrations, for CDDP and PTX, respectively. For the control group (SpHL), the corresponding volume of the SpHL-CDDP:SpHL-PTX was administered at a molar ratio of 1:3 in two doses. All injections were performed by the tail vein. The first day of treatment was considered day zero (D0) of the study. Antitumor efficacy was evaluated over six days by the determination of tumor volume.

The tumor volume (V) was evaluated each other day by the measurements of two orthogonal diameters (d1 and d2) with a slide calliper (Mitutoyo, MIP/E-103), where d1 and d2 were the smallest and the largest perpendicular diameters, respectively. It was calculated as follows: $v= (d1)^2 \times d_2 \times 0.5$.

Concerning the treatment toxicity, behavioral/clinical modifications, body weight, and mortality during treatment were observed. Six days after starting the therapeutic regimen, the mice were anaesthetized with a mixture of ketamine (80 mg/kg) and xylazine (15 mg/kg), and blood was collected by puncture of the brachial plexus in tubes with 10% w/v EDTA solution and then centrifuged at 1100 x g for 15 min to assess the plasma for biochemical tests. Urea and creatinine assays were conducted to investigate the renal function, while liver function was evaluated through measurements of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity, employing commercially kits (Labtest, Lagoa Santa, Brazil), and performed in the Bioplus BIO-2000 semiautomatic analyzer (São Paulo, Brazil).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 software. The normality and homogeneity of the variance analysis were verified by D'Agostino-Pearson's and Bartlett's tests, respectively. The difference among experimental groups was tested using the one-way analysis of variance (ANOVA), followed by Tukey's test. For non-parametric parameters, Kruskal-Wallis followed by Dunn's multiple comparison test. Differences were considered significant when P-values were lower or equal to 0.05 ($p \leq 0.05$).

RESULTS AND DISCUSSION

Physicochemical Characterization

The physicochemical parameters of the liposomal formulations are summarized in Table I. Average diameter values around 200 nm and vesicles of homogeneous size with PDI less than 0.3 were detected for all formulations evaluated. The size distribution by number showed approximately 99.8, 97.9, and 94.8% of the vesicles smaller than 200 nm for SpHL-PTX, SpHL-CDDP, and SpHL, respectively. As is known, passive targeting of liposomes can occur through the EPR effect; thus, small particles can take advantage of the overexpression of fenestrations in the neovasculature to extravasate to tumor sites and consequently achieve higher tumor uptake (Sawant, Torchilin, 2012; de Barros *et al*., 2013).

Zeta potential values close to the neutral range were observed for all formulations. These findings were similar to those obtained previously (Barbosa *et al*., 2015b; Leite *et al*., 2012). High encapsulation efficiency was obtained for PTX formulation (~ 90%) as those obtained in previous studies (Barbosa *et al*., 2015b; Monteiro *et al*., 2018). On the other hand, higher values (~30%) compared to previous studies were detected for CDDP formulation (Leite *et al*., 2012). ¬In previous studies, the reverse-phase evaporation method following the extrusion process through polycarbonate membranes with pore sizes of 0.4, 0.2, and 0.1 μm (5 cycles for each) was used to prepare CDDP-loaded liposome (Leite *et al*., 2012). The main disadvantage of the extrusion method is the product losses as well as the relatively small working volumes (<50 mL), which represent a limit for large-scale productions (Lombardo, Kiselev, 2022). Our present study chose the thin film hydration method and the high-intensity probe sonication, which have proven more efficient, practical, and less time-consuming than the previous method. Studies have shown that the molecular organization of the bilayers is determined by interactions between the constituents, which themselves do not depend on how the bilayer is formed (Lapinski *et al*., 2007). Thus, the sonication or extrusion process does not affect the molecular scale organization of liposomes. Based on this information, we can suggest that the higher encapsulation efficiency found in this study is a result of minimizing material loss during the process and a more effective encapsulation.

