UNIVERSIDADE FEDERAL DE PERNAMBUCO
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LABORATÓRIO DE IMUNOPATOLOGIA KEIZO-ASAMI
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LIPOSSOMAS CONVENCIONAIS E FURTIVOS CONTENDO β-LAPACHONA E COMPLEXOS DE INCLUSÃO β-LAPACHONA:2-HIDROXIPROPIL-β-CICLODEXTRINA: AVALIAÇÃO DA ATIVIDADE ANTIMICROBIANA E ANTIPROLIFERATIVA

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Setembro, 2012
Recife-PE

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Tese submetida ao Programa de Pós-Graduação em Ciências Biológicas do Centro de Ciências Biológicas da Universidade Federal de Pernambuco, como pré-requisito para obtenção do título de doutor.

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RESUMO

O presente estudo objetivou desenvolver e avaliar a atividade antimicrobiana e antiproliferativa de lipossomas convencionais e furtivos contendo β-lapachona (β-lap) ou complexos de inclusão β-lapachona:2-hidroxipropil-β-ciclodextrina (β-lap:HPβ-CD). Os lipossomas foram preparados através da técnica de hidratação do filme lipídico seguida de sonicação. A cinética de liberação *in vitro* foi realizada pelo método da diálise. A atividade antimicrobiana *in vitro* das formulações frente à *Staphylococcus aureus* resistente à meticilina/oxacilina (MRSA ATCC 33591), *S. aureus* sensível à meticilina/oxacilina (MSSA ATCC 29213), cepa hospitalar de *S. aureus* resistente à meticilina/oxacilina (MRSA), cepa comunitária de *S. aureus* resistente à meticilina/oxacilina (MRSA) e *Cryptococcus neoformans* foi determinada pelo método da microdiluição de acordo com o *Clinical and Laboratory Standards Institute* (CLSI). Ademais, a citotoxicidade dos lipossomas desenvolvidos foi avaliada frente a células de carcinoma de próstata (DU-145) utilizando o método do MTT, assim como as possíveis alterações celulares foram avaliadas através da microscopia confocal. Os lipossomas convencionais e furtivos contendo β-lap ou β-lap:HPβ-CD apresentaram tamanho médio de partículas variando de 88,7 ± 1,5 nm a 132,6 ± 3,3 nm, índice de polidispersão variando de 0,255 a 0,340 e eficiência de encapsulação variando de 97,4 ± 0,3 % a 99,2 ± 0,2 %. A cinética de liberação *in vitro* mostrou que as formulações neutras furtivas e as formulações convencionais contendo estearilamina encapsulando β-lap ou β-lap:HPβ-CD apresentaram um perfil de liberação semelhante, com efeitos *burst* em torno de 40% nas primeiras 4 h e velocidades de liberação em torno de 200 μg/h. Na atividade antimicrobiana evidenciou-se que a β-lap e β-lap:HPβ-CD apresentaram a mesma atividade com MIC variando de 1-2 mg/L e MBC variando de 1-16 mg/L frente às bactérias e MIC < 2 mg/L e MFC < 4 mg/L frente ao *Cryptococcus neoformans*. As formulações convencionais neutras mostraram-se inativas ou menos eficazes quando comparadas às demais formulações desenvolvidas. No estudo de citotoxicidade foi possível observar que a β-lap livre apresentou IC_{70} de 2,5 μM e os lipossomas apresentaram IC_{70} igual ou superior ao da molécula livre (2,5 μM, 3,3 μM ou 4,6 μM). A microscopia confocal revelou que a linhagem celular DU-145 exposta a β-lap apresentou alterações morfológicas como, por exemplo, figuras de mitose, condensação de cromatina, fragmentação nuclear, corpos apoptóticos e células gigantes. Neste contexto, a encapsulação de β-lap e complexo de inclusão β-lap:HPβ-CD em lipossomas pode futuramente permitir a utilização deste composto na terapia antibacteriana e antitumoral.

Palavras-chave: β-lapachona. Complexos de inclusão. Lipossomas. Atividade antimicrobiana. Atividade antiproliferativa.

ABSTRACT

The aim of this study was to evaluate the antimicrobial and antiproliferative activities of conventional and stealth liposomes encapsulating β-lapachone (β-lap) or 2-hydroxypropyl-β-cyclodextrin inclusion complex (β-lap:HPβ-CD). The liposomes were prepared using the hydration of the thin lipid film method followed by sonication. The *in vitro* release kinetics was performed using dialysis method. The *in vitro* antimicrobial activity against methicillin/oxacillin-resistant *Staphylococcus aureus* (MRSA ATCC 33591), methicillin/oxacillin-susceptible *Staphylococcus aureus* (MSSA ATCC 29213), methicillin/oxacillin-resistant *Staphylococcus aureus* hospital, community-acquired (MRSA) and *Cryptococcus neoformans* was evaluated by microdilution method according to the *Clinical and Laboratory Standards Institute* (CLSI). Furthermore, the cytotoxicity of liposomes against prostate carcinoma cells (DU-145) was evaluated using the MTT method and possible cellular changes were assessed by confocal microscopy. The conventional and stealth liposomes encapsulating β-lap or β-lap:HPβ-CD presented mean particle size ranged from 88.7 ± 1.5 nm to 132.6 ± 3.3 nm, polidispersity index ranged from 0.255 to 0.340 and drug encapsulation efficiency ranged from 97.4 ± 0.3 % to 99.2 ± 0.2%. Similar drug release profiles from neutral stealth liposomes and positively charged conventional liposomes encapsulating β-lap or β-lap:HPβ-CD were found, with burst effects around 40% in the first 4 h and rate constants around 200 μg/h. The antimicrobial activity test proved that β-lap and β-lap:HPβ-CD presented the same activity with MICs ranging from 1 to 2 mg/L and MBCs ranging from 1 to 16 mg/L against bacteria, and MIC < 2 mg/L and MFC < 4 mg/L against *Cryptococcus neoformans*. The conventional neutral liposome formulations were inative or less effective when compared with other liposomal formulations.The β-lap presents IC_{70} of 2.5 μM and β-lap-loaded liposomes had IC_{70} equal to or higher than the free molecule (2.5 μM, 3.3 μM or 4.6 μM). The Confocal microscopy revealed that the DU-145 cells exposed to β-lap presented morphological alterations such as mitotic figures, condensation of chromatin, nucleus fragmentation, apoptotic bodies and giant cells. Thus, the encapsulation of β-lap and β-lap:HPβ-CD inclusion complex into liposomes can further allow the use of this compound in the antibacterial and antitumor therapies.

Keywords: β-lapachone. Inclusion complex. Liposomes. Antimicrobial activity. Antiproliferative activity.

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LISTA DE ABREVIATURAS E SIGLAS

ATP	Adenosina tri-fosfato
ATCC	American Type Culture Collection
CD	Ciclodextrina
CGTase	Ciclodextrina-glicosiltransferase
CHOL	Colesterol
CLSI	Clinical and Laboratory Standards Institute
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CSF	Líquido cefalorraquidiano
DMSO	Dimetilsulfóxido
DOX	Doxorrubicina
D.P.	Desvio padrão
DSC	Calorimetria exploratória diferencial
DSPE-PEG2000	Diestearoil-fosfatidiletanolamina-polietilenoglicol 2000
EE	Eficiência de encapsulação
FDA	Food and Drug Administration
FBS	Soro bovino fetal
HPβ-CD	2- hidroxipropil-β-ciclodextrina
IC_{50}	Concentração para inibir 50% do crescimento celular
IC_{70}	Concentração para inibir 70% do crescimento celular
INCA	Instituto Nacional do Câncer
IV/IR	Espectroscopia de absorção na região do infravermelho
LAFEPE	Laboratório Farmacêutico do Estado de Pernambuco
LUV	Vesículas unilamelares grandes
MBC	Concentração Bactericida Mínima
MFC	Concentração Fungicida Mínima
MHB	Caldo Müeller-Hinton
MIC	Concentração Inibitória Mínima
MRSA	*Staphylococcus aureus* meticilina/oxacilina resistente
MSSA	*Staphylobcoccus aureus* meticilina/oxacilina sensível
MLV	Vesículas multilamelares grandes
NADH	Nicotinamida adenina dinucleotídeo

NAD(P)H	Nicotinamida adenina dinucleotídeo fosfato
NCL	Lipossoma convencional neutro
NQO1	NAD(P)H: quinona oxidorreductase 1
NSL	Lipossoma furtivo neutro
OMS	Organização Mundial de Saúde
OXA	Oxacilina
PC	Fosfatidilcolina de soja
PDI	Índice de polidipersão
PEG	Polietilenoglicol
PM	Peso molecular
RMN H^1/^{1}H-NMR	Ressonância magnética nuclear de prótons
ROS	Espécies reativas de oxigênio
RPMI	Roswell Park Memorial Institute
SA	Estearilamina
SACL	Lipossomas convencionais com carga positiva
SASL	Lipossomas furtivos com carga positiva
SDA	Ágar Sabouraud
SEM	Microscopia eletrônica de barredura
SNC	Sistema nervoso central
SUV	Vesículas unilamelares pequenas
TEM	Microscopia eletrônica de transmissão
TG	Análise termogravimétrica
TPT	Topotecano
UV	Ultravioleta
VAN	Vancomicina
α-CD	α-ciclodextrina
β-CD	β-ciclodextrina
β-lap	β-lapachona
β-lap:HPβ-CD	Complexo de inclusão de β-lapachona em 2-hidroxipropil-β-ciclodextrina
γ-CD	γ-ciclodextrina
Ø	Tamanho de partículas

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SUMÁRIO

1. INTRODUÇÃO

O Brasil é um país que apresenta uma importante fonte de riqueza natural e inclui-se entre os países de maior biodiversidade mundial, com mais de 1/5 de todas as espécies de vegetais e animais do planeta. Apesar deste potencial biológico, apenas uma pequena parcela desta biodiversidade tem sido explorada cientificamente quanto à produção de fármacos, extratos vegetais, dentre outros (TAKAKI et al., 2007).

Nos últimos anos muitas patologias de caráter infeccioso ou carcinogênico têm emergido e preocupado a sociedade. Desta forma, essas patologias têm estimulado os pesquisadores a buscar novos agentes antimicrobianos e anticancerígenos como uma estratégia importante para o estabelecimento de terapias alternativas (PETRAK, 2005; PEREIRA et al., 2006; PFALLER et al., 2006; ANDREOPOULOU et al., 2007).

A β-lapachona é uma *orto*-naftoquinona que pode ser obtida por semi-síntese a partir do lapachol isolado do caule de árvores das famílias *Bignoniaceae* e *Verbenaceae*, conhecidas como "ipês" (*Tabebuia* spp.). Estudos têm demonstrado que essa molécula apresenta diversas atividades biológicas, como uma potente ação antiproliferativa em várias linhagens de células humanas cancerígenas (PINK et al., 2000; PLANCHON et al., 2001), atividade antimicrobiana (PEREIRA et al., 2006; MEDEIROS et al., 2010; LOURENÇO et al., 2011), dentre outras (KUNG et al., 2008; BLANCO et al., 2010).

Apesar do seu potencial terapêutico, a β-lapachona apresenta limitações, como a baixa solubilidade em água (0,038 mg/mL ou 0,16 mM) (NASONGKLA et al., 2003) e toxicidade (OUGH et al., 2005). Desta forma, preparações farmacêuticas que visem aumentar sua solubilidade em água e melhorar sua biodisponibilidade são uma alternativa para garantir o uso terapêutico da β-lapachona. Neste contexto, as ciclodextrinas (CDs) podem ser utilizadas para a formação de complexos de inclusão com a β-lapachona com o objetivo de aumentar sua hidrossolubilidade.

Ciclodextrinas são oligossacarídeos cíclicos formados por moléculas de glicose unidas através de ligações α-1,4-glicosídicas (LOFTSSON; DUCHÊNE, 2007; GRILLO et al., 2008) que podem ser utilizadas para a formação de complexos de inclusão com uma variedade de moléculas (DAVIS; BREWSTER, 2004). A formação de complexos de inclusão proporciona uma melhora na estabilidade, maior hidrossolubilidade e maior biodisponibilidade do composto utilizado como princípio ativo (DUCHÊNE; PONCHEL; WOUESSIDJEWE, 1999; BOUDAD et

al., 2001; PASQUALI; BETTINI, 2008). Porém, as ciclodextrinas não são consideradas sistemas de liberação controlada de fármacos (FERNANDES et al., 2007). Desta forma, os nanocarreadores tais como lipossomas e nanopartículas constituem novas formas farmacêuticas para encapsulação da β-lapachona ou de seus complexos de inclusão com ciclodextrinas.

Liposomas são vesículas constituídas por uma ou mais bicamadas lipídicas, que isolam um ou vários compartimentos aquosos internos do meio externo (FRÉZARD et al., 2005; FANG et al., 2006). Na área da nanotecnologia farmacêutica, eles vêm sendo utilizados como sistemas de liberação controlada de fármacos e oferecem alternativas para superar inconvenientes farmacotécnicos inerentes à molécula em questão, como por exemplo, baixa solubilidade e toxicidade (BRANDL; GREGORIADIS, 1994; TORCHILIN, 2005; EDWARDS; BAEUMNER, 2006). Esses sistemas são concebidos para se obter uma concentração plasmática ou níveis de concentração tecidual de fármacos dentro da faixa terapêutica com liberação controlada (velocidade controlada), atingindo o efeito terapêutico desejado e evitando possíveis reações tóxicas dos compostos (TORCHILIN, 2005).

Tendo em vista as características físico-químicas da β-lapachona, os lipossomas apresentam aplicabilidades promissoras através da encapsulação dessa molécula. Assim, o presente trabalho apresenta caráter multidisciplinar envolvendo principalmente a Nanobiotecnologia Farmacêutica e a área da Biologia para o desenvolvimento de nanossistemas terapêuticos mais eficazes, com menor toxicidade e maior especificidade. Neste contexto, este estudo viabiliza a ampliação dos conhecimentos sobre a atividade de lipossomas convencionais e furtivos contendo β-lapachona e complexos de inclusão β-lap:CD como agentes antimicrobianos e antiproliferativos, utilizando ferramentas da nanotecnologia farmacêutica para aumentar a solubilidade, biodisponibilidade e consequentemente viabilizar a administração da β-lapachona.

2. REVISÃO DA LITERATURA

2.1 - Infecções bacterianas

Os microrganismos são capazes de provocar inúmeras patologias, que vão desde simples infecções localizadas e sem maiores consequências, até pneumonias ou infecções cardíacas podendo levar o paciente a óbito. A maioria dos medicamentos utilizados na sinfecções bacterianas são os antibióticos de origem natural e seus derivados semi-sintéticos. Esses medicamentos podem ser classificados em β-lactâmicos (penicilinas, cefalosporinas, carbapeninas, monobactâmicos, oxapeninas e sulfoxapeninas), tetraciclinas, aminoglicosídeos, macrolídeos, peptídicos cíclicos (glicopeptídeos, lipodepsipeptídeos), cloranfenicol, rifamicinas, dentre outros. Os antimicrobianos de origem sintética são classificados em sulfonamidas, fluoroquinolonas e oxazolidinonas. Os principais mecanismos de ação antimicrobiana destes agentes terapêuticos estão listados na Tabela 1 (GUIMARÃES; MOMESSO; PUPO, 2010).

