

**UNIVERSIDADE FEDERAL DE MINAS GERAIS
FACULDADE DE FARMÁCIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS**

FERNANDA ALVES BORATTO

DESENVOLVIMENTO DE FORMULAÇÃO LIPOSSOMAL CONTENDO ALFA-SUCCINATO DE TOCOFERILA E DOXORRUBICINA E INVESTIGAÇÃO DA EFICÁCIA ANTITUMORAL E TOXICIDADE

Belo Horizonte - MG

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Tese apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Faculdade de Farmácia da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do grau de Doutora em Ciências Farmacêuticas

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Belo Horizonte - MG

2020

Boratto, Fernanda Alves.

B726d Desenvolvimento de formulação lipossomal contendo alfa-succinato de tocoferila e doxorubicina e investigação da eficácia antitumoral e toxicidade / Fernanda Alves Boratto 2020.
121 f. : il.

Orientadora: Elaine Amaral Leite.
Coorientador: Lucas Antônio Miranda Ferreira.

Tese (doutorado) - Universidade Federal de Minas Gerais, Faculdade de Farmácia, Programa de Pós-Graduação em Ciências de Farmacêuticas.

1. Doxorubicina - Teses. 2. Alfa-tocoferol - Teses. 3. Lipossomos - Teses. 4. Câncer de mama - Teses. 5. Agentes antineoplásicos - Teses. I. Leite, Elaine Amaral. II. Ferreira, Lucas Antônio Miranda. III. Universidade Federal de Minas Gerais. Faculdade de Farmácia. IV. Título.

CDD: 616.994

Elaborado por Darlene Teresinha Schuler – CRB-6/1759



UNIVERSIDADE FEDERAL DE MINAS GERAIS
FACULDADE DE FARMACIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

FOLHA DE APROVAÇÃO

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TOCOFERILA E DOXORRUBICINA E INVESTIGAÇÃO DA EFICÁCIA ANTITUMORAL E TOXICIDADE**

FERNANDA ALVES BORATTO

Tese submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIAS FARMACÊUTICAS, como requisito para obtenção do grau de Doutora em CIÊNCIAS FARMACÊUTICAS, área de concentração CIÊNCIAS FARMACÊUTICAS.

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Belo Horizonte, 27 de agosto de 2020.



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Dedico esse trabalho aos meus pais, que sempre sonharam junto comigo e me deram toda a força e suporte que precisei.

AGRADECIMENTOS

Agradeço a Deus por me amparar e proteger em todos os momentos, naqueles em que houve dificuldade assim como naqueles em que houve sucesso e principalmente por ter colocado pessoas tão especiais em minha vida.

Aos meus pais, Beto e Nilma, por todo incentivo e esforço, sempre colocando meus sonhos à frente dos deles. Sou muito grata por tudo o que fizeram e fazem por mim. À minha mãe e melhor amiga, pela cumplicidade, por dividir comigo as aflições e os maiores sorrisos, por todo carinho e todas as orações para que eu tivesse condições de chegar ainda mais longe. Ao meu pai, meu herói, por me ensinar os valores mais nobres que existem, por todo o suporte e proteção e por acreditar que eu seria capaz de atingir esse tão sonhado objetivo.

Ao meu irmão, Igor, pela disponibilidade e desprendimento, pelo enorme coração, por me ajudar sempre que precisei.

À minha sobrinha e afilhada Malu, por ser o motivo da minha alegria. Por ser tão carinhosa e especial, meu tesouro mais precioso.

À minha querida Família Alves, por me proporcionar os momentos mais alegres, pela torcida pelo meu sucesso e pela presença constante. Em especial aos meus padrinhos Tio Antonio e Tia Marlene por todo o incentivo e carinho. À minha querida Tia Maria, por ser tão amorosa e especial.

Ao Gabriel, meu marido, pelo companheirismo, carinho e compreensão em todos os momentos. Obrigada por estar sempre ao meu lado, me dando apoio, conforto, e por me fazer feliz.

Aos meus sogros, Maciel e Paula, por me acolherem sempre com tanto carinho e zelo.

À Professora Elaine, minha orientadora, pelos ensinamentos e pelo exemplo de profissional. Admiro sua postura sempre correta e íntegra, mas ao mesmo tempo

com grande doçura. Sou extremamente grata pela oportunidade de me permitir evoluir e aprender em todos os aspectos. Certamente é para mim hoje, quem eu quero me espelhar na minha carreira profissional.

Ao Professor Lucas, meu co-orientador, pela presença constante e por ser tão atencioso. Por todos os conhecimentos compartilhados, sempre tão inteligente e sagaz. Me sinto honrada de ter sido sua aluna e de ter tido a chance de trabalhar com um profissional de tamanha excelência.

Aos Professores Geovanni, André, Adriano e Angelo pela colaboração e disponibilidade.

Aos demais colaboradores, Cristina e Eduardo, pela contribuição neste trabalho.

À Marina, por ter colaborado no primeiro artigo e pela ajuda nos estudos de morfologia nuclear, sempre disposta a me auxiliar com boa vontade e paciência, mesmo estando em outro país. Agradeço também por todas as experiências compartilhadas no LTF.

Ao LTF e todos os colegas que lá fiz, onde estive por 10 anos, desde a iniciação científica, por ser um lugar no qual pude aprender muito, me decepcionei algumas vezes, mas me superei em todas elas. Vou guardar na memória o quanto eu amadureci e o quanto foi importante ter passado por lá.

Às minhas grandes amigas, Letícia, Júlia, Rapha, Thayná e Angela pelo apoio de sempre, por acreditarem no meu potencial e por dividirem comigo inúmeros momentos maravilhosos há tantos anos.

À FAPEMIG pelo apoio financeiro que possibilitou a realização desse trabalho.

RESUMO

A doxorubicina desempenha um papel importante no tratamento do câncer de mama. Entretanto, a cardiotoxicidade e a baixa penetração em tumores sólidos são algumas limitações de seu uso. A encapsulação da doxorubicina em lipossomas permitiu a redução de efeitos adversos, embora não tenha trazido benefícios quanto a eficácia antitumoral quando comparada com o fármaco livre. Lipossomas pH-sensíveis contendo associações de agentes antitumorais podem ser uma estratégia para melhorar a eficiência da entrega dos fármacos a nível celular e assim melhorar a sua eficácia antitumoral. No presente trabalho, foram desenvolvidos lipossomas pH-sensíveis contendo alfa-succinato de tocoferila e doxorubicina (pHSL-TS-DOX) e o seu comportamento biológico em modelo experimental de tumor de mama foi comparado com uma formulação já utilizada na clínica. Foram obtidas vesículas esféricas de conteúdo homogêneo de diâmetro médio inferior a 200 nm, monodispersas, com potencial zeta próximo a neutralidade, teor de encapsulação acima de 90% e estáveis por até 30 dias. A formulação apresentou uma liberação controlada em pH 7,4 e uma alta taxa de liberação em pH 5,0, dada a sua pH-sensibilidade, também comprovada pela técnica de Espalhamento de raios-X a baixo ângulo. Nos estudos *in vitro*, foi observada uma maior taxa de captação celular da doxorubicina a partir da formulação desenvolvida, o que permitiu atingir níveis mais avançados de apoptose e bloqueio do ciclo celular do que a formulação comercial. pHSL-TS-DOX atingiu uma concentração plasmática de doxorubicina mais baixa em animais saudáveis, entretanto, em animais portadores de tumor, houve um maior acúmulo de doxorubicina tumoral, o que pode justificar seu melhor desempenho nos estudos de atividade antitumoral. Essa formulação demonstrou ser segura, uma vez que conferiu proteção cardíaca e hepática e não induziu perda de peso considerável e nem mielossupressão. Portanto, diante dos resultados obtidos, pHSL-TS-DOX apresenta-se como uma potencial alternativa para o tratamento do câncer de mama.

Palavras-chave: doxorubicina; alfa-succinato de tocoferila; lipossomas pH-sensíveis; câncer de mama; atividade antitumoral.

ABSTRACT

Doxorubicin plays an important role in breast cancer treatment. However, cardiotoxicity and low penetration in solid tumors are some limitations of its use. The encapsulation of doxorubicin in liposomes allowed the reduction of adverse effects, although it did not bring benefits in antitumor efficiency when compared to free drug. pH-sensitive liposomes containing associations of antitumor agents may be a strategy to improve the efficiency of drug delivery at cellular level and thus improving its antitumor efficiency. In the present work, pH-sensitive liposomes containing alpha-tocopheryl succinate and doxorubicin (pHSL-TS-DOX) were developed and their biological behavior in an experimental model of breast tumor was compared with a formulation already used in the clinic. Spherical vesicles of homogeneous content with an average diameter of less than 200 nm, monodisperse, with zeta potential close to neutrality, encapsulation content above 90% and stable for up to 30 days were obtained. The formulation showed a controlled release at pH 7.4 and a high release rate at pH 5.0, given its pH-sensitivity, also proven by Small-angle X-ray scattering. In *in vitro* studies, a higher rate of cell uptake of doxorubicin was observed from the developed formulation, which allowed more advanced levels of apoptosis and block of the cell cycle than the commercial formulation. pHSL-TS-DOX reached a lower plasma concentration of doxorubicin in healthy animals, however, in animals bearing tumor, there was a greater accumulation doxorubicin inside the tumor, which may justify its better performance in studies of antitumor activity. This formulation proved to be safe, since it conferred cardiac and liver protection and did not induce considerable weight loss or myelosuppression. Therefore, considering the obtained results, pHSL- TS-DOX is a potential alternative for the treatment of breast cancer.

Keywords: doxorubicin; alpha-tocopheryl succinate; pH-sensitive liposomes; breast cancer; antitumor activity.

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LISTA DE ABREVIATURAS

ATCC	<i>American Type Culture Collection</i>
CEUA	Comitê de ética de uso em animal
CHEMS	Hemissuccinato de colestera
CHOL	Colesterol
DMEM	<i>Dubelcco's modified Eagle's medium</i>
DMSO	Dimetilsulfóxido
DOPE	Dioleilfosfatidiletanolamina
DOX	Doxorrubicina
DSPE-PEG ₂₀₀₀	Diestearoilfosfatidiletanolamina acoplado ao polietilenoglicol 2000
EPR	Permeação e retenção aumentados
EPC	Fosfatidilcolina de ovo
FDA	<i>Food and Drug Administration</i>
HBS	Tampão HEPES-salina
HEPES	Ácido 4-(2-hidroxietil)-1-piperazina etanosulfônico
HER2	Receptor tipo 2 do fator de crescimento epidérmico humano
HSPC	Fosfatidilcolina de soja completamente hidrogenada
IC ₅₀	Concentração inibitória de 50%
INCA	Instituto Nacional de Câncer
ISO	<i>International Organization for Standardization</i>
npHSL-AS	Lipossomas não pH-sensíveis contendo sulfato de amônio
npHSL-DOX	Lipossomas não pH-sensíveis contendo doxorrubicina
npHSL-DOX-AS	Lipossomas não pH-sensíveis contendo doxorrubicina e sulfato de amônio, similares ao Doxil [®]
PDI	Índice de Polidispersão
PE	Fosfatidiletanolamina
PEG	Polietilenoglicol
pHSL-TS	Lipossomas pH-sensíveis contendo alfa-succinato de tocoferila
pHSL-TS-DOX	Lipossomas pH-sensíveis contendo alfa-succinato de tocoferila e doxorrubicina
pHSL-TS-DOX-AS	Lipossomas pH-sensíveis contendo alfa-succinato de tocoferila,

	doxorubicina e sulfato de amônio
RPMI	<i>Roswell Park Memorial Institute</i>
SAXS	Espalhamento de raios-X a baixo ângulo
SFB	Soro fetal bovino
EP	Teor de encapsulação
Tm	Temperatura de transição de fase
TS	Alfa-succinato de tocoferila
TPGS	Alfa-succinato de tocoferila acoplado ao polietilenoglicol 2000

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1 INTRODUÇÃO

A nanotecnologia tem sido amplamente utilizada no desenvolvimento de novas estratégias para administração de fármacos na terapia contra o câncer. Os nanossistemas apresentam grandes vantagens, tais como: direcionamento do fármaco para a região tumoral, melhoramento das propriedades dos fármacos como solubilidade, estabilidade, tempo de meia vida e acúmulo tumoral, diminuição da dose e de efeitos adversos, associação de fármacos sinérgicos, liberação controlada, aumento da eficácia e redução da toxicidade, dentre outras (LIU *et al.*, 2014; SHI *et al.*, 2017).

Ao utilizar essa plataforma de entrega, é ideal que o fármaco seja mantido dentro de um intervalo terapêutico desejado e que haja um direcionamento para uma região específica, enquanto simultaneamente ocorre a diminuição de seus níveis sistêmicos (LANGER, 2003). O Doxil[®] (Janssen Biotech, Inc; Johnson & Johnson, EUA), primeira formulação lipossomal aprovada pelo *Food and Drug Administration* (FDA) para uso clínico no tratamento do câncer (BARENHOLZ, 2012; SCHUTZ *et al.*, 2013), reduziu os principais efeitos adversos provocados pela doxorubicina (DOX) tais como cardiotoxicidade, mielossupressão, náuseas e vômitos (O'BRIEN *et al.*, 2004). Além disso, a concentração plasmática de DOX aumentou significativamente com a administração do Doxil[®] (BARENHOLZ, 2012). Apesar desses benefícios, essa formulação não trouxe ganhos em relação à eficácia antitumoral da DOX.

Uma estratégia que poderia contornar esse entrave é a adição de agentes responsivos a estímulos na composição dos nanossistemas, tornando-os capazes de ultrapassar barreiras biológicas e estabelecer uma entrega do fármaco a nível intracelular. Sabendo-se que o tecido tumoral apresenta um pH baixo (ligeiramente ácido), devido ao metabolismo celular, a inclusão de um componente sensível ao pH pode favorecer a liberação do fármaco na região tumoral, bem como a nível intracelular, dentro dos endossomas (FERREIRA *et al.*, 2013).

Além disso, a associação de agentes com potencial eficácia antitumoral também tem sido investigada. Alguns estudos têm demonstrado que o alfa-succinato de tocoferila

(TS) é capaz de aumentar a eficácia antitumoral da DOX, além de possuir baixa toxicidade contra células normais e habilidade de proteger essas células contra a toxicidade induzida por outros agentes quimioterápicos. Portanto, a combinação de TS e DOX pode ser uma estratégia em potencial para aumentar a eficácia antitumoral da DOX, bem como reduzir a toxicidade nos tecidos saudáveis (ZHANG *et al.*, 2011; NEOPHYTOU & CONSTANTINOU, 2015). Por outro lado, por ser uma substância carboxilada de caráter anfifílico, ao ser incorporado em uma formulação lipossomal contendo um derivado da fosfatidiletanolamina (PE) poderia promover repulsão eletrostática com o grupo fosfato do lípido, favorecendo a desestruturação da vesícula lipossomal em meio ácido.

Diante do exposto, a proposta deste trabalho foi obter uma formulação de lipossomas pH-sensíveis contendo TS e DOX, explorando diversas funções do TS: (1) como componente estrutural da bicamada lipídica; (2) lípido carboxilado capaz de estabelecer a pH sensibilidade, permitindo a desestabilização lipossomal com a liberação da DOX e dessa forma, favorecer a captação celular ou tumoral da DOX; (3) formação de um par iônico com a DOX, permitindo a sua encapsulação e retenção na vesícula e (4) agente antitumoral em associação com a DOX.

Este trabalho está dividido em dois capítulos, o primeiro trata do desenvolvimento e caracterização da formulação, bem como avaliação da eficácia antitumoral em modelo murino de tumor de mama e toxicidade. O segundo capítulo aborda estudos de farmacocinética, acúmulo de DOX tumoral, captação celular e análise de apoptose e ciclo celular.

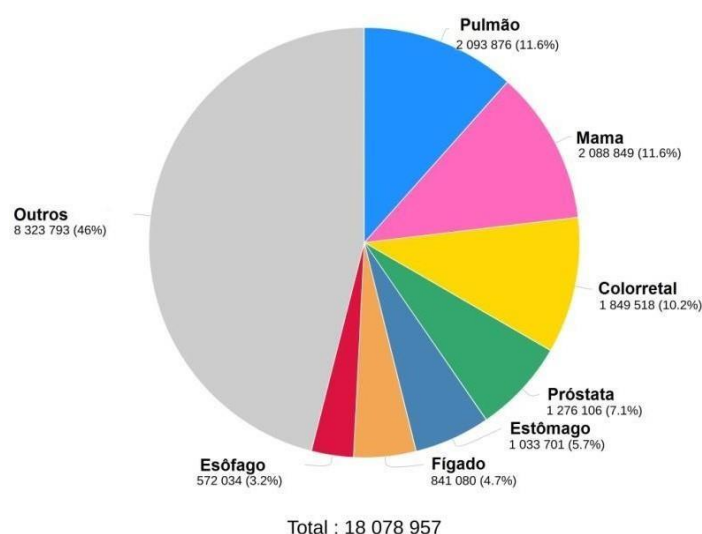
2 REVISÃO DA LITERATURA

Câncer

No ano de 2018 ocorreram 9,6 milhões de mortes em decorrência do câncer, representando a segunda causa de morte a nível mundial, atrás apenas das doenças cardiovasculares. Atualmente, uma em cada seis mortes ocorre devido a essa doença (WHO, 2020).

O projeto GLOBOCAN realizado pela Agência Internacional para Pesquisa em Câncer estimou que, no ano de 2018, ocorreram 18,1 milhões de casos novos em todo o mundo (WHO, 2020). Dentre os tipos de câncer mais incidentes estão o de pulmão, mama, colorretal e próstata (**Figura 1**). Para o ano de 2040, são estimados 29,5 milhões de casos. A estimativa de novos casos de câncer tende a aumentar no futuro não somente devido ao aumento da população mundial, mas também pelo aumento da expectativa de vida. A incidência de câncer aumenta com a idade provavelmente devido ao acúmulo de riscos combinado com a tendência dos mecanismos de reparo celular serem menos eficazes à medida que a pessoa envelhece (INCA, 2020; WHO, 2020).

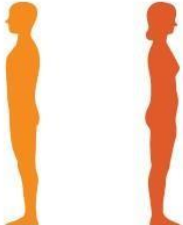
Figura 1: Estimativa do número de novos casos de câncer em 2018 no mundo



Adaptado de GLOBOCAN, 2018

No Brasil, estimativas do Instituto Nacional do Câncer (INCA) para o ano de 2018 apontaram a ocorrência de aproximadamente 600 mil casos novos de câncer. Os tipos mais incidentes em homens foram os cânceres de próstata, traqueia, brônquio e pulmão, cólon e reto e estômago. Nas mulheres, os de mama, cólon e reto, colo do útero e traqueia, brônquio e pulmão foram os mais incidentes. Esses dados não incluem o câncer de pele do tipo não-melanoma (INCA, 2020).

Figura 2: Estimativa do número de novos casos de câncer em 2018 no Brasil

Localização Primária	Casos	%			Localização Primária	Casos	%
Próstata	68.220	31,7%		Homens Mulheres	Mama Feminina	59.700	29,5%
Traqueia, Brônquio e Pulmão	18.740	8,7%			Cólon e Reto	18.980	9,4%
Cólon e Reto	17.380	8,1%			Colo do Útero	16.370	8,1%
Estômago	13.540	6,3%			Traqueia, Brônquio e Pulmão	12.530	6,2%
Cavidade Oral	11.200	5,2%			Glândula Tireoide	8.040	4,0%
Esôfago	8.240	3,8%			Estômago	7.750	3,8%
Bexiga	6.690	3,1%			Corpo do Útero	6.600	3,3%
Laringe	6.390	3,0%			Ovário	6.150	3,0%
Leucemias	5.940	2,8%			Sistema Nervoso Central	5.510	2,7%
Sistema Nervoso Central	5.810	2,7%			Leucemias	4.860	2,4%

Adaptado de Estimativa 2018 - Incidência de câncer no Brasil

Conceitualmente, câncer é um termo genérico para um grupo de doenças que tem como característica principal o crescimento descontrolado de células anormais que podem invadir partes adjacentes do organismo e se espalhar para outros órgãos, originando metástase (WHO, 2020). A transformação de células normais em tumorais ocorre por um processo de vários estágios que geralmente progride de uma lesão pré-cancerosa para um tumor maligno. Essas alterações são o resultado da interação entre fatores genéticos e agentes externos.

Dentre os fatores genéticos podem ocorrer danos mutagênicos em genes essenciais para a regulação da proliferação celular, como oncogenes (RAS, MYC, EGFR, e outros), genes supressores de tumor (codificam proteínas como p21, p53 e pRB, dentre outras), genes de reparo do DNA (MSH2, BRCA e CHK2, etc.) e genes que regulam a apoptose (genes que codificam caspases, p53 e Bax, etc.) (CROCE, 2008; TANAKA *et al.*, 2013; LAHTZ & PFEIFER, 2011; KIRAZ *et al.*, 2016; WONG; CHEN, R.; CHEN, B., 2014; NEAL & YU, 2010).

Com relação aos agentes externos, esses podem ser cancerígenos físicos, tais como radiação ultravioleta e ionizante; cancerígenos químicos, como amianto, componentes da fumaça do tabaco, aflatoxina (contaminante de alimentos) e arsênico (contaminante de água potável) e cancerígenos biológicos, como infecções de certos vírus (vírus Epstein Bar, vírus da Hepatite C, vírus do papiloma humano) e bactérias (como *Helicobacter pylori*) (WHO, 2020; TANAKA *et al.*, 2013). Além desses, fatores relacionados ao estilo de vida como sedentarismo, má alimentação,

consumo de álcool e uso de tabaco podem contribuir para o aumento do risco em desenvolver a doença.

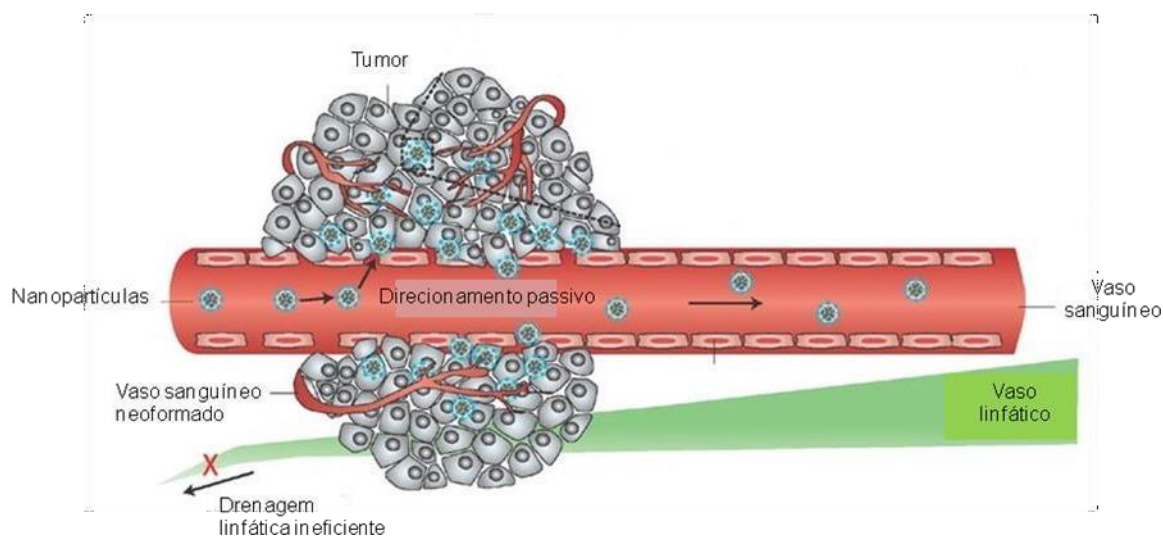
Para que um tumor maligno se desenvolva, é necessário que a célula adquira certas características, como: sinalização proliferativa autossustentada, evasão da apoptose, capacidade de invasão, indução de angiogênese e resistência à morte, entre outras (NEAL & YU, 2010; HANAHAN & WEINBERG, 2011).

Os tumores malignos podem ser classificados como tumores sólidos ou hematológicos. Os tumores sólidos apresentam características fisiopatológicas peculiares tais como hipervascularização, arquitetura vascular incompleta, secreção de fatores de permeabilidade vascular e ausência de um sistema linfático efetivo (MAEDA *et al.*, 2000). Em função da intensa proliferação celular e do crescimento tumoral, a vasculatura normal presente em sua vizinhança não é suficiente para fornecer toda a demanda de oxigênio necessária para a sua proliferação, formando regiões de hipóxia. A hipóxia estimula a secreção de fatores de crescimento pró-angiogênicos e enzimas proteolíticas que provocam o surgimento de novos vasos sanguíneos a partir dos capilares adjacentes ou de células tronco endoteliais circulantes (VERBRIDGE *et al.*, 2010). Esse processo, conhecido como angiogênese, promove o rápido desenvolvimento de novos vasos sanguíneos, os quais apresentam arquitetura irregular, epitélio descontínuo e normalmente são desprovidos de membrana basal (BERTRAND *et al.*, 2014).

Além disso, sob certas circunstâncias de inflamação/hipóxia (comum em tumores, infartos e infecções), o revestimento endotelial dos vasos sanguíneos torna-se mais permeável do que em tecidos saudáveis (TORCHILIN, 2011). A permeabilidade vascular aumentada, devido à formação de vasos irregulares, também é resultado de uma resposta inflamatória provocada inicialmente pelo tumor, que leva à liberação de citocinas que promovem um aumento da permeabilidade com a finalidade de facilitar a diapedese das células de defesa (MAEDA, 2010). Essas alterações conjuntas caracterizam o efeito de permeabilidade e retenção aumentadas (EPR). Assim sendo, partículas de tamanho variando de 10 a 500 nm conseguem deixar o leito vascular e se acumulam dentro do espaço intersticial, permanecendo nesse local, devido à ineficiente drenagem linfática. Caso essas

partículas estejam carregadas com um agente terapêutico, esse pode ser liberado e se acumular na região afetada (TORCHILIN, 2011) (Figura 3).