TABLE I - Morphological characteristics evaluated as measures for the normal development at different time points

Formulations / Parameters	SpHL	SpHL-PTX	SpHL-CDDP
Average diameter (nm)	200 ± 63	197 ± 22	193 ± 14
PDI	0.25 ± 0.05	0.23 ± 0.03	0.22 ± 0.04
Zeta potential (mV)	-3.3 ± 0.3	-2.9 ± 0.3	-2.9 ± 0.6
EE (%)	-	84 ± 2	31 ± 2

Data are expressed as mean ± standard deviation (*n*=3). Abbreviations - PDI: polydispersity index; EE: encapsulation efficiency; SpHL: pH-senstive liposomes; PTX: paclitaxel; CDDP: cisplatin.

It is well-described that PEG-modified liposomes show a fixed aqueous layer thickness (FALT) around the vesicle due to the interaction between PEG and water, which prevents the opsonization of the vesicles. This fact can provoke a reduced uptake by the mononuclear phagocyte system and may affect biodistribution profiles. Studies have shown that FALT can be determined by zeta potential measurements in media with different ionic concentrations. This is significant as the FALT parameter decreases notably with increasing NaCl concentration, leading to alterations in the PEG conformation on the particle's surface. At least two conformations can be identified as mushrooms (isolated grafts) and brushes (extended chain conformations) (Sadzuka *et al*., 2002). This parameter was calculated for SpHL, SpHL-PTX, and SpHL-CDDP formulations (Table II) to compare the influence of the drugs on the FALT. Data clearly showed that with increasing NaCl concentration, the absolute value of the zeta potential of liposomes decreased. However, there was no significant difference in the average FALT values calculated. All formulations showed values near 2 nm and FALT values between 0.73 and 2.52 nm are characteristic of PEG chains with mushroom structure (Sadzuka *et al*., 2002). Based on the FALT values found for both formulations, we can assume that SpHL-PTX and SpHL-CDDP will display similar biological behavior, which is essential to guarantee a simultaneous delivery of the drugs to the tumor. The co-delivery of PTX and CDDP would be expected since both liposomes have the same composition and very similar physicochemical parameters. In addition, previous studies from our group showed that this type of nanosystem presents a favorable biodistribution with suitable tumor accumulation and a high tumor-to-regular tissue ratio (Carvalho Júnior *et al*., 2007; Monteiro *et al*., 2018).

TABLE II - Zeta potential data of PEG-functionalized liposomes measured in ionic solutions containing different NaCl concentrations and fixed aqueous layer thickness determined

Diluents	SpHL	SpHL-PTX	SpHL-CDDP
Milli-Q water	-32.7 ± 0.3	-31.6 ± 2.2	-36.1 ± 4.4
NaCl 0.005 mol/L	-25.5 ± 8.0	-23.4 ± 3.5	-29.5 ± 1.9
NaCl 0.01 mol/L	-21.6 ± 3.2	-19.5 ± 2.7	-23.5 ± 1.5
NaCl 0.02 mol/L	-18.5 ± 1.5	-14.6 ± 1.3	-17.3 ± 1.3
NaCl 0.15 mol/L	-3.3 ± 0.3	-3.4 ± 0.4	-2.9 ± 0.4
FALT (nm)	1.85 ± 0.12	1.79 ± 0.09	2.06 ± 0.08

Data are expressed as mean ± standard deviation (n=3). Abbreviations – SpHL: pH-sensitive liposome; PTX: paclitaxel; CDDP: cisplatin

Cytotoxicity and synergism evaluation

The sulforhodamine B cell viability assay was performed to investigate whether liposome encapsulation impacted the drug cytotoxicity. These cells were incubated with free drugs, SpHL-CDDP, SpHL-PTX, or SpHL, and analyzed for viability after 48 h of treatment. The data obtained, expressed as cell viability (percentage), are shown in Figure 1, and the IC50 values can be seen in Table III. Firstly, no significant cytotoxic activity was detected for the SpHL treatment (data not shown). All treatments showed a concentration-dependent relationship between drug concentration and cell viability. A similar cytotoxic effect was observed for CDDP treatment, either in free form or encapsulated into liposomes (Figure 1A).