Tabela 1 – Principais mecanismos de ação dos agentes antimicrobianos.

Antibióticos	**Alvo**	**Mecanismo de ação**
β-lactâmicos (penicilinas, cefalosporinas, carbapêmicos, monobactâmicos)	Enzima transpeptidase	Inibição da formação de ligação cruzada entre cadeias de peptideoglicano, impedindo a formação correta da parede celular bacteriana.
β-lactâmicos (oxapeninas, sulfoxapeninas)	Enzima β-lactamase	Inibição da enzima de resistência bacteriana, que degrada antibióticos β-lactâmicos.
Macrolídeos, lincosamidas, estreptograminas (dalfopristina e quinupristina), cloranfenicol, oxazolidinonas (linezolida)	Subunidade 50S ribossômica	Inibição da síntese protéica bacteriana.
Aminoglicosídeos, tetraciclinas	Subunidade 30S ribossômica	Inibição da síntese protéica bacteriana.
Glicopeptídeos (vancomicina, teicoplanina)	Dipeptídeo terminal D-Ala-D-Ala do peptideoglicano	Complexação com as cadeias peptídicas não ligadas e bloqueio da transpeptidação, impedindo a formação correta da parede celular bacteriana.
Peptídeos não ribossomais (bacitracina, gramicidina C, polimixina B)	Membrana plasmática	Afetam permeabilidade da membrana bacteriana por facilitarem o movimento descontrolado de íons através da membrana.
Lipodepsipeptídeos (daptomicina)	Membrana plasmática	Afeta permeabilidade da membrana bacteriana e bloqueia síntese de ácido lipoteicóico, componente da membrana externa de bactérias Gram positivas.
Rifampicina	RNA polimerase dependente de DNA	Inibição da síntese de RNA.
Fluoroquinolonas	Enzima DNA girase	Bloqueio da replicação e reparo do DNA.
Sulfonamidas	Enzima di-hidropteroato sintetase	Bloqueio da formação de cofatores do ácido fólico, importantes para síntese de ácidos nucléicos.

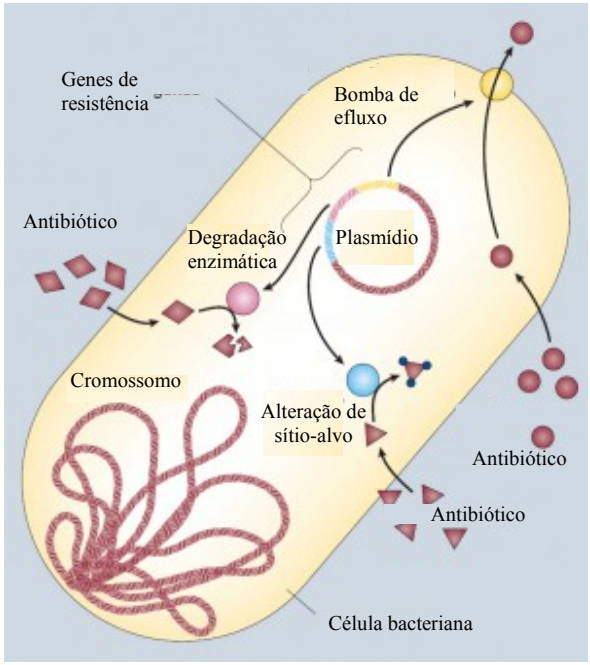
Fonte: Adaptado de GUIMARÃES; MOMESSO; PUPO, 2010.

Durante a última década as infecções microbianas, principalmente as ocasionadas por microrganismos multirresistentes, têm sido descritas como causa frequente de morbidade e mortalidade entre os pacientes criticamente enfermos, como os imunodeprimidos ou em estado pós-operatório (KLUGMAN, 2002; BEDINI et al., 2007; SOOD et al., 2008; LOURENÇO et al., 2011). Dados da literatura identificam níveis crescentes de resistência bacteriana e de bactérias multirresistentes. Infecções que há décadas atrás eram tratadas com protocolos terapêuticos de rotina, hoje apresentam difícil tratamento, assim como altas taxas de mortalidade (THEURETZBACHER, 2011).

Para os antimicrobianos atuarem de forma eficaz eles devem chegar ao seu alvo bacteriano e se acumular em concentrações adequadas para que possam atuar durante o período de tempo necessário. Ao longo dos anos, as bactérias desenvolveram mecanismos capazes de provocar resistência aos diversos agentes antimicrobianos, dentre eles: produção de bomba de efluxo do fármaco, destruição ou modificação do fármaco e prevenção da interação fármaco – alvo (Figura 1) (ALEKSHUN; LEVY, 2007; DAVIN-RÉGLI et al., 2008; NIKAIDO, 2009).

A bomba de efluxo compõe um mecanismo de resistência que ocorre quando o fármaco é bombeado para o meio externo mais rápido do que é capaz de se difundir na célula bacteriana, por isso a concentração intrabacteriana é ineficaz. Como pode ser observado, este mecanismo impede que o antimicrobiano se acumule no compartimento desejado, porém deixa este antibiótico inalterado (WALSH, 2000). Desta forma, uma segunda estratégia de resistência é a destruição ou modificação do fármaco. Este mecanismo envolve estratégias químicas de inativação, que incluem hidrólise, transferência de grupo através de acetiltransferases, fosforilação, glicosilação, assim como mecanismos de óxido-redução (WRIGHT, 2005). A terceira estratégia de resistência consiste na prevenção da interação fármaco-alvo através da alteração do sítio de ligação dos antibióticos, que podem resultar de mutações espontâneas ou induzidas por genes bacterianos. Além disso, a aquisição da resistência pode ocorrer por transferência de genes de resistência de outros organismos por alguma forma de recombinação genética (conjugação, transdução ou transformação) (LAMBERT, 2005).

Figura 1 - Mecanismos de resistência bacteriana.



Fonte: Adaptado de http://scienceofacne.com/pt/how-do-bacteria-become-resistant-to-antibiotics/

Atualmente uma grande diversidade de bactérias, sejam elas Gram-positivas ou Gram-negativas, apresentam vários mecanismos de resistência aos agentes antimicrobianos (Tabela 2) (ALEKSHUN; LEVY, 2007).

A pesquisa e desenvolvimento de medicamentos antibacterianos não fornecem opções de novos antibióticos que possam conflitar diretamente com a contínua difusão de patógenos multirresistentes. Desta forma, várias campanhas e iniciativas internacionais estão estimulando o desenvolvimento de novas tecnologias de produção de agentes antibacterianos através da integração entre indústria farmacêutica, universidades e pequenas empresas (THEURETZBACHER, 2012).

Tabela 2 - Características gerais das bactérias multirresistentes.

Microrganismos	**Infecções**	**Resistência aos antibióticos**	**Fármacos utilizados no tratamento das bactérias multiressistentes**
Pseudomonas aeruginosa	Pulmão e feridas	β-lactâmicos, fluoroquinolonas e aminoglicosídeos	Colistina
Acinetobacter spp.	Pulmão, feridas, ossos e sangue	β-lactâmicos, fluoroquinolonas e aminoglicosídeos	Colistina e tigeciclina
Escherichia coli e *Klebsiella pneumoniae* produtoras de β-lactamases de espectro estendido	Tratourinário, biliar, gastrointestinal, pulmão e sangue	β-lactâmicos, fluoroquinolonas e aminoglicosídeos	Colistina (*Klebsiella pneumoniae)* e tigeciclina
Enterococcus vancomicina-resistentes	Sangue, coração e região intra-abdominal	Vancomicina	Linezolida, daptomicina
Staphylococcus aureus meticilina/oxacilina-resistentes	Pele, tecidos moles, tratorespiratório e sangue	β-lactâmicos, fluoroquinolonas e macrolídeos	Linezolida, daptomicina, tigecilina e vancomicina
Streptococcus pneumoniae multirresistente	Orelha, pulmão, sangue e flúidos cérebro-espinhais	β-lactâmicos, macrolídeos e tetraciclinas	Fluoroquinolonas e tigeciclina

Fonte: Adaptado de ALEKSHUN; LEVY, 2007.

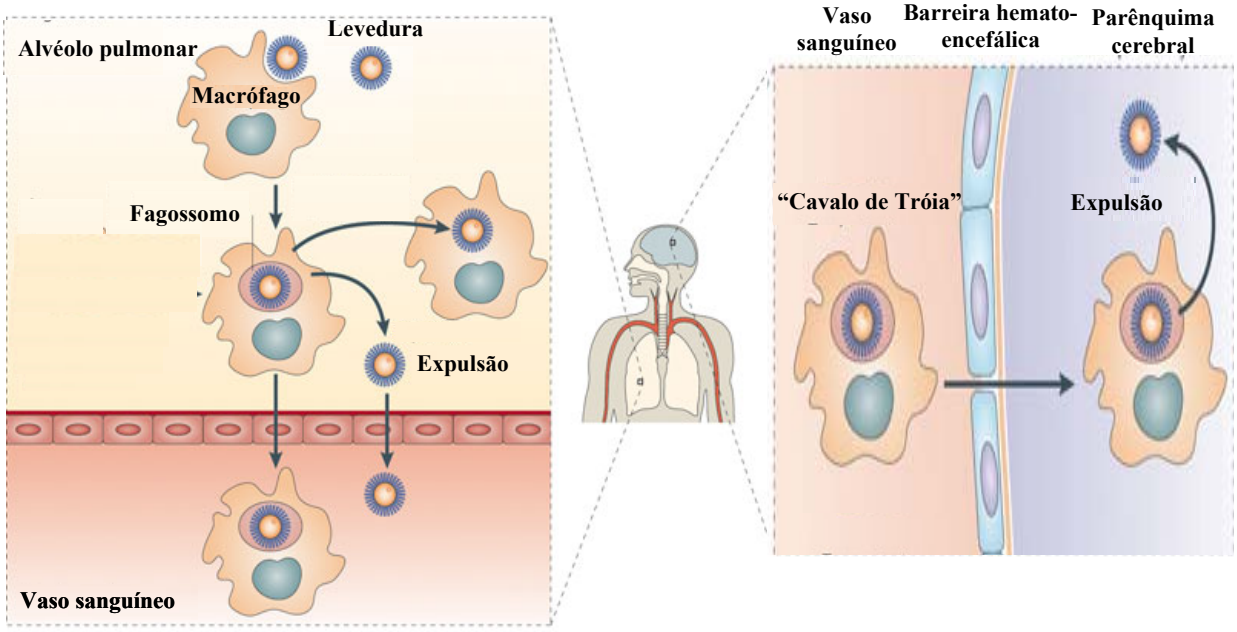
2.2 - Infecções fúngicas

Nas últimas décadas, a frequência de infecções fúngicas oportunistas tem aumentado significativamente (TRICK et al., 2002; PFALLE; DIEKEMA, 2004; WISPLINGHOFF et al., 2004; ALBUQUERQUE; RODRIGUES, 2012). Este aumento está associado à morbidade e mortalidade (GUDLAUGSSON et al., 2003; PAPPAS et al., 2003; WISPLINGHOFF et al., 2004; MORGAN et al., 2005), principalmente em pacientes que fazem parte do grupo de risco para o desenvolvimento de infecções fúngicas graves, isto é, pacientes que apresentam alguma imunossupressão, como transplantados de medula óssea e sangue, portadores do vírus HIV ou

doença neoplásica, pacientes com idade avançada ou ainda pacientes que recebem terapia imunossupressora, assim como os bebês prematuros (BADDLEY et al., 2001; BLUMBERG et al., 2001; KAUFFMAN, 2001; TRICK et al., 2002; KAUFFMAN, 2004; PFALLE; DIEKEMA, 2004; ROILIDES et al., 2004; WISPLINGHOFF et al., 2004).

As principais micoses oportunistas são: candidíase, criptococose e aspergilose (SABOL; GUMBO, 2008; FARIA et al., 2011). A criptococose é uma micose causada por espécies do gênero *Cryptococcus* principalmente pelo *Cryptococcus neoformans*, levedura capsulada com uma ampla distribuição mundial, que causa infecção disseminada, principalmente em pacientes imunodeprimidos (RODERO et al., 2000; OSUNA et al., 2008; SINGH et al., 2008). A principal forma de aquisição da criptococose ocorre através da inalação de estruturas fúngicas desta levedura. Após serem inaladas, as células fúngicas, no pulmão, são facilmente fagocitadas por macrófagos alveolares e outras células fagocíticas tais como neutrófilos. Na ausência de depuração imunológica, o fungo prolifera, tanto a nível intracelular quanto extracelular (FELDMESSER et al., 2000). As células fúngicas podem escapar dos fagossomos por um mecanismo de expulsão, que mantém a viabilidade das células (ALVAREZ; CASADEVALL, 2006; MA et al., 2006). O processo de expulsão também pode resultar em movimento do fungo de célula a célula quando resulta na passagem da levedura entre macrófagos adjacentes (ALVAREZ; CASADEVALL, 2007; MA et al., 2007). As leveduras livres ou aquelas presentes em células fagocíticas entram na corrente sanguínea podendo se disseminar para outros órgãos e atingir o sistema nervoso central (CHARLIER et al., 2009; SHI et al., 2010). Quando as células fúngicas estão nos microcapilares do cérebro, elas atravessam o endotélio por meio de um mecanismo de "cavalo de Tróia", isto é, dentro de células fagocíticas (CHARLIER et al., 2009), ocorrendo a expulsão da levedura para o parênquima cerebral (Figura 2). Neste contexto, *Cryptococcus neoformans* é uma das principais causas de meningite em pacientes portadores de HIV (KEELE et al., 2001; PARK et al., 2009).

Figura 2 - Interação de células fúngicas com células fagocíticas e difusão dos fungos através da barreira hemato-encefálica.



Fonte: Adaptado de KRONSTAD et al., 2011.

Durante os últimos 40 anos, a anfotericina B tem sido o fármaco padrão utilizado para o tratamento de infecções fúngicas sistêmicas, porém este medicamento, mesmo exibindo um largo espectro de atividade, apresenta utilização limitada devido ao perfil de toxicidade (KEELE et al., 2001), assim como já foram relatados isolados de *Cryptococcus neoformans* resistentes ao tratamento com anfotericina B (PERFECT et al., 2010; CÓRDOBA; AFELTRA; VITALE, 2011).

2.3 - Câncer

O termo câncer é uma denominação genérica que se dá aos processos neoplásicos malignos, com morbidade e mortalidade elevadas e crescente prevalência. O câncer agrupa um conjunto de mais de 100 doenças que têm em comum o crescimento desordenado de células, que invadem tecidos e órgãos. Essas células se dividem rapidamente e tendem a ser muito agressivas e incontroláveis, determinando a formação de tumores malignos, que podem espalhar-se para outras regiões do corpo (INCA, 2012). Esta patologia é crônica e normalmente apresenta grande

impacto para a população em geral, pois é responsável por muitos casos de mortalidade em todo o mundo, atingindo países desenvolvidos e em desenvolvimento (BRUNHEROTTI, 2007).