Figura 3: Efeito EPR – Aumento da permeabilidade no tecido tumoral devido a *gaps* intercelulares em vasos sanguíneos defeituosos, permitindo o extravasamento de nanopartículas e retenção dessas devido à ineficiente drenagem linfática



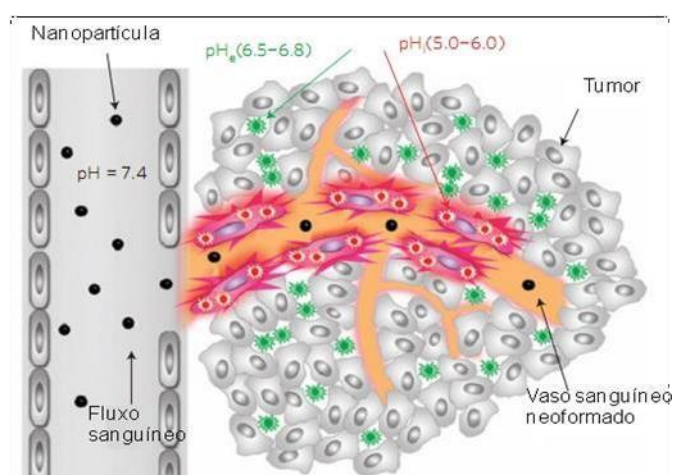
Adaptado de PEER *et al.*, 2007

Apesar do efeito EPR ser um mecanismo amplamente aceito e ter sido estudado intensamente nos últimos anos, novas vias de acumulação no tumor vem sendo propostas, como a transcitose. Diversos autores já descreveram esse processo (LIU *et al.*, 2019; LIU X *et al.*, 2019; PADIT *et al.*, 2020) e, recentemente, foi realizado um estudo bastante completo por Sindwhani e colaboradores (2020) testando diversas linhagens celulares. Nesse estudo, foi demonstrado que os *gaps* intercelulares presentes no endotélio tumoral não são suficientes para que ocorra o extravasamento e acúmulo de nanopartículas no interstício tumoral. Por outro lado, as células endoteliais realizam o processo de transcitose, envolvendo as seguintes etapas: captação ativa, transporte intracelular e exocitose. Esse processo permite a internalização de nanopartículas no tumor, promovendo um maior acúmulo dessas na região tumoral.

Além disso, sabe-se que tecidos tumorais apresentam um pH mais baixo (aproximadamente 6,5) do que tecidos normais (pH 7,4) e essa diferença pode ser

utilizada como base para o desenvolvimento de nanocarreadores responsivos ao pH (GUAN *et al.*, 2013) (**Figura 4**). A redução do pH tumoral pode ser explicada pela má organização das estruturas vasculares dentro dos tecidos tumorais, que resulta em um fluxo sanguíneo heterogêneo e uma entrega de nutrientes de forma não uniforme. Isso força as células presentes em regiões pobremente perfundidas a produzirem energia por meio de um aumento na taxa de glicólise anaeróbica, que, por sua vez, leva a um aumento na produção de ácido lático. Uma vez que o sistema linfático é ineficiente nessas regiões, o ácido lático não é removido adequadamente, resultando em uma redução do pH no interstício tumoral (FERREIRA *et al.*, 2013). Além disso, algumas organelas celulares também apresentam pH reduzido, como é o caso dos endossomos (pH entre 5 e 6), que podem também ser explorados para a entrega de fármacos. Assim, estratégias terapêuticas de direcionamento e que sejam responsivas às variações de pH são promissoras para contornar os grandes desafios associados ao tratamento do câncer (VISVADER, 2011).

Figura 4: Valores de pH na região tumoral



Adaptado de WANG *et al.*, 2013

Câncer de mama

O câncer de mama é o tipo de câncer mais frequentemente diagnosticado em mulheres no mundo, afetando 2,1 milhões de mulheres a cada ano causando o maior número de mortes relacionadas ao câncer entre as mulheres. Em 2018, estimou-se que 627.000 mulheres morreram de câncer de mama, correspondendo a

aproximadamente 15% de todas as mortes por câncer entre mulheres. Embora as taxas de câncer de mama sejam mais altas entre as mulheres em regiões mais desenvolvidas, elas estão aumentando em quase todas as regiões do mundo (WHO, 2020). No Brasil, a estimativa para o ano de 2018 foi de 59.700 novos casos, com um risco estimado de 56,33 casos a cada 100 mil mulheres (INCA, 2020).

A etiologia do câncer de mama é multifatorial, resultado de interação entre fatores genéticos com hábitos de vida, comportamento reprodutivo e meio ambiente. Alguns fatores de risco para o desenvolvimento do câncer de mama são bem conhecidos, como: envelhecimento, fatores relacionados à vida reprodutiva da mulher, histórico familiar, uso de alguns anticoncepcionais e terapia de reposição hormonal na menopausa, consumo de bebidas alcoólicas, excesso de peso, sedentarismo, exposição à radiação ionizante e alta densidade do tecido mamário (INCA, 2020).

Cerca de 5-10% dos casos de câncer de mama podem ser considerados hereditários, dentre esses, pelo menos, 30% são atribuídos a mutações nos genes BRCA-1 e BRCA-2. Outros genes têm surgido como alvos susceptíveis para o câncer de mama, tais como TP53, PTEN, CHEK2, ATM e PALB2 (ECONOMOPOULOU; DIMITRIADIS; PSYRRI, 2015; AMERICAN CANCER SOCIETY, 2020).

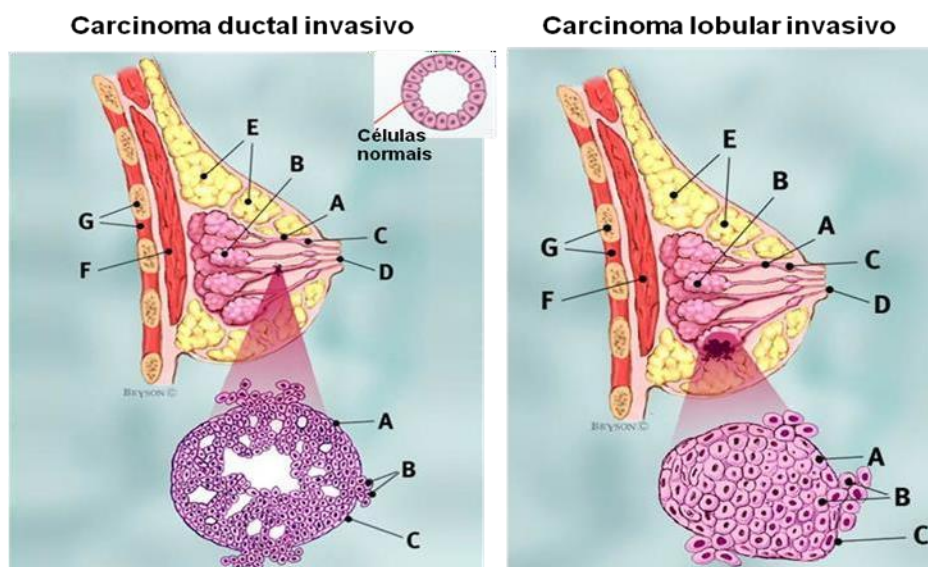
Apesar do câncer de mama ser altamente curável quando se tem um diagnóstico precoce, essa doença se torna incurável e às vezes até fatal caso ocorra metástase em órgãos distantes. Mais de 90% das mortes decorrentes do câncer de mama não são causadas pelo tumor primário, mas ocorrem como um resultado da metástase (CAO *et al.*, 2015).

A metástase é um processo complexo que compreende várias etapas, incluindo a invasão e penetração das células tumorais do tumor primário para a circulação sanguínea, sobrevivência no sangue e nos vasos linfáticos, extravasamento para tecidos distantes e proliferação nesses órgãos. A capacidade das células tumorais se desenvolverem em órgãos distantes ao tumor primário depende de complexas interações entre as células disseminadas e o microambiente tecidual dos órgãos invadidos e pode estar relacionada à produção e liberação de fatores de

crescimento, quimiocinas, moléculas guias pelo órgão-alvo ou tumor (FOKAS *et al.*, 2007). Os sítios de metástases mais comuns, provenientes de tumor de mama primário, são pulmões, ossos, fígado e cérebro (CAO *et al.*, 2015).

O câncer de mama compreende tumores heterogêneos com diferentes características clínicas, cursos de doenças e respostas a tratamentos específicos. A patologia clássica organiza os tumores de mama em várias categorias, com base na sua morfologia geral e organização estrutural. Os tipos mais comuns são o carcinoma ductal invasivo (cerca de 75% dos casos) e o carcinoma lobular invasivo (cerca de 10% dos casos) (**Figura 5**). Juntas, essas duas categorias e suas combinações formam a grande maioria (cerca de 90%) dos cânceres de mama, enquanto outros tipos menos comuns como medular, neuroendócrino, tubular, apócrino, metaplásico, mucinoso, inflamatório e tipos micropapilares, representam os 10% restantes (BERTOS & PARK, 2011).

Figura 5: Principais tipos de câncer de mama. Na figura da mama: A – ductos, B – lóbulos, C – porção dilatada do ducto, D – mamilo, E – tecido adiposo, F – músculo peitoral, G – caixa torácica. Na figura ampliada: A – células normais, B – células rompendo a membrana basal, C – membrana basal



Adaptado de breastcancer.org

O carcinoma ductal invasivo surge inicialmente nos ductos mamários. Pode ocorrer de algumas células conseguirem atravessar os ductos e posteriormente proliferarem

no tecido adiposo adjacente. Ao atingir esse ponto, essas células podem se espalhar para outras partes do corpo através do sistema linfático e da circulação sanguínea, formando metástases. Cerca de 8 em 10 cânceres de mama invasivos são do tipo carcinoma ductal. O carcinoma lobular invasivo, por sua vez, tem origem nas glândulas mamárias e, assim como o ductal invasivo, pode também provocar metástases (ARPINO *et al.*, 2004; CRISTOFANILLI *et al.*, 2005; VLUG *et al.*, 2014).

Do ponto de vista clínico, o câncer de mama pode ser subdividido em três grupos principais: 1) tumores que expressam receptores de estrogênio e/ou receptores de progesterona, que apresentam um melhor prognóstico, uma vez que as células dependem de estrógenos para seu crescimento e, dessa forma, antiestrógenos como o tamoxifeno, ou inibidores da aromatase são opções de tratamento; 2) tumores com expressão amplificada do receptor do tipo 2 do fator de crescimento epidermal humano (HER2), que também tem prognóstico favorável devido à possibilidade do uso de agentes terapêuticos direcionados a esse receptor, como o anticorpo monoclonal trastuzumabe; 3) câncer de mama triplo-negativo, devido à falta de expressão dos receptores de estrogênio, progesterona e uma expressão de HER2 normal ou negativa, sendo a quimioterapia convencional e a radioterapia as principais alternativas terapêuticas (HIGGINS & BASELGA, 2011; TCGA, 2012; CHEN, 2013).

Dentre os tipos de câncer de mama citados acima, o triplo-negativo é o que apresenta o pior prognóstico (BERTOS & PARK, 2011). Esses tumores correspondem à cerca de 15% de todos os cânceres de mama invasivos e geralmente apresentam histologicamente menor proporção de arranjos tubulares, alto grau de pleomorfismo nuclear e elevado índice mitótico (FOULKES *et al.*, 2010).

Dentre as linhagens celulares de tumor de mama triplo-negativo utilizadas experimentalmente podemos destacar a MDA-MB-231 (tumor de mama humano) e a 4T1 (tumor de mama murino). Ambas fornecem um padrão de tumor bastante agressivo. A MDA-MB-231, uma linhagem de grau 3, contém uma morfologia anormal e é considerada pouco diferenciada, pois não possui uma estrutura ou padrão arquitetônico do tecido. Enquanto que a 4T1 mimetiza um tumor de mama

humano em estágio IV, com presença de tumor primário com metástase em linfonodos e órgãos distantes (HERO *et al.*, 2019; KAUR *et al.*, 2012).

Atualmente, o câncer de mama é considerado uma doença tratável, sendo que diversas classes de fármacos antitumorais têm sido empregadas. Dentre elas, destacam-se as antraciclinas (como a DOX e a epirrubicina) e os taxanos (como o paclitaxel e o docetaxel) para o tratamento de câncer de mama em estágios iniciais. Esses fármacos podem ser utilizados isoladamente ou em terapias combinadas com outros quimioterápicos como o fluorouracil, ciclofosfamida e a carboplatina (AMERICAN CANCER SOCIETY, 2020). Para o tratamento do câncer de mama HER2 positivo, por exemplo, pode ser utilizado o trastuzumabe geralmente associado com um taxano. Outros fármacos já são aprovados pelo FDA na imunoterapia para o tratamento do câncer de mama, como everolimus, toremifeno, fulvestrant, lapatinibe, etc. Na quimioterapia convencional, os fármacos mais comumente utilizados no tratamento do câncer de mama em estágio avançado são: docetaxel, paclitaxel, derivados da platina (cisplatina, carboplatina), vinorelbina, capecitabina, DOX lipossomal (Doxil[®]), gencitabina, ixabepilona, nanopartículas de paclitaxel (Abraxane[®]) e eribulina (AMERICAN CANCER SOCIETY, 2020).

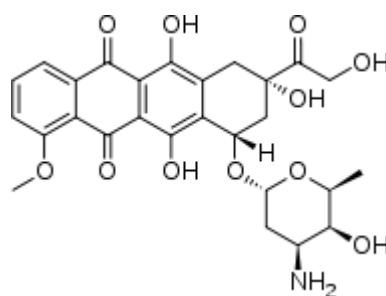
As antraciclinas, em especial a DOX, estão entre os fármacos mais eficazes no tratamento do câncer de mama. As taxas de resposta clínica para a DOX como agente isolado são em geral de 40 a 65% podendo ser superiores a 85% em associações com taxanos ou com ciclofosfamida e 5-fluorouracil (HUNT, 2008; CASTIGLIONE & PICCART, 2009).

Doxorrubicina

A DOX é um fármaco citotóxico pertencente à classe das antraciclinas, que apresenta uma notável eficácia antitumoral. A DOX foi isolada a partir de uma espécie de bactéria, *Streptomyces peucetius* (YANG *et al.*, 2014) e foi utilizada pela primeira vez em ensaios clínicos na década de 60. Atualmente, a DOX permanece como um agente quimioterápico de linha de frente no tratamento do câncer (WAKHARDE *et al.*, 2018).

A estrutura da DOX, representada na **Figura 6**, apresenta uma porção aglicídica que consiste de quatro anéis planares com grupos adjacentes aos anéis quinona-hidroquinona, um substituinte metoxila no primeiro anel e um grupo α -hidroxicetona no quarto anel. O componente glicídico (daunosamina) está ligado a um dos anéis por uma ligação glicosídica (TACAR; SRIAMORNSAK; DASS, 2012).

Figura 6: Estrutura química da doxorubicina



A DOX é utilizada principalmente no tratamento de leucemias, linfomas, mama, útero, ovário e pulmão, mas também mostra-se eficaz contra o câncer de tireoide, pâncreas, estômago e osteosarcoma (TACAR; SRIAMORNSAK; DASS, 2012). Embora sua atividade citotóxica seja pronunciada, a baixa especificidade, a alta toxicidade e o desenvolvimento de resistência são alguns fatores que limitam o uso na quimioterapia (MINOTTI *et al.*, 2004).

A DOX apresenta baixa biodisponibilidade oral, baixa permeabilidade, sofre hidrólise ácida no estômago e é suscetível ao citocromo P450. Está disponível no mercado na forma injetável como solução (Adriamicina[®] e Rubex[®]) ou dispersão lipossomal (Doxil[®], Myocet[®] e Lipodox[®]) (ZHAO *et al.*, 2018).

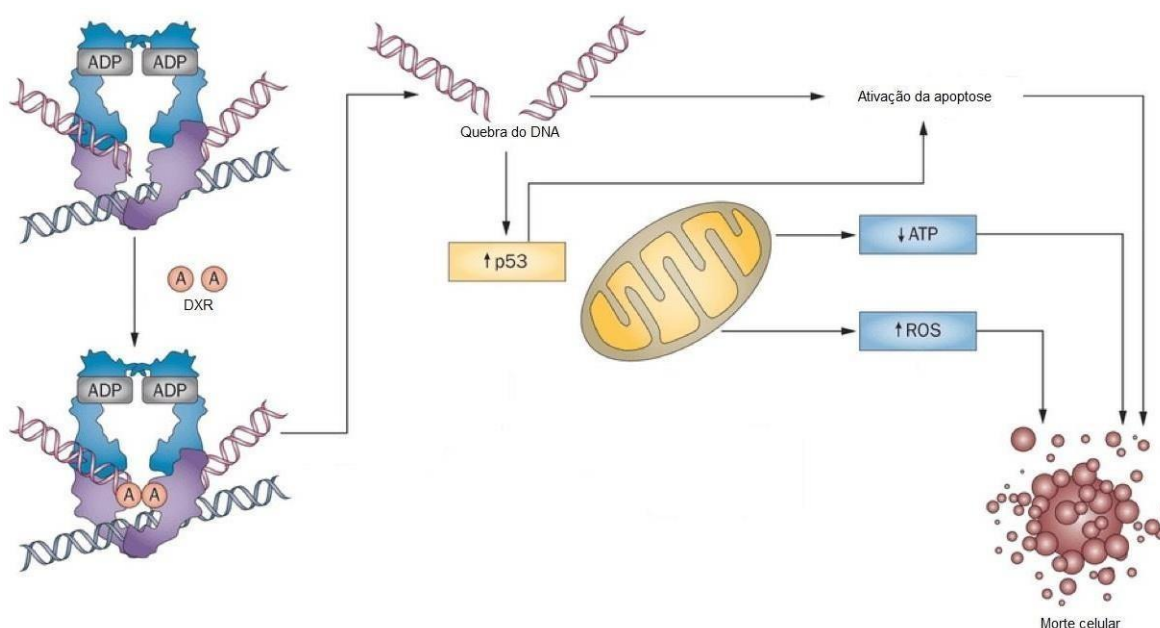
Vários mecanismos vêm sendo atribuídos a sua atividade citotóxica, como: (i) intercalação com o DNA nuclear e com o DNA mitocondrial, resultando na inibição da biossíntese de DNA; (ii) ligação e estabilização do complexo DNA- topoisomerase II, impedindo a passagem dessa enzima através da fita de DNA, e consequentemente, levando a quebra nas cadeias do DNA; (iii) liberação direta do citocromo c mitocondrial, e subsequente, sinalização de apoptose; (iv) formação de radicais livres resultantes de stress oxidativo, que pode causar peroxidação lipídica e dano ao DNA (SEMPKOWSKI, *et al.* 2014).

A translocação da DOX do citoplasma para o núcleo das células proliferativas tem sido atribuída à presença dos proteassomas, que são complexos de proteinases intracelulares identificados em várias células eucarióticas. A DOX exibe uma alta afinidade de ligação aos proteassomas citoplasmáticos, formando um complexo que por sua vez é transportado para o núcleo por meio de um poro nuclear dependente de ATP. Uma vez dentro do núcleo, ocorre a dissociação do complexo já que a DOX possui maior afinidade pelo DNA do que pelo proteassoma, permitindo que ocorram as interações desse fármaco com o DNA (SEMPKOWSKI *et al.*, 2014).

Juntamente com a intercalação com o DNA, a interferência na ação da topoisomerase II, enzima que regula o empacotamento do DNA, permanece como o mecanismo mais convincente da citotoxicidade da DOX. Primeiramente, ocorre a sua ligação ao DNA, a clivagem das duas fitas, a passagem de uma das fitas de DNA através do sítio clivado de maneira dependente de ATP, e então a junção das fitas no sítio original de clivagem (SEMPKOWSKI *et al.*, 2014). Dessa forma, a topoisomerase II pode causar uma quebra transitória da dupla fita de DNA, que posteriormente é selada depois de alterar o estado de torção da cadeia (MINOTTI *et al.*, 2004). A DOX age estabilizando o intermediário da reação na qual as cadeias de DNA são cortadas, e posteriormente, ligadas covalentemente aos resíduos de tirosina da topoisomerase II, eventualmente impedindo a selagem do DNA. A formação e a estabilidade do complexo ternário formado pela DOX-DNA-topoisomerase II depende de determinantes estruturais definidos (**Figura 7**). O sistema de anéis planares é importante para a intercalação com o DNA, uma vez que os anéis que contêm os grupos quinona e hidroquinona fazem ligações com determinados pares de bases, enquanto o anel contendo o substituinte metoxila passa através do sítio de intercalação. A parte externa da molécula, que não participa da intercalação (o resíduo glicídico e o anel ligado ao mesmo) parece desempenhar um papel importante na formação e estabilização do complexo ternário (MINOTTI *et al.*, 2004). Ao estabilizar esse complexo, a DOX impede que a dupla hélice seja liberada e, conseqüentemente, ocorre a interrupção do processo de replicação. Esse complexo estável prejudica a habilidade das células de reparar lesões no DNA e pode eventualmente promover uma sinalização para desencadeamento de apoptose (SEMPKOWSKI *et al.*, 2014).

A atividade da DOX também pode estar associada com a formação de radicais livres. A adição de um elétron na porção quinona da DOX resulta na formação de um radical de semiquinona, mediado por enzimas NADPH-redutases. Esse radical formado reage com oxigênio rapidamente gerando superóxido e peróxido de hidrogênio, causando posteriormente dano ao DNA. Além disso, a DOX é um quelante de ferro e o complexo DOX-ferro é capaz de catalisar a conversão do peróxido de hidrogênio em radicais hidroxilas altamente reativos. Radicais livres podem interagir com lípidos insaturados induzindo peroxidação lipídica, além de oxidar grupos tióis de proteínas funcionais. Assim, a DOX induz a liberação de radicais livres causando stress oxidativo, resultando em dano ao DNA e morte celular (YANG *et al.*, 2014; SEMPKOWSKI *et al.*, 2014; MINOTTI *et al.*, 2004).

Figura 7: Mecanismo de ação da DOX por meio da formação do complexo com o DNA e com a topoisomerase II, levando a quebra do DNA, seguida do aumento nos níveis de p53, ocasionando na ativação da apoptose. A DOX também induz a redução nos níveis de ATP e aumento de ROS, induzindo a morte celular.



Apesar da DOX ser uma das antraciclinas mais largamente utilizada no tratamento de vários tipos de câncer, seu uso tem sido dificultado devido aos efeitos adversos como mielossupressão, náuseas e vômitos, efeitos mucocutâneos (estomatite, alopecia), toxicidade hepática, renal e, principalmente, cardiotoxicidade. Esse efeito cumulativo e dose-dependente pode provocar insuficiência cardíaca congestiva

irreversível e cardiomiopatias dilatáveis, o que limita consideravelmente a sua utilização. A dose acumulativa máxima permitida é de 550 mg/m² (BARENHOLZ, 2012).

Os efeitos cardiotóxicos da DOX têm sido atribuídos a vários fatores, que resultam da sua habilidade em produzir espécies reativas de oxigênio que levam a interrupção da homeostasia do cálcio resultando no *stress* oxidativo, dano mitocondrial, necrose e a indução de morte celular por vias apoptóticas. O tratamento utilizando DOX ainda pode causar disfunção contrátil, afetando as proteínas responsáveis pela contração de forma direta ou pela interrupção da homeostasia do cálcio celular. Alguns fatores contribuem para a depressão da função contrátil como a subregulação da liberação de cálcio do retículo sarcoplasmático e da bomba de cálcio ATPase e a diminuição da entrada de cálcio no retículo (GHARANEI *et al.*, 2014).

Tem sido proposto que o aumento no *stress* oxidativo poderia levar a mudanças subcelulares no miocárdio, incluindo perda de miofibrilas e vacuolização das células miocárdicas. Outro mecanismo é o atraso na oxidação de ácidos graxos de cadeia longa no coração, levando a uma deficiência no suprimento energético (SEMPKOWSKI *et al.*, 2014). Além disso, alguns estudos têm mostrado que a morte dos cardiomiócitos induzida pela DOX está associada com o aumento da expressão e da ativação da proteína pró-apoptótica p53 (GHARANEI *et al.*, 2014).

Outro órgão que pode ser seriamente afetado é o fígado com aproximadamente 40% dos pacientes que utilizam DOX sofrendo lesão no tecido hepático. Devido a sua atividade de metabolização e desintoxicação, o fígado acaba recebendo um acúmulo da DOX administrada. Após ser metabolizada, uma grande quantidade de espécies reativas de oxigênio é produzida, o que, por sua vez, pode provocar dano ao DNA, peroxidação lipídica, diminuição dos níveis de vitamina E e glutathiona culminando em um desequilíbrio nos processos oxidativos. O resultado final da toxicidade da DOX é a diminuição nos níveis de fosfato inorgânico, ATP, ADP e AMP podendo causar uma condição patológica nos hepatócitos. Um transportador dependente de ATP é necessário para realizar o efluxo da DOX na célula, impedindo que compartimentos intracelulares a acumulem, obtendo assim uma homeostase. Entretanto, para que esse mecanismo funcione, é necessário um aporte constante

de ATP, e, uma vez que a DOX provoca redução dos níveis de ATP, esse processo é prejudicado (TACAR; SRIAMORNSAK; DASS, 2012).

O uso clínico da DOX na sua forma livre é significativamente prejudicado devido a sua toxicidade cardíaca e por um número de efeitos adversos agudos e subagudos. Os lipossomas podem contornar estes problemas e melhorar o índice terapêutico da DOX e, simultaneamente, reduzir os volumes de distribuição, reduzindo assim a toxicidade, e aumentar o acúmulo em células tumorais (SEMPKOWSKI *et al.*, 2014).

A terapia combinada pode ser bastante vantajosa uma vez que une dois ou mais fármacos que contém atividade antitumoral e que atuam de forma sinérgica. Algumas associações já foram estabelecidas com sucesso utilizando a DOX, tais como: DOX e ciclofosfamida; DOX, ciclofosfamida e docetaxel; DOX, ciclofosfamida e fluoracila; DOX, ciclofosfamida e cisplatina, bem como DOX e paclitaxel (AMERICAN CANCER SOCIETY, 2020). Mais recentemente, a combinação da DOX com o alfa-succinato de tocoferila (TS), um derivado da vitamina E, tem sido descrita (ZHANG *et al.*, 2011).

Alfa-Succinato de tocoferila

A vitamina E engloba uma família de oito isoformas naturais que têm exibido diversas propriedades terapêuticas em inúmeros estudos nas últimas décadas. Estudos *in vitro* e *in vivo* têm destacado suas propriedades antioxidante, anti-inflamatória, neuroprotetora e antitrombótica. As isoformas naturais da vitamina E têm demonstrado também ação preventiva e terapêutica contra diversos tipos de câncer (NEOPHYTOU & CONSTANTINOU, 2015).

Estruturalmente, a vitamina E (**Figura 8**) apresenta uma porção contendo dois anéis (um fenólico e um heterocíclico) e uma porção contendo uma cadeia lateral que pode ser saturada ou insaturada, no caso dos tocoferóis ou tocotrienóis, respectivamente. Para esses grupos, existem quatro variações possíveis de metilação no anel fenólico, denominados alfa, beta, delta e gama (CONSTANTINOU, C.; PAPAS; CONSTANTINOU, A., 2008).