On the other hand, SpHL-PTX showed cytotoxic effects significantly higher than those verified for the

free PTX at concentrations of 2.50 and 0.04 μM (Figure 1B). There was no significant difference between the IC50 values for free and encapsulated drugs. However, our results indicated a higher cytotoxic activity from the treatment with PTX than CDDP, as evidenced by lower IC50 values. This fact suggests higher sensitivity to PTX treatment. The IC50 values for PTX and CDDP reported in the literature differ significantly among studies ranging from nM to μM. The IC50 values of PTX for the 4T1 cell line are reported to be between 50 nM to 16.52 μM, a range determined through the use of a variety of different colorimetric assay methods (WTS-1, MTT, and sulforhodamine assays) (Varan *et al*., 2021; Zuo *et al*., 2021; Gupta, Gupta, Srivastava, 2019). For CDDP, the IC50 values ranged from 10.6 to 341 μM using an MTT assay (Yerlikaya *et al*., 2013; Li *et al*., 2014; Moammeri *et al*., 2022). No data for these drugs loaded in liposomes were found, indicating the novelty of the study.

The next step was to evaluate the combination

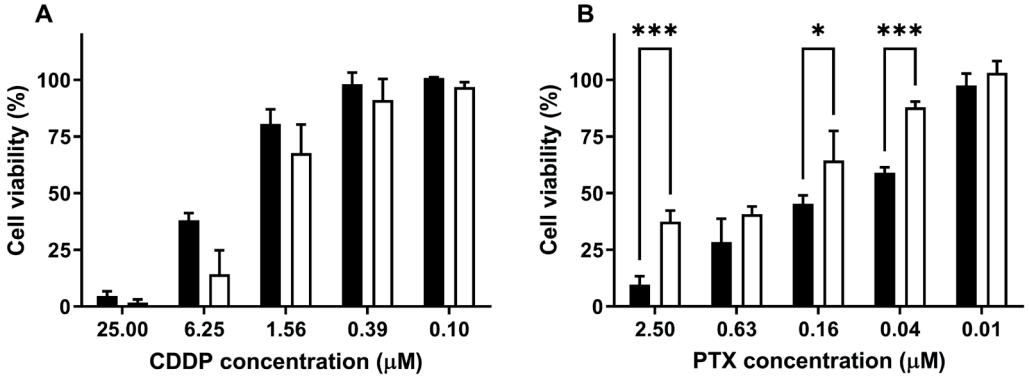


FIGURE 1 - Cytotoxicity activity on 4T1 cancer cells. The cell line was exposed to free (white bars) or liposome-encapsulated (black bars) cisplatin (A) and paclitaxel (B) at different concentrations for 48 hours, and cell viability was determined by sulforhodamine B assay.
Note: Data are expressed as mean ± error of three independent experiments (*n*=3). * p<0.05, *** p<0.001, Tukey's test.

TABLE III - IC_{50} values of free or liposome-encapsulated cisplatin and paclitaxel on 4T1 cells

	[a]IC_{50} (μM)	[b]CI 95%
Free CDDP	2.5	2.3 - 3.2
SpHL-CDDP	5.8	5.3 - 6.5
Free PTX	0.8	0.5 - 1.0
SpHL-PTX	0.4	0.1 - 0.6

[a] IC50: inhibitory concentration of 50 % cell growth was calculated through a nonlinear fit-curve (log of sample concentration versus normalized response - variable slope).

[b] CI 95 %: 95% confidence interval.