No território brasileiro, o câncer já figura como a segunda causa de morte na população, precedido apenas pelas doenças cardiovasculares. As estimativas para o ano de 2012, válidas também para o ano de 2013, apontam que ocorrerão 518.510 casos novos desta doença (INCA, 2012). Nas últimas décadas o câncer de próstata tem emergido como uma das doenças mais comuns entre os homens sendo considerada a segunda causa de morte por câncer em homens. Apesar das melhorias no tratamento do câncer, os regimes quimioterápicos existentes que usam agentes anticancerígenos clássicos apresentam limitações que incluem índice terapêutico baixo, com consequente dose terapêutica limitada, além da inexistência de especificidade para células cancerígenas. Desta forma, os tratamentos convencionais, radioterapia e quimioterapia, não garantem que as células normais não sejam atingidas pelos fármacos não seletivos (HARLEY et al., 2008; NISHIYAMA; EGUCHI, 2009).

Existem diversos medicamentos antitumorais aprovados pelo FDA (Tabela 3), porém a resistência ao tratamento quimioterápico e os efeitos colaterais são os maiores obstáculos de uma quimioterapia bem-sucedida. Essa resistência resulta em uma resposta terapêutica incompleta, recorrente e que muitas vezes acarreta metástase (WANG et al., 2011). Numerosos antineoplásicos exibem alta citotoxicidade não-seletiva e baixo índice terapêutico, o que têm impulsionado pesquisas não apenas para o desenvolvimento de novos fármacos, mas também novas formas inovadoras para otimizar a utilização dos fármacos já existentes (JULIANO; DAOUD, 1990; PETRO; DESIMONE, 2010).

2.4 - β-lapachona

2.4.1 - Características gerais da β-lapachona

A β-lapachona [3,4-diidro-2,2-dimetil-2H-naftol (1,2-b)pirano-5,6-diona ($C_{15}H_{14}O_3$, PM 242,3)] é uma *orto*-naftoquinona (Figura 3) que pode ser obtida naturalmente da casca de plantas da espécie *Tabebuia avellanedae*, conhecida popularmente como Ipê-roxo ou Pau d'arco, árvore nativa da América do Sul com alta frequência no Brasil (ALVES et al., 2008). Porém, em larga escala, a β-lapachona (β-lap) é obtida por semi-síntese a partir do lapachol, substância amarela também obtida das mesmas plantas, utilizando ácido sulfúrico (LI; AVERBOUK; PARDEE, 1993).

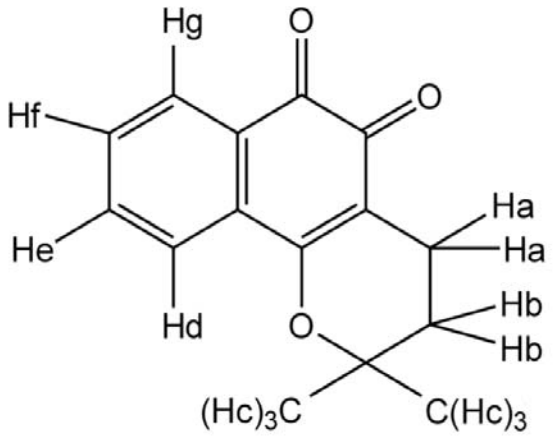
Tabela 3 - Fármacos antitumorais utilizados na terapia.

Fármacos que provocam dano irreversível ao DNA	**Fármacos que provocam dano reversível ao DNA**
Doxorrubicina (Antraciclina)	Trastuzumab (Anticorpo monoclonal)
Daunorrubicina (Antraciclina)	Sunitinibin (Inibidor de tirosina quinase)
Epirrubicina (Antraciclina)	Lapatinibin (Inibidor de tirosina quinase)
Idarrubicina (Antraciclina)	
Ciclofosfamida (Agente alquilante)	

Fonte: Adaptado de http://www.medscape.org/viewarticle/727012_6

Na metade do século passado, as naftoquinonas do Ipê-roxo foram estudadas pioneiramente pelo Instituto de Antibióticos da Universidade Federal de Pernambuco, com destaque para o lapachol, antigamente comercializado como medicamento pelo Laboratório Farmacêutico do Estado de Pernambuco (LAFEPE), porém este medicamento foi retirado do mercado devido a sua toxicidade. Vale salientar que este composto e seus análogos e derivados continuam sendo estudados nas pesquisas científicas (OLIVEIRA et al., 2001; SILVA; FERREIRA; SOUZA, 2003; YAMASHITA et al., 2009).

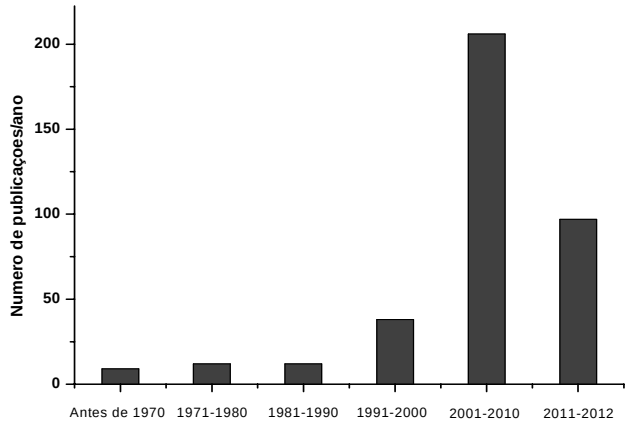
Figura 3 - Estrutura química da β-lapachona.



Fonte: CAVALCANTI et al., 2011.

Ao longo dos anos, houve um aumento significativo do interesse científico pela avaliação das atividades e propriedades da β-lap como pode ser observado na Figura 4. A β-lap tem sido, desde a década de 70 (DOCAMPO; CRUZ; BOVERIS,1979), alvo de estudos no mundo todo devido ao excelente potencial farmacológico *in vitro* e *in vivo* (LI et al., 1999; LI et al., 2002; BLANCO et al., 2010).

Figura 4 - Crescimento do número de publicações referentes à β-lapachona e seus análogos ou derivados.



Fonte: Dados adquiridos do Scopus^{TM}; 2012.

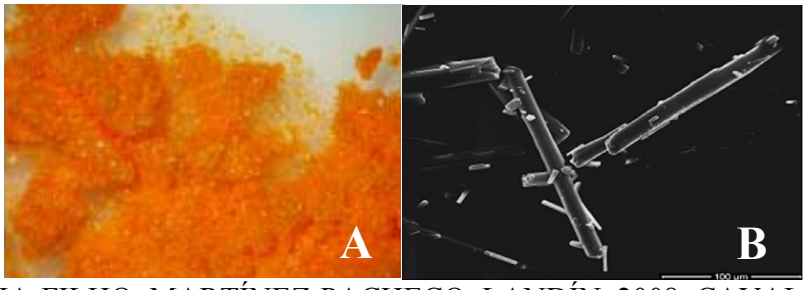
A β-lapachona caracteriza-se por ser uma substância de caráter hidrofóbico, sendo pouco solúvel em água (0,038 mg/mL ou 0,16 mM) (NASONGKLA et al., 2003), porém muito solúvel na maioria dos solventes orgânicos. A β-lapachona possui uma forma bem definida de cristais aciculares, laranja-avermelhado, visíveis a olho nu, assim como na microcopia óptica e eletrônica (Figura 5). O ponto de fusão da β-lap varia entre 154 e 156 ºC e seu peso molecular é 242,3 (ALVES et al., 2008).

2.4.2 - Atividades farmacológicas da β-lapachona

A β-lapachona tem sido uma das naftoquinonas mais extensivamente estudadas, pois apresenta diferentes atividades farmacológicas com potencial terapêutico tais como: antibacteriana (OLIVEIRA et al., 2001; PEREIRA et al., 2006), antifúngica (GUIRAUD et al., 1994; MEDEIROS et al., 2010), tripanossomicida (LOPES et al., 1978; GOULART et al., 1997; MOURA et al., 2001; SILVA; FERREIRA; SOUZA, 2003), antiviral (SCHUERCH; WEHRLI,

1978; SCAFFBER-SABBA et al., 1984), antiinflamatória (MOON et al., 2007), cicatrizante (KUNG et al., 2008) e antineoplásica (WOO; CHOI, 2005).

Figura 5 – Aspectos macroscópico (A) e microscópico (B) dos cristais da β-lapachona.



Fonte: CUNHA-FILHO; MARTÍNEZ-PACHECO; LANDÍN, 2008; CAVALCANTI et al., 2011.

2.4.2.1 - Atividade antimicrobiana da β-lapachona

Na literatura, a β-lapachona e seus derivados já apresentaram atividade antimicrobiana frente a várias cepas bacterianas. Cruz, DoCampo e Boveris (1978) realizaram um dos primeiros experimentos relacionados a esta atividade e encontraram concentração inibitória mínima (MIC) de 2,4 mg/L e 4,3 mg/L para os isolados de *Bacillus subtilis* e *Bacillus stearothermophilus*, respectivamente, indicando que o *Bacillus subtilis* foi duas vezes mais sensível que o *Bacillus stearothermophilus.*

Em 2001, Oliveira e colaboradores avaliaram a atividade antimicrobiana de alguns derivados do lapachol frente a isolados clínicos de *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterobacter* spp., *Escherichia coli*, *Klebsiella* spp., *Proteus mirabilis* e *Pseudomonas aeruginosa.* Neste estudo foi possível observar que os derivados apresentaram atividade frente ao *S. aureus, S. epidermidis*, assim como para os bacilos Gram-negativos não fermentadores. Os resultados de MIC para o *S. aureus* ATCC 29213 (MSSA) indicaram que os derivados do lapachol (~128 mg/L) apresentam maior atividade que a própria molécula (~256 mg/L), mas foram menos ativas que a oxacilina (0,25 mg/L) e vancomicina (2 µg/mL), antibióticos atualmente utilizados na clínica.

Riffel e colaboradores (2002) observaram que isolados clínicos de *Staphylococcus aureus* oxacilina/meticilina resistentes (MRSA) apresentaram sensibilidade a algumas naftoquinonas, mesmo apresentando resistência aos antimicrobianos comerciais, exceto para a vancomicina. A

maioria dos compostos apresentou valores de MIC entre 30-125 mg/L, porém alguns derivados apresentaram valores de MIC superiores a 500 mg/L. Nos testes para determinação da concentração bactericida mínima (MBC), os valores foram superiores a 500 mg/L para todos os compostos testados, desta forma as naftoquinonas testadas apresentaram apenas atividade bacteriostática.

Em 2006, Pereira e colaboradores determinaram os valores de MIC da β-lapachona e de alguns derivados frente à MRSA. A citotoxicidade em culturas celulares de rim de macaco (BSC-40) e a irritabilidade dérmica primária *in vivo* em coelhos saudáveis também foram realizadas. Neste estudo a β-lapachona apresentou MIC de 8 mg/L. Os compostos se apresentaram atóxicos, quando aplicados na forma de preparações tópicas em coelhos saudáveis, porém eles apresentaram efeito tóxico em células eucarióticas, sugerindo assim que sejam realizadas modificações estruturais ou encapsulação do composto em sistemas de liberação controlada como estratégia importante para produzir novas moléculas ou sistemas menos tóxicos.

Nos experimentos de Lourenço e colaboradores (2011) a β-lapachona apresentou MIC de 8 mg/L frente a isolados hospitalares de *Enterococcus faecalis* resistentes, resultado promissor uma vez que o cloranfenicol, um antibiótico disponível na clínica, apresentou MIC de 12 mg/L.

Na literatura, também foi evidenciada a atividade antifúngica do lapachol, da β-lapachona e de seus derivados e análogos. Souza e colaboradores (2008) avaliaram a atividade do lapachol, da β-lapachona e de alguns derivados frente à *Fusarium oxysporum*, um fungo de solo de grande importância agronômica. De todas as substâncias testadas, β-lapachona, seguido por lapachol, são os mais ativos na redução do crescimento deste fungo. O lapachol foi o composto que apresentou as melhores características para uso agronômico, uma vez que não alterou o percentual de germinação de sementes comerciais de alface, mas inibe o crescimento fúngico, evitando o tombamento de mudas (causado pelo fungo *Fusarium oxysporum*). Vale salientar que este fungo tem importância agronômica, porém ele tem emergido como patógeno oportunista causando infecções disseminadas em pacientes imunocomprometidos (BOUTATI; ANAISSIE, 1997; O'DONNELL et al., 2004).

Em 2010, Medeiros e colaboradores investigaram a atividade *in vivo* da β-lapachona contra infecção disseminada por *Cryptococcus neoformans*. Neste trabalho, camundongos Swiss foram imunossuprimidos e posteriormente infeccionados por via intravenosa com *C. neoformans*. Sete dias após a infecção, os camundongos foram tratados com β-lapachona (10 mg/kg, iv) por 7

e 14 dias. A anfotericina B (0,5 mg/kg) foi utilizada como fármaco de comparação e um grupo adicional recebeu apenas tampão fosfato (PBS). Após tratamento, foi possível observar que a β-lapachona diminuiu a carga fúngica em cerca de 10^4 vezes no pulmão e no cérebro após 14 dias de infecção, quando comparado com o grupo que recebeu apenas PBS. A atividade da β-lapachona foi semelhante a da anfotericina B, porém ela ainda apresentou toxicidade local. Desta forma, este estudo destaca a possível utilização de β-lapachona para o tratamento de criptococose disseminada.

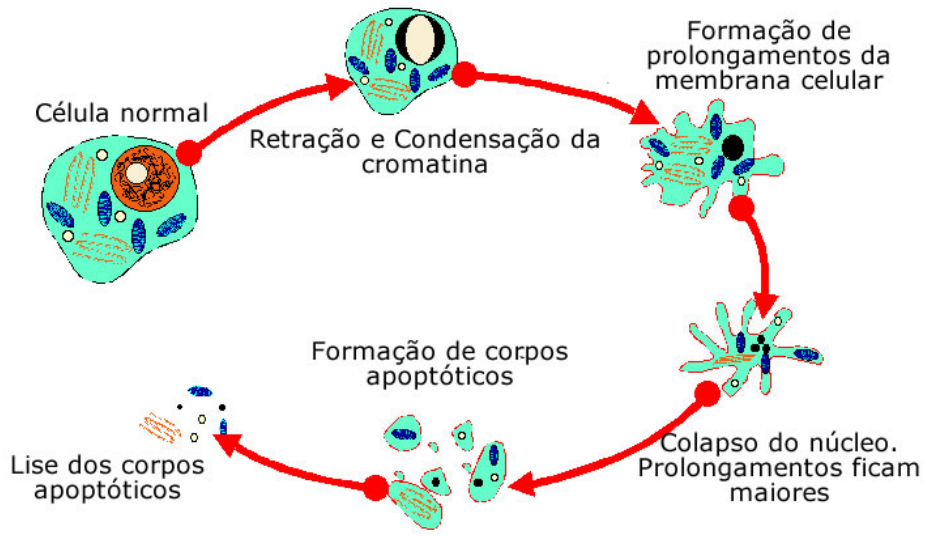
O mecanismo de ação antimicrobiana da β-lap ainda não foi largamente estudado na literatura, porém estudos indicam que as naftoquinonas podem causar estresse oxidativo em células bacterianas devido a produção de ânion superóxidos e peróxido de hidrogênio (RAVELO, ESTEVEZ-BRAUN, PÉREZ-SACAU, 2003; PEREIRA et al., 2006).