É possível obter análogos da vitamina E por meio da substituição da hidroxila na posição C₆ do anel fenólico (**Figura 8A**) por uma ligação éster de um grupo succinato ou acetato. Esses análogos são compostos semissintéticos que apresentam atividades biológicas próprias. Entretanto, dependendo da ligação química ou do grupo usado na substituição, a saída do grupo hidroxila poderá implicar na perda da atividade antioxidante (ZHAO; NEUZIL; WU, 2009). Um desses análogos, o TS (**Figura 9**), tem sido foco de extensa pesquisa como um composto com atividade antitumoral na forma isolada ou em combinação com outros agentes quimioterápicos tais como cisplatina, tamoxifeno, paclitaxel e DOX (CONSTANTINOU, C.; PAPAS; CONSTANTINOU, A., 2008; NEUZIL *et al.*, 2001; KANAI *et al.*, 2010).

Figura 8: Estrutura química das isoformas da Vitamina E. (A) Tocoferóis e (B) Tocotrienóis. A seta representa o carbono C₆, o qual é substituído no alfa-succinato de tocoferila.

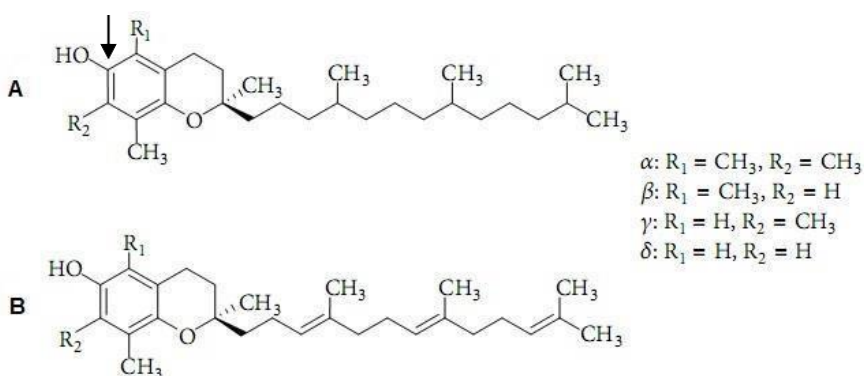
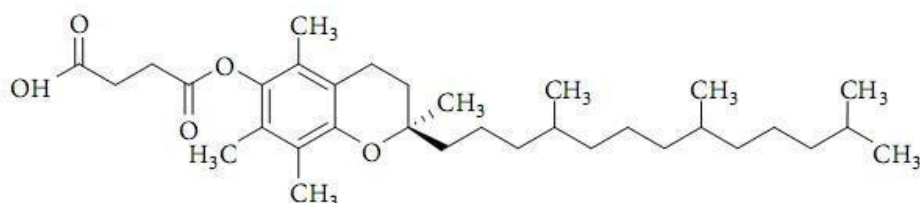


Figura 9: Estrutura química do alfa-succinato de tocoferila



O primeiro estudo que relatou o uso do TS como um agente antiproliferativo foi publicado em 1982, quando Prasad & Edwards-Prasad testaram diversos análogos

do alfa-tocoferol para avaliar a captação e a inibição do crescimento de células de melanoma *in vitro*. Eles mostraram que o TS foi o único análogo capaz de inibir o crescimento dessas células. Nas décadas seguintes, estudos *in vitro* em diversas linhagens celulares confirmaram os efeitos antiproliferativo e apoptótico do TS. Alguns estudos demonstraram inibição do crescimento por ação do TS em várias linhagens celulares, como: neuroblastoma, melanoma, linfoma, leucemia, câncer de mama, cólon, próstata, pulmão e estômago (SWETTENHAM *et al.*, 2005; PRASAD & EDWARDS-PRASAD, 1982; TURLEY *et al.*, 1995; TURLEY *et al.*, 1997; ZU, HAWTHORN, IP, 2005; WU *et al.*, 2001; HRZENJAK *et al.*, 2004; KOUDELKA *et al.*, 2015). Estudos *in vivo* comprovaram o efeito antitumoral do TS no câncer de mama, pulmão, próstata e melanoma (MALAFA & NEITZEL, 2000; MALAFA *et al.*, 2002; QUIN *et al.*, 2005; ZHANG, ALTUWAIJRI, YEH, 2004).

Os efeitos antitumorais do TS têm sido relacionados com a indução de apoptose, prevenção contra a angiogênese, inibição da proliferação celular, bloqueio do ciclo de divisão celular e interrupção da síntese de DNA (HAMA *et al.*, 2012; WANG *et al.*, 2006). Uma importante atividade atribuída ao TS é a sua capacidade de provocar desestabilização mitocondrial precedente a apoptose. O TS mimetiza o domínio BH3 e bloqueia Bcl-2 ou Bcl-xL, proteínas antiapoptóticas, evitando que essas substâncias se liguem a Bax e Bak, tornando-as disponíveis para formar poros na membrana externa da mitocôndria. Conseqüentemente, na presença de excesso do mimético de BH3, quando Bax e Bak são ativadas pela sinalização de apoptose, elas estarão livres para aumentar a permeabilidade da membrana mitocondrial, dessa forma, levando a indução de apoptose (NEUZIL *et al.*, 2007; PALAO-SUAY *et al.*, 2015).

A associação do TS com um direcionador mitocondrial (trifenilfosfônio) induziu apoptose em maior extensão do que a molécula isolada em diversas linhagens, além de apresentar eficácia antitumoral *in vivo* em modelo de tumor colorretal humano (HCT116) (DONG *et al.*, 2011).

Apesar da atividade antitumoral apresentada pelo TS, nenhuma formulação contendo essa substância para aplicação clínica foi ainda relatada. Entretanto, novos sistemas de entrega de fármacos como lipossomas, nanopartículas e micelas vêm sendo desenvolvidos para aprimorar a entrega do TS.

Hama e colaboradores (2012) desenvolveram lipossomas contendo fosfatidilcolina de ovo (EPC) e TS que apresentaram indução de apoptose *in vitro* e supressão do crescimento tumoral *in vivo* em linhagem de melanoma murino (B16-F1).

Um sistema micelar composto por quitosana, TS e paclitaxel sintetizado por Liang e colaboradores (2012) demonstrou citotoxicidade *in vitro* contra uma linhagem de mama humana (MCF-7) e eficácia antitumoral *in vivo* em um modelo de tumor cervical (U14).

Outro derivado da vitamina E que vem sendo utilizado em nanossistemas é o TS associado ao polietilenoglicol (TPGS). Esse derivado está presente em diversos tipos de formulações como micelas, lipossomas e nanopartículas, atuando como agente promotor da absorção, emulsificante, solubilizante, promotor de permeação e inibidor do transportador P-gp (NEOPHYTOU & CONSTANTINOU, 2015).

Sun e colaboradores (2014) desenvolveram nanopartículas poliméricas contendo poli ácido lático co-ácido glicólico (PLGA), paclitaxel e TPGS, as quais se acumularam preferencialmente em um modelo de tumor de pulmão humano (A549) e foram capazes de inibir significativamente o crescimento tumoral.

De forma geral, o desenvolvimento de nanossistemas contendo derivados da vitamina E tem como objetivo aumentar a biodisponibilidade destes ou promover um efeito sinérgico junto a outro agente antitumoral (NEOPHYTOU & CONSTANTINOU, 2015).

Sistemas nanoestruturados de liberação de fármacos

De acordo com a Organização Internacional para Padronização (*International Organization for Standardization* - ISO), nanopartículas são objetos que apresentam todas as suas dimensões externas na faixa de 1 a 100 nm (ISO, 2015). Os sistemas nanoestruturados de liberação de fármacos são partículas nanométricas compostas usualmente por substâncias atóxicas e biodegradáveis e apresentam uma capacidade de encapsulação de substâncias variadas (PATEL *et al.*, 2013). Eles podem contribuir significativamente para a melhora da estabilidade e da absorção do

fármaco, facilitando a sua passagem através de diversas barreiras existentes no organismo. Além de poderem apresentar uma circulação prolongada no sangue, habilidade de acumular na região de interesse e responsividade ao estímulo local, como mudanças de temperatura e/ou pH, resultando, por exemplo, em uma liberação acelerada do fármaco (TORCHILIN, 2012). Podem fornecer maior eficácia terapêutica e diminuição da toxicidade (SAWANT & TORCHILIN, 2012). O uso desses sistemas pode ainda superar entraves de alguns fármacos como baixa solubilidade em água, baixa biodisponibilidade e distribuição inespecífica pelo organismo (TORCHILIN, 2014).

A oncologia representa o campo da medicina no qual a nanotecnologia tem feito as maiores contribuições nas últimas décadas. Os lipossomas foram a primeira classe de nanocarreadores aprovados para uso clínico no tratamento do câncer e juntamente com outros nanocarreadores constituídos por lipídes, ainda representam uma grande proporção em estágios clínicos (SHI *et al.*, 2017). Essas formulações podem, preferencialmente, realizar a entrega de fármacos quimioterápicos no sítio tumoral devido ao seu tamanho reduzido e a circulação sistêmica prolongada.

Existem diversos tipos de nanossistemas, como lipossomas, nanopartículas poliméricas, micelas, nanopartículas de sílica ou de ouro, nanotubos de carbono, nanopartículas lipídicas sólidas e dendrímeros. Dentre eles, destacam-se as formulações lipossomais, com o lipossoma contendo DOX (Doxil[®]) sendo o primeiro sistema nanoestruturado disponível comercialmente para tratamento do câncer (SAKAMOTO *et al.*, 2010).

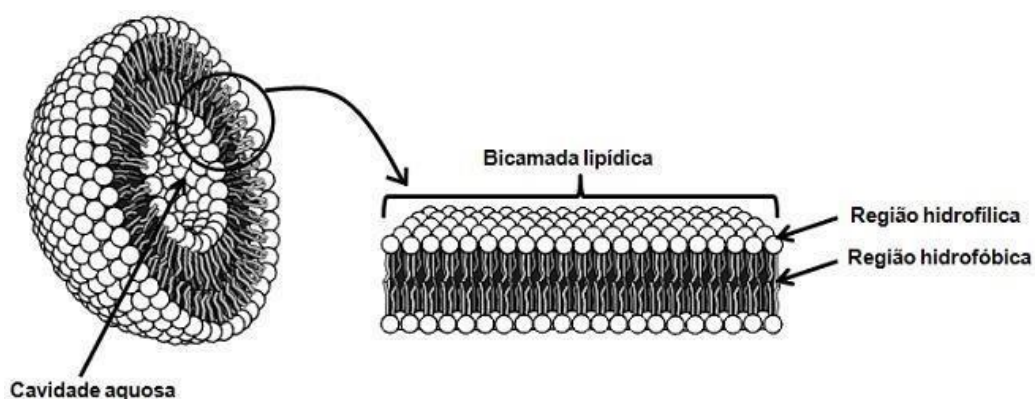
Lipossomas

Lipossomas foram primeiramente descritos por Bangham e colaboradores (1965), como modelos de membranas biológicas. São definidos como vesículas esféricas compostas por uma ou várias bicamadas lipídicas, envolvendo um compartimento aquoso (**Figura 10**) (NEW, 1990). A característica típica da organização dos lipídes em bicamadas é decorrente da natureza anfifílica dos mesmos com a presença de um grupo polar ligado covalentemente a duas cadeias hidrofóbicas. Quando esses

lípidos são expostos a um meio aquoso, interações entre eles (interações hidrofílicas entre os grupos polares e interações de Van der Waals entre as cadeias hidrocarbonadas) levam a formação espontânea de bicamadas fechadas denominadas lamelas (BHAL *et al.*, 2012).

Dentre as vantagens do uso de lipossomas como veículos de entrega de fármacos, podemos destacar a biocompatibilidade, biodegradabilidade, não imunogenicidade e elevada capacidade de encapsular fármacos tanto hidrofílicos quanto lipofílicos (BISWAS *et al.*, 2013). Moléculas hidrofílicas são encapsuladas na sua cavidade interna aquosa, onde estão presentes os grupos polares dos fosfolípidos. As substâncias lipofílicas são acomodadas na região apolar da bicamada. Substâncias anfífilas também podem ser encapsuladas, alojando-se ao longo de toda sua extensão, interagindo com a região apolar e polar (NEW, 1990).

Figura 10: Representação esquemática da estrutura dos lipossomas



De forma geral, quanto à composição e ao mecanismo de liberação intracelular, as vesículas podem ser classificadas como convencionais, de circulação prolongada, de superfície modificada e polimórficas, incluindo nessa classe, os lipossomas pH-sensíveis e termossensíveis (BATISTA *et al.*, 2007).

Os lipossomas convencionais são considerados estruturalmente mais simples, pois são compostos apenas por fosfolípides, como por exemplo, a EPC e colesterol (VEMURI & RHODES, 1995). Em meio biológico, os lipossomas convencionais são reconhecidos pelo sistema fagocitário mononuclear (SFM), sendo, então, rapidamente removidos da circulação sanguínea (VEMURI & RHODES, 1995). Ocorre rápida adsorção de proteínas séricas, as opsoninas, ocasionando na captura das vesículas e culminando no acúmulo em órgãos ricos em macrófagos como fígado, baço e medula óssea (ALLEN & HANSEN, 1991).

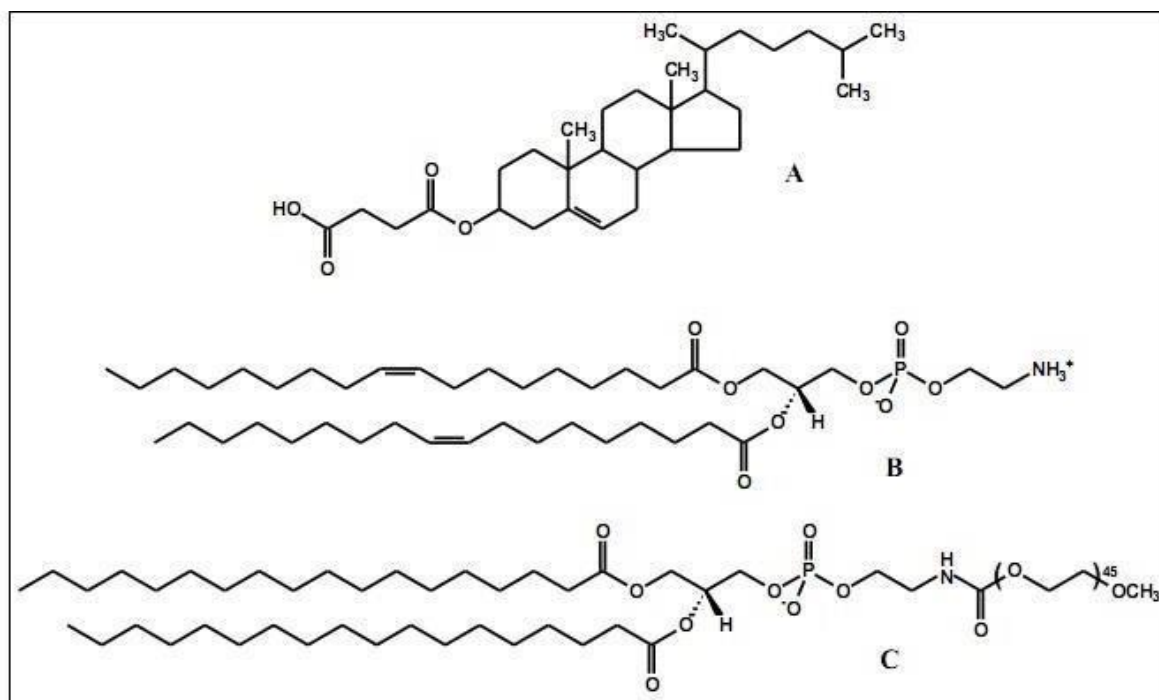
No ano de 1990, Klibanov e colaboradores demonstraram que a incorporação de um polímero hidrofílico, o polietilenoglicol (PEG), na bicamada lipídica aumentou significativamente o tempo de circulação sanguínea de lipossomas. O PEG é capaz de alterar a interação da vesícula com o ambiente, sendo o efeito mais importante a diminuição da captura pelos macrófagos e o prolongamento do seu tempo na circulação. A presença de grupos hidrofílicos na superfície dos lipossomas leva à formação de uma camada aquosa no entorno das vesículas, aumento das interações repulsivas entre lipossomas e componentes do sangue e formação de uma barreira estérica para a penetração de opsoninas, impedindo o reconhecimento e a destruição pelas células do SFM (KLIBANOV *et al.*, 1990; MARUYAMA, 2011). Esses lipossomas, denominados lipossomas de circulação prolongada, permitem uma distribuição do fármaco para outros órgãos além daqueles do SFM (FONTES *et al.*, 2005; TORCHILIN, 2007).

Apesar das vantagens obtidas por meio da adição de polímeros hidrofílicos na superfície das vesículas, esses sistemas podem apresentar uma lenta liberação do fármaco encapsulado ou não serem capazes de fundir com a membrana endossomal após a internalização celular. Diante disso, lipossomas polimórficos vêm sendo desenvolvidos a fim de superar esses entraves. Esses lipossomas sofrem alterações na membrana quando submetidos a variações de pH (lipossomas pH-sensíveis) ou variações na temperatura (lipossomas termosensíveis), por exemplo (LOPES *et al.*, 2013).

Os lipossomas pH-sensíveis são estáveis em pH fisiológico, porém após entrarem em contato com o meio ácido existente na região extracelular de tecidos tumorais e dentro do compartimento endossomal, se desestabilizam, permitindo a liberação do material encapsulado no citoplasma (FERREIRA *et al.*, 2013). Esses sistemas são constituídos por fosfolípides derivados da fosfatidiletanolamina (PE), como por exemplo, a dioleilfosfatidiletanolamina (DOPE), os quais se organizam em meio aquoso, à temperatura ambiente, sob a forma hexagonal, não sendo capazes de se apresentar na forma de vesículas (SIEGEL, 1986). Diferentemente da maioria dos fosfolípides, a DOPE possui uma cabeça polar pequena e pouco hidratada que ocupa um pequeno volume, quando comparado com o das cadeias de hidrocarbonetos, resultando em uma geometria molecular cônica. Esse formato favorece o estabelecimento de fortes interações intermoleculares entre os grupos amino e fosfato da sua cabeça polar, o que explica a tendência dessas moléculas a adquirirem a forma hexagonal invertida. A formação de lipossomas com esses fosfolípides requer a adição de agentes estabilizantes, normalmente lípides carboxilados, como o hemisuccinato de colesterila (CHEMS), que em pH fisiológico encontram-se sob a forma ionizada (**Figura 11**). O TS, por ser um composto carboxilado e estruturalmente relacionado ao CHEMS, também poderia ser utilizado como um agente estabilizante em formulações lipossomais pH-sensíveis.

Esses estabilizantes são capazes de se inserir entre as moléculas de fosfolípides, e o aparecimento de repulsões eletrostáticas entre os grupamentos carboxila e os grupos fosfato dos fosfolípides, favorecem a organização lamelar possibilitando a formação dos lipossomas. A exposição dos lipossomas pH-sensíveis a um meio ácido, como no interior dos endossomas, resulta na protonação dos agentes estabilizantes, com consequente perda da repulsão eletrostática, desestabilização das vesículas e a liberação do material encapsulado (OLIVEIRA *et al.*, 2000; SIMÕES *et al.*, 2004; SUDIMACK *et al.*, 2002).

Figura 11: Estruturas químicas de CHEMS (A), DOPE (B) e DSPE-PEG₂₀₀₀ (C)



Abreviações: CHEMS: hemissuccinato de colesterila; DOPE: dioleilfosfatidiletanolamina; DSPE-PEG₂₀₀₀: diestearoilfosfatidiletanolamina acoplado ao polietilenoglicol 2000.

É interessante mencionar que o TS forma por si só nanovesículas e apresenta potencial como nanocarreador. Entretanto, as vesículas formadas exclusivamente por TS são instáveis e para melhorar a estabilidade, a adição de outro lípide como a fosfatidilcolina tem sido preconizada (HAMA *et al.*, 2012). A pH sensibilidade de lipossomas contendo TS também já foi descrita (JIZOMOTO *et al.*, 1994; REHMAN *et al.*, 2018).

O preparo de lipossomas contendo TS e encapsulando a DOX se revela com uma alternativa interessante. O TS pode contribuir para a pH sensibilidade, bem como para aumentar a eficácia antitumoral da DOX.

Sistemas nanoestruturados contendo doxorubicina e alfa-succinato de tocoferila

Diversos estudos na literatura já comprovaram os benefícios da associação entre derivados da vitamina E e DOX. Em 2008, Cao e colaboradores sintetizaram um conjugado de DOX e TPGS que apresentou maior captação em células de câncer de

mama humano (MCF-7) e glioma (C6), além de apresentar menor valor de IC₅₀ comparado com a forma livre.

Zhang e colaboradores, em 2011, relataram ganho na eficácia antitumoral em células de câncer de estômago humano (SGC-7901) com a associação entre o TS e a DOX. O TS foi capaz de promover aumento nos níveis intracelulares de DOX por meio da promoção do influxo de DOX e supressão do efluxo. A combinação entre DOX e TS também induziu aumento nos níveis de expressão de Fas e Bax e quebra da caspase-8 e caspase-9, levando a apoptose ativada pela mitocôndria, induzida por Fas/caspase-8 e Bax. Esses estudos serviram de base para o desenvolvimento de sistemas nanoestruturados contendo essas substâncias.

Wang e colaboradores (2012) sintetizaram um copolímero de TPGS acoplado a lisina para a obtenção de micelas contendo DOX. Foi possível obter a reversão da resistência à DOX em linhagens de tumor de mama resistente (MCF-7/Adr) e houve maior inibição do crescimento tumoral em modelo murino (4T1).

Estudos anteriores do nosso grupo de pesquisa demonstraram que a co-administração de TS e DOX em nanopartículas lipídicas sólidas promoveu citotoxicidade em linhagens resistentes de tumor de ovário e mama (NCI/Adr e MCF-7/Adr) em modelo de esferóide (OLIVEIRA *et al.*, 2016). Posteriormente, foi desenvolvida uma formulação de carreadores lipídicos nanoestruturados contendo TS e DOX (de razão sinérgica 4:1) que foi capaz de se acumular preferencialmente no tumor e ainda apresentou eficácia antitumoral *in vivo* em modelo de mama murino (4T1) (FERNANDES *et al.*, 2018).

Em 2019, Song e colaboradores desenvolveram uma formulação lipossomal composta por EPC, colesterol e TS contendo DOX e um inibidor de fosfolipase D (5 - fluoro-2-indolil descloroalopemida). Essa formulação foi capaz de prevenir a iniciação, bem como a progressão de metástase em modelo de tumor de mama humano.

Embora diversos sistemas nanoestruturados contendo TS e DOX tenham sido desenvolvidos com comprovada eficácia antitumoral e reduzida toxicidade em tecidos saudáveis, todos ainda permanecem na fase experimental seja *in vitro* ou *in*

vivo. Diante disso, a proposta do presente estudo foi desenvolver, avaliar os parâmetros físico-químicos e o comportamento biológico *in vitro* e *in vivo* de uma formulação pH-sensível contendo DOX e TS, com o objetivo de aumentar a captação intratumoral e, conseqüentemente, a eficácia antitumoral bem como reduzir a incidência de efeitos adversos em relação ao fármaco livre ou Doxil®. É ideal que esta formulação apresente características físico-químicas adequadas, seja estável, segura, com menor incidência de efeitos adversos e, finalmente, apresente uma eficácia antitumoral satisfatória.

3 OBJETIVO

Objetivo Geral

Desenvolver e caracterizar uma formulação lipossomal composta por TS e DOX e avaliar a sua eficácia antitumoral como alternativa para o tratamento do câncer de mama.

Objetivos específicos

- Preparar uma formulação lipossomal pH-sensível contendo TS e DOX e analisar suas características físico-químicas;
- Avaliar um método alternativo de encapsulação de DOX nos lipossomas na ausência de sulfato de amônio (método clássico por gradiente remoto);
- Avaliar a pH-sensibilidade da formulação por meio de análises de difração de raio X a baixo ângulo (SAXS) e estudo de liberação da DOX;
- Avaliar a estabilidade da formulação desenvolvida por um período de 30 dias;
- Realizar uma análise morfológica da formulação por meio de criomicroscopia;
- Avaliar a citotoxicidade da formulação frente às células tumorais das linhagens celulares 4T1 (tumor de mama murino) e MDA-MB-231 (tumor de mama humano);
- Avaliar a eficácia antitumoral *in vivo* da formulação em modelo de tumor de mama murino 4T1;
- Avaliar a toxicidade da formulação *in vivo* por meio de testes bioquímicos e hematológicos e análise histológica;
- Realizar um estudo de farmacocinética da formulação;
- Avaliar a captação celular e o acúmulo tumoral da formulação;
- Realizar estudos de morfologia nuclear;
- Avaliar a taxa de apoptose e o perfil do ciclo celular induzido pela formulação.

4 PARTE EXPERIMENTAL, RESULTADOS E DISCUSSÃO

Artigo 1 - Alfa-succinato de tocoferila melhora a encapsulação, a pH-sensibilidade, a atividade antitumoral e reduz a toxicidade de lipossomas contendo doxorubicina

Boratto FA, Franco MS, Barros ALB, Cassali GD, Malachias A, Ferreira LAM, Leite EA. Alpha-tocopheryl succinate improves encapsulation, pH-sensitivity, antitumor activity and reduces toxicity of doxorubicin-loaded liposomes. *Eur J Pharm Sci.* 2020; 144: 1-12. doi: 10.1016/j.ejps.2019.105205.

ABSTRACT

Doxorubicin (DOX) plays an important role in cancer treatment; however, high cardiotoxicity and low penetration in solid tumors are the main limitations of its use. Liposomal formulations have been developed to attenuate the DOX toxicity, but the technological enhancement of the liposomal formulation as well as the addition of another agent with antitumor properties, like alpha-tocopheryl succinate (TS), a semi-synthetic analog of vitamin E, could certainly bring benefits. Thus, in this study, it was proposed the development of liposomes composed of DOX and TS (pHSL-TS-DOX). A new DOX encapsulation method, without using the classic ammonium sulfate gradient with high encapsulation percentage was developed. Analysis of Small Angle X-ray Scattering (SAXS) and release study proved the pH-sensitivity of the developed formulation. It was observed stabilization of tumor growth using pHSL-TS-DOX when compared to free DOX. The toxicity tests showed the safety of this formulation since it allowed body weight initial recovery after the treatment and harmless to heart and liver, main target organs of DOX toxicity. The developed formulation also avoided the occurrence of myelosuppression, a typical adverse effect of DOX. Therefore, pHSL-TS-DOX is a promising alternative for the treatment of breast cancer since it has adequate antitumor activity and a safe toxicity profile.