Abbreviations – SpHL: pH-sensitive liposomes; PTX: paclitaxel; CDDP: cisplatin.

effect (synergy, additivity, or antagonism) since it can be affected by the associated drugs ratio (Franco, Roque, Oliveira, 2019). Further, studies have shown that drugs that may cause mitochondrial damage, like PTX, have a synergistic effect with drugs that damage the DNA as CDDP (Bidkar, Sanpui, Ghosh, 2017; Nejati-Koshki *et al*., 2014). Hence, the combination cytotoxicity was tested by median-effect analysis using the CalcuSyn software in order to determine the combination index values for the different ratios. Regarding the combination effect for the mixture of free drugs, IC_{50} and IC_{50}x2 showed additive effects, while other combined free drugs led to antagonism (CI > 2.0). Strong synergism (CI = 0.52) was observed only for the treatment with SpHL-PTX:SpHL-CDDP at concentration 1.6:4.8 μM, 1:3 molar ratio (Figure 2). Previous studies reported *in vitro* data of PTX:CDDP combinations suggesting synergistic or antagonistic effects between these agents for different cell lines, besides cytotoxic effect appeared to be cell-line, time-exposure, and concentration-dependent (Uggioni *et al*., 2022; Feng *et al*., 2015; Gao *et al*., 2021; Cai *et al*., 2015). On the other hand, studies have also shown that the combined use of therapeutic agents associated with nanosystems may increase therapeutic efficacy and reduce side effects (Uggioni *et al*., 2022). The results of the present study are in accordance with these previous data and allow us to suggest that the combination of these drugs might result in an increased *in vivo* therapeutic efficacy.

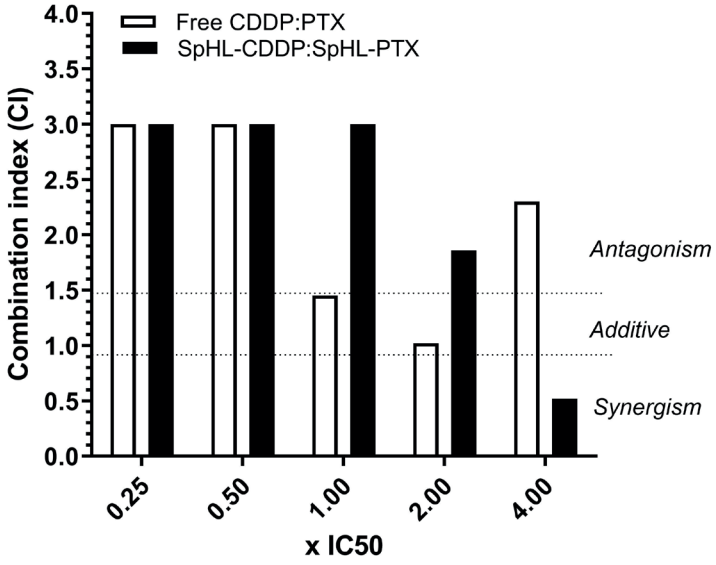


FIGURE 2 - Combination index values of PTX:CDDP in free or encapsulated form at five concentrations (0.25, 0.50, 1.0, 2.0, and 4.0 x IC50) combined at a molar ratio of 1:3 and adopted from the IC_{50} value of the PTX liposome. Range of CI: > 0.9 synergism; 0.9-1.45 additive; >1.45 antagonism.

Note: To improve the figure's readability, the values on the Y-axis were restricted to a maximum of 3.0.

In vivo antitumor efficacy and toxicity

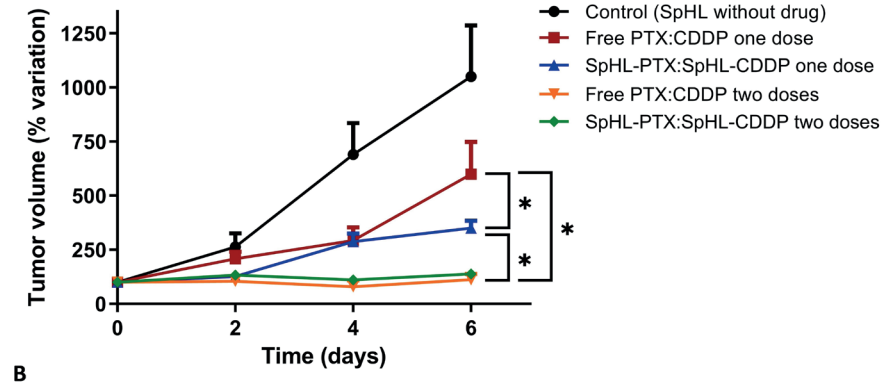
In order to confirm the synergistic effect observed *in vitro*, free and encapsulated drugs were administered in a murine triple-negative breast cancer model (4T1). Triple-negative breast cancer is an aggressive disease and usually requires a highly potent regimen to reduce tumor growth (Wang *et al*., 2022). In this study, we choose to evaluate the treatment regimens in 4T1 breast cancer since it is considered a model that shares substantial molecular characteristics with human cancer with high aggressiveness and high rate of cell proliferation (Arroyo-Crespo *et al*., 2019). Two regimen protocols were tested: a single dose protocol (7.5 mg/