2.4.2.2 - Atividades antiproliferativa e antitumoral da β-lapachona

2.4.2.2.1 - Atividade antiproliferativa da β-lapachona

A β-lapachona atua frente a diferentes linhagens de células tumorais *in vitro*, especialmente em células de próstata (LI et al., 1999; BLANCO et al., 2007; DONG et al., 2009), mama (SIEGEL; ROSS, 2000), ovário (LI et al., 1999; WOO; CHOI, 2005), fígado (LAI et al., 1998), pâncreas (OUGH et al., 2005), pulmão (BLANCO et al., 2007) e células de mieloma múltiplo (LI et al., 2000). No entanto, o mecanismo de ação exato da morte celular provocada pela β-lapachona ainda é desconhecido (RÍOS-LUCI et al., 2012). Na verdade, a apoptose (Figura 6) (LEE et al., 2011), necrose (SUN et al., 2006) ou autofagia (PARK; CHOI; KWON, 2011) foram observados em células tratadas com esta molécula. Apesar da enzima nicotinamida adenina dinucleotídeo (fosfato):quinona oxidorredutase 1 (NQO1) (LI et al., 2011) ser o alvo biológico mais provável para a β-lapachona, topoisomerase I (PARDEE et al., 2002) e topoisomerase II (KRISHNAN et al., 2001) também foram identificadas como possíveis alvos biológicos.

Figura 6 - Mecanismo de morte celular por apoptose.



Fonte: http://www.infoescola.com/wp-content/uploads/2010/06/apoptose.jpg

A β-lapachona apresenta efeito citotóxico significativamente reforçado pela presença de NQO1, uma proteína altamente expressa em uma variedade de cânceres humanos, incluindo os de pulmão (BELINSKY; JAISWAL, 1993), próstata (LOGSDON et al., 2003), pâncreas (LEWIS et al., 2005) e mama (SIEGEL; ROSS, 2000). NQO1 catalisa a redução de dois elétrons das quinonas usando nicotinamida adenina dinucleotídeo fosfato - NAD(P)H - ou nicotinamida adenina dinucleotídeo (NADH) como doador resultando na forma hidroquinona (PLANCHON et al., 2001). A β-lap reduzida em dois elétrons é instável e oxida de volta à forma original de β-lap, que é novamente reduzida pela NQO1. Esse ciclo entre as formas de quinona e hidroquinona da β-lap provoca grave redução de NAD(P)H e NADH, resultando na depleção de adenosina tri-fosfato (ATP), liberação de citocromo C da mitocôndria e um aumento na Ca^{2+} citosólico (SUZUKI et al., 2006). Estes distúrbios metabólicos intracelulares resultam em morte celular por apoptose (PINK et al., 2000). A formação de espécies reativas de oxigênio (ROS), durante a redução e oxidação de β-lap, também foi relatada como causadora de morte celular (PINK et al., 2000; PARDEE et al., 2002). Siegel e Ross (2000) observaram que as células tumorais possuem teores mais elevados de NQO1 do que as células normais. Pink e colaboradores (2000) mostraram evidências que indicam a participação da enzima NQO1 no processo de ativação da β-lapachona na apoptose, aumentando assim a sua citotoxicidade. Eles constataram que ao

adicionar nas culturas celulares um inibidor de NQO1 (dicoumarol) a apoptose era bloqueada. Ough e colaboradores (2005) observaram que a β-lapachona diminui a viabilidade em células cancerígenas de pâncreas, pois estas células apresentam alta expressão de NQO1. Planchon e colaboradores (2001) observaram que a expressão dessa enzima em células prostáticas cancerígenas é o ponto chave determinante para a apoptose e letalidade após exposição a β-lap. Em seus experimentos estas células foram mais sensíveis a β-lap que as células normais. Desta forma, esse mecanismo de ação poderia ser uma estratégia para o tratamento seletivo de tumores com alta expressão de NQO1.

A β-lapachona também atua como inibidora das topoisomerases I e II. A incubação direta desta substância com a topoisomerase I, antes da adição de DNA como substrato, aumenta drasticamente o efeito inibitório, sugerindo a interação direta da β-lap com a topoisomerase I. Este modo de atuação difere em relação ao de outras substâncias inibidoras das topoisomerases, como, por exemplo, a Camptotecina que é DNA dependente (LI; AVERBOUK; PARDEE, 1993; SILVA; FERREIRA; SOUZA, 2003). A β-lap pode também agir diretamente na topoisomerase II (CHOI; KANG; YOO, 2003; SILVA; FERREIRA; SOUZA, 2003) inibindo sua atividade, que resulta em citotoxicidade, devido ao fato desta enzima ser crítica para o funcionamento normal de qualquer célula, pois ela mantém a integridade do DNA, reparando-o quando danificado (WHITACRE et al., 1997).

Vários estudos foram realizados para determinar a concentração mínima da β-lap necessária para inibir 50% do crescimento celular (IC_{50}). A tabela 4 mostra a IC_{50} desta molécula frente a diversas linhagens celulares de câncer.

2.4.2.2.2 – Atividade antitumoral da β-lapachona

Em 1999, Li e colaboradores estudaram a atividade antitumoral da β-lap (25–50 mg/kg) e do taxol (1 mg/kg) isolados, assim como em terapia combinada (25–50 mg/kg de β-lap após 24h + 1 mg/kg de taxol) em camundongos fêmeas que apresentavam tumor de ovário humano (36M2). Neste estudo foi possível observar uma pronunciada diminuição do tumor (75%) após o tratamento com a β-lap sozinha (50 mg/kg). Os camundongos tratados apenas com taxol apresentaram um efeito menor com redução de 60% do tumor. Além disso, em ambos os casos, houve uma redução considerável na dimensão dos nódulos tumorais e na quantidade de ascite. Com a combinação de β-lapachona e taxol, não houve ascite, assim como havia poucos nódulos

tumorais. Além disso, os animais se apresentaram saudáveis (sem perda de peso ou anormalidades grosseiras em órgãos internos).

Tabela 4 - Avaliação da citotoxicidade da β-lapachona frente a diferentes linhagens de células cancerígenas humanas.

Linhagem celular	**IC_{50}**	**Referências**
Câncer de próstata (DU-145)	0,72 µg/mL (3 µM)	DONG et al., 2009.
Câncer de próstata (PC-3)	0,36 µg/mL (1,5 µM)	DONG et al., 2009.
Câncer de próstata (LNCaP NQO1+)	0,65 µg/mL (2,7 µM)	DONG et al., 2009.
Câncer de próstata (LNCaP NQO1-)	2,8 µg/mL (11,8 µM)	DONG et al., 2009.
Câncer de colo-retal (HCT-8)	0,20 µg/mL (0,83 µM)	SILVA-JÚNIOR et al., 2007.
Câncer do SNC (SF-295)	0,22 µg/mL (0,91 µM)	SILVA-JÚNIOR et al., 2007.
Câncer leucêmico (HL-60)	0,40 µg/mL (1,65 µM)	SILVA-JÚNIOR et al., 2007.
Câncer de mama (MDAMB-435)	0,06 µg/mL (0,25 µM)	SILVA-JÚNIOR et al., 2007.
Câncer de mama (MCF-7)	0,41 µg/mL (1,7 µM)	NASONGKLA et al., 2003.
Câncer de mama (T47D:AD18)	0,48 µg/mL (2 µM)	PLANCHON et al., 1995.

Ough e colaboradores (2005) administraram a β-lap (50 mg/kg) e os complexos de inclusão β-lap:2-hidroxipropil-β-ciclodextrina (50 mg/kg ou 75 mg/kg) em camundongos com tumor pancreático (MIA PaCa-2). Esse estudo demonstrou que a β-lap administrada em uma única injeção intratumoral inibiu o crescimento do tumor. No entanto, quando β-lap é complexada com 2-hidroxipropil-β-ciclodextrina e administrada diretamente no tumor, o crescimento do tumor é dramaticamente reduzido. Por sua vez, quando a β-lap é administrada sistemicamente através de injeções intraperitoniais, ela apresentou um ligeiro, mas não

significativo efeito na inibição do crescimento do tumor. Porém, a toxicidade foi aumentada quando as doses foram administradas duas vezes por dia resultando em óbito de 2/3 dos animais durante ou logo após o tratamento.

Em estudo mais recente, Blanco e colaboradores (2010) analisaram as respostas de morbidade e mortalidade de camundongos fêmeas com tumor de pulmão humano (A549) tratadas com complexos de inclusão β-lap:HPβ-CD e micelas contendo β-lap. Injeções intravenosas na dose de 30mg/kg de β-lap:HPβ-CD não resultaram em morte dos animais, porém efeitos colaterais moderados foram observados, com animais que apresentaram dificuldade de respirar e marcha irregular. Estes sintomas foram mais intensos em doses de 40 e 50 mg/kg do complexo de inclusão, produzindo contrações musculares graves, dificuldades respiratórias e letalidade, em alguns casos. A dose de 60 mg/kg de β-lap:HPβ-CD resultou em 100% de mortalidade. Em contraste, doses que variaram de 30 a 50 mg/kg de β-lap nas micelas não resultou em morte e apresentou efeitos colaterais significativamente menores. Os camundongos que receberam 40 e 50 mg/kg de β-lap nas micelas apresentaram leve ou moderada dificuldade para respirar e marcha irregular. A dose de 60 mg/kg de β-lap nas micelas provocou reações severas nos camundongos, porém só houve morte de aproximadamente 40% dos animais. Como resultado destes estudos, conclui-se que as doses ótimas de β-lap nas micelas estão na faixa de 30 a 50 mg/kg e há uma vantagem evidente na administração da β-lap nas micelas quando comparada com a sua administração na forma de complexo de inclusão.

Estes resultados apontam a β-lap como um novo e potente agente antimicrobiano e antitumoral, porém por apresentar baixa solubilidade em água e toxicidade, a sua aplicação terapêutica é limitada. Desta forma a utilização de compostos que aumentem sua solubilidade é uma alternativa para ultrapassar esta limitação. Neste contexto, as ciclodextrinas (CDs) podem ser utilizadas para a formação de complexos de inclusão com a β-lap.

2.5 - Ciclodextrinas

2.5.1 - Características gerais das ciclodextrinas

As CDs foram descobertas há mais de 100 anos, quando em 1891 foram produzidas pela primeira vez por Villier através da digestão do amido pelo *Bacillus amylobacter* (VAN DER VEEN et al., 2000). Esta digestão é promovida pela enzima Ciclodextrina-glicosiltransferase (CGTase) que, atualmente, pode ser obtida por diferentes microrganismos (CHAROENLAP et

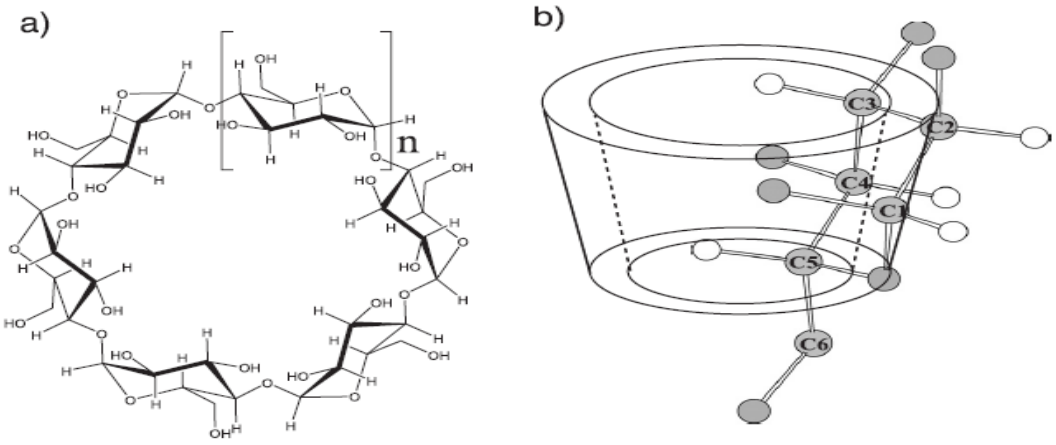
al., 2004; CUCOLO et al., 2006).

Apesar do trabalho pioneiro de Villiers, as CDs foram primeiramente detalhadas por Schardinger em 1903, que descreveu o processo de preparação e isolamento das mesmas. Entre 1903 e 1911 o referido autor identificou a α e β-CD (VEIGA; PECORELLI; RIBEIRO, 2006a).

De 1930 a 1970 foram realizados estudos sistemáticos sobre CDs. Um grupo formado por Freudenberg e outros estudiosos concluiu, na segunda metade da década de 30, que as CDs apresentam estrutura cíclica e identificaram a γ-ciclodextrina (FREUDENBERG et al., 1936). Em 1938, Freudenberg e colaboradores descobriram que as CDs apresentam uma cavidade central e seus pesos moleculares foram determinados. Cramer em 1954 descreveu a estrutura básica e as características físico-químicas da α, β e γ-ciclodextrina, como, por exemplo, estrutura molecular, tamanho de cavidade, solubilidade e habilidade de formar complexos de inclusão. Ao final da década de 60, o domínio do método de preparação de CDs em escala laboratorial levou a estudos aprofundados visando particularmente suas possíveis aplicações. No entanto, a obtenção das CDs ainda apresentava custo elevado e sua utilização em humanos era discutível, uma vez que aparentemente apresentavam toxicidade elevada (SZEJTLI, 1998).

Ciclodextrinas (CDs) são oligossacarídeos cíclicos formados por moléculas de glicose unidas através de ligações glicosídicas α-1,4 (Figura 7) (CHALLA et al., 2005; GRILLO et al., 2008). As CDs apresentam-se na forma de "cones truncados" com o lado mais largo formado pelas hidroxilas secundárias em C-2 e C-3 e a face mais estreita constituída pelas hidroxilas primárias ligadas em C-6. Os átomos de oxigênio envolvidos nas ligações glicosídicas (em C-1 e C-4) e os átomos de hidrogênio ligados em C-3 e C-5 determinam o caráter hidrofóbico da cavidade das CDs. A presença das hidroxilas livres na parte externa das CDs confere a essas moléculas um caráter hidrofílico (BRITTO; NASCIMENTO-JR; SANTOS, 2004).

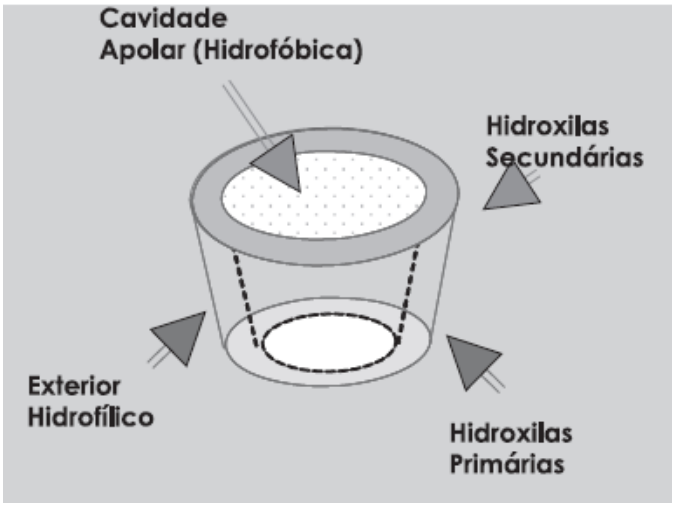
Figura 7 – Ciclodextrina. (a) Estrutura geral das ciclodextrinas (CD's). (b) Representação esquemática da estrutura tridimensional das CD's, mostrando as características estruturais definidas pelo arranjo das unidades de glicose.