Keywords: breast cancer, doxorubicin, alpha-tocopheryl succinate, antitumor activity, cytotoxicity, pH-sensitivity

1. Introduction

The use of nanoparticulate systems as carriers of antineoplastic drugs has been an alternative to overcome the challenges associated with cancer therapy, providing greater drug accumulation in the tumor and reducing toxicity (BERTRAND *et al.*, 2014; PEER *et al.*, 2007; SCHUTZ *et al.*, 2013). Doxorubicin (DOX) is one of the most used antitumor drugs in cancer treatment; however, high cardiotoxicity and low penetration in solid tumors have been widely described as the main limitations of its use (PRIMEAU *et al.*, 2005; TACAR *et al.*, 2012). Doxil[®] (Janssen Biotech, Inc., Johnson & Johnson, USA) was the first liposomal formulation containing DOX approved by the Food and Drug Administration for clinical use in cancer treatment (BARENHOLZ, 2012; SCHUTZ *et al.*, 2013). Significant reductions in the cardiotoxicity, myelosuppression, nausea, vomiting, and alopecia were the major advantages associated with Doxil[®] compared to free DOX (O'BRIEN *et al.*, 2004). Nowadays, other liposomal formulations containing DOX are approved or in an advanced stage of clinical study for cancer treatment (BULBAKE *et al.*, 2017). Despite these formulations have shown efficacy comparable to free DOX and reduced toxicity, side effects were also reported with their use.

The combination of DOX with other drugs has also been proposed as an alternative to improve therapeutic efficacy; however, this strategy could increase the side effects. Some semi-synthetic vitamin E analogs, such as alpha-tocopheryl succinate (TS), have been studied as a potential antitumor agent administered alone or in combination with other chemotherapeutics such as cisplatin, tamoxifen, paclitaxel, and DOX (CONSTANTINO *et al.*, 2008, KANAI *et al.*, 2010, PRASAD *et al.*, 1994).

The first study reporting the use of TS as an antiproliferative agent was published in 1982 by Prasad & Edwards-Prasad. They found that TS was capable of inhibiting the growth of melanoma cells. In the following decades, *in vitro* and *in vivo* studies confirmed the antitumor effect of TS on several types of cancer, such as breast, lung,

prostate, and melanoma (MALAFA *et al.*, 2002; MALAFA & NEITZEL, 2000; QUIN *et al.*, 2005; ZHANG, ALTUWAIJRI, YEH, 2004). The antitumor effect of TS consists in the induction of apoptosis, inhibition of the cell proliferation, blocking the cell division cycle and disruption of DNA synthesis (HAMA *et al.*, 2012; NEUZIL *et al.*, 2001; NEUZIL *et al.*, 2007; PALAO SAY *et al.*, 2015; WANG *et al.*, 2006).

Therefore, the combination of TS and DOX may be a potential strategy in order to increase the antitumor activity of DOX as well as reduce toxicity in healthy tissues (FERNANDES *et al.*, 2018; NEOPHYTOU & CONSTANTINO, 2015; OLIVEIRA *et al.*, 2016a; OLIVEIRA *et al.*, 2016b; ZHANG *et al.*, 2011).

Zhang *et al.* (2011) reported a gain in the cytotoxicity against human stomach cancer cells by combining TS and DOX. TS was able to promote an increase in intracellular DOX levels by promoting DOX influx and suppression of efflux. In addition, TS demonstrated low toxicity against normal cells and protected these cells against the toxicity induced by other chemotherapeutic agents.

Previous studies, developed by our research group, demonstrated the ability of TS to improve the encapsulation of DOX into lipid nanocarriers increasing simultaneously the cytotoxicity against resistant human breast carcinoma cell line (MCF-7/Adr) and resistant human ovarian carcinoma cell line (NCI/Adr) in monolayer and 3-D spheroid models (OLIVEIRA *et al.*, 2016a; OLIVEIRA *et al.*, 2016b). In addition, *in vivo* assays demonstrated high antitumor activity for the nanocarriers containing TS and DOX compared to free DOX (FERNANDES *et al.*, 2018).

Song *et. al* (2019) developed liposomes composed of egg phosphatidylcholine, cholesterol and TS loaded with DOX and 5-fluoro-2-indolyl deschlorohalopemide, an inhibitor of phospholipase D. *In vivo* studies showed this liposomal formulation prevented metastasis of breast cancer.

Rehman *et. al* (2018) have also described the encapsulation of DOX in liposomal formulations using different phosphatidylethanolamine (PE) derivatives in association with TS by passive and active loading methods. However, the liposomes are not formed by the passive method and the use of an active loading method resulted in

low DOX encapsulation, except for palmitoyloleoylphosphatidyl ethanolamine. Thus, no adequate formulation containing these substances for clinical application has been reported.

In the present study, we developed pH-sensitive liposomes containing DOX and TS using a passive loading method. These liposomes were designed to release the encapsulated drug when submitted to acidic environments, either in endosomal vesicles or in the extracellular tumor matrix. The developed formulation was characterized by dynamic light scattering (DLS), zeta potential, and Small Angle X-ray Scattering (SAXS). Then, *in vitro* and *in vivo* biological behavior in experimental models of breast cancer was evaluated.

2. Materials and methods

Materials

Dioleoylphosphatidylethanolamine (DOPE), egg phosphatidylcholine (EPC), hydrogenated soybean phosphatidylcholine (HSPC), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (DSPE-PEG₂₀₀₀) were purchased from Lipoid (Ludwigshafen, Germany). 4-(2-hydroxyethyl)-1-piperazine ethanosulphonic acid (HEPES), cholesterol, ammonium sulfate, alpha-tocopheryl succinate (TS), trypsin, and sulforhodamine B were purchased from Sigma-Aldrich (Steinheim, Germany). Doxorubicin hydrochloride (DOX) was purchased from ACIC Chemicals (Ontario, Canada). Chloroform, anhydrous monobasic potassium phosphate, and dimethylsulfoxide (DMSO) were purchased from Synth (São Paulo, Brazil). Sodium hydroxide was purchased from Vetec Química Fina Ltda (Rio de Janeiro, Brazil). HPLC grade methanol was purchased from Tedia (Fairfield, USA). CellTiter-Blue® Cell Viability Assay kit (CTB) was purchased from Promega (Madison, USA). 4T1 and MDA-MB-231 tumor cell lines were purchased from the ATCC (Manassas, USA). Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute 1640 (RPMI-1640), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Invitrogen (São Paulo, Brazil). Xylazine and ketamine solutions were acquired from Hertape Calier Saúde Animal S/A (Juatuba,

Brazil) and Vetbrands Agroline (Campo Grande, Brazil), respectively. Any other chemicals used in this work were analytical grade.

Liposomes preparation

The formulations were prepared by hydration of lipid film in the presence or not of ammonium sulfate. The formulations were composed of DOPE or EPC, as structural lipids, TS and DSPE-PEG₂₀₀₀ at different ratios (Table 1). Briefly, aliquots of the lipids, in chloroform, were transferred to a round bottom flask and the solvent was removed under reduced pressure until a thin lipid film was obtained. Then, NaOH solution (0.1 M) at a TS/NaOH (mol/mol) ratio of 1:1 was added to promote TS complete ionization, and the film was hydrated with ammonium sulfate solution (300 mmol/L, pH 7.4) or HEPES-saline buffer pH 7.4 (HBS) at room temperature.

For liposomal formulations hydrated with ammonium sulfate (pHSL-TS-DOX-AS), the calibration was performed by extrusion through polycarbonate membranes with pores sizes of 0.4, 0.2, and 0.1 μm . Thereafter, the suspension was subjected to ultracentrifugation at 350,000 \times g, 4°C for 180 minutes (OPTIMA L-80XP, Beckman Coulter–Fullerton, CA, USA) in order to remove the ammonium sulfate present in the external medium. Then, the pellet was reconstituted with HBS pH 7.4, and 1.0 mL of this formulation was incubated with 2.0 mg of lyophilized DOX for 2h at 4°C.

On the other hand, for liposomal formulations hydrated with HBS pH 7.4 (pHSL-TS), the calibration was performed by sonication using an ultrasonic homogenization apparatus (CPX 500, Cole Parmer, Vernon Hills, USA) with high power probe for 3 minutes in an ice bath and 21% amplitude. Then, 1.0 mL of concentrated DOX solution (10 mg/mL) was added. The resulting dispersion was maintained for 2 h under refrigeration at 4°C. In both cases, untrapped DOX was eliminated by ultracentrifugation and the pellet was resuspended with HBS.

Non-pH-sensitive liposomes (npHSL-DOX-SA) composed of HSPC, CHOL, and DSPE-PEG₂₀₀₀ at the total lipid concentration of 20 mmol/L, molar ratio 57:38:5, respectively, were also prepared in order to evaluate the advantages of our pH-sensitive formulation over a similar formulation already in the market. npHSL-DOX-

AS was prepared by thin film hydration method followed by extrusion and the DOX incubation was performed for one hour at 60 °C by the ammonium sulfate gradient method.

Table 1 – Formulation composition

Formulations	Lipid Concentration (mmol/L)	Lipid Ratio (%)				Ammonium sulfate
		DOPE	EPC	TS	DSPE-PEG ₂₀₀₀	
pHSL-TS-DOX-AS	20	65	-	30	5	Yes
	40	55	-	40	5	Yes
pHSL-TS-DOX	40	55	-	40	5	No
npHSL-DOX	40	-	95	-	5	No

Abbreviations: DOPE: dioleoylphosphatidylethanolamine; EPC: egg phosphatidylcholine; TS: alpha tocopheryl succinate; DSPE-PEG₂₀₀₀: distearoylphosphatidylethanolamine coupled to polyethylene glycol 2000; DOX: doxorubicin; pHSL-TS-DOX-AS: pH-sensitive liposomes containing alpha tocopheryl succinate, doxorubicin and ammonium sulfate; pHSL-TS-DOX: pH-sensitive liposomes containing alpha tocopheryl succinate and doxorubicin; npHSL-DOX: non-pH sensitive liposomes containing doxorubicin .

Liposomes characterization

Liposomes were characterized according to their mean diameter, polydispersity index (PDI), zeta potential, and encapsulation percentage (EP). The average diameter and PDI were determined by DLS at 25°C at a 90° angle using monomodal analysis. The zeta potential was evaluated by DLS associated with the electrophoretic mobility at an angle of 90°. All samples were diluted (1:30) with HBS pH 7.4 and the measurements were performed in triplicate using the Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK).

The entrapped DOX concentration was determined by HPLC using fluorimetric detection. HPLC analyses were performed in a Waters chromatographer (Waters Instruments, 1200 series, Milford, USA). The separation was performed using a 250 x 4.6 mm, 5 µm column (Merck, ACE® 250-4.6 C8, Aberdeen, Scotland). The eluent

system consisted of methanol:phosphate buffer 0.01 mol/L pH 3.0 (65:35 v/v, respectively), and the flow rate was of 1.0 mL/min. Samples were injected, and the eluate was monitored at excitation/emission wavelengths of 477/555 nm. The EP was calculated according to the following equation:

$$EP = \frac{[DOX]_{PurifiedLip} \times 100}{[DOX]_{TotalLip}} \quad (1)$$

Abbreviations: EP: encapsulation percentage, $[DOX]_{PurifiedLip}$: doxorubicin concentration after liposome purification, $[DOX]_{TotalLip}$: doxorubicin concentration before liposome purification.

Release studies

A release study was assessed in two different pH conditions: 5.0 and 7.4. An aliquot of 1.0 mL of the formulations (pHSL-TS-DOX-AS and pHSL-TS-DOX) and 1.0 mL of HBS pH 7.4 or 5.0 were transferred to a CelluSep® 14 kDa dialysis bag. This dialysis membrane was incubated in 50 mL HBS pH 7.4 or 5.0 under magnetic stirring at 37°C. At several time intervals (1, 2, 3, 4, 6, 8, and 24h), 1.0 mL of the dialysis fluid was withdrawn and replaced by 1.0 mL of fresh medium. The quantification of DOX released from liposomes was performed by HPLC. The values were plotted as cumulative percentage of drug release.

Storage stability test

The physicochemical stability of the formulation pHSL-TS-DOX was studied concerning the average diameter, zeta potential, PDI, and EP at 15 and 30 days after preparation. The formulation (n = 3 batches) was maintained at 4°C and aliquots of the sample were submitted to overnight dialysis (dialysis bag CelluSep® 14 kDa) to remove the released DOX. Then, the purified formulation was analyzed and the values for each parameter were compared with those obtained at time of preparation (day 0).

Cryo-Transmission Electron Microscopy

To obtain images of pHSL-TS-DOX, cryo-TEM analysis was performed using a FEI Tecnai Spirit G2-12 (FEI, Hillsboro, OR, USA) electron microscope operating at 120 kV. An aliquot of 3 μ L of the sample was deposited into the carbon grid previously discharged. Grids were blotted with filter paper for 5 s and vitrified by plunging into liquid ethane. The vitrified samples were stored in liquid nitrogen prior to cryo-TEM analysis.

Evaluation of Supramolecular Interactions by Small-Angle X-ray Scattering

Analysis (SAXS)

pHSL-TS and pHSL-TS-DOX were submitted to ultracentrifugation for 180 minutes at 350,000 \times g at 4 °C (OPTIMA L-80XP, Beckman Coulter–Fullerton, CA, USA). The obtained pellets were resuspended in HBS pH 7.4 or HBS pH 5.0 or RPMI-1640 containing 10% (v/v) FBS and were kept for 4 h. Then, the samples were again submitted to ultracentrifugation, and the pellet was analyzed by SmallAngle X-ray Scattering (SAXS). SAXS analysis was performed on a beamline D1B-SAXS1 instrument at Brazilian Synchrotron Light Laboratory (LNLS, Campinas, Brazil), at a fixed X-ray wavelength of 0.1488 nm. SAXS measurements of the samples were detected using a 300K Pilatus detector providing a range of 0.15–4.0 nm⁻¹, where $q = (4\pi/\lambda) \sin \theta$ and θ is the scattering angle. Liposomes were deposited on metal rings, which were sealed by a polyimide film (Kapton). The lattice parameter (a) was calculated using the formula $a = 2\pi/q$, which was determined for the first-order peaks of all packing structure types analyzed in this study.

Cell culture

4T1 (murine breast tumor) and MDA-MB-231 (human breast tumor) cell lines were cultured with RPMI-1640 and DMEM culture medium, respectively, supplemented with FBS (10% v/v), penicillin (1% w/v), and streptomycin (1% v/v). Cultures were maintained in a humidified incubator containing 5% CO₂ at 37°C. Upon reaching the

confluence stage, the cells were trypsinized and an aliquot was transferred to another flask containing complete culture medium for subculture.

Cell viability study

Cell viability was assayed with the CellTiter-Blue® Cell Viability Assay kit (CTB). 4T1 and MDA-MB-231 cells were plated in 96-well culture plates at a density of 1×10^4 cells/well. After 24h of incubation, DOX, DOX plus TS solution, and pHSL-TS-DOX were added to the wells (DOX concentration range of 0.25 to 16 μM). The drug solutions were prepared by dissolving DOX in purified water and TS in ethanol 95%. Treatment-free wells were maintained as control. The cells were maintained in a humidified incubator containing 5% CO_2 at 37°C and, after incubation of 24h for 4T1 or 48h for MDA-MB-231, the wells were washed with phosphate-buffered saline (PBS) three times. Then, CTB reagent diluted in culture medium (1:12) was added to each well and the plate was incubated for 2 h. Finally, the fluorescence intensity was measured using the Cytation 5 equipment (Biotek®, Winooski, USA) and the control group was considered as 100% of viability. Data were expressed as the percentage of cell viability for three independent experiments, being represented as the mean \pm standard deviation. Subsequently, the concentration of DOX required to promote 50% inhibition of cell viability (IC_{50}) was determined.

Cell proliferation study

4T1 or MDA-MB-231 cells (1×10^4 cells/well) were seeded in 96-well plates and maintained at 37°C and 5% CO_2 . After 24h, two procedures were performed in parallel: a plate, as control, was fixed with trichloroacetic acid (TCA) and another plate was treated with DOX, DOX plus TS, and pHSL-TS-DOX (DOX concentration range of 0.25 to 16 μM) and incubated for 24 or 48h, according to the lineage. After incubation, trichloroacetic acid (TCA) was added to each well and the plate was incubated at 4° C for 1 h. After washing the plates with distilled water and thorough drying at room temperature, 100 μL of the solution of sulforhodamine B (0.057% w/v)

was added to each well and the plates were maintained for 1 h at room temperature. Next, the plates were washed with an acetic acid solution (1% v/v) in order to remove the non-protein bound dye. Tris-base solution (10 mmol/L and pH 10.5) was added to solubilize the dye bound to the cellular proteins and the absorbance was acquired on a Spectra Max Plux 384 spectrophotometer, from Molecular Devices®, using Softmax Pro 6.51® software at 510 nm wavelength. The following equation was used to calculate the percentage of cell proliferation:

$$\text{Cell proliferation (\%)} = \frac{\text{Abs}_{\text{treatment}} - \text{AverageAbs}_{\text{prolifcontrol}} \times 100}{\text{AverageAbs}_{\text{control}} - \text{AverageAbs}_{\text{prolifcontrol}}} \quad (2)$$

Antitumor activity study

In vivo studies were conducted under the approval of the local Ethics Committee on Animal Use (CEUA) (Protocol # 168/2018) following the National Institutes of Health guide for the care and use of Laboratory Animals. BALB/c mice (female, 7-8 weeks old, 21.9 ± 1.4 g) were kept in an environment with controlled light cycle and ventilation and allowed free access to food and water. Mice received, subcutaneously, into the left thigh, aliquots (100 μ L) of 2.5×10^6 4T1 cells. After one week of inoculation, mice were randomly divided into four groups (5 - 7 mice per group), and received different treatments: HSB pH 7.4, free DOX, npHSL-DOX-AS, and pHSL-TS-DOX via tail vein injection at a dose of 5.0 mg DOX/kg every two days in a total of four administrations.

The antitumor efficacy was determined by the tumor volume measurement and calculation of the relative tumor volume (RTV) and the inhibition ratio (IR) (LEITE *et al.*, 2012). The tumor volume (TV) was monitored every two days, from the first day of treatment (D0) until four days after the end of the treatment (D10). It was calculated according to the following equation (3), where d_1 and d_2 represent the major and minor perpendicular diameters, respectively (ROLLAND, 2009). RTV and IR were calculated on day 10 according to equations 4 and 5.

$$TV = 0.5 \times (d_1 \times d_2^2) \quad (3)$$

$$RTV = \frac{TVon D10}{TVon D0} \quad (4)$$

$$IR(\%) = 1 - \frac{\text{Mean RTV of drug - treated group}}{\text{Mean RTV of control group}} \times 100 \quad (5)$$

On the fourth day after the end of the treatment, animals were anesthetized 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 containing anticoagulant (0.18% w/v EDTA) for hematological analysis. Then, the blood was centrifuged (2000 x g for 10 minutes) and the plasma obtained was used for biochemical analysis. Hematology analyses were determined using an automated analyzer (Hemovet[®] 2300) and parameters evaluated included red blood cell counts, hemoglobin, hematocrit, total and differential white blood cells, and platelet counts. Biochemical tests were performed in the Bioplus BIO-2000 semiautomatic analyzer (São Paulo, Brazil) using commercial kits (Labtest, Lagoa Santa, Brazil). The following parameters were analyzed: aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea and creatinine. Monitoring body weight was also used as a parameter for evaluation of toxicity. After blood collection, the mice were euthanized and the heart, spleen, and tumor were removed for histopathological evaluation.

Statistical analysis

Normality and homoscedasticity of variance were evaluated by the Kolmogorov-Smirnov test and Bartlett, respectively. Variables without normal distribution were transformed by equations: $y = \log(\text{variable})$ or $y = \sqrt{\text{variable}}$. The difference between experimental groups was tested by one-way ANOVA followed by Tukey's test or Student's unpaired t-test. For all analyses, it was adopted the confidence range of 95%, and differences were considered significant when the p-value was less than 0.05 ($p < 0.05$).

3. Results and discussion

Liposome characterization

The average diameter, PDI, and zeta potential data obtained for different formulations are presented in Table 2.

Table 2 - Average diameter, PDI, zeta potential, and EP of liposomal formulations

Formulation	Average diameter (nm)	PDI	Zeta potential (mV)
pHSL-TS-DOX-AS (with 30% TS)	158 ± 35	0.17 ± 0.01	- 4.0 ± 0.3
pHSL-TS-DOX-AS (with 40% TS)	172 ± 11	0.17 ± 0.03	- 2.5 ± 0.8
pHSL-TS-DOX	151 ± 18	0.20 ± 0.08	- 2.7 ± 0.8
npHSL-DOX	106 ± 11	0.20 ± 0.05	- 1.0 ± 0.2

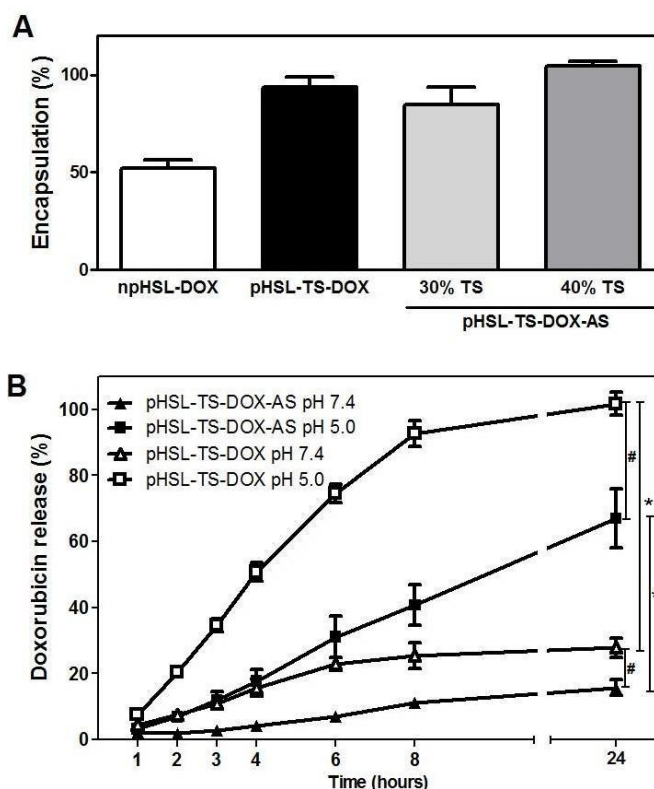
Notes: Results expressed as the mean ± standard deviation (n = 3). npHSL-DOX was prepared with EPC as structural lipid since any formulation containing DOPE would need to add a stabilizing agent which could interfere in the DOX encapsulation.

Abbreviations: TS: alpha tocopheryl succinate; PDI: polydispersity index; pHSL-TS-DOX-AS: pH-sensitive liposomes containing alpha tocopheryl succinate, doxorubicin, and ammonium sulfate; pHSL-TS-DOX: pH-sensitive liposomes containing alpha tocopheryl succinate and doxorubicin; npHSL-DOX: non-pH sensitive liposomes containing doxorubicin.

Firstly, pHSL-TS-DOX-AS was prepared with 30% of TS at a lipid concentration of 20 mmol/L. Average diameter value less than 200 nm, and a PDI value indicative of monodisperse vesicles population (< 0.3) were obtained. Zeta potential values were close to neutrality. It can be attributed to the presence of DSPE-PEG on the surface of the vesicles. The long PEG chains arranged on the vesicles' surface promote hydrodynamic resistance, which might decrease the electrophoretic mobility and

mask the surface charge, resulting in the zeta potential near neutrality. A high DOX encapsulation content ($84.6 \pm 15.4\%$) could be verified (Table 2, **Figure 1A**). To obtain a formulation containing a high TS amount, the proportion of this compound was altered to 40% and the lipid concentration to 40 mmol/L. No significant difference in the physicochemical parameters was observed. In addition, the influence of the presence of ammonium sulfate was investigated. High values of EP were obtained for both preparations: $104.7 \pm 4.1\%$ and $93.4 \pm 10.5\%$ for pHSL-TS-DOX-AS and pHSL-TS-DOX, respectively (**Figure 1A**). The high encapsulation obtained by using ammonium sulfate is often explained by the precipitation of DOX into the liposomal vesicle resulting in an insoluble salt, DOX sulfate (GABIZON; SHMEEDA; GRENADER, 2012). However, high values were also obtained for liposomes prepared without ammonium sulfate and it can be attributed to the presence of TS. One possible reason would be the formation of an ionic pair between DOX and TS, leading to the greater encapsulation of DOX in the liposomes. To investigate this hypothesis, a liposomal formulation without TS and ammonium sulfate was also prepared and evaluated (npHSL-DOX). The npHSL-DOX formulation presented a reduction of average diameter, although PDI and zeta potential values had been similar to those obtained for other formulations (Table 2). In contrast, a significantly lower EP ($\sim 50\%$) than those observed for other formulations was obtained ($p < 0.05$) (**Figure 1A**). These findings are in line with previously obtained data, which showed that the encapsulation of DOX in lipid nanocarriers increased in the presence of TS (OLIVEIRA *et al.*, 2016a).

Figure 1 – Encapsulation percentage of formulations (A). Doxorubicin release from liposomes containing or not ammonium sulfate at different pH conditions (B). Results expressed as the mean \pm SD (n = 3). ** Represents significant difference between pH 7.4 and pH 5.0 for the same formulation ($p < 0.05$). # Represents significant difference for different formulation ($p < 0.05$).



Following, pHSL-TS-DOX-AS and pHSL-TS-DOX formulations were assessed for *in vitro* DOX release at pH 7.4 and 5.0. The data are shown in **Figure 1B**. At physiological pH, a low release of DOX from pHSL-TS-DOX-AS was observed, reaching 17% up to 24 h. In contrast, at pH 5.0, a release of 40% of DOX occurs within 8h and approximately 67% was released up to 24 h. For pHSL-TS-DOX, a release profile significantly higher in both pH conditions was observed ($p < 0.05$). At pH 7.4, around 24% of DOX was released in the first 8h, followed by a *plateau*. In contrast, at pH 5.0, a rapid release was verified (~92%) until 8 h, reaching 100% at the end of the study. The more rapid release of DOX from liposomes at pH 5.0 when compared to pH 7.4 might be explained by the TS protonation, inducing the destabilization of the lipid membrane, enhancing, consequently, the membrane permeability and the DOX release. Another possible scenario would be the fact that

the acid medium provides the protonation of the DOX enhancing its release from the formulation.