kg of PTX and 8.0 mg/kg of CDDP) and a dual dose protocol resulting in a cumulative dose of 11.5 and 12.0 mg/kg for PTX and CDDP, respectively. It is essential to underscore that the molar ratio PTX: CDDP was 1:3 in both treatments. The doses of 8.0mg/kg and 7.5mg/kg were chosen from previous *in vivo* studies, in which these drugs individually were tested against Ehrlich and breast cancer with promising results (Leite *et al*., 2012; Monteiro *et al*., 2019). Thus, in the first step of the study, the mice were treated with these combined doses. Afterward, to improve the antitumor efficacy in the 4T1 breast cancer model, a dose 1.5-fold higher separated into two administrations was tested.

Antitumor efficacy assessed by tumor volume variation over time is shown in Figure 3A. Besides, regression analysis was performed to detect the changes in the tumor growth curves after treatments. The models that best fit and their respective determination coefficients are shown in Figure 3B. As depicted in Figure 3A, the tumor volume in the SpHL control group increased rapidly over time. Tumor volume was significantly reduced in all treated groups compared to the control group ($p<0.05$). In addition, tumor growth inhibition at D6 proved to be higher in mice treated with a single dose of SpHL-PTX: SpHL-CDDP than those treated with free PTX:CDDP.

On the other hand, tumor growth was significantly decreased when two doses of free or encapsulated drugs were used. Both treatments had similar tumor growth profiles with no significant change in the tumor volume throughout the experiment. These data are in agreement with *in vitro* studies and suggest that the synergistic effect between taxanes and platinum salts is dose-dependent.

A



B

Treatments	Dose(mg/kg)	Regression model	R^2
SpHL	-	y = 178.86x - 35.95	0.9922
PTX:CDDP	7.5:8.0	$y = 6.07x^2 + 57.59x + 17.26$	0.9678
SpHL-PTX:SpHL-CDDP	7.5:8.0	y = 60.61x + 9.36	0.9748
PTX:CDDP	11.5:12.0	NA	NA
SpHL-PTX:SpHL-CDDP	11.5:12.0	NA	NA

FIGURE 3 - *In vivo* antitumor efficacy. (A) Variation of tumor volume growth curves in mice after intravenous free or encapsulated drugs administration at ratio 1:3 (PTX: CDDP). (B) A regression analysis of the data on antitumor efficacy shows a regression model and determination coefficient (R^2). Different mathematical model fits indicated significant differences between groups. The absence of the equation from the regression analysis for groups treated with two doses is justified by the determination coefficients lower than 0.5.

Note: Data are presented as the mean ± error standard. *$p < 0.05$, ANOVA followed by Tukey's test.NA means not adjustable. Abbreviations – SpHL: pH-sensitive liposome; PTX: paclitaxel; CDDP: cisplatin.

The toxicity of each treatment regimen was monitored using the loss of animal body weight, behavioral/clinical, and mortality. Clinical toxicity signs (prostration and intense piloerection) were more pronounced in mice that received the free drugs. A mortality rate of 57% (4/7) was observed for mice treated with free drugs at a dose of 11.5 mg/kg and 12.0 mg/kg of PTX and CDDP, respectively, while the treatment with free drugs at a dose of 7.5 mg/kg and 8.0 mg/kg of PTX and CDDP induced to death of 28% (2/7) of mice. In contrast, the absence of death (100% survival rate) was found for mice treated with the mixture of drugs encapsulated into liposomes, even at the highest dose (Figure 4A).