Fonte: Adaptado de BRITTO; NASCIMENTO-JR; SANTOS, 2004.

Elas apresentam uma cavidade interna hidrofóbica e uma superfície externa hidrofílica que são capazes de interagir com uma ampla variedade de compostos (Figura 8).

Figura 8 - Forma "cone truncado" das CDs e localização dos grupos de hidroxilas primárias e secundárias.



Fonte: RAMA et al., 2005.

2.5.2 - Classificação das ciclodextrinas

As CDs podem ser classificadas quanto à dimensão da cavidade interna que é determinada pelo número de unidades de glicose. Segundo esta classificação existem três tipos de CDs: α, β e γ (FRACETO et al., 2007; GRAMMENOS et al., 2009). Assim, as α, β e γ CDs são classificadas como naturais e possuem seis, sete e oito unidades de glicose e diâmetro de aproximadamente 5,3, 6,5 e 8,4 Å, respectivamente (Tabela 5) (Figura 9) (DEL VALLE, 2004).

Tabela 5 – Propriedades físico-químicas das ciclodextrinas naturais.

Propriedades	**Ciclodextrinas**		
	α	**β**	**γ**
N° de unidades de glicopiranose	6	7	8
Peso molecular (g/mol)	972	1135	1297
Solubilidade em água (g/100 mL a 25 °C)	14,5	1,85	23,2
Diâmetro da cavidade (Å)	4,7 – 5,3	6,0 – 6,5	7,5 – 8,3
Volume da cavidade (Å)	174	262	427
Fórmula empírica	$C_{36}H_{60}O_{30}$	$C_{42}H_{70}O_{35}$	$C_{48}H_{80}O_{40}$

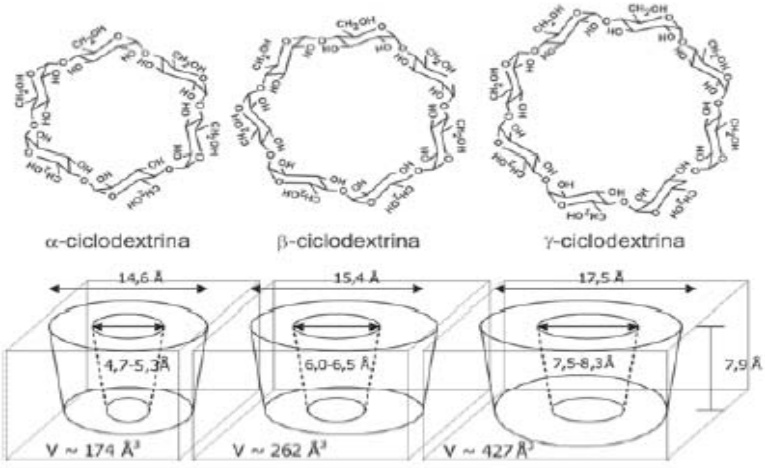
Fonte: Adaptado de SALTÃO; VEIGA, 2001.

Por muitos anos as ciclodextrinas têm sido extensivamente utilizadas como potentes agentes acentuadores da solubilidade, modificando a liberação e melhorando a biodisponibilidade de fármacos fracamente hidrossolúveis (DÍAZ-RODRÍGUEZ; LANDIN, 2012). Embora as CDs naturais sejam muito utilizadas no desenvolvimento de formulações farmacêuticas, elas apresentam algumas propriedades menos adequadas enquanto veículos de fármacos. A β-ciclodextrina, por exemplo, apresenta uma solubilidade aquosa bastante reduzida devido à estrutura rígida resultante da formação de pontes de hidrogênio intramoleculares entre os seus grupos hidroxilo secundários. Esta limitação incentivou o desenvolvimento de CDs quimicamente modificadas, que oferecem maior solubilidade, menor toxicidade e a possibilidade de aumentar a capacidade de inclusão dos seus derivados (DUCHÊNE; PONCHEL; WOUESSIDJEWE, 1999; HIRAYAMA; UEKAMA, 1999; CALABRÒ et al., 2004; VEIGA; PECORELLI; RIBEIRO, 2006a). Após esta observação, vários derivados das CDs surgiram como, por exemplo, derivados hidroxipropil da β e γ-ciclodextrina.

Veiga (1996) referiu à obtenção dos derivados das ciclodextrinas utilizando grupamentos

metila, etila, carboximetila, hidroxietila, hidroxipropila, dentre outros. A 2-hidroxipropil-β-ciclodextrina (HPβ-CD) é um dos derivados das CDs que apresenta atenção especial pela sua alta solubilidade, baixa toxicidade e maior cavidade hidrofóbica em comparação as outras ciclodextrinas da mesma família, fazendo desta CD uma forte candidata para utilização em formulações farmacêuticas (MISIUK; ZALEWSKA, 2009).

Figura 9 - Estrutura molecular e dimensões da cavidade das α, β e γ-ciclodextrinas.



Fonte: VENTURINI et al., 2008.

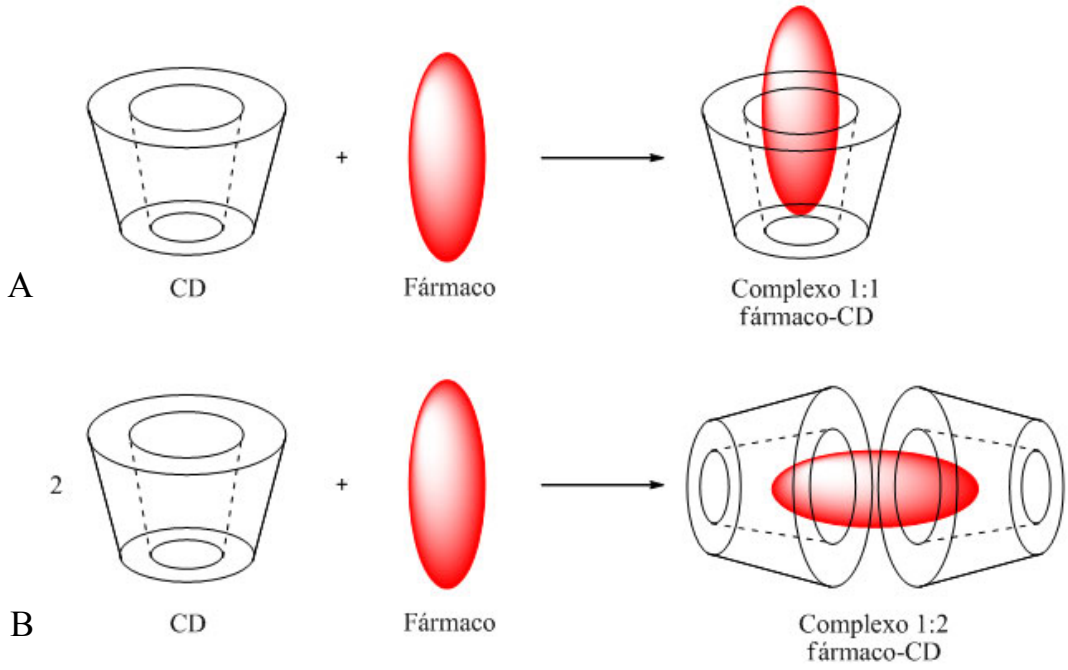
2.5.3 - Complexos de inclusão com ciclodextrinas

Devido à sua estrutura peculiar, as CDs podem ser consideradas cones abertos em ambas as extremidades, o que lhes permite a inclusão de uma enorme variedade de moléculas hóspedes, na sua cavidade central hidrofóbica (ZHANG et al., 2009), formando assim complexos de inclusão com vários compostos (DAVIS; BREWSTER, 2004). Os complexos de inclusão são formados por duas ou mais moléculas onde uma delas, a hospedeira, inclui total ou parcialmente uma molécula hóspede, sem o estabelecimento de ligações covalentes (VEIGA; PECORELLI; RIBEIRO, 2006b). O complexo obtido exibe novas características físico-químicas quando comparadas às moléculas livres. A formação deste complexo proporciona uma melhora na estabilidade, maior hidrossolubilidade e maior biodisponibilidade do composto utilizado como princípio ativo (DUCHÊNE; PONCHEL; WOUESSIDJEWE, 1999; BOUDAD et al., 2001;

PASQUALI; BETTINI, 2008). Nestes complexos a molécula hóspede se aloja na cavidade interna hidrofóbica das ciclodextrinas (DEL VALLE, 2004).

Quanto à estequiometria do complexo de inclusão, são considerados quatro tipos mais comuns de complexo CD:substrato (1:1, 1:2, 2:1 e 2:2), dependendo do tamanho e aspecto estrutural do substrato em relação à cavidade da CD (Figura 10) (VENTURINI et al., 2008). Os diferentes tipos de complexos surgem das diferenças que o substrato em potencial apresenta para se adaptar à cavidade da CD e este ajuste espacial é considerado o fator mais importante para reger o processo de complexação (HARATA, 1998). Desta forma, para moléculascom baixo peso molecular é mais fácil formar complexos com α- e β-CD devido à compatibilidade do volume do substrato e da CD.

Figura 10 - Estequiometria da ciclodextrina e do fármaco (A: complexos de inclusão 1:1; B: complexos de inclusão 1:2).

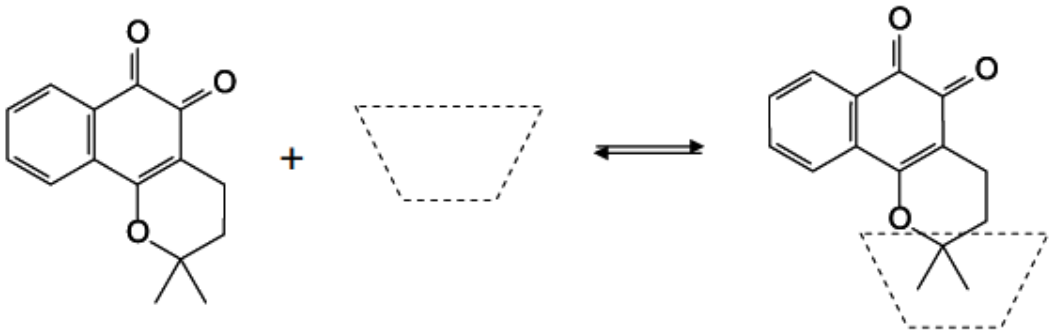


Fonte: http://www.portaldosfarmacos.ccs.ufrj.br/resenhas_ciclodextrinas.html

As extremidades da cavidade da CD são abertas de tal forma que o substrato pode entrar nesta cavidade por ambos os lados (Figura 11). Em solução aquosa, a cavidade levemente apolar é ocupada por moléculas de água que são energeticamente menos favoráveis que moléculas

apolares, dada a natureza da interação polar-apolar e, portanto, podem ser facilmente substituídas por um substrato que seja menos polar que a água (VEIGA; PECORELLI; RIBEIRO, 2006b). Considera-se que a força motriz para a complexação seja a substituição das moléculas de água de alta entalpia por substratos apropriados, desta forma a polaridade dos compostos é um fator condicionante da formação do complexo (SZEJTLI, 1998).

Figura 11 - Representação esquemática da formação do complexo de inclusão da β-lapachona com uma ciclodextrina.



Fonte: ABREU et al., 2007.

A formação de complexos de inclusão altera significativamente as características do substrato, como por exemplo, aumento da solubilidade de compostos, estabilização de substâncias sensíveis à luz, calor e oxidação, proteção da degradação de substâncias por microrganismos, mascaramento de corantes ou pigmentos e fixação de substâncias muito voláteis (VENTURIRI et al., 2008). O método de formação do complexo tem grande influência no composto final obtido, desta forma, é importante que seja realizada uma seleção da melhor metodologia levando-se em consideração não só o complexo final obtido, como também simplicidade do método, baixo custo, rendimento elevado, rapidez e, principalmente, facilidade de transposição de escala. Há diversos métodos de preparação dos complexos propostos na literatura, mas ainda não há regras gerais ou métodos universais para a formação de complexos de inclusão, isso porque cada composto a ser complexado é um caso particular, ou seja, possui características específicas e por isso as condições ideais podem variar de acordo com as características tanto do composto utilizado como da CD (MURA et al., 1999). De maneira geral os métodos de formação dos complexos mais utilizados são co-evaporação, co-precipitação e

Freeze-Drying (BARBATO et al., 2003; MANOLIKAR; SAWANT, 2003; TEIXEIRA et al., 2003; DEVARAKONDA et al., 2005; ZIGONE; RUBESSA, 2005).

2.5.4 - Métodos de caracterização dos complexos de inclusão

As propriedades físico-químicas do fármaco e da CD livre são relativamente diferentes das que possuem estes compostos quando estão complexados. São muitas as metodologias que podem ser utilizadas para a investigação dos complexos de inclusão, apresentando todas elas características próprias que permitem não só detectar a formação do complexo de inclusão, como também conhecer a sua estrutura química e interações que se estabelecem entre os diferentes componentes que o constituem. Partindo deste conceito, qualquer metodologia que tenha sensibilidade suficiente para medir estas diferenças pode ser utilizada para caracterizar estes complexos. Os métodos de caracterização mais utilizados são: Espectroscopia de Ressonância Magnética Nuclear de Prótons (RMN H^1), Análise Termogravimétrica (TG), Calorimetria Exploratória Diferencial (DSC), Espectroscopia de Absorção na Região do Infravermelho (IV), Difração de raio-X e Espectroscopia Raman. Além destes métodos de caracterização dos complexos de inclusão, existe ainda o estudo de solubilidade de fases e a eficiência de complexação (VEIGA; PECORELLI; RIBEIRO, 2006c).

2.5.5 - Aplicação das ciclodextrinas

As CDs estão sendo utilizadas nas mais variadas áreas, dentre elas, na indústria alimentícia, cosmética e farmacêutica (VENTURINI et al., 2008). Na indústria alimentícia ela reduz odores e sabores indesejáveis; na cosmética, ela diminui irritação local, propicia ação prolongada e também diminui odores e na farmacêutica, ela é muito utilizada para aumentar a solubilidade de fármacos fracamente hidrossolúveis, assim como aumentar a estabilidade (LOFTSSON; DUCHÊNE, 2007). As CDs que podem atualmente ser comercializadas na forma de produtos farmacêuticos estão citadas na Tabela 6.

As ciclodextrinas são conhecidas por permitirem a incorporação molecular de fármacos com características hidrofóbicas, alterando-lhes a solubilidade e, em alguns casos, melhorando a biodisponibilidade. Porém a estrutura química da ciclodextrina, seu peso molecular (>972 Da) e seu baixo coeficiente de partição são características que tornam este composto pouco permeável às membranas biológicas (LIPINSKI et al., 2001). Além disso, a capacidade destes complexos

funcionarem como sistemas de vetorização de fármacos por si só é inexistente (FERNANDES et al., 2007). Desta forma a utilização de sistemas de liberação controlada de fármacos seria uma alternativa para ultrapassar estas limitações.

Tabela 6 - Produtos farmacêuticos contendo ciclodextrinas comercializados mundialmente. α-ciclodextrina (α-CD), β-ciclodextrina (β-CD), 2-hidroxipropil-β-ciclodextrina (HP-βCD), sulfobutiléter-β-ciclodextrina (SBE-βCD), 2-hidroxipropil-γ-ciclodextrina (HP-γCD).