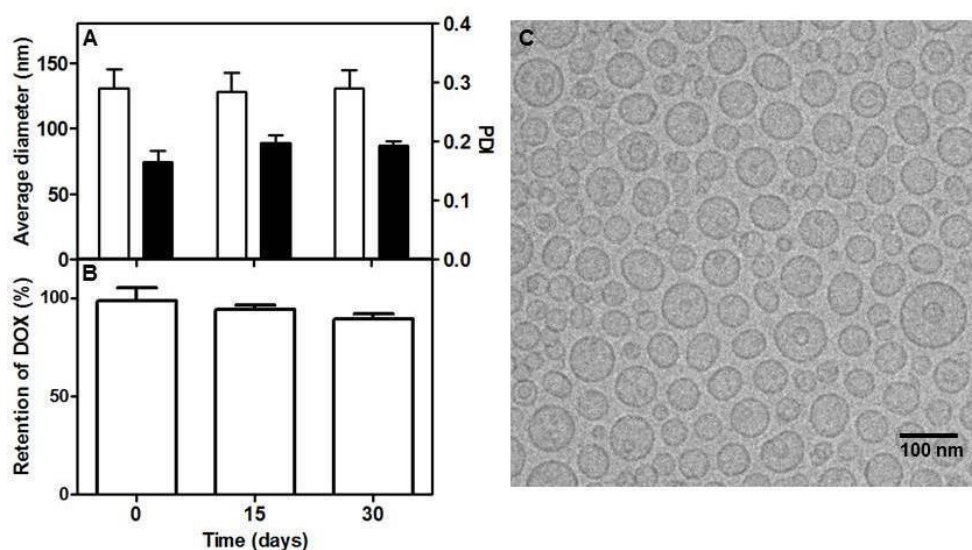
The higher DOX release from pHSL-TS-DOX than occurred in pHSL-TS-DOX-AS ($p < 0.05$) could be attributed to the DOX state inside the liposome. In this case, the DOX would be soluble and thus more available to be released, while in pHSL-TS-DOX-AS it could be precipitated and therefore its release would be hindered. In both formulations, it is possible to note higher release rates when subjected to an acid medium, indicating that they are responsive to pH variation. These data are in agreement with those obtained by Xu *et al.* (2017) that also showed high DOX release rates in acid pH for liposomes containing an ion-pair between cholesteryl hemisuccinate and DOX.

It is known PE derivatives cannot easily form a lamellar phase due to molecular geometry (FAN *et al.*, 2017), thus to form liposomes, a stabilizing agent should be added. In physiological pH, PE derivatives present a conical geometry that prevents from being organized in lamellar form. Rehman *et al.* (2018) investigated various lipid compositions using PE derivatives in association with TS loading DOX and observed the influence on pH-sensitivity. The use of a passive loading method wasn't able to promote the formation of vesicles. Although the pH sensitivity has been observed after loading of DOX by active method, the encapsulation of DOX was usually low. On the other hand, our data suggested that the TS acted properly as a stabilizing agent, allowing the production of liposomal vesicles with a low diameter. The encapsulation method proposed, without using the ammonium sulfate gradient, brought benefits since high encapsulation percentage and enhanced the DOX release, which could be more available to exert its antitumoral activity. Besides its structural importance in the system being studied, it is also necessary to investigate TS collaboration for antitumor activity associated with DOX in pHSL-TS-DOX.

In the short-term stability test, no significant variation in the parameters was observed, indicating no loss of formulation stability (**Figure 2A and 2B**). No significant change in zeta potential was also verified (data not shown). These results suggest no aggregation or fusion phenomena that could alter the average diameter and unwanted release of the encapsulated DOX. Morphological analysis of the pHSL-TS-DOX vesicles was performed by cryo-TEM (**Figure 2C**). In general, the

vesicles are spherical, unilamellar, and with reasonably uniform diameter. There was no evidence of crystals inside the vesicles typical coffee bean as it has been demonstrated in formulations containing ammonium sulfate and DOX (BARENHOLZ, 2012; GABIZON; SHMEEDA; GRENADER, 2012). Thus, these findings reinforce our hypothesis that DOX is present in the lipid bilayer interacting with TS.

Figure 2 – Storage stability evaluation through (A) average diameter (white bars) and PDI (black bars), and (B) retention percentage of pHSL-TS-DOX. (C) Cryo-TEM photomicrographs obtained for pHSL-TS-DOX. There were no differences in the average diameter, PDI, and retention percentage during the study ($p>0.05$). These analyses were performed by one-way ANOVA followed by Tukey's test.



SAXS analysis

In order to investigate the pH sensitivity of pHSL-TS-DOX, SAXS analyses were conducted at 37°C after resuspension in HBS at pH 7.4, and 5.0. As a control, pHSL-TS formulation (without DOX) was also evaluated (**Figure 3A**). At pH 7.4, a lamellar domain in both diffraction patterns could be observed. For pHSL-TS, the sequence of SAXS peaks presented $q = 0.48, 0.98, \text{ and } 1.42 \text{ nm}^{-1}$, while q values for the sequence of peaks for pHSL-TS-DOX were found at $0.52, 1.00, \text{ and } 1.49 \text{ nm}^{-1}$ (**Figure 3B**). Both sequences showed a periodicity of Bragg reflections with ratios close to 1, 2, and 3. Lattice spacing values of 13.1 and 12.1 nm, for pHSL-TS and

pHSL-TS-DOX, respectively, were determined. These findings are characteristic of lamellar regions enriched with DSPE-PEG₂₀₀₀ (MONTEIRO *et al.*, 2018) and demonstrate that the presence of DOX does not alter the lamellar organization.

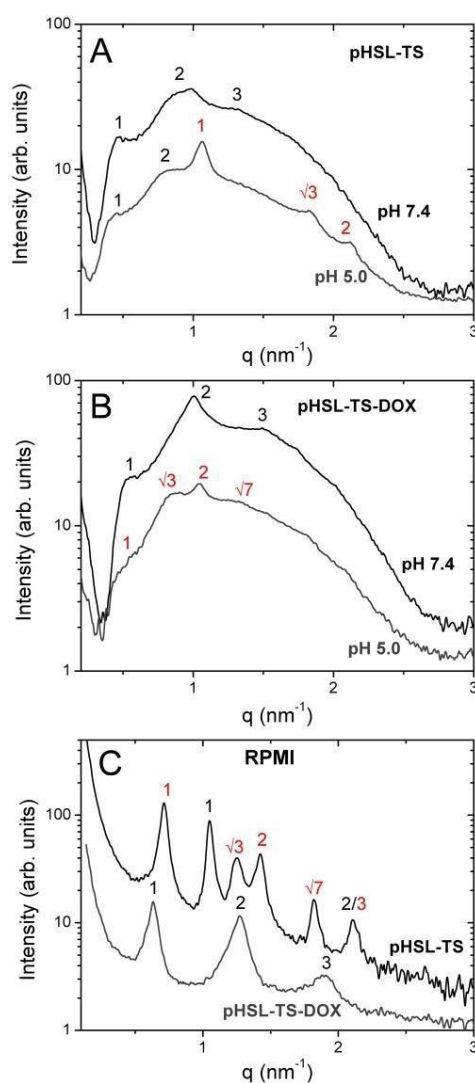
At pH 5.0, the coexistence of two domains was observed for pHSL-TS. The first sequence of peaks presented q values equal to 0.44 and 0.86 nm⁻¹ with Bragg indexes of nearly 1 and 2, and lattice parameter of 14.3 nm, showing a lamellar phase organization of DOPE molecules. A second non-lamellar order (hexagonal phase) showed SAXS peaks at q 1.06, 1.82, and 2.12 nm⁻¹, with Bragg index ratios close to 1, $\sqrt{3}$, 2 (**Figure 3A**). It is believed that under conditions of acid pH, the protonation of the TS occurs, leading to a loss of stabilization of the lipid bilayer. The presence of two patterns of conformation (lamellar and hexagonal) in the same sample may represent a transition process, in which some vesicles have their organization impaired by the acidic condition, leading to hexagonal conformation, while other vesicles remain in the lamellar phase.

On the other hand, for pHSL-TS-DOX, a single sequence of peaks, located at q = 0.49, 0.84, 1.04, and 1.32 nm⁻¹, with Bragg reflection indices of 1, $\sqrt{3}$, 2, $\sqrt{7}$ was verified (**Figure 3B**), suggesting a hexagonal phase organization of DOPE molecules. This result corroborates the high release rate of DOX obtained in the release study at pH 5.0. As previously mentioned, at this condition, the protonation of the TS occurs, which consequently increases the permeability of the membrane and ends up allowing the DOX release. The large intensity humps observed in Figures 3A and 3B denote the presence of disordered material, where only the average first-neighbor correlation is observed. The signal from ordered material phases sits on top of this hump, indicating the coexistence of a long-range ordered volume of material and a short-range (poorly ordered) volume.

Analyses of both formulations, pHSL-TS and pHSL-TS-DOX, were also performed in RPMI-1640 culture medium at pH 7.4 at 37°C (**Figure 3C**). The blank formulation showed the coexistence of two domains. The first order type was defined at q equal to 0.71, 1.24, 1.42, 1.83, and 2.10 with Bragg reflection indices of approximately 1, $\sqrt{3}$, 2, $\sqrt{7}$, 3, characteristic of DOPE molecules in the hexagonal organization. The second order type was found to present a lamellar domain exhibiting Bragg index ratios of 1, and 2 (q = 1.04 and 2.10). This data suggests a possible interaction between pHLS-TS and culture medium components, leading to a destabilization of

the lipid bilayer. On the other hand, the analysis carried out on pHSL-TS-DOX revealed the presence of just one lamellar domain corresponding to Bragg reflection position ratios close to 1, 2, 3 (q values equal to 0.63, 1.27, and 1.90). Thus, it can be inferred that the presence of DOX brings stability to the formulation in relation to the interactions with components of the culture medium. This finding might represent an *in vivo* advantage since less alteration in the blood circulation can occur, maintaining greater integrity until the therapeutic target.

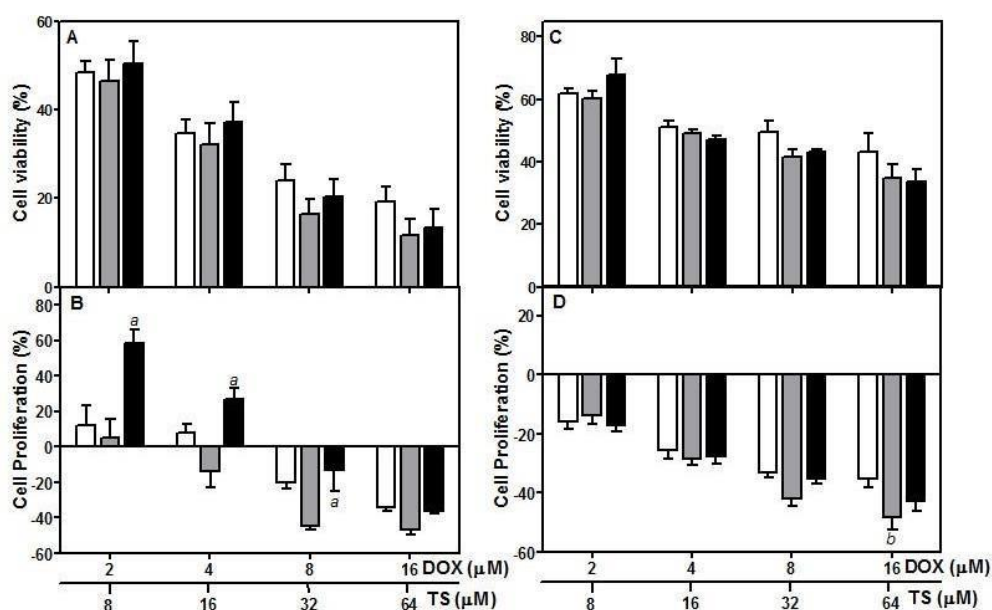
Figure 3 – SAXS patterns of pHSL-TS (A) and pHSL-TS-DOX (B) prepared at 37°C in HBS at pH 7.4 and 5.0, and RPMI (C) at pH 7.4. The indexes (peak labels) represent phases that can be identified by the periodicity of the Bragg reflections.



Cell viability and proliferation

In order to determine the cytotoxic activity of the formulations, breast cancer cell lines (4T1 and MBA-MB-231) were used. The cells were incubated with free DOX, free DOX plus TS, or pHSL-TS-DOX and the viability and proliferation cell were evaluated as showed in **Figure 4**. Cell viability was found to be 100% after treatment with negative control and blank liposomal formulation (pHSL-TS, data not shown).

Figure 4 – Cell viability and proliferation of 4T1 (A and B, respectively) and MDA-MB-231 (C and D) lineages against treatments with DOX (white), DOX plus TS (gray), and pHSL-TS-DOX (black). Results expressed as mean \pm SEM. ^a Represents a significant difference compared to DOX+TS; ^b Represents a significant difference compared to free DOX.



For both cell lines, clear response and a dose-dependent decrease in the cell viability were observed for all groups, however, no significant difference was verified among the treatments (**Figure 4A and C**). At the concentrations evaluated, MDA-MB-231 showed to be more resistant than 4T1, since a percentage of low viability was not achieved, even at the highest concentration.

Concerning the cell proliferation, the treatment with pHSL-TS-DOX against 4T1 cell showed a significant difference when compared with TS plus DOX, except at the highest concentration. Surprisingly, the cell proliferation for pHSL-TS-DOX increased when compared to TS plus DOX and could be attributed to a higher delivery of TS, which, because its lipophilicity, can perfuse more easily through the plasmatic membrane than in its encapsulated form and thus, it could perform better together with DOX. For the MDA-MB-231 cell line, cell proliferation rates were similar for all treatments.

Table 3 shows the IC₅₀ values obtained for the 4T1 and MDA-MB-231. Similar IC₅₀ values were found among treatments for each of the strains ($p > 0.05$). Therefore, it can be inferred that the delivery of DOX and TS at the intracellular level is not impaired when they are inside the liposome since there is no difference in the cytotoxicity of the formulation and the drugs in its free form. Previous studies have reported that cytotoxicity data against sensitive cells are generally conflicting and the activity differences between free and encapsulated DOX are subtle (OLIVEIRA *et al.*, 2016a).

Table 3 – IC₅₀ of 4T1 and MDA-MB-231 cell lines submitted to different treatments

Cell line	IC ₅₀ (µM)		
	DOX	DOX plus TS	pHSL-TS-DOX
4T1	1.3 ± 0.2	1.1 ± 0.4	1.2 ± 0.5
MDA-MB-231	8.3 ± 5.6	4.4 ± 1.1	4.6 ± 0.1

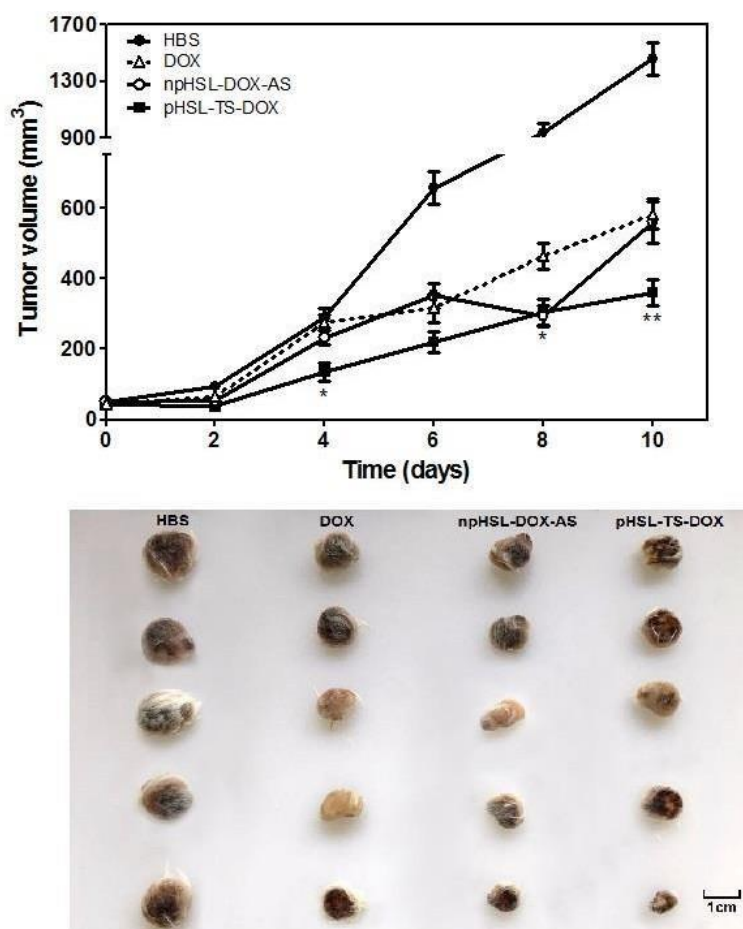
Notes: Results expressed as mean ± SD. There was no significant difference between treatments ($p > 0.05$). These analyses were performed by one-way ANOVA followed by Tukey's test.

Abbreviations: IC₅₀: concentration that inhibited cell proliferation by 50% when compared to that untreated cells; DOX: free doxorubicin; TS: alpha tocopheryl succinate; DOX plus TS: free doxorubicin plus free alpha tocopheryl succinate; pHSL-TS-DOX: pH-sensitive liposomes containing alpha tocopheryl succinate and doxorubicin.

Antitumor activity evaluation

The antitumor activity of free or liposomal DOX (pHSL-TS-DOX and npHSL-DOX-AS) was monitored by tumor volume measurement over time. The tumor volume, measured every other day, in 4T1 breast tumor-bearing mice treated with HBS, free DOX, npHSL-DOX-AS, or pHSL-TS-DOX is shown in **Figure 5A**.

Figure 5 - Tumor volume in 4T1 bearing mice (A) and photographs of the tumors after dissection (B) treated with HBS, free DOX, npHSL-DOX-AS, and pHSL-TS-DOX. All results were expressed as the mean \pm SEM. * Represents a significant difference against group treated with DOX. ** Represents a significant difference against group treated with DOX and npHSL-DOX-AS. ($p < 0.05$). These analyses were performed by one-way ANOVA followed by Tukey's test.



The control group, treated with HBS, presented a rapid tumor growth profile reaching a tumor volume of approximately 1500 mm³ at day 10, given the aggressiveness and

high rate of cell proliferation of the studied lineage. Lower tumor volume was obtained in the groups treated with formulations containing DOX. However, the group treated with pHSL-TS-DOX presented greater control of the tumor growth and from day 4 and until the end of the study it is noticed a smaller tumor volume compared to the group treated with DOX (except for day 6). The mean tumor volume, obtained on the tenth day, for animals treated with pHSL-TS-DOX ($360 \pm 81 \text{ mm}^3$) was significantly lower ($p < 0.05$) than those observed for the groups treated with DOX ($580 \pm 103 \text{ mm}^3$) and npHSL-DOX-AS ($561 \pm 140 \text{ mm}^3$). As demonstrated by Silva et al. (2016), long-circulating pH-sensitive liposomes containing DOX are able to accumulate more efficiently in mice-induced solid tumors, compared to long-circulating non-pH-sensitive liposomes containing DOX and free DOX. This could be evidence for better control in tumor growth provided by pHSL-TS-DOX compared to npHSL-TS-DOX-AS besides the presence of TS that could act synergistically with DOX.

Figure 5B represents the tumors removed from the mice after the treatments. Tumors of the HBS-treated group were noticeably larger and an irregular shape was observed. Tumors from DOX and npHSL-DOX-AS treated groups showed more similarity. Tumors of the pHSL-TS-DOX-treated group were smaller; more rounded and a concave surface with evidence of tissue loss in the central region was verified.

RTV and IR were calculated using the volume tumor values (Table 4). A higher RTV value was observed for the control group, indicating tumor growth during the course of the study. The treatments with free DOX and npHSL-DOX-AS resulted in an intermediate value of RTV, while pHSL-TS-DOX treatment showed the smaller RTV value, indicating a low rate of tumor growth. The treatment with pHSL-TS-DOX also led to higher IR (71.6%) compared to the groups treated with npHSL-DOX-AS (63.7%) and free DOX (52.8%). These data demonstrate the effect of TS in potentializing the antitumor activity of DOX against 4T1 cells.

Table 4 - Determination of RTV and IR obtained after administration of HBS, free DOX, npHSL-DOX-AS, and pHSL-TS-DOX

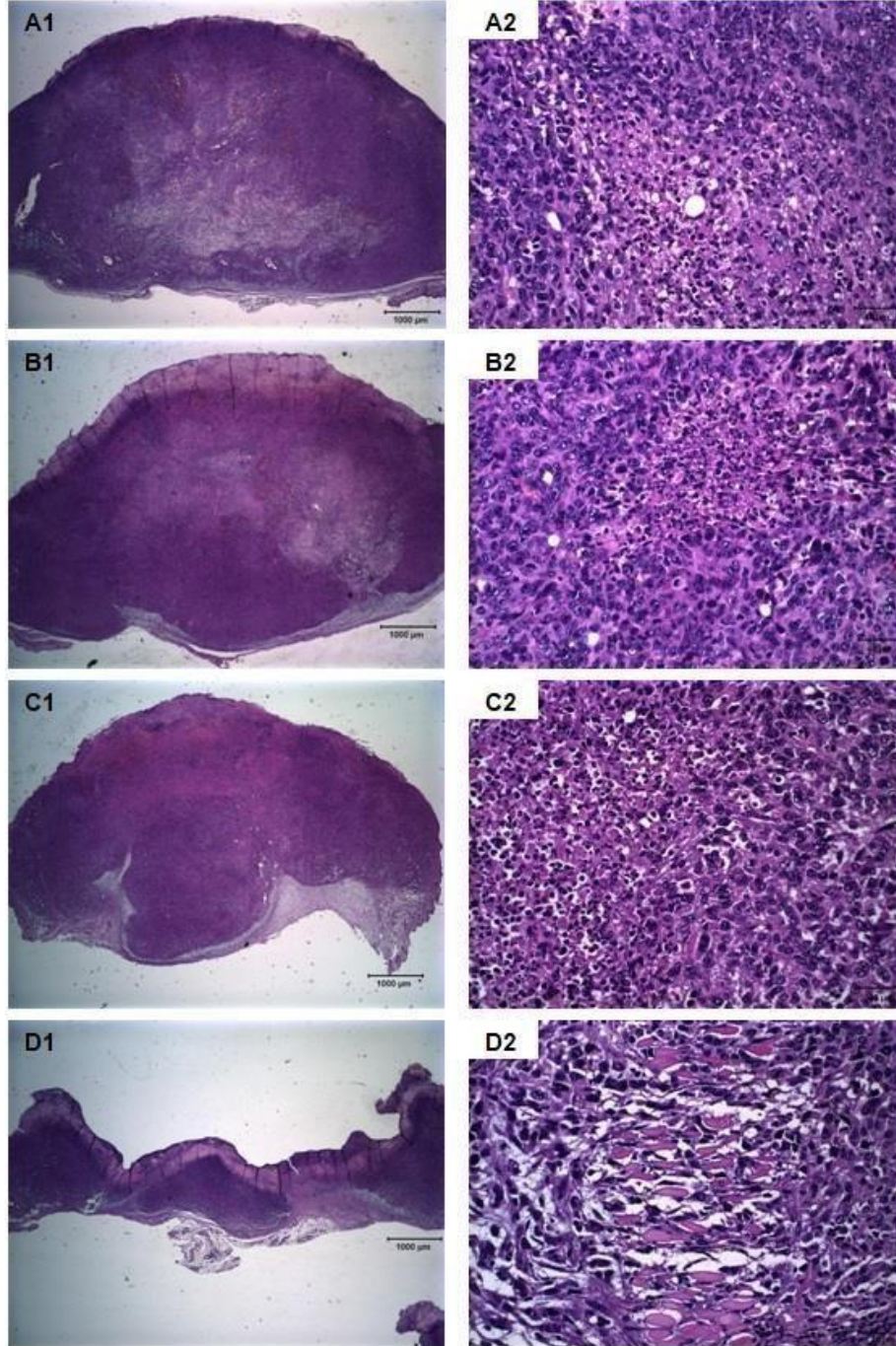
Treatment	RTV	IR (%)
HBS	31.4 ± 7.9 ^a	-
DOX	14.8 ± 6.7	52.8
npHSL-DOX-AS	11.4 ± 3.6	63.7
pHSL-TS-DOX	8.9 ± 1.5	71.6

Notes: Results expressed as the mean ± SD. ^a Represents a significant difference against the other groups ($p < 0.05$). These analyses were performed by one-way ANOVA followed by Tukey's test.

Abbreviations: HBS: HEPES-buffered saline; DOX: free doxorubicin; npHSL-DOX-AS: non-pH sensitive liposomes containing doxorubicin and ammonium sulfate; pHSL-TS-DOX: pH-sensitive liposomes containing alpha tocopheryl succinate and doxorubicin; RTV: relative tumor volume; IR: inhibition ratio.

Histopathological analyses were carried out in the tumors for all experimental groups (**Figure 6**). Tumors treated with HBS (control) showed large areas of necrosis in the central region as well as extensive areas of viable cells, with mitotic figures indicating a high rate of cell proliferation. Tumors treated with DOX presented extensive areas of viable cells, while npHSL-DOX-AS-treated group showed larger areas of necrosis, surrounded by viable cells. On the other hand, the group treated with pHSL-TS-DOX presented isolated areas of viable cells surrounded by granulation tissue with angiogenesis indicative of a tissue repair. This process involves the laying down of acellular fibrous tissue to replace the region of lost cells due to the treatment. The fibrous tissue is laid down by fibroblasts, which migrate to the injured area, proliferate and secrete collagen under the influence of numerous growth factors and cytokines. All these findings with the presence of new blood vessels constitute the granulation tissue (KRAFTS, 2010). The treatment with pHSL-TS-DOX was able to eliminate the vast majority of cancerous cells allowing the development of a granulation tissue containing collagen fibers which may result in a resolving process.