Signs of toxicity related to body weight loss were observed for all groups treated either with free or encapsulated drugs (Figure 4B). For the group treated with 7.5 mg/kg of PTX and 8.0 mg/kg of CDDP, the highest variation occurred at D2 for SpHL-PTX: SpHL-CDDP; however, the mice recovered the body weight from D4. By contrast, the treatment at cumulative doses equal to 11.5 mg/kg and 12.0 mg/kg of PTX and CDDP, respectively, caused pronounced systemic toxicity to mice, inducing a loss of body weight higher

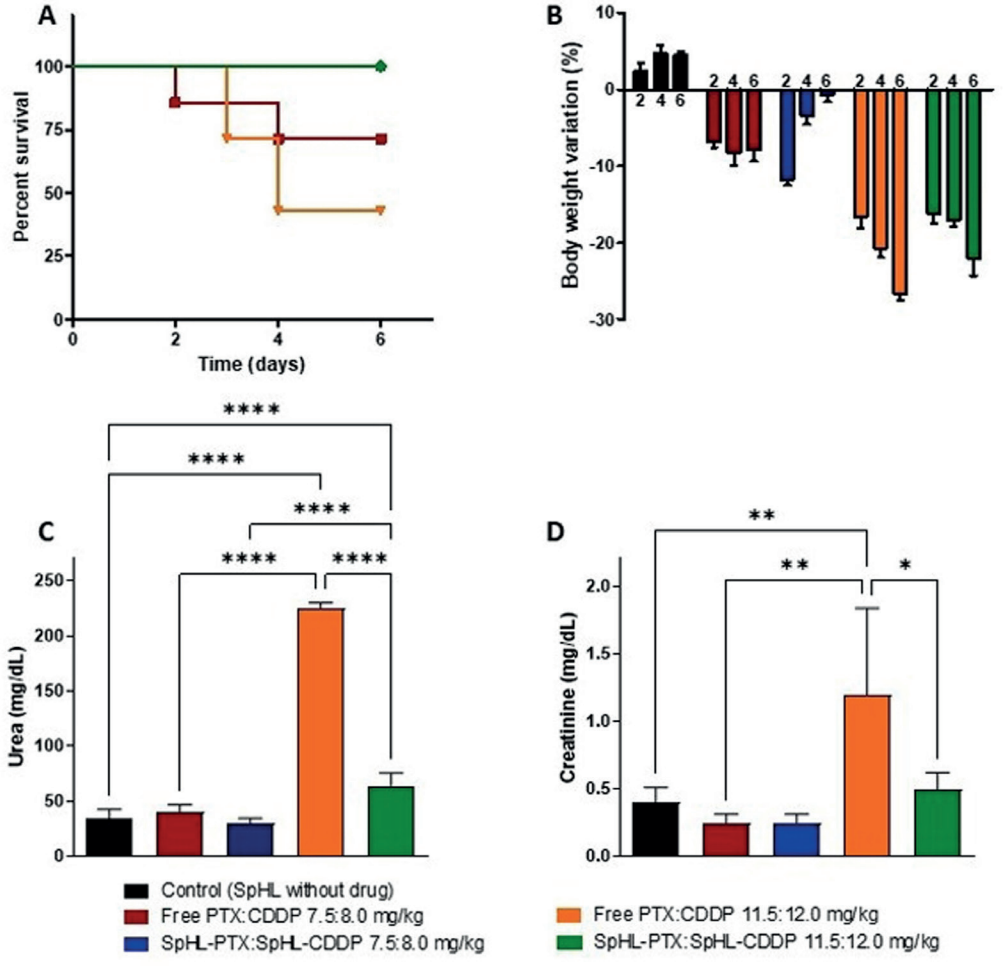


FIGURE 4 - Kaplan–Meier survival curves (A), body weight changes (B), blood urea (C), and creatinine level (D) of 4T1-bearing mice after intravenous treatment with mixtures of PTX and CDDP in free form or encapsulated into liposomes.