Fármacos/ciclodextrina	**Aplicação terapêutica**	**Nome comercial**
α-CD		
Alprostadil	Tratamento da disfunção erétil	Caverject Dual
β-CD		
Cetirzina	Agente antibacteriano	Cetrizin
Dexametasona	Antiinflamatório	Glymesason
Nimesulida	Antiinflamatório	Nimedex
HP-βCD		
Indometacina	Antiinflamatório	Indocid
Itraconazol	Agente antifúngico	Sporanox
Mitomicina	Agente anticancerígeno	MitoExtra
SBE-βCD		
Voriconazol	Agente antifúngico	Vfend
HP-γCD		
Diclofenaco sódico	Anti-inflamatório	Voltaren Ophtha

Fonte: Adaptado de KURKOV; LOFTSSON, 2012.

2.6 - Nanotecnologia Farmacêutica e Sistemas de Liberação Controlada de Fármacos

A nanotecnologia farmacêutica é a área das ciências farmacêuticas envolvida no desenvolvimento, caracterização e aplicação de sistemas terapêuticos em escala nanométrica ou micrométrica. A nanotecnologia possui um incrível potencial para revolucionar diagnóstico e terapêutica, ao desenvolver engenhosos nanodispositivos (PARK, 2007). Estudos de tais sistemas têm sido realizados ativamente no mundo com o propósito de direcionar e controlar a liberação de fármacos (SAKATA et al., 2007).

A tecnologia associada à modificação da liberação de fármacos, ou de outras substâncias bioativas, a partir de preparações farmacêuticas sofreu um incremento notório nas últimas

décadas na tentativa de maximizar as vantagens inerentes às formas farmacêuticas de liberação controlada (DAS; DAS, 2003). Uma ampla variedade de sistemas, visando condicionar a velocidade e o local de liberação dos fármacos, tem sido objeto de investigação na área da indústria farmacêutica (PIMENTEL et al., 2007).

Sistemas de liberação controlada de fármacos são concebidos para se obter uma concentração plasmática ou níveis de concentração tecidual de fármacos dentro da faixa terapêutica com liberação controlada (velocidade controlada), atingindo o efeito terapêutico desejado e evitando possíveis reações tóxicas inerentes ao composto. Estes sistemas podem retardar a liberação do princípio ativo, sustentar a sua liberação e/ou direcioná-lo a sítios específicos de ação (células, órgãos, microrganismos). Na terapia medicamentosa utilizando formas farmacêuticas convencionais como soluções, suspensões, emulsões, cápsulas, etc.; torna-se difícil manter as concentrações plasmáticas de muitos fármacos em nível terapêutico por longo período de tempo, isto porque essas apresentações farmacêuticas normalmente liberam todo seu conteúdo de imediato gerando inicialmente um pico máximo de concentração plasmática que pode atingir níveis tóxicos, e logo após uma dose subterapêutica, o que proporciona flutuações aleatórias da biodisponibilidade do princípio ativo (LEE; ROBINSON, 2004; BOISSEAU; LOUBATON, 2011).

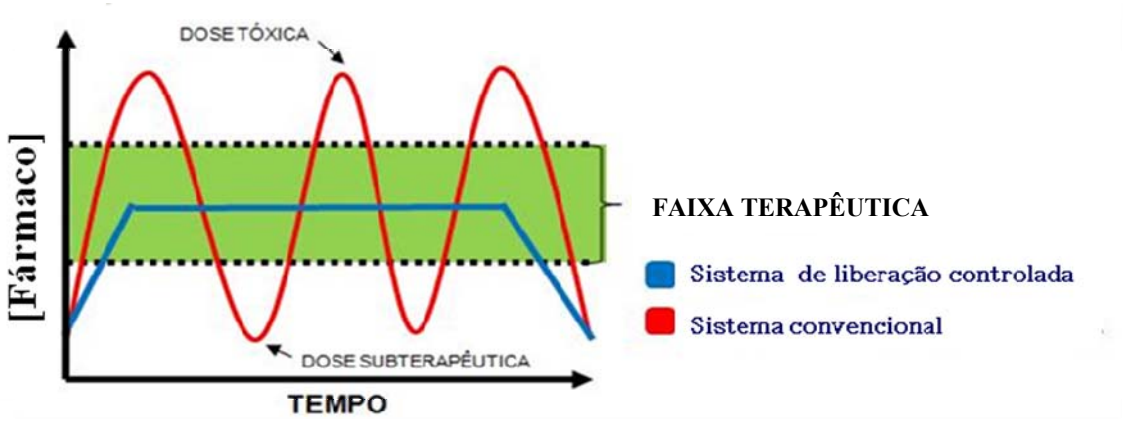
Desta forma, o principal objetivo de uma forma farmacêutica de liberação controlada é manter constante a concentração plasmática do fármaco na faixa terapêutica, eliminando as variações que geralmente são observadas no decorrer do tratamento como a falta de efetividade (dose subterapêutica) ou efeitos tóxicos da substância ativa (Figura 12).

As principais vantagens dos sistemas de liberação controlada de fármacos são (GULATI et al., 1998; TORCHILIN, 2005; PIMENTEL et al., 2007):

- Proteger o conteúdo encapsulado;
- Possibilitar a incorporação tanto de substâncias hidrofílicas quanto lipofílicas;
- Promover a vetorização do fármaco;
- Promover liberação controlada do princípio ativo ao longo do tempo;
- Manter os níveis plasmáticos do fármaco em concentração constante dentro da faixa terapêutica;

- Reduzir o número de administrações do fármaco;
- Reduzir a toxicidade devido à menor liberação do princípio ativo em tecidos saudáveis;
- Melhorar a adesão do paciente à terapêutica.

Figura 12 - Farmacocinética de sistemas farmacêuticos convencionais e sistemas de liberação controlada de fármacos.



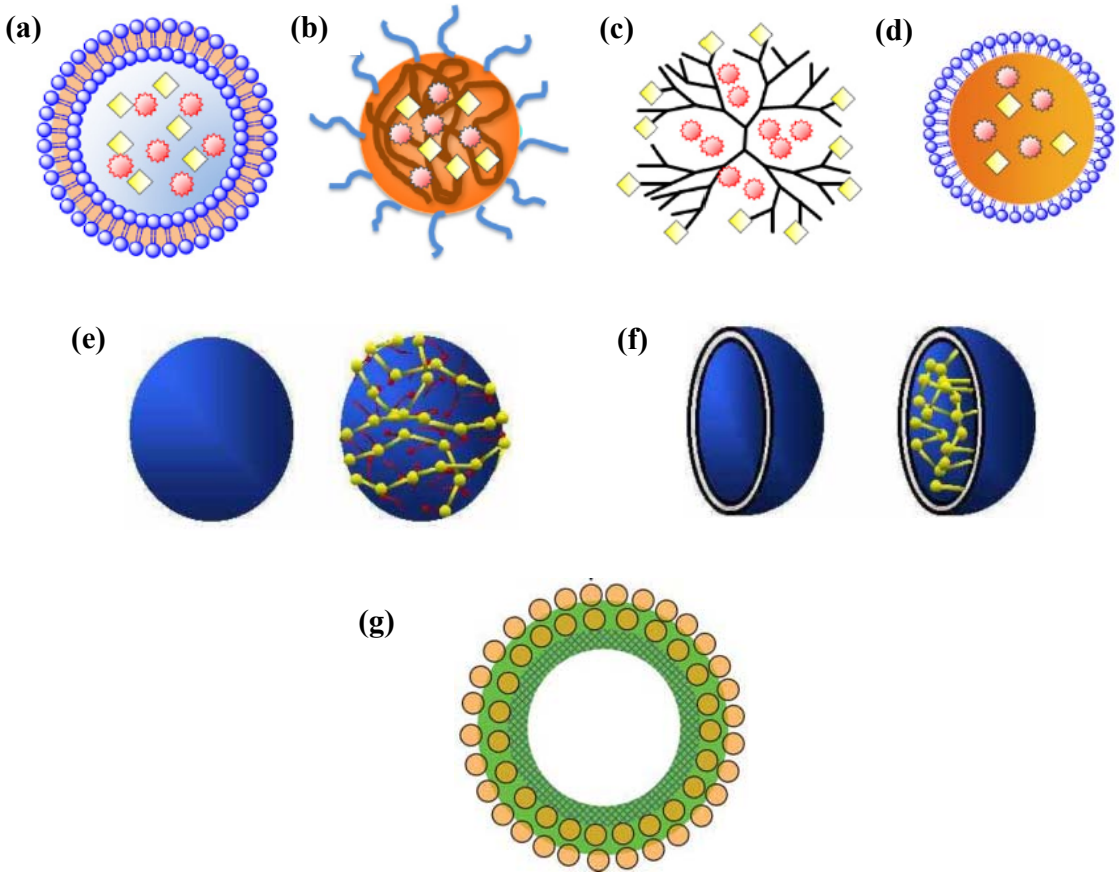
Fonte: Adaptado de CAMPOS, 2011.

As principais formas farmacêuticas de liberação controlada são dendrímeros, micelas, nanoemulsões, micropartículas (microesferas e microcápsulas), nanopartículas (nanoesferas e nanocápsulas), lipossomas, dentre outros (Figura 13) (VERMA; GARG, 2001).

2.6.1 - Lipossomas

Alec Bangham há mais de 40 anos com sua observação pioneira de que fosfolipídios em soluções aquosas podem formar estruturas fechadas em bicamadas, permitiu que a pesquisa relacionada aos lipossomas percorresse um longo caminho, convertendo-os de simples objetos de pesquisa biofísica em carreadores terapêuticos para numerosas aplicações clínicas (FRÉZARD et al., 2005; TORCHILIN, 2005). Na década de 70, Gregory Gregoriadis começou a investigar a capacidade de encapsulação de fármacos por vesículas lipossomais e sua posterior liberação e propôs pela primeira vez a utilização de lipossomas como sistema transportador de fármacos, mantendo desde então um papel preponderante no desenvolvimento desta área (FRÉZARD et al., 2005).

Figura 13 - Representação esquemática de sistemas de liberação controlada: (a) lipossoma, (b) micela polimérica, (c) dendrímero, (d) nanoemulsão oleosa, (e) nanoesfera, (f) nanocápsula e (g) micropartícula. ◇ e ○ representam os diferentes tipos de fármacos.

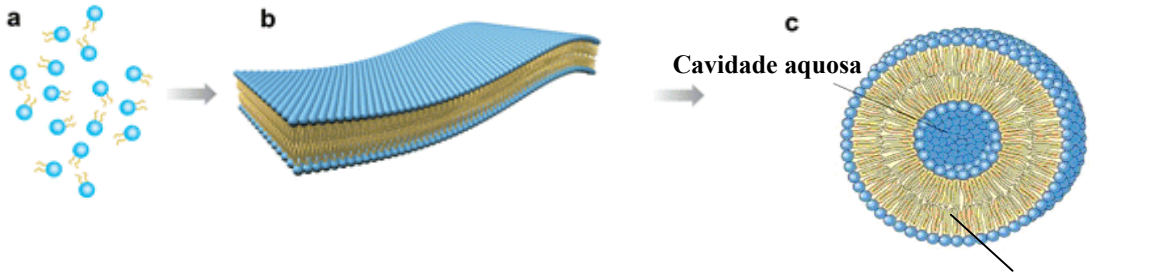


Fonte: Adaptado de YU et al., 2006; Adaptado de SOPPIMATH et al., 2011; Adaptado de HU; ZHANG, 2012.

Os lipossomas, descobertos em 1963, são vesículas aquosas microscópicas de tamanhos variados (em escalas de nm e μm) com uma ou mais bicamadas lipídicas concêntricas (TORCHILIN, 2005) (Figura 14). Eles podem encapsular substâncias hidrofílicas e/ou lipofílicas, sendo que as primeiras ficam no compartimento aquoso e as lipofílicas inseridas ou adsorvidas na bicamada lipídica (GILLET et al., 2009). Os lipossomas possuem algumas vantagens em relação a outros sistemas transportadores por serem biodegradáveis, biocompatíveis e não imunogênicos. Além disso, são sistemas altamente versáteis, cujo tamanho, número de lamelas, superfície e

composição lipídica podem ser manipulados de acordo com as propriedades físico-químicas do fármaco (BRANDL; GREGORIADIS, 1994; TORCHILIN, 2005; EDWARDS; BAEUMNER, 2006).

Figura 14 - Esquema da formação dos lipossomas: (a) fosfolipídio, (b) bicamada lipídica e (c) lipossoma.



Fonte: Adaptado de JESORKA; ORWAR, 2008.

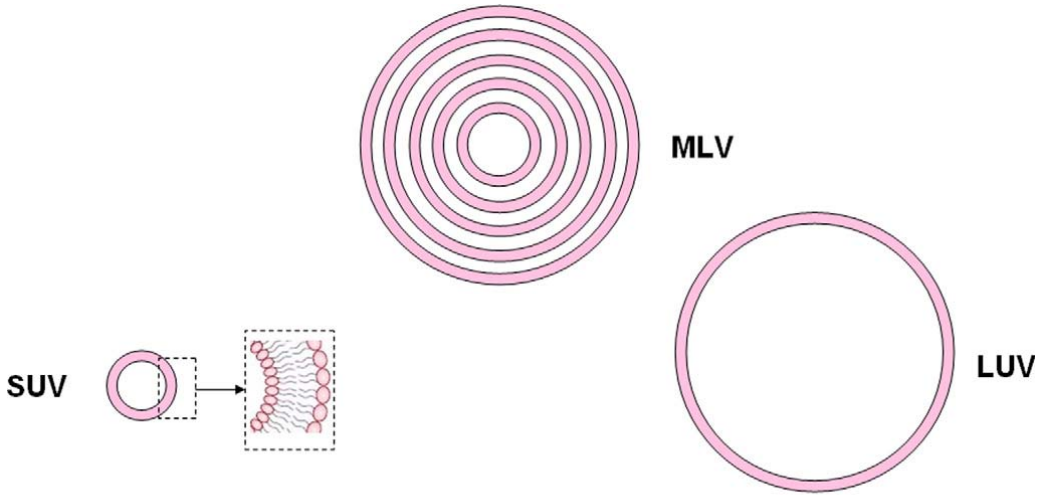
Estas vesículas são constituídas basicamente por fosfolipídios e esteróis (VEMURI; RHODES, 1995). Os fosfolipídios mais utilizados nas formulações de lipossomas são os que apresentam forma cilíndrica como a fosfatidilcolina, fosfatidilserina, fosfatidilglicerol e esfingomielina, que tendem a formar uma bicamada estável em solução aquosa. As fosfatidilcolinas são as mais empregadas nas preparações lipossomais, pois apresentam grande estabilidade frente a variações de pH ou da concentração de sal no meio (BATISTA; CARVALHO; SANTOS-MAGALHÃES, 2007). Os fosfolipídios são substâncias graxas de origem natural ou sintética; sendo os principais componentes das membranas celulares apresentando a capacidade de auto-organização quando em soluções. Dentre os esteróis, o colesterol, muito presente nas membranas celulares de mamíferos, destaca-se. Mesmo incapaz de se organizar em bicamadas, esse lipídio pode ser incorporado em membranas de fosfolipídios para modular a fluidez da membrana fosfolipídica, reduzindo a permeabilidade da bicamada e melhorando a estabilidade da membrana em fluidos biológicos (TORRES, 2008).