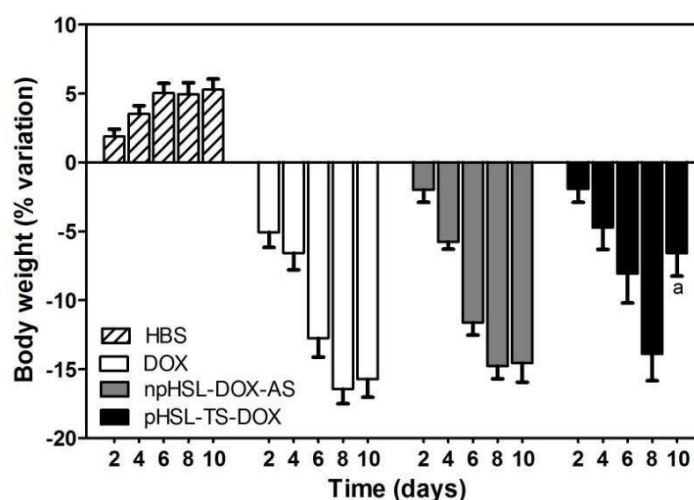
Figure 6 - Representative photomicrographs of 4T1 line breast tumors implanted in BALB/c mice treated with HBS (A1, A2), free DOX (B1, B2), npHSL-DOX-AS (C1, C2) or pHSL-TS-DOX (D1, D2). Hematoxylin-eosin staining, original magnification x 2 (A1, B1, C1, and D1) and x 40 (A2, B2, C2, and D2).



Toxicity evaluation

The animal's body weight was monitored every two days throughout the study (Figure 7). For animals in the control group (HBS), it was observed weight gain over time, which may be a result of the tumor growth. For the group treated with DOX, there was weight loss throughout the study period, from day 6 begins to present a critical weight loss (12.6%) reaching up to the loss of 16% on day 10. The variation percentage of body weight in the npHSL-DOX-AS-treated group was quite similar to that observed in the DOX-treated group. The group treated with pHSL-TS-DOX showed a maximum body weight loss on day 8 (13.8%), but on day 10 it was possible to observe a beginning of body weight recovery, significantly different from DOX and npHSL-DOX-AS ($p < 0.05$). This result indicates that the treatment with pHSL-TS-DOX allowed a start of body weight recuperation; probably it could restore the animal's normal condition in a long-term, which could indicate less toxicity than the other treatments.

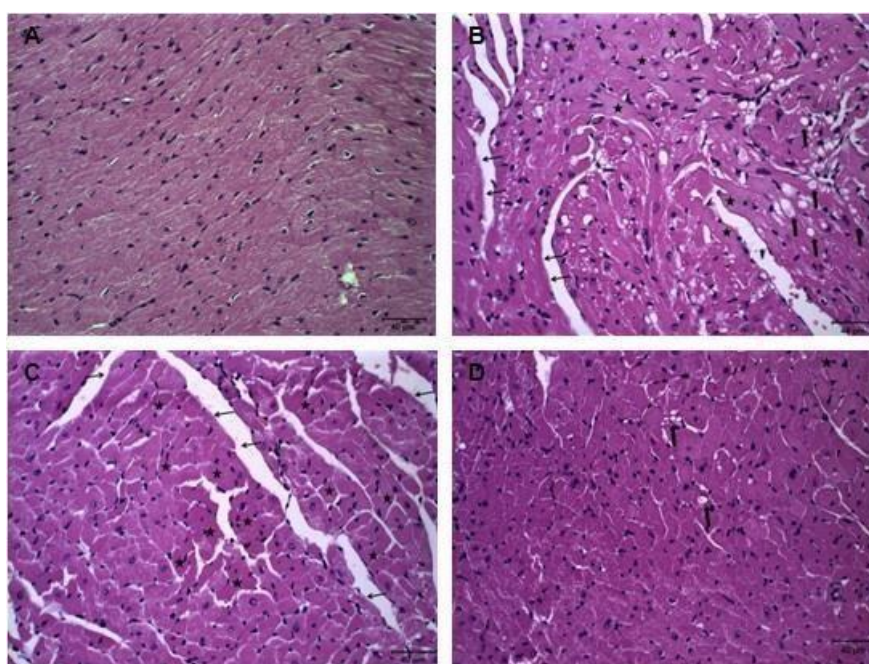
Figure 7 - Body weight variation of BALB/c mice bearing 4T1 breast tumor line submitted to different treatments. Results expressed as mean \pm SEM. ^a Represents a significant difference against the group treated with DOX and npHSL-DOX-AS ($p < 0.05$). These analyses were performed by one-way ANOVA followed by Tukey's test.



In addition, histological analyses of the heart were performed at the end of the treatment period. The cardiac tissue of animals treated with HBS demonstrated normal tissue architecture and cardiac fibers of usual thickness (Figure 8). In the

group treated with free DOX, large areas of vacuolization, swollen fibers, diffuse hyaline degeneration, and loss of striation were detected, indicating injury due to DOX cardiotoxicity. Its cardiotoxic effects result from the production of reactive oxygen species, leading to oxidative stress and mitochondrial damage (GHARANEI *et al.*, 2014). The increase in oxidative stress could lead to subcellular changes in the myocardium, including loss of myofibrils and vacuolation of myocardial cells (SEMPKOWSKI *et al.*, 2014). One of the strategies to minimize DOX cardiac toxicity is its encapsulation in nanosystems, to direct DOX to the tumor region and avoid spreading to other organs, including the heart. In the group treated with npH SL-DOX-AS, focal hyaline degeneration was observed and some swollen fibers, whereas in the group treated with pHSL-TS-DOX, vacuoles, and hyaline degeneration were rare. Furthermore, no loss of tissue striation and preservation of normal cardiac tissue could be verified, indicating greater protection against the cardiotoxic effects of DOX.

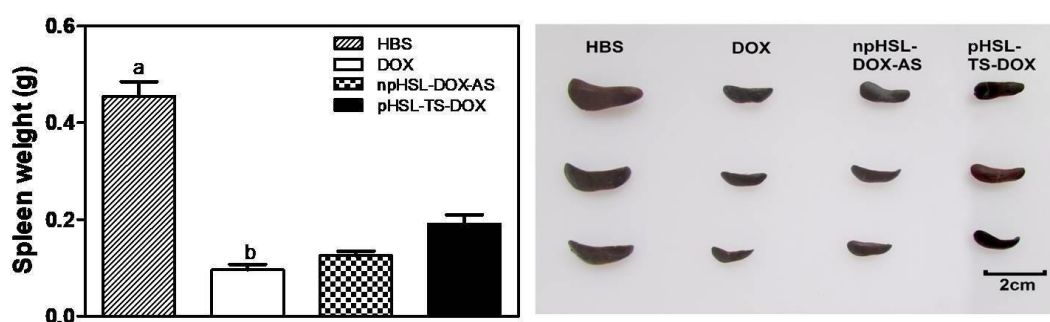
Figure 8 - Photomicrographs of the cardiac tissue of BALB/c mice treated with HBS (A), free DOX (B), npHSL-DOX-AS (C), and pHSL-TS-DOX (D). Hematoxylin-eosin stain, original increase x 40. Thick arrows indicate vacuolization, while thin arrows indicate swollen cardiac fibers and black stars indicate hyaline degeneration.



The animal's spleen was also removed and weighed. It was observed a higher weight of HBS-treated group compared to other groups (**Figure 9**). It can be explained by the splenomegaly induced by 4T1 cells, which has already been

described. The splenomegaly in 4T1 tumor-bearing mice could be the result of the increased number of splenic granulocytes, which probably come from the expanded peripheral blood granulocyte pool and/or from extramedullary granulopoiesis (DUPRE AND HUNTER JR., 2007). The other groups treated with free or encapsulated DOX showed similar spleen weights.

Figure 9 - Spleen weight of BALB/c mice bearing 4T1 breast tumor line submitted to different treatments (A) and photographs of the spleens after dissection (B). Results expressed as mean \pm SEM. ^a Represents a significant difference against the other groups. ^b Represents a significant difference against group treated with pHSL-TS-DOX ($p < 0.05$). These analyses were performed by one-way ANOVA followed by Tukey's test.



The hematological analysis of 4T1-bearing mice allowed observing leukocytosis in the control group due to tumor growth, which leads to greater production of defense cells (Table 5). In tumors from the 4T1 lineage, it has been observed an increase of granulocytes in the blood, characteristic of a leukemoid reaction, due to the progression of tumor volume (DUPRE AND HUNTER JR., 2007). This reaction could be related to many features, including splenomegaly, which was observed for this group as mentioned above. The neutrophil and basophil count also corroborates with this fact. In the DOX and npHSLDOX-AS-treated groups, a reduction in total leukocytes numbers can be attributed to the myelosuppressive activity of DOX, one of its most serious adverse effects (BARENHOLZ, 2012). However, this was not observed for the group treated with pHSL-TS-DOX, which conferred a protective effect, avoiding a marked reduction in total leukocytes. The same pattern described above was also observed for lymphocyte counts. The neutrophil to lymphocyte ratio (NLR) indicates, particularly in breast cancer, a prediction of the patient survival: a high NLR is generally associated with a poor prognosis (FARIA *et. al*, 2016). As it

can be noted in Table 5, the untreated group had the highest NLR, indicating a poor prognosis for animals in this group.

Table 5 - White blood cell, neutrophil, lymphocyte, basophil, and monocyte count and neutrophil to lymphocyte ratio of BALB/c evaluated in mice bearing 4T1 breast tumor after different treatments.

Parameters	Treatments			
	HBS	DOX	npHSL-DOX-AS	pHSL-TS-DOX
Total leucocyte ($10^9/L$)	30.3 ± 9.9^a	3.0 ± 1.6	2.4 ± 0.7	5.8 ± 0.9^b
Neutrophils ($10^9/L$)	19.4 ± 1.4^a	1.7 ± 0.1	1.3 ± 0.1	3.0 ± 0.3^b
Lymphocytes ($10^9/L$)	9.5 ± 1.6^a	1.2 ± 0.8	1.0 ± 0.1	2.6 ± 0.3^b
Basophils ($10^9/L$)	0.6 ± 0.4^a	0.03 ± 0.03	0.07 ± 0.04	0.07 ± 0.06
Monocytes ($10^9/L$)	0.8 ± 0.6^a	0.1 ± 0.1	0.08 ± 0.03	0.1 ± 0.1
NLR	2.1 ± 0.4^a	1.4 ± 0.2	1.3 ± 0.2	1.2 ± 0.2

Note: Results expressed as mean \pm SD. ^a Represents a significant difference compared to other groups. ^b Represents a significant difference compared to the group treated with DOX and npHSL-DOX-AS ($p < 0.05$). These analyses were performed by one-way ANOVA followed by Tukey's test.

Abbreviations: HBS: HEPES-buffered saline; DOX: free doxorubicin; npHSL-DOX-AS: non-pH sensitive liposomes containing doxorubicin and ammonium sulfate; pHSL-TS-DOX: pH-sensitive liposomes containing alpha tocopheryl succinate and doxorubicin.

NLR: neutrophil to lymphocyte ratio.

Concerning the erythrocytic series, no alteration in the red blood cell count, hemoglobin and hematocrit as well as platelet count could be observed after the treatments containing DOX (Table 6).

Table 6 – Red blood cell and plaquet count of BALB/c mice bearing 4T1 breast tumor after different treatments.

Parameters	Treatments			
	HBS	DOX	npHSL-DOX-AS	pHSL-TS-DOX
Red blood cells ($10^{12}/L$)	5.2 ± 0.5	5.4 ± 0.6	5.2 ± 0.7	4.8 ± 0.8
Hemoglobin (g/L)	9.4 ± 1.3	9.3 ± 1.4	9.1 ± 1.6	8.2 ± 1.9
Hematocrit (%)	25.1 ± 2.6	25.4 ± 2.9	24.8 ± 3.5	22.9 ± 4.1
Platelets ($10^9/L$)	216.3 ± 73.9	262.4 ± 123.8	169.4 ± 66.2	172.0 ± 53.5

Note: Results expressed as mean \pm SD. These analyses were performed by one-way ANOVA followed by Tukey's test.

Abbreviations: HBS: HEPES-buffered saline; DOX: free doxorubicin; npHSL-DOX-AS: non-pH sensitive liposomes containing doxorubicin and ammonium sulfate; pHSL-TS-DOX: pH-sensitive liposomes containing alpha tocopheryl succinate and doxorubicin.

Table 7 shows the results of the biochemical analysis. Parameters indicative of renal (urea and creatinine), and hepatic (AST and ALT) toxicity were evaluated. Both renal parameters were similar for all the treatments, indicating no renal toxicity. A 2.2-fold increase in AST levels was observed in animals receiving npHSL-DOX-AS compared to those belonging to the HBS group, while no significant difference was observed between pHSL-TS-DOX and the control group. A similar pattern was observed for ALT levels. This could indicate hepatic toxicity induced by npHSL-DOX-AS. In contrast, pHSL-TS-DOX did not induce hepatic toxicity, which is harmless to the liver. The liver is a common site for DOX-induced cell death and tissue damage. The metabolization of high concentrations of DOX results in the production of a vast number of ROS. Consequently, ROS causes an excessive amount of damage ranging from DNA damage, production of lipid peroxidation and decreasing vitamin E levels (TACAR, 2012). Since TS is a vitamin E analog and when is hydrolyzed it recover its antioxidant activity, it could avoid the hepatic toxicity in pHSL-TS-DOX. The AST is also a cardiac marker (ERTEN *et al.*, 2019) and its increase may indicate a cardiac injury. As mentioned above, the group treated with npHSL-DOX-AS presented the highest AST level, which could point to a cardiac effect. Differently, pHSL-TS-DOX presented an AST level similar to the control group, suggesting no cardiac toxicity.

Table 7 - Biochemical parameters of BALB/c mice bearing 4T1 breast tumor after different treatments.

Parameters	Treatments			
	HBS	DOX	npHSL-DOX-AS	pHSL-TS-DOX
Urea (mg/dL)	70.9 ± 11.8	86.3 ± 15.9	68.4 ± 11.9	83.7 ± 8.9
Creatinine (mg/dL)	0.36 ± 0.05	0.42 ± 0.09	0.39 ± 0.08	0.38 ± 0.08
AST (U/L)	96.3 ± 22.4	125.4 ± 19.6	210.0 ± 55.6 ^a	82.3 ± 4.9
ALT (U/L)	26.8 ± 12.3	49.9 ± 9.2 ^c	50.8 ± 17.4 ^b	26.5 ± 7.6

Note: Results expressed as mean ± SD. ^a Represents a significant difference against the other groups. ^b Represents a significant difference against group treated with HBS and pHSL-TS-DOX. ^c Represents a significant difference against group treated with pHSL-TS-DOX ($p < 0.05$). These analyses were performed by one-way ANOVA followed by Tukey's test.

Abbreviations: HBS: HEPES-buffered saline; DOX: free doxorubicin; npHSL-DOX-AS: non-pH sensitive liposomes containing doxorubicin and ammonium sulfate; pHSL-TS-DOX: pH-sensitive liposomes containing alpha tocopheryl succinate and doxorubicin; AST: aspartate aminotransferase; ALT: alanine aminotransferase

4 Conclusions

Through the development of pHSL-TS-DOX, it was possible to obtain a new and efficient DOX encapsulation method in the absence of ammonium sulfate, improving even the DOX release profile. By the release tests and SAXS can be demonstrated the pH-sensitivity of the system, which destabilizes in an acidic environment, favoring the release of DOX. The SAXS analyses also demonstrated the stability of the DOX-containing formulation in the culture medium, which may suggest an increase in stiffness of the lipid bilayer containing TS and DOX. The pHSL-TS-DOX formulation proved to be efficient against the 4T1-induced breast tumor since it was able to stagnate tumor growth and allow restoration of repair tissue in the region. This formulation was shown to be safe, did not cause marked weight loss in the animals and was still harmless to the heart and liver, thus proving to be a promising alternative for treatment against breast cancer.

Acknowledgment

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Brazil) and Coordenação de Aperfeiçoamento de Pessoal de

Nível Superior (CAPES-Brazil). The authors would like to thank Laboratório Nacional de Luz Síncroton (LNLS-Campinas-Brazil) for the SAXS studies. In addition, BORATTO, F.A. is grateful to Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG-Brazil) for providing a scholarship.

4.2 Artigo 2 - Lipossomas compostos por alfa-succinato de tocoferila e doxorubicina melhoram o acúmulo tumoral em modelo de tumor de mama murino

Artigo em fase final de redação, a ser submetido na *Journal of Pharmaceutical Sciences*

ABSTRACT

Liposomes composed by a rigid bilayer have high plasma stability, however, they can present serious difficulties in releasing the encapsulated drug as well as being internalized by the tumor cell. On the other hand, fusogenic liposomes may fuse with the plasmatic membrane and release encapsulated material directly into the cytoplasm. In a previous study, fusogenic liposomes composed of alpha-tocopheryl succinate (TS) and doxorubicin (DOX) were developed (pHSL-TS-DOX). These were able to stabilize tumor growth and reduce the toxicity when compared to a commercial formulation. In the present study, we investigated whether cell uptake or DOX accumulation in the tumor could justify the better performance of the pHSL-TS-DOX formulation. Release, deformability and DOX plasmatic concentration studies were also carried out. Data from studies of apoptosis, cell cycle and nuclear morphology have shown that the induction of cell death caused by pHSL-TS-DOX occurred more quickly. Higher DOX cell uptake and tumor accumulation were observed when pHSL-TS-DOX was administered, demonstrating better drug delivery ability. Therefore, better DOX uptake as well as tumor accumulation explain the great antitumor activity previously demonstrated for this formulation.

Keywords: doxorubicin, alpha-tocopheryl succinate, pH-sensitivity, liposomes, cell uptake, tumor accumulation, breast cancer

1 Introduction

The anthracycline antibiotic doxorubicin (DOX) has a broad spectrum of activity against different types of cancer and is often used as a single agent or in therapeutic combination regimens (WAKHARDE *et al.*, 2018). Doxil[®] (Janssen Biotech, Inc., Johnson & Johnson, USA), the first FDA-approved nano-drug of DOX, demonstrated a reduction in cardiotoxicity and myelosuppression, which are severe limitations of the DOX treatment (BARENHOLZ, 2012; TACAR *et al.*, 2012; SCHUTZ *et al.*, 2013). The rationale behind the use of Doxil[®] was based on (i) prolonging the drug circulation time and avoid clearance by cells of the mononuclear phagocytic system by using a PEGylated liposome, (ii) high and stable remote loading of DOX driven by a transmembrane ammonium sulfate gradient, and (iii) lipid bilayer composed of the high-T_m (53°C) saturated phosphatidylcholine (BARENHOLZ, 2012; CAGEL, 2016).

Despite all the advantages of Doxil[®], other modern strategies in the nanosystems area, whether through the use of polymorphic lipids or techniques of drug encapsulation without using a remote gradient, as well as the association with more than one component that presents antitumor activity, may be beneficial (SERCOMBE *et al.*, 2020).

Our group developed recently a new pH-sensitive liposome containing DOX and alpha-tocopheryl succinate (TS) (pHSL-TS-DOX) (BORATTO *et al.*, 2020). pH-sensitive liposomes have been designed to be less stable in an acidic environment, as the tumor tissue and intracellular endosomes (pH about 6.5 and 5.0-6.0, respectively), than normal tissues (pH 7.4). This system contains a polymorphic lipid capable of forming a lamellar bilayer at physiological pH. When submitted to an acidic environment, it undergoes destabilization, changes to a hexagonal phase, and then releases the vesicle content (GUAN, 2013; FERREIRA *et al.*, 2013).

In our previous work, we obtained a new and efficient DOX encapsulation method in the absence of ammonium sulfate (a passive loading method), with high encapsulation percentage and improving the DOX release profile. The formulation proved to be more efficient in controlling the tumor growth in a 4T1 breast tumor-induced model than a non-pH-sensitive formulation (similar to Doxil[®], npHSL-DOX-AS). Additionally, it was shown to be safe, harmless to the heart and liver, and avoided myelosuppression (BORATTO *et al.*, 2020). All these benefits over a well-established formulation used in the clinic motivate us to pursue further biological studies to a better comprehension of the pharmacokinetic features of this formulation as well as its cellular uptake, tumor accumulation, and possible effects on the cell cycle.

2 Materials and methods

Materials

Dioleoylphosphatidylethanolamine (DOPE), hydrogenated soybean phosphatidylcholine (HSPC), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (DSPE-PEG₂₀₀₀) were purchased from Lipoid (Ludwigshafen, Germany). 4-(2-hydroxyethyl)-1-piperazine ethanosulphonic acid (HEPES), cholesterol, ammonium sulfate, alpha-tocopheryl succinate (TS), and trypsin were acquired from Sigma-Aldrich (Steinheim, Germany). Doxorubicin hydrochloride (DOX) was purchased from ACIC Chemicals (Ontario, Canada). Chloroform and anhydrous monobasic potassium phosphate were supplied by Synth (São Paulo, Brazil). Sodium hydroxide was purchased from Vetec Química Fina Ltda (Rio de Janeiro, Brazil). HPLC grade methanol was purchased from Tedia (Fairfield, USA). 4T1 tumor cell line was purchased from the ATCC (Manassas, USA). Roswell Park Memorial Institute 1640 (RPMI-1640), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Invitrogen (São Paulo, Brazil). Xylazine and ketamine solutions were acquired from Hertape Calier Saúde Animal S/A (Juatuba, Brazil) and Vetbrands Agroline (Campo Grande, Brazil), respectively. All other chemicals used in this work were analytical grade.

Liposomes preparation

The pHSL-TS-DOX was prepared by hydration of lipid film as described previously (BORATTO *et al.*, 2020). Briefly, chloroform aliquots of DOPE, TS, and DSPE-PEG₂₀₀₀ (molar ratio of 55:40:5, 40 mmol/L) were transferred to a round bottom flask, and the solvent was removed under reduced pressure until a formation of a thin lipid film. Then, a NaOH solution was added to promote TS complete ionization, and the film was hydrated with HEPES-saline buffer pH 7.4 (HBS). The obtained vesicles were calibrated by sonication using an ultrasonic homogenization apparatus (CPX 500, Cole Parmer, Vernon Hills, USA) with a high power probe for 3 min in an ice bath and 21% amplitude. Then, 1.0 mL of concentrated DOX solution (10 mg/mL) was added. The resulting dispersion was maintained for 2 h under refrigeration at 4°C.

npHSL-DOX-AS, composed of HSPC, CHOL, and DSPE-PEG₂₀₀₀ at the total lipid concentration of 20 mmol/L, molar ratio 57:38:5, respectively, were also prepared in order to evaluate the advantages of pHSL-TS-DOX over a non pH-responsive liposome, similar to the commercially available formulation. npHSL-DOX-AS was prepared by the film hydration method followed by extrusion, and the DOX incubation was performed for one hour at 60 °C by the ammonium sulfate gradient method.

Liposomes characterization

Liposomes were characterized according to their mean diameter and polydispersity index (PDI) by dynamic light scattering (DLS) at 25°C at a 90° angle using monomodal analysis; the zeta potential was evaluated by DLS associated with the electrophoretic mobility at an angle of 90°. The measurements were performed in triplicate using the Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK).

The entrapped DOX concentration was determined by high performance liquid chromatography (HPLC) using fluorimetric detection in a Waters chromatographer (Waters Instruments, 1200 series, Milford, USA). The separation was performed using a 250 x 4.6 mm, 5 µm column (Merck, ACE® 250-4.6 C8, Aberdeen, Scotland). The eluent system consisted of methanol:phosphate buffer 0.01 mol/L pH 3.0 (65:35

v/v, respectively), and the flow rate was 1.0 mL/min. Samples were injected, and the eluate was monitored at excitation/emission wavelengths of 477/555 nm. The EP was calculated according to the following equation:

$$EP = \frac{[DOX]_{PurifiedLip} \times 100}{[DOX]_{TotalLip}} \quad (1)$$

Abbreviations: EP: encapsulation percentage, $[DOX]_{PurifiedLip}$: doxorubicin concentration after liposome purification, $[DOX]_{TotalLip}$: doxorubicin concentration before liposome purification.

Release studies

One milliliter of pHSL-TS-DOX, npHSL-DOX-AS, or free DOX was transferred to a CelluSep® 14 kDa dialysis bag along with 1.0 mL of RPMI pH 7.4. This dialysis membrane was dropped into an external phase with 25 mL or 50 mL of RPMI pH 7.4 under magnetic stirring at 37°C. At several time intervals (1, 2, 3, 4, 6, 8, and 24h), 1.0 mL of the dialysis fluid was withdrawn and replaced by 1.0 mL of fresh medium. An aliquot of this fluid was added acetonitrile, and then it was homogenized and centrifuged at 9,400 x g for 15 min. The supernatant was used for DOX quantification by HPLC. The values were plotted as the cumulative percentage of drug release.

Deformability test

The deformability of pHSL-TS and npHSL-AS (both without DOX) was assessed by the alteration of the vesicle size. The formulations were passed once in a polycarbonate membrane of pore size of 100 nm. After, the extrusion membrane used was turned 180° and the vehicle of each formulation was passed, making a backwash. The mean diameter of the formulation before and after extrusion and the backwash liquid was measured by DLS.

In vivo studies

DOX plasmatic concentration studies

In vivo studies were conducted under the approval of the local Ethics Committee on Animal Use (CEUA) (Protocol # 63/2019) following the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Groups of four healthy BALB/c female mice (19 – 25g) received a dose equivalent of 5 mg/kg of DOX. The treatments were as follows: free DOX, pHSL-TS-DOX, and npHSL-DOX-AS via the tail vein injection. At 0.5, 1, 4, and 24 h post-injection, blood was collected from anesthetized mice by puncture of the brachial plexus in tubes containing an anticoagulant (0.18% w/v EDTA) (method adapted from MUSSI *et al.*, 2015). Then, the blood was centrifuged (1,200 x g, for 10 min), and the plasma was used for DOX quantification by HPLC. The samples were diluted before the analysis as follows: 125 μ L of plasma of mice treated with free DOX, pHSL-TS-DOX or npHSL-DOX-AS were added to an eppendorf with daunorubicin (internal standard) and 150 μ L of acetonitrile. The suspensions were homogenized and centrifuged at 9,400 x g for 15 min. The supernatants containing the extracted DOX were used for quantification. A six-point standard curve (10, 100, 200, 300, 400, and 500 ng/mL) vs. area under curve ratio DOX/daunorubicin was previously prepared using plasma of mice. Data obtained by linear regression were $y = 0.0033 x + 0.0557$ and $r = 0.9890$.

Quantification of DOX in the tumor

BALB/c female mice (22 – 25g) received, subcutaneously, into the left thigh, aliquots (100 μ L) of 1.0×10^6 4T1 cells. After eleven days of inoculation, they were randomly divided into three groups (n=4). The animals received a single dose (the equivalent of 20 mg/kg of DOX) of free DOX, pHSL-TS-DOX, or npHSL-DOX-AS via the tail vein. At 4 h post-injection, the animals were anesthetized and euthanized by blood puncture, and the tumors were collected and weighted. Then, tumors were homogenized with 300 μ L of acetonitrile in Ultra Turrax T-25 homogenizer (Ika Labortechnik, Germany), and 50 μ L of Triton X-100 10% v/v, 50 μ L of distilled water and 300 μ L of acidified isopropanol (0.75 N) were added (method adapted from LAGINHA *et al.*, 2005). The preparation was maintained overnight at -20°C for DOX

extraction. Afterward, samples were warmed to room temperature, vortexed for 5 min, centrifuged at 9,400 x g for 15 min. For DOX quantification by HPLC, 125 μ L of the supernatant obtained were added to an eppendorf with daunorubicin (internal standard) and 150 μ L of acetonitrile.