Note: Data are displayed as mean ± error standard. *, ** and **** P-values less than 0.05, 0.01 and 0.0001, respectively were set as the significance level by the ANOVA, followed by Tukey' test.

than 20% at D6. Because of this, the experiments were finished at this point, and the surviving mice from free or encapsulated drug treatment were euthanized.

Biochemical indicators of nephrotoxicity and hepatotoxicity were also evaluated for survival mice (Figures 4C and D and Table IV). Figure 4C and D also show renal (urea and creatinine) parameters. Changes were not observed in serum urea concentration for mice that received the combined treatment at a dose of 7.5:8.0 mg/kg either in free or encapsulated form. On the other hand, the urea levels significantly increased in animals receiving the combinations at a dose of 11.5:12.0 mg/kg compared to the control group. This increase was around 6.6-fold for animals treated with free PTX:CDDP combination. The same dose of SpHL-PTX:SpHL-CDDP presented urea levels 2.0-fold higher than the control group and significantly decreased the urea level compared to free PTX:CDDP. Concerning the creatinine levels, significant enhancement was detected only in mice receiving free PTX:CDDP combination at a dose of 11.5:12.0 mg/kg. Other groups showed creatinine levels comparable to the saline group. It is worth noting that urea and creatinine levels after SpHL-PTX:SpHL-CDDP treatment at the highest dose were significantly reduced compared to free PTX:CDDP.

Regarding the clinical chemistry parameters indicators of hepatic toxicity (Table IV), an increase in serum enzymes (AST and ALT) was detected in mice treated with the highest combination dose in free or encapsulated form compared to the control group. No significant clinical alteration was observed after treatment with a combination at the lowest dose in free or encapsulated liposome form. Although mice submitted to free PTX:CDDP and SpHL-PTX:SpHL-CDDP at a dose of 11.5:12.0 mg/kg showed increased levels of AST and ALT, the AST/ALT ratio remained constant in all groups treated compared to the control group. Previous studies reported that the combined chemotherapy with PTX and CDDP induced a transient elevation of ALT and/or AST (Wang, Su, Yin, 2017).

In vivo, the results showed improved antitumor

TABLE IV - AST and ALT levels of 4T1-bearing mice treated with the PTX:CDDP combination in the free or encapsulated form

Treatments	AST (U/L)	ALT (U/L)
Control (SpHL without drug)	110 ± 24	24 ± 2
Free PTX:CDDP 7.5:8.0 mg/kg	97 ± 13	24 ± 3
SpHL-PTX:SpHL-CDDP 7.5:8.0 mg/kg	101 ± 27	23 ± 4
Free PTX:CDDP 11.5:12.0 mg/kg	202 ± 25*	42 ± 7*
SpHL-PTX:SpHL-CDDP 11.5:12.0 mg/kg	227 ± 24*	51 ± 10*

* represents *P*-values less than 0.05, Kruskal-Wallis' test followed by Dunn's multiple comparison test. Data are displayed as mean ± error standard. Abbreviations – AST: aspartate aminotransferase; ALT: alanine aminotransferase.

Abbreviations – SpHL: pH-sensitive liposomes; PTX: paclitaxel; CDDP: cisplatin.

efficacy, a significant survival benefit, and reduced systemic toxicity when the combined encapsulated drugs were used, even in the highest dose. Clinical trials have provided evidence that progression-free survival was better and adverse effects were more common after combining CDDP and PTX compared to CDDP and gemcitabine (Li *et al*., 2019; Wang *et al*., 2022). In this study, we were able to demonstrate that regimen protocols with drugs encapsulated into SpHL significantly decrease toxicity and exhibit high potency for the treatment of triple-negative breast cancer compared to association with free drugs.

CONCLUSION

In conclusion, the combined therapy with PTX and CDDP encapsulated into pH-sensitive liposomes achieved greater efficacy with manageable toxicity, and hence, it can be an option as a first-line treatment of breast cancer.

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DATA AVAILABILITY STATEMENT

Data available from the corresponding author upon reasonable request.

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