2.6.2 - Classificação dos lipossomas

Os lipossomas podem conter uma única bicamada lipídica ou bicamadas múltiplas em torno do compartimento aquoso interno e, portanto, são classificados em unilamelares e

multilamelares, respectivamente. Quanto ao tamanho e número de lamelas, podem ser classificados como vesículas multilamelares grandes (MLV - multilamellar large vesicles) que são lipossomas de tamanho em escala micrométrica que possuem vários compartimentos aquosos e lipofílicos intercalados; vesículas unilamelares grandes (LUV - large unilamellar vesicles) que são lipossomas de tamanho em escala de micrômetros possuindo um único compartimento interno aquoso e um lipofílico (bicamada lipídica); e os lipossomas em escala nanométrica que possuem um compartimento interno aquoso e um lipofílico que são classificados como vesículas unilamelares pequenas (SUV - small unilamellar vesicles) (Figura 15) (BATISTA; CARVALHO; SANTOS-MAGALHÃES, 2007).

Figura 15 - Classificação dos lipossomas quanto ao tamanho e número de lamelas. SUV: vesículas unilamelares pequenas. LUV: vesículas unilamelares grandes. MLV: vesículas multilamelares grandes.



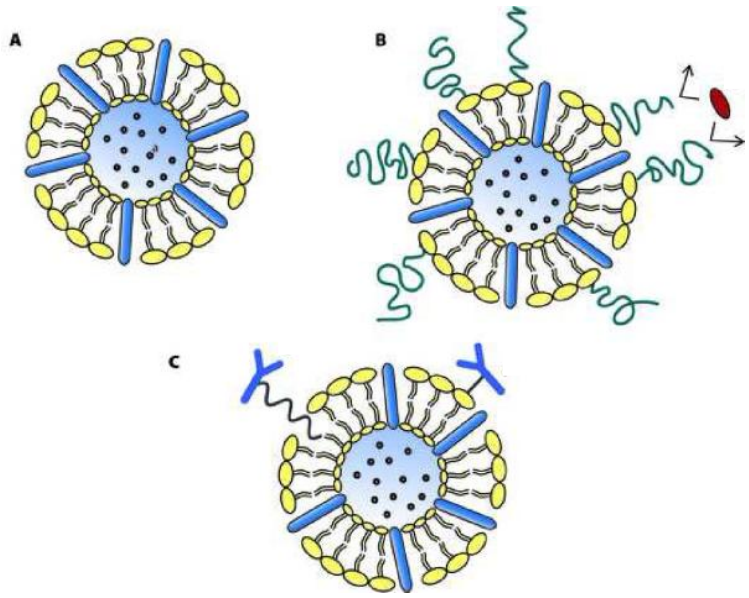
Fonte: Adaptado de YANG et al., 2011.

De acordo com a composição química os lipossomas podem ser classificados em convencionais, furtivos ou Stealth®, sítio-específicos e catiônicos (Figura 16).

Lipossomas convencionais são compostos de fosfolipídios e colesterol, além de um lipídio com carga negativa ou positiva para evitar a agregação das vesículas, aumentando a estabilidade em suspensão. *In vivo* os lipossomas convencionais são rapidamente removidos da circulação devido à adsorção das opsoninas na superfície desses nanocarreadores, desencadeando o seu

reconhecimento e captura pelo sistema fagocitário mononuclear (PINTO-ALPHANDARY; ANDREMONT; COUVREUR, 2000; BATISTA; CARVALHO; SANTOS-MAGALHÃES, 2007). Essa captura está diretamente relacionada ao tamanho das vesículas e sua superfície hidrofóbica, o que resulta em uma curta permanência desses lipossomas na corrente sanguínea após o contato com componentes do sangue (VEMURI; RHODES, 1995; PINTO-ALPHANDARY; ANDREMONT; COUVREUR, 2000; BATISTA; CARVALHO; SANTOS-MAGALHÃES, 2007). Devido a essa captura, os lipossomas convencionais são potentes candidatos a carrear fármacos que vão agir nos macrófagos, porém a sua rápida eliminação compromete a aplicação desses lipossomas no tratamento de várias patologias.

Figura 16 - Classificação dos lipossomas quanto à composição química. (A) lipossomas convencionais, (B) lipossomas furtivos e (C) lipossomas sítio-específicos.

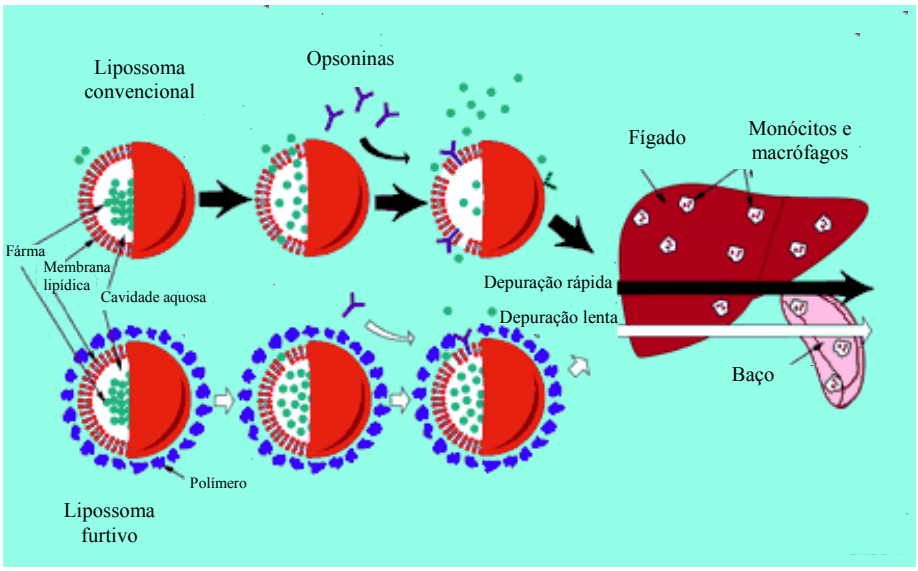


Fonte: BENNEWITZ; SALTZMAN, 2009.

Os lipossomas furtivos, conhecidos como "Stealth®", apresentam superfície hidrofílica, ocasionando impedimento estérico para a ligação das opsoninas. Desta forma, há a inibição do processo de reconhecimento molecular e a captura pelas células do sistema fagocitário mononuclear, principalmente as células de Kupffer no fígado, e consequentemente esses sistemas permanecem por um período de tempo maior na circulação (Figura 17) (ABU LILA et al., 2009;

LI et al., 2009; PASUT; VERONESE, 2009). Sistemas de longa circulação são obtidos por diferentes métodos, incluindo o revestimento da superfície do lipossoma com componentes hidrofílicos naturais ou polímeros hidrofílicos sintéticos, especificamente o polietilenoglicol (PEG) (TORCHILIN, 2005; MARKOVSKY et al., 2012).

Figura 17 - Depuração de lipossomas convencionais e furtivos.



Fonte: Adaptado de ZAMBONI, 2005.

Na tentativa de aumentar a especificidade de interação de lipossomas com células alvo e elevar a concentração do fármaco liberado nestas células, a pesquisa neste campo é direcionada ao desenvolvimento de lipossomas sítio-específicos. Esses sistemas utilizam ligantes acoplados em sua superfície, que conferem seletividade para direcionar o fármaco encapsulado no sítio de ação desejado (SAPRA; ALLEN, 2003). Alguns exemplos de ligantes de reconhecimento são os anticorpos, glicopeptídeos, polissacarídeos, proteínas virais e lectinas. Estes constituintes são ligados covalentemente à superfície dos lipossomas a fim de carreá-los para o local específico de ação (EDWARDS; BAEUMNER, 2006; BATISTA; CARVALHO; SANTOS-MAGALHÃES, 2007).

Os lipossomas catiônicos, como o nome sugere, são lipossomas que apresentam carga positiva na superfície e são utilizados para terapia gênica. Os lipídios catiônicos interagem com o DNA, que possui carga negativa (CHRISTENSEN et al., 2007).

2.6.3 - Método de preparação dos lipossomas

A larga aceitação dos lipossomas para os mais diversos fins criou a necessidade de desenvolver métodos de preparação eficientes, reprodutíveis e com a simplicidade possível, à escala laboratorial e industrial (SANTOS; CASTANHO, 2002).

Neste contexto, existem vários métodos de preparação, como extrusão, evaporação em fase-reversa, microfluidização e hidratação do filme lipídico. No método da hidratação do filme lipídico, os lipídios são solubilizados em solvente orgânico, em seguida, forma-se o filme lipídico pela evaporação do(s) solvente(s). Então, a fase aquosa é adicionada formando espontaneamente as vesículas. O diâmetro médio dos lipossomas obtidos por esse método é bastante elevado sendo apropriado para produzir MLVs (FATTAL; COUVREUR; PUISIEUX, 1993; WATWE; BELLARE, 1995; SANTOS; CASTANHO, 2002; FRÉZARD et al., 2005). Os lipossomas obtidos pelo método da hidratação do filme lipídico podem ser submetidos à ação de ultrassom com ajuda de uma sonda ou banho. A energia liberada promove a diminuição do raio dos MLVs tornando-os SUVs (FATTAL; COUVREUR; PUISIEUX, 1993; WATWE; BELLARE, 1995; ANDRADE et al., 2004).

2.6.4 - Aplicações farmacêuticas dos lipossomas

A utilização de lipossomas como sistemas de liberação controlada no tratamento de várias patologias tem sido explorado extensivamente por mais de 20 anos (AGRAWAL; GUPTA, 2000). Considerando os mecanismos de resistência bacteriana, o desenvolvimento de sistemas coloidais de liberação controlada de fármacos, como os lipossomas, para o carreamento de antibióticos, poderia aumentar sua atuação em cepas não resistentes e superar essa resistência, no caso de cepas resistentes. Lipossomas e bactérias podem interagir diretamente por processos de fusão, levando à liberação do antibiótico encapsulado diretamente ao alvo bacteriano (SACHETELLI et al., 2000).

Algumas limitações nas formulações existentes no mercado têm motivado o desenvolvimento de alternativas que possam vir a contribuir ao melhor aproveitamento dos sistemas de liberação controlada de medicamentos. Neste sentido, Rukholm e colaboradores (2006) compararam a atividade bactericida da gentamicina livre e encapsulada em lipossomas frente a isolados clínicos de *Pseudomonas aeruginosa*. Neste estudo foi possível observar que o MIC para os lipossomas contendo gentamicina (1 mg/L) foram significativamente mais baixo

quando comparado ao da gentamicina livre (4 mg/L). Além disso, os lipossomas contendo gentamicina apresentaram um tempo necessário para matar o microrganismo igual ou inferior ao da molécula livre.

Estudos com MiKasome®, lipossoma furtivo contendo amicacina, em animais e em humanos revelou um aumento na meia-vida do fármaco no plasma em comparação a amicacina livre (FIELDING; LEWIS; MOON-MCDERMOTT, 1998; FIELDING et al., 1999). Em modelos animais de infecção, MiKasome® exibiu um aumento de eficácia terapêutica no que diz respeito à sobrevivência dos animais quando comparado com a amicacina livre (XIONG et al., 1999).

Nicolosi e colaboradores (2010) encapsularam a vancomicina em lipossomas fusogênicos e avaliaram a ação deste fármaco frente a bactérias Gram-negativas. Os sistemas desenvolvidos apresentaram MIC de 6 μg/mL para os isolados clínicos de *Escherichia coli* e *Acinetobacter baumannii*. Nesse estudo, o antibiótico livre e encapsulado nos lipossomas não fusogênicos não apresentaram nenhuma atividade contra as mesmas bactérias. Assim, esta estratégia tecnológica pode ser proposta como uma forma potencialmente bem sucedida para ampliar o espectro de atividade da vancomicina.

Em 2011, Pumerantz e colaboradores avaliaram a viabilidade de MRSA dentro dos macrófagos tratados com lipossomas convencionais e furtivos contendo vancomicina. Em contraste com os resultados da vancomicina livre, o tratamento com os lipossomas convencionais contendo vancomicina resultou em uma concentração deste antibiótico no interior dos macrófagos suficiente para exercer um efeito bactericida contra o MRSA. Por outro lado, o tratamento de macrófagos infectados com os lipossomas furtivos não resultou em qualquer impacto sobre a sobrevivência do MRSA e esta ausência de efeito inibitório, provavelmente, pode ter ocorrido devido à fagocitose um pouco mais lenta dos sistemas furtivos.

Os lipossomas podem ainda encapsular agentes antifúngicos possibilitando a administração de medicamentos que antes não poderiam ser utilizados na terapêutica devido a sua toxicidade, como, por exemplo, a anfotericina B. AmBisome® (Nexstar Pharmaceuticals Inc., San Dimas, CA), lipossoma furtivo contendo anfotericina B, tem sido amplamente utilizada com sucesso em modelos animais e em pacientes com infecções fúngicas com risco de morte (TOLLEMAR; KLINGSPOR; RINGDÉN, 2001).

Além das aplicações da área da microbiologia, os lipossomas também são utilizados na terapia contra o câncer. De acordo com a Organização Mundial de Saúde (OMS), constatou-se

que anualmente 10 milhões de casos de câncer são diagnosticados em todo o mundo e seis milhões de pessoas morrem devido a esta patologia. Neste contexto, os lipossomas podem contribuir para a terapêutica do câncer, já que esta patologia ainda apresenta limitações quanto ao seu tratamento, como por exemplo, os efeitos colaterais e as repetidas administrações.

Desde as observações pioneiras de Alec Bangham há aproximadamente 40 anos, os lipossomas têm percorrido um longo caminho para se tornar um sistema de liberação controlada de escolha para numerosas aplicações farmacêuticas. O avanço real no desenvolvimento na área da nanotecnologia durante os últimos 15 anos resultou na aprovação de vários sistemas lipossomais e no aparecimento de muitas tecnologias e produtos biomédicos envolvendo lipossomas (TORCHILIN, 2005).

Os lipossomas são utilizados na terapia contra o câncer, com significantes vantagens, como uma maior retenção local do fármaco, e evasão às várias barreiras fisiológicas para a entrega, incluindo a alta pressão intersticial presente na maioria dos tumores (Figura 18) (BAO et al., 2006). Esses sistemas aumentam a eficácia antitumoral e tolerabilidade, reduzem a toxicidade dos fármacos, assim como diminuem a probabilidade do desenvolvimento de resistência (JACK; ZHANG, 2012; SLINGERLAND et al., 2012).