In vitro studies

Cell culture

4T1 cell line (murine breast tumor) was cultured with RPMI-1640 culture medium, supplemented with FBS (10% v/v), penicillin (1% w/v), and streptomycin (1% v/v). The culture was maintained in a humidified incubator containing 5% CO₂ at 37°C. Upon reaching the confluence stage, the cells were trypsinized, and an aliquot was transferred to another flask containing complete culture medium for subculture.

Cellular uptake

4T1 cells (1 x 10⁶ cells/well) were seeded in 12-well plates and maintained at 37°C and 5% CO₂. After 24 h, the culture medium was removed and the treatments were added. The cells were treated with free DOX, pHSL-TS-DOX, and npHSL-DOX-AS (DOX concentration of 1 μ mol/L) and incubated for 1, 2, or 8 h. After incubation, the medium was removed, and the cells were washed with PBS. Then, the cells were trypsinized, homogenized and centrifuged at 160 x g for 5 min. The *pellet* was resuspended with 1.0 mL of isopropanol:methanol (1:4 v/v). The dispersion was submitted to homogenization in an ultrasound bath for 15 min, centrifuged at 1,200 x g for 15 min. The supernatant was used for DOX quantification by HPLC. The cellular uptake was calculated by the ratio of the DOX obtained in the quantification of each well and the DOX used in cell treatment (1 μ mol/L).

Analysis of apoptosis

Initially, 4T1 cells were seeded on a 12-well plate and incubated at 37°C and 5% CO₂ in a density of 300,000 cells/well. After incubation, free DOX, pHSL-TS-DOX, and npHSL-DOX-AS were added to the wells at DOX concentration of 1 μ M or 2 μ M.

After 4 or 8 h, the culture medium was removed and the cells were washed with PBS buffer, trypsinized, homogenized with RPMI, and centrifuged for 5 min at 160 x g. Under the *pellet* was added the binding buffer, 2.5 μ L of Annexin V solution, and 2.5 μ L of Propidium iodide (PI) (Annexin V - FITC Apoptosis Detection Kit - Sigma Aldrich). The plate was incubated for 20 min protected from light. A total of 50,000 events was recorded, and the analysis was performed on a flow cytometer (LSR Fortessa BD Biosciences) according to the manufacturer's instructions.

Analysis of cell cycle

300,000 cells/well of 4T1 cells were seeded on a 12-well plate and incubated for 24 h at 37°C and 5% CO₂. After incubation, free DOX, pHSL-TS-DOX, and npHSL-DOX-AS were added to the wells at DOX concentration of 1 μ M. After 8 h, the culture medium was removed and the cells were washed with PBS buffer, trypsinized, homogenized with RPMI, and centrifuged for 5 min at 160 x g. The pellet was homogenized with 1 mL of ethyl alcohol 70% v/v and incubated for 30 min at 4°C. Then, the cells were centrifuged for 5 min at 290 x g and the *pellet* was resuspended with a solution containing RNase, PI, and PBS. The cell dispersion was incubated for 15 min at 37°C and protected from light. A total of 50,000 events was recorded on a flow cytometer (LSR Fortessa BD Biosciences) according to the manufacturer's instructions.

Nuclear morphometric analyses

4T1 cells were plated at a density of 2.5×10^5 cells/well in 6-well plates and incubated at 37 °C for 24 h. After incubation time, cells were treated with free DOX, pHSL-TS-DOX, or npHSL-DOX-AS (DOX concentration of 1 μ M) for 24 h (method adapted from FRANCO *et al.*, 2019). Afterward, the cells were fixed with formaldehyde 3.7% (v/v) for 10 min and stained with Hoescht 33342 (0.2 μ g/mL) solution for 10 min protected from light. The fluorescence images of nuclei were captured using a microscope AxioVert 25 with a fluorescence module Fluo HBO 50 connected to the Axio Cam MRC camera (Zeiss, Oberkochen, Germany). A total of 100 nuclei per treatment was analyzed using the Software Image J 1.50i (National

Institutes of Health, Bethesda, USA) and the plugin “NII_Plugin” available at <http://www.ufrgs.br/labsinal/NMA/>.

Statistical analysis

Normality of variance was evaluated by the Kolmogorov-Smirnov or D’Agostino and Pearson test. Variables without normal distribution were transformed by equation: $y = \log$ or $y = \log(\text{variable} + 200)$. The difference between experimental groups was tested by one-way ANOVA followed by Tukey’s test. For all analyses, it was adopted the confidence range of 95%, and differences were considered significant when the p-value was lower than or equal to 0.05 ($p \leq 0.05$).

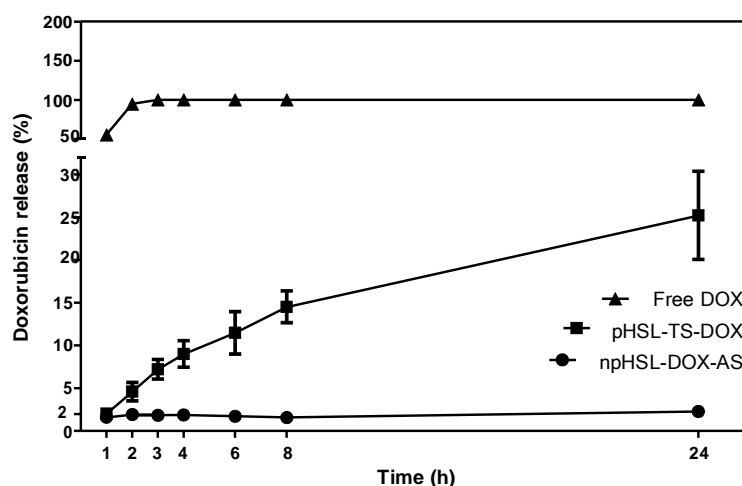
3 Results

Liposomes characterization

The average diameter for pHSL-TS-DOX and npHSL-DOX-AS was 164 ± 22 nm (PDI 0.23 ± 0.06), and 142 ± 15 nm (PDI 0.05 ± 0.03), respectively, indicating monodisperse vesicles population (PDI < 0.3). Zeta potential values were close to neutrality (-2.7 ± 0.8 mV and -2.2 ± 1.0 mV) as expected, due to the presence of DSPE-PEG₂₀₀₀ (HEURTAULT *et al.*, 2003; BORATTO *et al.*, 2020). A high DOX encapsulation content ($97.2 \pm 9.0\%$ and $98.4 \pm 6.0\%$) could be achieved in both cases.

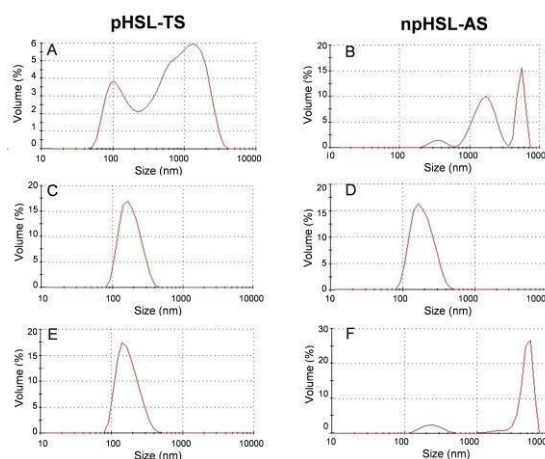
The pHSL-TS-DOX and npHSL-DOX-AS were also assessed for *in vitro* DOX release at pH 7.4 in RPMI. The data are shown in **Figure 1**. pHSL-TS-DOX showed a sustained release profile. The percentage of released DOX increased with time, reaching a maximum release of around 25% after 24 h. By contrast, DOX release from npHSL-DOX-AS was much lower than that observed for pHSL-TS-DOX and the rate of release remained practically constant (around 2%) throughout the study. The free DOX dissolution profile was also analyzed for comparison. After 2 h, 100% of the free DOX has already dissolved.

Figure 1 - Doxorubicin release/dissolution at pH 7.4 in RPMI. Results expressed as the mean \pm SD (n = 3).



Considering the deformability study, as can be seen in **Figure 2**, a heterogeneous distribution of average diameters could be observed for pHSL-TS and npHSL-AS formulations before extrusion. After extruding, a defined peak (around 200nm) was observed in both formulations. In the backwash analysis of npHSL-AS there were two populations of liposomes, including one close to 5000 nm, which represented approximately 74% of the particles. This result suggests that the majority of npHSL-AS was unable to cross the membrane. On the other hand, pHSL-TS showed only one vesicle's population.

Figure 2 – Representative profile of size distribution of pHSL-TS and npHSL-AS evaluated before extrusion (A and B), after extrusion (C and D) and in backwash (E and F) by DLS analysis.



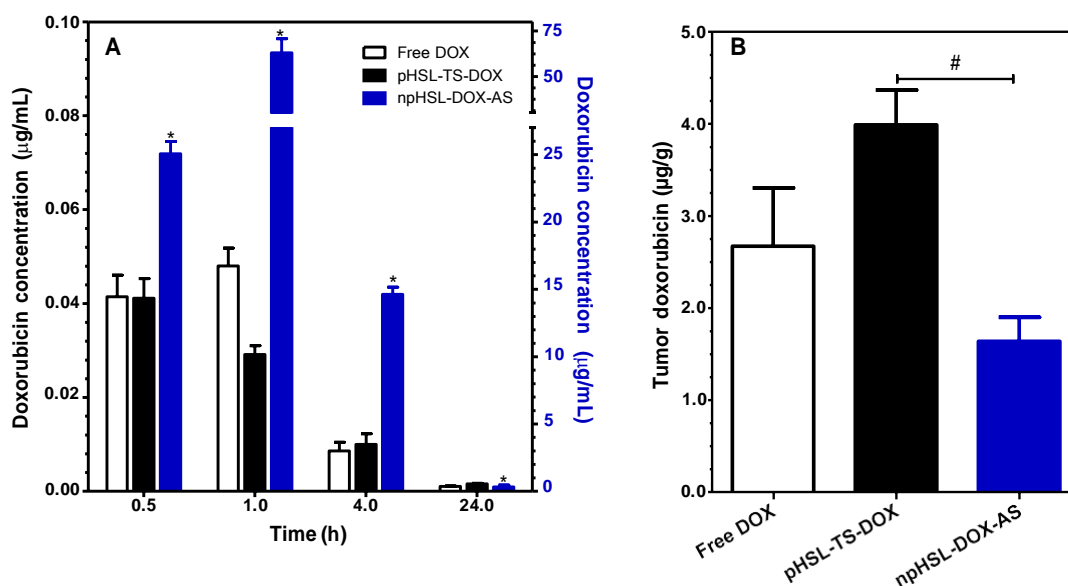
DOX plasmatic concentration studies

Firstly, we performed a DOX plasmatic concentration study in healthy mice treated with free DOX, pHSL-TS-DOX, or npHSL-DOX-AS. Data of DOX concentration in plasma of the animals are shown in **Figure 3A**. Free DOX and pHSL-TS-DOX showed similar profiles of plasma concentration ranging from $4.14 \times 10^{-2} \pm 0.92 \times 10^{-2} \mu\text{g/mL}$ and $4.11 \times 10^{-2} \pm 0.84 \times 10^{-2} \mu\text{g/mL}$ at 30 min to $0.11 \times 10^{-2} \pm 0.02 \times 10^{-2} \mu\text{g/mL}$ and $0.16 \times 10^{-2} \pm 0.03 \times 10^{-2} \mu\text{g/mL}$ at 24 h respectively. In contrast, npHSL-DOX-AS was able to retain higher plasma DOX concentration throughout the experiment: $25.061 \pm 1.823 \mu\text{g/mL}$ at 30 min to $0.334 \pm 0.269 \mu\text{g/mL}$ at 24 h.

Tumor accumulation

Figure 3B represents the tumor accumulation of DOX. Tumors extracted from mice treated with pHSL-TS-DOX showed that DOX concentration was 2.4 and 1.5-fold higher than npHSL-DOX-AS and free DOX, respectively. On the other hand, no significant difference was observed between free DOX and pHSL-TS-DOX or npHSL-DOX-AS.

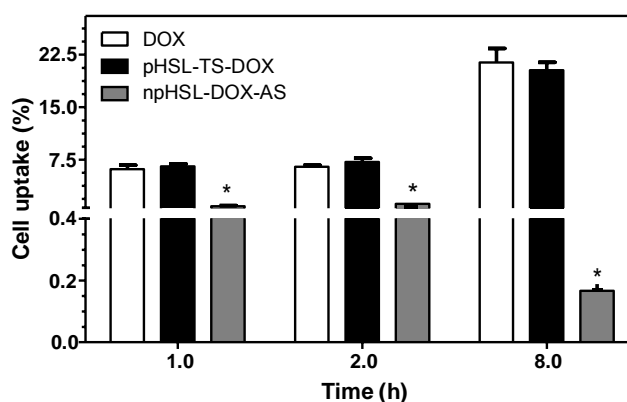
Figure 3 - (A) Evaluation of DOX concentration in plasma after administration of free DOX, pHSL-TS-DOX, and npHSL-DOX-AS. The scale of free DOX and pHSL-TS-DOX should be verified on the left Y-axis; while npHSL-DOX-AS, on the right Y-axis. **(B)** DOX concentration in tumor evaluated 4 hours after administration of free DOX, pHSL-TS-DOX, and npHSL-DOX-AS. Results expressed as the mean \pm SD (n = 4). *Represents a significant difference compared to other groups; # Represents a significant difference compared to npHSL-DOX-AS ($p \leq 0.05$). The analyses were performed by one-way ANOVA followed by Tukey's test.



Cellular uptake

The cellular uptake study evaluated the DOX concentration upon cell internalization after 1, 2, or 8 h of incubation (**Figure 4**). Both DOX and pHSL-TS-DOX showed similar profiles in all times investigated. DOX maximum uptakes of $21.4\% \pm 3.4$, for free DOX, and $20.2\% \pm 1.9$, for pHSL-TS-DOX, were obtained after 8 h of treatment. The treatment with npHSL-DOX-AS induced less DOX internalization in all studied times comparing with the other treatments ($p \leq 0.05$). Its maximum DOX uptake ($1.2\% \pm 0.3$) occurred at 2 h.

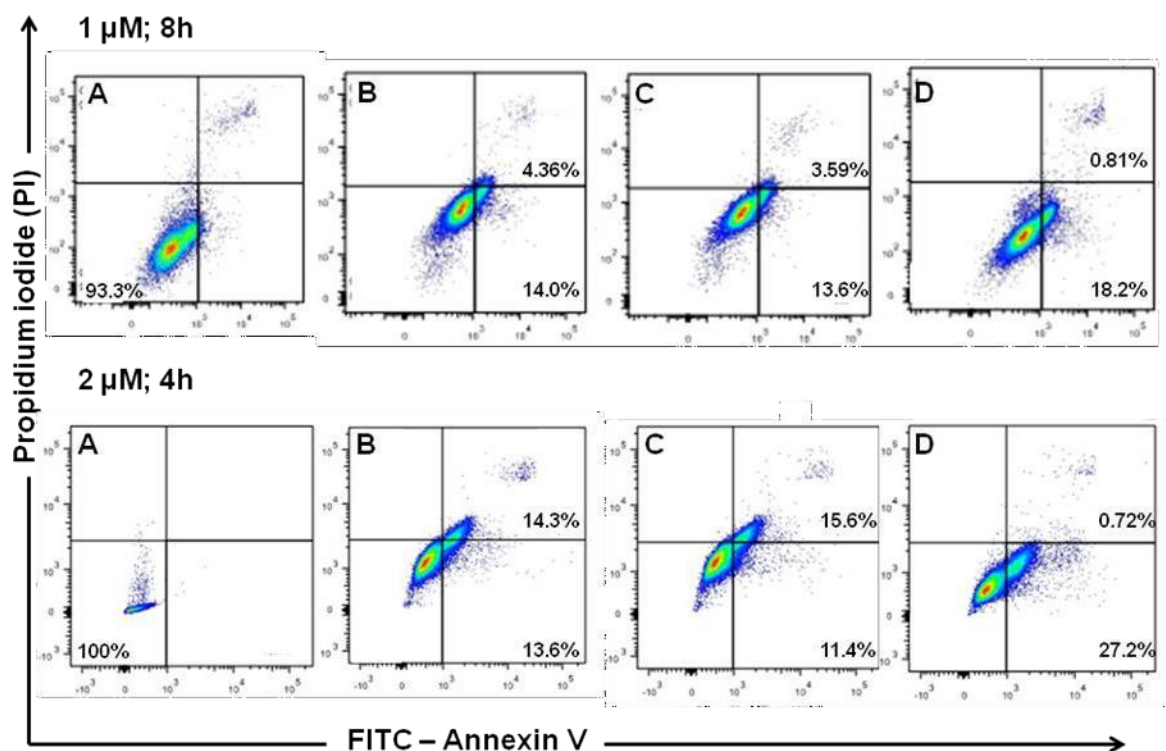
Figure 4 – Percentage of DOX uptake in 4T1 cells treated with free DOX, pHSL-TS-DOX, and npHSL-DOX-AS evaluated after 1, 2, or 8 hours of incubation. *Represents a significant difference against DOX and pHSL-TS-DOX ($p \leq 0.05$). Analyses performed by one-way ANOVA followed by Tukey's test.



Apoptosis analysis

The quantification of apoptosis was assessed by flow cytometry with FITC-Annexin V and PI labeling. Firstly, a DOX concentration of 1 μM and 8 h of incubation were used. In this condition, the level of early apoptosis between the treatments was similar, while the late apoptosis showed to be more intense in free DOX and pHSL-TS-DOX than npHSL-DOX-AS (**Figure 5**). Similar pattern was obtained when 2 μM of DOX and 4 h of incubation were used. However, in this condition, the difference between the late apoptosis of free DOX and pHSL-TS-DOX (14.3% and 15.6%, respectively), and npHSL-DOX-AS (0.72%) was more expressive.

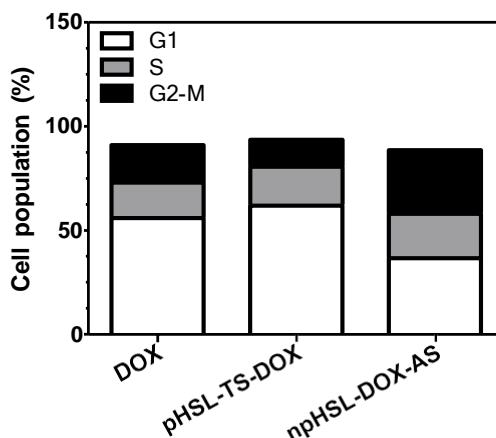
Figure 5 – Analysis of the cell death profile of 4T1 cells treated with 1 μM of DOX and incubated for 8 hours, and treated with 2 μM of DOX and incubated for 4 hours, and stained with PI and FITC-Annexin V. Control group (not treated cells) (A), free DOX (B), pHSL-TS-DOX (C), and npHSL-DOX-AS (D).



Cell cycle analysis

The cell percentage in each stage of cell division in free DOX and pHSL-TS-DOX was close (G1: 55.9 and 61.9%, S: 17.1 and 18.7%, G2-M: 18.0 and 12.9%, respectively). The cells treated with npHSL-DOX-AS showed a different pattern in blocking cell division (G1: 36.7%, S: 21.3% and G2-M: 30.6%), more balanced distribution between the cell division stages were observed (**Figure 6**).

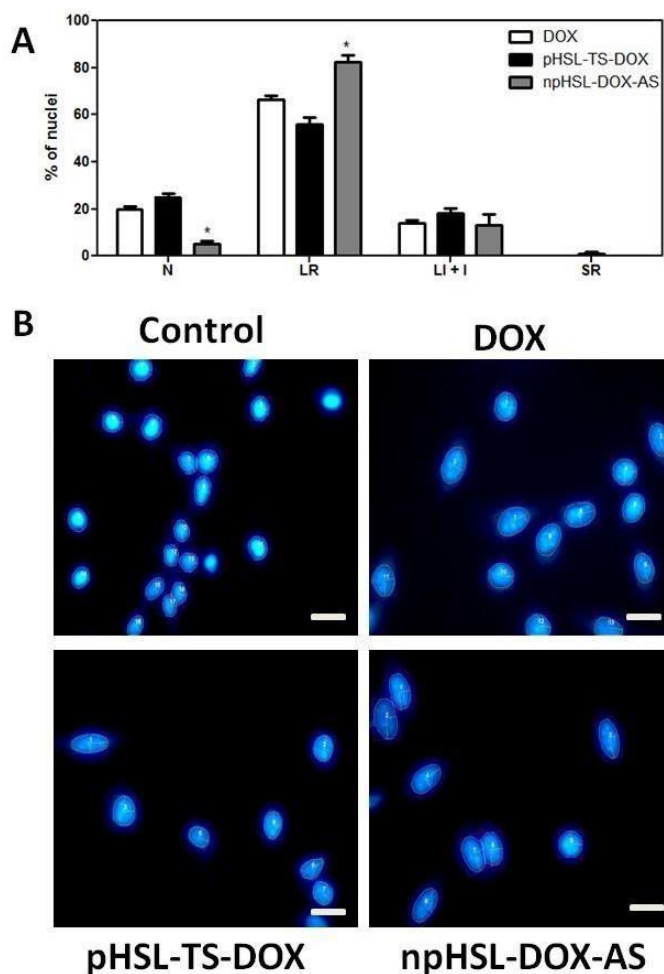
Figure 6 – Percentage of 4T1 cells in each stage of the cell cycle after treatment with DOX, pHSL-TS-DOX, and npHSL-DOX-AS.



Nuclear morphology

Nuclear morphology was assessed after treatment with free DOX, pHSL-TS-DOX, and npHSL-DOX-AS, according to the “Nuclear Morphometric Analysis Tool” developed by Filippi-Chiela and co-workers (2012). It was defined six patterns of nuclear morphology related to biological events: normal (N), irregular (I, mitotic catastrophe or other nuclear damaging events), small regular (SR, apoptosis), small (S, mitosis), small irregular (SI, mitosis with damage or nuclear fragments), large regular (LR, senescence), and large irregular (LI, mitotic catastrophe or other nuclear damaging events). Free DOX and pHSL-TS-DOX showed similar percentages of normal nuclei, $19.7 \pm 2.5\%$ and $25 \pm 3\%$, respectively, while only $5 \pm 3\%$ could be observed for npHSL-DOX-AS ($p < 0.05$). Similar percentages of LR nuclei, indicative of senescence, were obtained for free DOX and pHSL-TS-DOX ($66.7 \pm 2.5\%$ and $56 \pm 5\%$, respectively), while it was detected $82.3 \pm 5.5\%$ of LR nuclei after npHSL-DOX-AS treatment ($p < 0.05$). All treatments resulted in similar percentages of LI + I nuclei, around 15% (**Figure 7A**). **Figure 7B** presents fluorescence photomicrographs of 4T1 stained nuclei. Smaller nuclei with a more uniform sizes and shapes could be observed in the control group. Free DOX and pHSL-TS-DOX groups presented some rounded nuclei as well as some elliptic with similar sizes, and npHSL-DOX-AS presented larger nuclei compared to the other groups.

Figure 7 – (A) Nuclear morphometric distribution of 4T1 nuclei submitted to different treatments for 24 h. Data represent the mean \pm SD (n=3). *Represents significant difference against DOX and pHSL-TS-DOX ($p \leq 0.05$). Analyses performed by one-way ANOVA followed by Tukey's test. (B) Fluorescence photomicrographs of 4T1 stained with Hoescht 33342 after different treatments. Amplification 40x, scale bar = 20 μ m.



4 Discussion

In our previous work, we developed a new pH-sensitive liposomal formulation containing TS and DOX for breast tumor treatment. This formulation was able to promote the tumor growth stabilization in a murine breast tumor model and demonstrated a better safety profile since it did not induce cardiac and hepatic toxicity as well as myelosuppression, the main limitations in DOX use. All these parameters were compared with a formulation already used in the clinic (npHSL-

DOX-AS, which has the same composition of Doxil[®]), and superior performance of pHSL-TS-DOX was observed (BORATTO *et al.*, 2020). Given these results, it became interesting to investigate what factors could contribute to making pHSL-TS-DOX more advantageous. As both formulations had PEG in this composition, which would confer long circulation time and similar physicochemical characteristics (mean diameter, PDI, zeta potential, and adequate percentage of DOX encapsulation), our first hypothesis focused on investigating the pharmacokinetic profile of these formulations administered to healthy mice. DOX concentration obtained in plasma of animals treated with npHSL-DOX-AS was substantially higher than that observed for free DOX and pHSL-TS-DOX at all times studied (**Figure 3A**). After 24 h post-injection, the DOX plasma concentration was 200-times higher from the animals treated with npHSL-DOX-AS than that obtained for pHSL-TS-DOX. The substantially increased amount of plasmatic DOX after npHSL-DOX-AS administration has been described. Gabizon *et al.* (2003) reported a significant increase in AUC (60-fold) and plasma concentration of DOX (hundred-fold) in animals treated with the liposomal drug compared with the free drug, many hours after injection. Besides, the AUC (21.60-fold) and half time life (4.51-fold) of a formulation similar to Doxil[®] were higher than PEGylated liposomal formulation containing an ion pair between DOX and CHEMS (Xu *et al.*, 2017). This considerable difference was related to the insoluble form of the drug inside the liposomal formulation similar to Doxil[®], which could minimize the intraliposomal osmotic pressure and help to maintain the liposome integrity, avoiding DOX release. On the other hand, in pHSL-TS-DOX, we supposed that DOX forms an ionic pair with TS and it is soluble and more available to be released, favoring its elimination from the blood, reaching peripheral organs and the tumor to a greater extent.

Unlike the results obtained in plasma, there was a higher tumor accumulation of DOX in animals treated with pHSL-TS-DOX than npHSL-DOX-AS ($p < 0.05$), as can be seen in **Figure 3B**. This finding could explain a better performance in the antitumor activity previously proven for pHSL-TS-DOX (BORATTO *et al.*, 2020). Since they are pegylated formulations, with similar diameters and PDI, both would have the same capacity to accumulate in the tumor. Taken together these results allowed us to infer that npHSL-DOX-AS remained intact for a long time in the bloodstream, causing higher DOX plasma levels; however, it could not reach the tumor region in an

adequate concentration to exert its antitumor activity, either due to inefficient internalization or release of the drug. Sindhvani *et al.* (2020) demonstrated that 97% of nanoparticles are internalized in the tumor by active trans-endothelial transport and not only by passive transport (EPR effect). For this process, a previous fusion of the membrane of the endothelial cell with the liposomal membrane is necessary to occur transcytosis. As npHSL-DOX-AS has a very rigid lipid bilayer, it would have more difficulties to be previously internalized in the endothelial cell.

In agreement with the release study, pHSL-TS-DOX showed a higher DOX release compared to npHSL-DOX-AS and presented a sustained drug release during the study. After 24 h of analysis around 25% of the drug was released from pHSL-TS-DOX, while only 2% from the npHSL-DOX-AS. Possibly the lipid composition of pHSL-TS-DOX could bring a more fluid membrane, which could contribute to more permeability through the lipid bilayer. In fact, in our previous work, we detected 100% of the drug released from pHSL-TS-DOX in HBS buffer at pH 5.0, proving its pH-sensitivity (BORATTO *et al.*, 2020).

The deformability test data are also in agreement with this hypothesis. For pHSL-TS, the presence of a homogeneous peak around 200 nm after extrusion and in the backwash chart suggests that the pHSL-TS composition caused a higher degree of vesicles flexibility and deformability since the particles were able to break down into smaller particles as passing through the membrane, resulting in a population of small vesicles. While in npHSL-AS there is a homogeneous peak around 200 nm after extrusion; however, in the backwash, higher particles, observed as a peak around 5000nm, could not be broken and were retained on the extrusion membrane. This results suggests that pHSL-TS has a more elastic bilayer and less rigid, which could justify the higher ability to release DOX properly. The more rigidity of the npHSL-DOX-AS liposomal membrane may be related to the structural lipid. The long saturated fatty acyl chains of HSPC interact themselves by van der Waals interactions resulting in a high degree of packaging, which requires more energy to break it and explains its high phase transition temperature (BARENHOLZ, 2012). Lipid compounds containing high phase transition temperature (above 37°C) form less fluid and less leaky lipid bilayers (YINGCHONCHAROEN *et al.*, 2016). Based on these finding, it can infer that both the rigidity in the lipid bilayer and the presence of

DOX as the insoluble salt could be obstacles for the drug release as well as less tumor uptake, consequently resulting in less antitumor activity *in vivo* for npHSL-DOX-AS.

The cellular uptake study suggested again a difficulty of npHSL-DOX-AS being internalized by the tumor cell since the rate of DOX uptake after treatment with this formulation was minimal. Otherwise, pHSL-TS-DOX showed a maximum DOX uptake (around 20%) after 8 h of treatment, demonstrating its greater capacity to deliver the drug at the intracellular level. DOPE, structural lipid in this formulation, has fusogenic propriety, which could facilitate the cell internalization of the liposome (KOLASINAC *et al.*, 2018). This component could allow the fusion between the lipid bilayer and the cell membrane (AKBARZADEH *et al.*, 2013). The low hydration of its polar head group increases the lipophilicity of the liposomal membrane and reduces the energy of interaction between lipid bilayers (EPAND *et al.*, 1996). Another possible mechanism could occur through endocytosis, followed by the destabilization of the liposome at the endosomal level and subsequent release of its content (FERREIRA *et al.*, 2013).

It is also worth noting that although the amount of released DOX from its free form was undoubtedly superior to that provided by the formulation, the cellular uptake of both was the same (**Figures 1 and 4**). The internalization of DOX in the liposomal formulation by endocytosis could evade transport mechanisms, preventing the elimination from the cell (BOZZUTO *et al.*, 2015). In addition, a previous study has described that the TS was able to promote an increase in the influx of DOX and suppress the efflux (ZHANG *et al.*, 2011).

As pHSL-TS-DOX showed advantageous *in vivo* and *in vitro* studies, we performed other *in vitro* studies to evaluate if the encapsulation of DOX could alter its activity. The apoptosis quantification demonstrated that both free DOX and pHSL-TS-DOX presented the same profile, in both conditions tested, differently of npHSL-DOX-AS. Although the cumulative cell population in early and late apoptosis is very close among all the treatments, free DOX and pHSL-TS-DOX treatments showed a higher percentage of the cell population in late apoptosis than npHSL-DOX-AS. This result

could suggest that the process of cell death after free DOX and pHSL-TS-DOX treatments seems to happen faster compared with npHSL-DOX-AS. Also, it is in line with the similar amount of internalized DOX from free form and pHSL-TS-DOX and the lower uptake rate obtained from npHSL-DOX-AS. Laginha *et al.* (2005) reported that a formulation similar to npHSL-DOX-AS, when administered to 4T1 tumor-bearing mice, reached a maximum concentration in the nucleus of tumor cells after 96 h, unlike a liposomal formulation with more fluid lipid bilayer reached in 4 h.

About the cell cycle, free DOX and pHSL-TS-DOX induced the blocking of the cell division in the previous stages, mainly in G1. For npHSL-DOX-AS treatment, there was a more balanced distribution between the cell division phases and the higher percentage of the cell was observed in the G2-M stage (**Figure 6**). The arrest in the G1 phase suggests early detection of damage in genetic material, which gives the cells time to repair the critical damage before DNA replication occurs, avoiding the propagation of genetic lesions to progeny cells and activating the apoptotic pathway. Thus, arresting tumor cells in G1 can slow the proliferation of tumor cells, and the induction of apoptosis may have more impact on the growth and death of tumor cells (MARONI *et al.*, 2012; MONTEIRO *et al.*, 2019). This could also explain the results obtained in the study of nuclear morphology. Treatments with free DOX and pHSL-TS-DOX generated a similar percentage of normal nuclei, around 20%. Both treatments induced blocking the cell cycle at G1, which increases the probability of DNA repair mechanisms to act, restoring the condition and then resulting in normal cell nuclei. On the other hand, the senescence of the most cells may be due to the mechanism of action of DOX-induced DNA damage, which includes DNA alkylation, DNA cross-linking, and production of ROS (BIELAK-ZMIJEWSKA *et al.*, 2013; GEWIRTZ, 1999; ELMORE *et al.*, 2002).

5 Conclusion

The purpose of this work was to clarify the possible reasons that justify the superior performance of pHSL-TS-DOX compared to a similar formulation already used in the clinic. pHSL-TS-DOX presents a more fluid and permeable lipid bilayer, being more capable of releasing DOX. In addition, this formulation is pH-sensitive and contains a

fusogenic structural lipid, which allows the drug internalization at the cellular level to a greater extent, either by fusion with the cell membrane or by endocytosis and destabilization of the vesicle at the endosomal pH. pHSL-TS-DOX seems to present a mechanism of action very similar to that of free DOX and different from the formulation in the clinical use, showing gains in the antitumor activity. Under the *in vivo* perspective, it could accumulate in the tumor region and destabilize itself in the interstitium, which presents a more acidic pH, releasing DOX to be later internalized. All of these factors together are important to understand the reasons why this formulation is highly effective, stable, safe, and therefore promising for the treatment of breast cancer.

5 DISCUSSÃO GERAL

Os lipossomas responsivos a estímulos representam uma grande evolução na área de nanossistemas, uma vez que buscam a interação entre o sistema e o meio, superando obstáculos previamente existentes com o uso de lipossomas convencionais (BOZUTTO & MOLINARI, 2015). Dentre esses, os sistemas pH sensíveis representam uma estratégia importante no tratamento do câncer, já que são capazes de explorar características típicas do tecido tumoral, como a acidez no interstício e, dessa forma, aprimorar o direcionamento do fármaco para a região tumoral (LIU *et al.*, 2014). Lipossomas pH-sensíveis são compostos principalmente por lípides derivados da PE. Para que ocorra a formação de lamelas a partir desses derivados, há a necessidade de utilizar agentes estabilizantes que exerçam uma força de repulsão entre as moléculas do lípide estrutural, permitindo dessa forma a obtenção de vesículas. Um exemplo de lípide estabilizante carboxilado comumente utilizado em lipossomas pH-sensíveis é o CHEMS (FAN *et al.*, 2017). A fim de explorar uma nova alternativa, além de possivelmente agregar outras vantagens ao sistema, pensou-se em utilizar o TS como estabilizante de uma formulação pH sensível.

Jizimoto e colaboradores (1994) descreveram a capacidade de se obter nanossistemas pH-sensíveis utilizando o TS como estabilizante a partir de uma formulação lipossomal composta por 1-palmitoil-2-olcoil-fosfatidiletanolamina (POPE), TS e colesterol. Em 2018, Rehman e colaboradores testaram diversos lípides e combinações utilizando o TS em lipossomas pH-sensíveis, investigando a encapsulação de DOX. A combinação de DOPE:TS não foi capaz de encapsular a DOX por um método passivo, resultando na não formação de vesículas.

No presente trabalho, foi proposto o desenvolvimento de uma formulação lipossomal contendo TS e DOX para aplicação no tratamento do câncer de mama. Primeiramente, a proporção dos componentes da formulação em relação a quantidade de TS foi otimizada. Foi possível aumentar o aporte de TS sem prejudicar as características físico-químicas da formulação. De forma simultânea, foi investigada a possibilidade de remover o sulfato de amônio e promover um novo

método de encapsulação da DOX, originando pHSL-TS-DOX. O método de encapsulação da DOX por gradiente remoto de sulfato de amônio já é bem estabelecido na literatura e garante altas taxas de encapsulação (GABIZON; SHMEEDA; GRENADER, 2012). Entretanto, nesse método há a formação de um sal insolúvel, o sulfato de DOX, que pode ser um entrave para a liberação do fármaco. Foi obtido um elevado teor de encapsulação da DOX, sem utilizar o método por gradiente remoto de sulfato de amônio, resultando em um novo método bastante eficaz. Ao comparar com o teor de uma formulação isenta de TS e sulfato de amônio, foi possível observar a importância da presença do TS para a encapsulação da DOX. Supostamente, ocorre uma interação eletrostática entre o TS e a DOX, permitindo a encapsulação do fármaco, bem como sua retenção, conforme demonstrado no estudo de estabilidade. Essa interação é suficiente para manter a DOX e permitir que esta permaneça solúvel e, portanto, mais disponível para ser liberada. De fato, na imagem obtida por criomicroscopia, são observadas vesículas de interior liso, homogêneo e plano, sem visualização de nenhum material eletrodensito que indique algum cristal, diferentemente da imagem típica de grão de café obtida para lipossomas similares ao Doxil[®], nos quais a DOX está precipitada (BARENHOLZ, 2012; GABIZON; SHMEEDA; GRENADER, 2012). A formação de um par iônico entre TS e DOX foi previamente descrita por Oliveira e colaboradores (2016), como uma estratégia química para modificar a lipofilicidade de fármacos ionizáveis e, dessa forma, aumentar a encapsulação e retenção de fármacos hidrofílicos em carreadores lipídicos nanoestruturados.

pHSL-TS-DOX apresentou diâmetro médio, índice de polidispersão, potencial zeta e teor encapsulação adequados para administração *in vivo* e, ainda, foi observada a manutenção desses parâmetros físico-químicos por até um mês, demonstrando-se a estabilidade dessa formulação nas condições de armazenamento avaliadas. pHSL-TS-DOX apresentou uma liberação controlada em HBS pH 7.4, enquanto em pH 5.0, houve uma maior taxa de liberação de DOX, demonstrando sua pH sensibilidade, a qual também foi confirmada por SAXS. Estudos de liberação realizados em meio de cultura (RPMI pH 7,4) demonstraram o mesmo perfil de liberação de pHSL-TS-DOX obtido anteriormente em HBS pH 7,4. Em marcante contraste, npHSL-DOX-AS (similar ao Doxil[®]) apresentou taxas baixíssimas de liberação da DOX por todo o tempo do estudo, demonstrando sua resistência em liberar a DOX. Além de a DOX

estar precipitada em npHSL-DOX-AS, outro fator que poderia prejudicar a sua liberação poderia ser a rigidez da membrana lipossomal (YINGCHONCHAROEN *et al.*, 2016). Diferentemente de pHSL-TS-DOX, que é composto por uma bicamada mais fluida e, conseqüentemente, mais permeável, permitindo a difusão da DOX para o meio externo. Esses resultados nos permitiram sugerir que o TS apresenta papéis bem definidos na formulação como componente estrutural, agente estabilizante, contra íon da DOX, permitindo sua encapsulação/retenção e, ao mesmo tempo, sua liberação adequada.

Tendo em vista esses resultados físico-químicos promissores para o sistema desenvolvido, as etapas seguintes consistiram na avaliação do comportamento biológico *in vitro* e *in vivo*. Inicialmente, foi realizado um estudo de viabilidade celular para as linhagens de tumor de mama murino e humana, 4T1 e MDA-MB-231, respectivamente. Não foram observadas diferenças significativas entre os tratamentos (DOX, DOX mais TS e pHSL-TS-DOX), sugerindo que a encapsulação da DOX nos lipossomas não prejudicou a sua atividade a nível celular para ambas as linhagens. De fato, ao ser realizado o estudo de captação celular na linhagem 4T1 (a ser discutido mais adiante), foram obtidas taxas de captação de DOX similares nas células tratadas com DOX e pHSL-TS-DOX. Uma vez que DOX e pHSL-TS-DOX apresentaram um comportamento *in vitro* bastante semelhante nesses ensaios, na sequência, foi estudado se a administração *in vivo* destes tratamentos poderia resultar em comportamentos biológicos diferentes, principalmente quanto a eficácia antitumoral e toxicidade.

Como demonstrado no estudo de eficácia antitumoral, pHSL-TS-DOX foi capaz de estabilizar o crescimento tumoral, resultando em uma manutenção do volume tumoral com o tempo, diferentemente de npHSL-DOX-AS e DOX ($p < 0.05$), nos quais foi observado aumento do tamanho tumor em função do tempo. Além disso, essa formulação apresentou um elevado índice de inibição do crescimento (IR) de 71,6%, o maior dentre os tratamentos utilizados. As imagens histológicas do tumor de animais tratados com pHSL-TS-DOX mostraram a presença de uma vasta área composta de tecido granuloso, indicativo de processo resolutivo e apenas uma área pequena isolada de células tumorais viáveis, o que reforçou a elevada eficácia desse tratamento frente aos demais. Aliado a isso, pHSL-TS-DOX mostrou ser uma

formulação segura, uma vez que não induziu uma drástica perda de peso nos animais, não provocou mielossupressão e foi capaz de conferir uma proteção ao tecido cardíaco e hepático, alvos típicos da toxicidade induzida pela DOX.

Esse conjunto de resultados demonstraram portanto uma série de vantagens trazidas pela formulação desenvolvida (pHSL-TS-DOX) ao ser comparada com uma formulação já bem estabelecida (npHSL-DOX-AS). Ambas as formulações apresentam características físico-químicas semelhantes (diâmetro médio, potencial zeta e teor de encapsulação), entretanto, apresentam comportamentos biológicos distintos. Diante deste cenário, tornou-se interessante investigar as possíveis razões que poderiam esclarecer os benefícios gerados por pHSL-TS-DOX.

Inicialmente, a concentração plasmática de DOX após tratamento com DOX livre, pHSL-TS-DOX e npHSL-DOX-AS em animais sadios foi quantificada. Foram observadas concentrações plasmáticas de DOX nos animais tratados com npHSL-DOX-AS bastante superiores em relação aos outros tratamentos. Diversos estudos na literatura já relataram esse fato (GABIZON *et al.*, 2003; LAGINHA *et al.*, 2005; XU *et al.*, 2017). Aparentemente, essa formulação por apresentar uma bicamada bastante rígida, mantém sua estabilidade na circulação sanguínea e é capaz de permanecer por mais tempo, atingindo elevadas concentrações plasmáticas de DOX. Apesar disso, essa formulação apresentou uma eficácia antitumoral inferior àquela induzida por pHSL-TS-DOX. Uma provável explicação para esse comportamento seria a baixa taxa de captação tumoral de DOX induzida por npHSL-DOX-AS, a qual foi 2.4 vezes menor do que aquela observada para pHSL-TS-DOX. Como mencionado anteriormente, por apresentarem características físico-químicas semelhantes e serem formulações peguiladas, esperava-se que ambas tivessem a mesma capacidade de sofrerem o efeito EPR, se extravasarem pelos *gaps* endoteliais e então acumularem no tumor. No entanto, isso não foi observado. Sindhwani e colaboradores (2020) demonstraram que os *gaps* inter-endoteliais são responsáveis por uma parcela muito pequena do transporte de nanopartículas em tumores sólidos, diferentemente do que se acreditava. Ao invés disso, cerca de 97% das nanopartículas são transportadas para o interior do tumor por um processo através das células endoteliais, a transcitose, que ocorre em maior frequência do que os *gaps* endoteliais. Esse processo é metabolicamente ativo e requer um

rearranjo do citoesqueleto e da membrana celular das células endoteliais com a formação de estruturas vesiculares que conseguem captar nanopartículas.

Por outro lado, a concentração plasmática detectada nos animais tratados com pHSL-TS-DOX foi significativamente inferior. Nos estudos de SAXS foi demonstrado que a formulação pHSL-TS-DOX mantém sua organização lamelar de forma estável ao ser submetida em meio de cultura na presença de proteínas. Entretanto, por apresentar uma bicamada mais fluida e permeável, pode ser que parte da DOX seja liberada da vesícula com o tempo e então sofra eliminação renal, acarretando em uma concentração plasmática menor em animais saudáveis. Possivelmente parte da DOX pode estar interagindo com o TS na superfície da vesícula lipossomal ou mesmo no interior da bicamada lipídica e isso pode tornar a DOX mais disponível para ser liberada. Apesar de pHSL-TS-DOX ter apresentado uma menor concentração plasmática de DOX, a sua captação tumoral foi superior, demonstrando que no animal portador de tumor, essa formulação é capaz de se acumular preferencialmente na região tumoral e, conseqüentemente, ser internalizada para então exercer sua atividade a nível intracelular.

Uma maior taxa de captação celular de DOX foi observada para pHSL-TS-DOX comparada a npHSL-DOX-AS. No entanto, essa foi similar a da DOX livre, o que corrobora com o estudo de viabilidade celular. Apresentar a mesma taxa de captação é um indicativo de que a entrega de DOX para o meio intracelular não foi prejudicada quando está na forma encapsulada, o que, conseqüentemente, poderia resultar em mesmo perfil de citotoxicidade. Aliado a isso, pHSL-TS-DOX tem em sua composição um lípido estrutural com propriedades fusogênicas, o DOPE, e essa característica poderia facilitar a fusão da bicamada lipídica com a membrana celular, permitindo a captação da DOX. Diversos fatores podem contribuir para a internalização celular de um nanossistema, como por exemplo, diâmetro, formato, carga superficial, hidrofobicidade, funcionalização e elasticidade/deformabilidade (BEHZADI *et al.*, 2017; FOROOZANDEH & AZIZ, 2018). Alguns estudos já demonstraram que nanossistemas menos rígidos e/ou mais elásticos são internalizados de forma mais eficiente do que nanossistemas rígidos (TANG *et al.*, 2015; YI & GAO, 2017).

O desempenho da formulação desenvolvida a nível celular avaliado por meio dos estudos de apoptose, ciclo celular e morfologia nuclear demonstrou semelhança

entre os grupos pHSL-TS-DOX e DOX, o que está em consonância com os dados obtidos nos estudos de viabilidade. Comparando-se ambos com npHSL-DOX-AS, pode-se perceber que as porcentagens da população de células que estão no estágio de apoptose tardia são maiores, indicando que em um mesmo intervalo de tempo, são capazes de induzir apoptose em estágios mais avançados do que npHSL-DOX-AS. Como visto anteriormente, pHSL-TS-DOX e DOX apresentam maior taxa de captação celular e com isso, conseguem atingir níveis de apoptose tardia. Novamente um perfil do ciclo celular semelhante entre pHSL-TS-DOX e DOX, com bloqueio em fases mais iniciais do ciclo foi observado, indicando que esse processo ocorre de maneira mais rápida. Esse bloqueio em G1 aumenta a probabilidade de que o mecanismo de reparo no DNA seja bem sucedido, e com isso a célula pode retornar ao seu fenótipo normal, cerca de 20% das células observadas após esses tratamentos eram normais pelo estudo de morfologia nuclear. As demais células, em sua maior parte, estavam em estágio de senescência, provavelmente induzida pelo mecanismo de ação da DOX.

Diante dos resultados obtidos, conclui-se que a superioridade na eficácia antitumoral provocada por pHSL-TS-DOX pode ser explicada por diversos fatores:

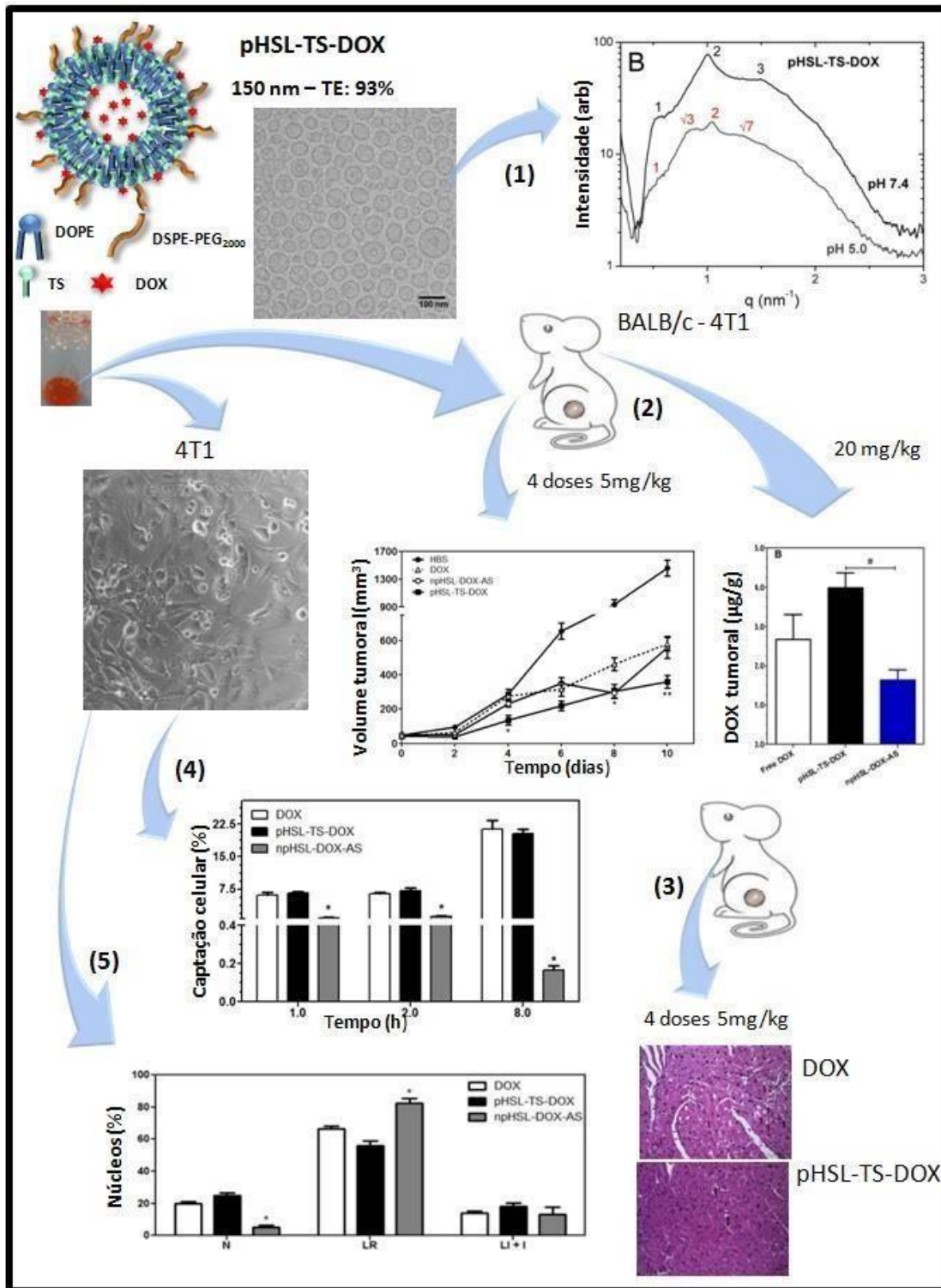
- (i) a presença de uma bicamada lipídica fluida e um lípido fusogênico que favorecem a internalização pela célula tumoral e conseqüentemente o acúmulo no tecido tumoral;
- (ii) a formação de um par iônico entre a DOX e o TS, permite a liberação da DOX de forma adequada;
- (iii) por se tratar de uma formulação pH-sensível, a liberação da DOX no interstício tumoral ou no interior do endossoma após a internalização é favorecida.

O esquema apresentado na **Figura 12** destaca os principais resultados obtidos nesse trabalho:

- 1) Foi possível desenvolver uma nanoformulação pH-sensível contendo vesículas esféricas, conteúdo homogêneo e teor de encapsulação adequados para administração *in vivo*;
- 2) A formulação desenvolvida demonstrou maior acúmulo na região tumoral que resultou em ótimo desempenho no controle do crescimento tumoral, em modelo de tumor de mama murino;

- 3) pHSL-TS-DOX trouxe benefícios quanto a toxicidade, permitindo o início de recuperação de peso dos animais, não apresentando toxicidade cardíaca e hepática tampouco mielossupressão;
- 4) pHSL-TS-DOX apresentou uma taxa de captação celular semelhante a do fármaco livre, indicando sua eficiência no processo de internalização;
- 5) Células tratadas com pHSL-TS-DOX apresentaram morfologia nuclear indicativa de senescência tal como a DOX livre, demonstrando sua atividade citotóxica.

Figura 12 - Resumo dos principais resultados obtidos no trabalho



6 CONCLUSÃO

Nesse trabalho foi desenvolvida uma nova formulação contendo TS e DOX que demonstrou estabilidade, eficácia e segurança frente a um modelo experimental de tumor de mama mostrando-se superior a uma formulação já utilizada na clínica. Dessa forma, trata-se de uma formulação promissora já que conseguiu superar diversos entraves de uma formulação já estabelecida além de ter demonstrado melhor eficácia antitumoral. Estudos futuros de mecanismo de ação poderão esclarecer com maior eficiência como essa formulação atua em sua atividade antitumoral. Ainda, estudos utilizando outras linhagens celulares derivadas de outros tipos de câncer, são interessantes para investigar a potencialidade do uso desse sistema além do tumor de mama.

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