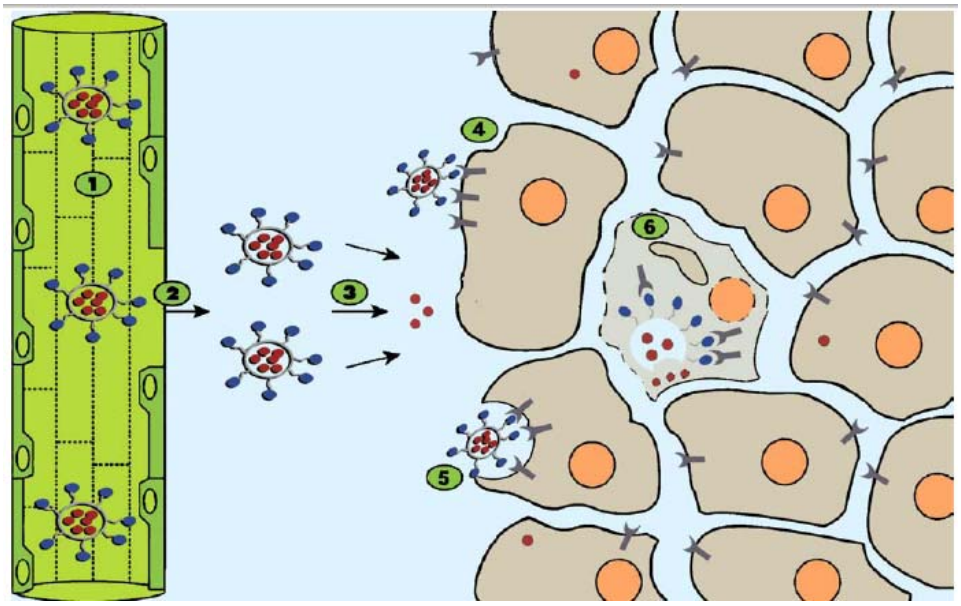
Doxil®, lipossoma furtivo contendo Doxorrubicina, foi o primeiro nanomedicamento aprovado pela FDA (BARENHOLZ, 2012). Atualmente há no mercado outros fármacos antineoplásicos encapsulados em lipossomas como, por exemplo, o DaunoXome® (lipossoma peguilado contendo daunorrubicina), Peg-Intron® (lipossoma contendo IFN-α-2b) e Caelyx® (lipossoma peguilado contendo cloridrato de doxorrubicina) que já são amplamente utilizados clinicamente (BUKOWSKI et al., 2002; TORCHILIN, 2006; SALZBERG et al., 2007).

Estudos clínicos de fase I do Doxil® e de avaliação antitumoral deste medicamento em pacientes com tumores sólidos revelaram uma mudança importante no perfil de toxicidade da doxorrubicina, caracterizada por toxicidade mucocutânea dose dependente, mielossupressão leve, alopecia mínima, mas não ocorreu toxicidade cardíaca aparente (clínica e funcional) (UZIELY et al., 1995).

Em estudos clínicos de fase II realizados por Muggia e colaboradores (1997) foi possível observar que o Doxil® não induziu náuseas, perda de cabelo ou necrose extravasante. Os lipossomas contendo doxorrubicina apresentaram toxicidade mínima e atividade significativa contra o câncer de ovário. Gordon e colaboradores (2001), em estudos clínicos de fase III,

observaram que o Doxil® é uma opção para o tratamento de pacientes com carcinoma epitelial de ovário.

Figura 18 - Representação esquemática da chegada dos lipossomas nas células tumorais. Os pontos azuis representam os ligantes sítio-específicos dos lipossomas. Os pontos vermelhos representam o fármaco encapsulado na fase aquosa do lipossoma. Os passos de 1 a 3 são comuns a todos os lipossomas, sejam eles sítio-específicos ou não. Os passos de 4 a 6 são específicos para os lipossomas sítio-específicos. (1) Passagem dos lipossomas pela microvasculatura do tumor; (2) aumento da permeabilidade vascular no tecido tumoral, permitindo que os lipossomas de tamanho reduzido extravasem e atinjam o líquido intersticial do tumor; (3) o fármaco é gradualmente liberado a partir de lipossomas e entra nas células tumorais como fármaco livre para exercer um efeito citotóxico; (4) os lipossomas sítio-específicos se ligam a seus receptores de membrana nas células tumorais; (5) os lipossomas são internalizados pelas células; (6) os lipossomas internalizados liberam o fármaco no citosol permitindo que ele exercer o seu efeito citotóxico.



Fonte: GABIZON et al., 2004.

Estudos clínicos de fase I/II realizados por Gill e colaboradores (1995) revelaram que o DaunoXome® apresenta melhor perfil farmacocinético em comparação a daunorrubicina livre. O

DaunoXome® foi bem tolerado pelos pacientes e apresentou atividade antitumoral significativa em pacientes com Sarcoma de Kaposi. Em 1996, Gill e colaboradores realizaram estudos clínicos de fase III do DaunoXome® em pacientes com Sarcoma de Kaposi. Eles observaram que o tratamento com lipossomas contendo daunorrubicina foi comparável ao regime de referência para o tratamento desta patologia (doxorrubicina, bleomicina e vincristina). Desta forma, DaunoXome® se mostrou uma terapia eficaz e segura para o tratamento do Sarcoma de Kaposi.

Zervas e colaboradores (2004) avaliaram a eficácia e toxicidade do tratamento combinado de lipossomas contendo vincristina, doxorubicina e dexametasona associado à talidomida, administrado em regime ambulatorial como tratamento inicial citorredutor em pacientes com mieloma sintomático que não foram previamente tratados. Neste experimento, dos 39 pacientes, 74% responderam ao tratamento, dos quais 10% obtiveram resposta completa e 64% resposta parcial. Três pacientes (8%) apresentaram uma resposta menos evidente e 7 (18%) foram classificados como não-respondedores. Neste contexto, a combinação do lipossoma contendo vincristina, doxorubicina e dexametasona associado à talidomida é um eficaz e relativamente bem tolerado tratamento inicial citorredutor.

Em 2007, Andreopoulou e colaboradores observaram que a manutenção a longo prazo do tratamento utilizando lipossomas peguilados contendo Doxorrubicina (PLD, Caelyx/DOXIL) em portadoras de câncer de ovário epitelial recorrente é benéfica e segura. Eles avaliaram 16 pacientes com câncer ovariano recorrente ou na trompa de Falópio que receberam PLD por mais de 1 ano. Todas as pacientes apresentaram estabilização da doença, assim como foi notável a ausência de mielossupressão cumulativa.

Os nanocarreadores farmacêuticos podem ainda ser utilizados para fins de diagnóstico e imagem, pois permitem a biodistribuição do agente de contraste em tempo real. Os agentes de contraste encapsulados em lipossomas têm sido utilizados para diagnóstico experimental por imagem de fígado, baço, cérebro, sistema cardiovascular, tumores, inflamações e infecções (TORCHILIN, 2005; TORCHILIN, 2006).

Diante dos resultados apresentados nos trabalhos desta revisão, acreditamos no potencial terapêutico da β-lapachona encapsulada em sistemas de liberação controlada, tais como os lipossomas. Adicionalmente, a formação dos complexos de inclusão β-lapachona:HPβ-CD podem fornecer uma nova estratégia para melhorar a solubilidade e biodisposnibilidade deste fármaco.

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4. OBJETIVOS

4.1 – Geral

O objetivo do presente trabalho consiste em desenvolver e avaliar a atividade antimicrobiana e antiproliferativa de lipossomas convencionais e furtivos contendo β-lapachona (β-lap) e complexos de inclusão β-lapachona:2-hidroxipropil-β-ciclodextrina (β-lap:HPβ-CD).

4.2 – Específicos

- Preparar complexos de inclusão da β-lapachona em 2-hidroxipropil-β-ciclodextrina (β-lap:HPβ-CD);
- Obter lipossomas convencionais e furtivos contendo β-lapachona e β-lap:HPβ-CD;
- Avaliar a estabilidade acelerada e a longo prazo das formulações lipossomais;
- Determinar a taxa de encapsulação da β-lapachona em lipossomas;
- Realizar ensaios de cinética de liberação *in vitro* da β-lapachona a partir dos lipossomas;
- Avaliar a atividade antibacteriana *in vitro* da β-lapachona livre e encapsulada em lipossomas convencionais e furtivos;
- Avaliar a atividade antifúngica *in vitro* da β-lapachona encapsulada em lipossomas convencionais e furtivos;
- Avaliar a atividade antiproliferativa da β-lapachona livre e encapsulada em lipossomas convencionais e furtivos em células de carcinoma de próstata (DU-145).

5. CAPÍTULO 1

A ser submetido ao Antimicrobial Agents and Chemotherapy

Conventional and stealth liposomes encapsulating β-lapachone or β-lapachone:2-hydroxypropyl-β-cyclodextrin: a comparative study of stability, *in vitro* release kinetics and antimicrobial activity

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Abstract

The aim of this study was to evaluate the stability, *in vitro* release kinetics and antimicrobial activity of conventional and stealth liposomes encapsulating β-lapachone (β-lap) or β-lap:2-hydroxypropyl-β-cyclodextrin inclusion complexes (β-lap:HPβ-CD). Liposomes were prepared using the hydration of thin lipid film and the *in vitro* release kinetics was performed using dialysis method. The *in vitro* antimicrobial activity against methicillin/oxacillin-resistant and -susceptible *Staphylococcus aureus* (MRSA and MSSA, respectively) was evaluated by microdilution method according to the *Clinical and Laboratory Standards Institute* (CLSI). The limposomes presented mean particle size ranged from 88.7 ± 1.5 nm to 132.6 ± 3.3 nm, polidispersity index ranged from 0.255 to 0.340 and drug encapsulation efficiency ranged from 97.4 ± 0.3 % to 99.2 ± 0.2%. The kinetics data showed that neutral stealth liposomes and positively charged conventional liposomes encapsulating β-lap or β-lap:HPβ-CD presented similar release profiles, with burst effect around 40% in the first 4 h and the release rate of β-lap were approximately 60% to 80%. β-lap and β-lap:HPβ-CD presented MICs from 1 to 2 mg/L for all microorganisms tested. MICs of the stable liposomes encapsulating β-lap or β-lap:HPβ-CD varied from 2 to 32 mg/L. Our results of β-lap and β-lap:HPβ-CD antimicrobial activities is comparable to linezolid, an antibiotic approved by the FDA and currently available in the market. Despite liposomes entrapped β-lap exhibit higher MIC values compared with the free molecule, these drug delivery systems have advantages that the free molecule could not provide. Emphasis should be done that β-lap as well as its liposomal formulations presented bacteriostatic and bactericidal activity. Thus β-lap, β-lap:HPβ-CD and the newly developed liposomes can significantly contribute to the treatment of infections caused by multiresistant bacteria, specially MRSA.

***Keywords*:** β-lapachone; 2-hydroxypropyl-β-cyclodextrin; inclusion complexes, liposomes; *in vitro* kinetics; antimicrobial activity.

1. Introduction

Global health data identified increasing levels of multiresistant bacteria. Infections that two or three decades ago were treated with therapeutic protocols routinely now appear with high mortality rates (1). The pandemic of multiresistant pathogens and their continuous distribution are irrefutable. The research and development of antibacterial agents do not provide a new arsenal of antibiotics, although various campaigns and international initiatives are encouraging the development of new technologies for production of antibacterial drugs through the integration of the pharmaceutical industry, universities and small businesses (2,3). According to the literature, the genus *Staphylococcus* is one of the principal human pathogens, which are related to a varied of hospital- and community-acquired infections worldwide (4,5). The treatment of bacterial infections is difficult and became a challenge as a result of the multiresistant bacteria (6,7). Vancomycin (VAN) was used successfully for many years for the treatment of infections caused by methicillin-resistant GRAM-positive bacteria but, due to the increase of resistance of these bacteria, some strains are resistant to VAN (8-10). Thus, a marked increase in GRAM-positive bacteria resistance against the traditional antimicrobial has become a worldwide concern and emphasizes the necessity of new drugs development.

β-lapachone (β-lap) is a compound which can be naturally extracted from the bark of *Tabebuia avellanedae*, popularly known as "Ipê roxo or Pau d'arco", a native tree of South America with high frequency in Brazil (11). This compound can also be produced by semi-direct chemical synthesis of lapachol, a yellow substance also obtained from the same plant species (12). β-lap has attracted increasing attention due to its different pharmacological activities with therapeutic potential, such as antibacterial (13-16), antifungal (17,18), trypanocidal (19), healing activity (20), as well as anticancer properties (21-24). Even β-lap presenting a significant pharmacological activity, their therapeutic application is limited due to its low water solubility (0.038 mg mL^{-1} or 0.16 mM) (25) and toxicity (26). This limitation had been overcome by forming inclusion complexes of β-lap with 2-hydroxypropyl-β-cyclodextrin (β-lap:HPβ-CD), which were able to increase in more than 300 fold the molecule solubility and it was observed in molecular modeling that β-lap was included in the cavity of HPβ-CD, with an intermolecular interaction energy of -23.67 kJ mol^{-1} (27).

Moreover, the encapsulation of β-lap well as β-lap:HPβ-CD in positively charged conventional liposomes has been carried out successfully (27). Liposomes are used to encapsulate

both hydrophobic molecules (bound to the lipid bilayer or 'dissolved' in the lipid phase) and hydrophilic molecules (encapsulated within the aqueous compartment). The type of the lipid used influences the formulation in order to obtain an appropriate liposomal system as a carrier for a given drug (28,29).

Depending on the lipids constituents, liposomes vesicles can have surface charge or not and this constitution can completely change formulation characteristics. Neutral conventional liposomes can be obtained by uncharged phospholipids, such as, phosphatidylcholine, and the cholesterol is include to increase the stability of phospholipid bilayers and decrease the leakage of liposomes (30). The positively charged conventional liposomes can be prepared by the addition of cationic lipid, such as stearylamine, to the above formulation (31,32). These conventional liposomes are know as interesting carriers to target drugs toward cells of immune systems, such as macgophages, once they are recognized by the mononuclear phagocyte system, and fastily removed from the circulation (33-35).

In this sense, PEGylated or "stealth" liposomes prolong circulation time, thus they are important systems for passive targeting. Stealth liposomes are obtained by different methods, including coating the liposomal surface with natural hydrophilic components or synthetic hydrophilic polymers, in particular polyethylene glycol (PEG) (36).

Therefore considering the mechanisms of bacterial resistance, the development of drug delivery systems, such as liposomes, for carrying antibiotics could increase their effectiveness on bacterial strains (37), because the liposomes and these microorganisms can interact directly by fusion processes leading to the release of the encapsulated antibiotic directly into the bacteria (38-40).

Thus, the present study was planned to prepare, characterize and compare the i*n vitro* antimicrobial activity of conventional and stealth liposomes encapsulating β-lap or β-lap:HPβ-CD.

2. Materials and Methods

2.1 Materials

β-lapachone, obtained from lapachol by a semi-synthetic route was supplied by Dr. Alexandre Góes (UFPE, Brazil). Cholesterol (CH), trehalose, stearylamine (SA), 2-hydroxypropyl-β-cyclodextrin (HPβ-CD) were purchased from Sigma-Aldrich (St Louis, USA);

soybean phosphatidylcholine (PC) (Lipoid S 100®) and distearoyl phosphatidylethanolamine-polyethylene glycol 2000 (DSPE-PEG2000) were obtained from Lipoid GMBH (Ludwigshafen, Germany). Solvents and other chemicals were supplied by Merck (Darmstadt, Germany).

2.2 Methodology

2.2.1 Preparation of conventional and stealth liposomes encapsulating β-lap or β-lap:HPβ-CD

The choice of lipid composition and drug/lipid ratio (1:28.5) used in this study was based on our previous studies. We have successfully entrapped β-lap and β-lap:HPβ-CD in positively charged conventional liposomes (SACL), leading to stable formulations (27). Based on these findings new conventional and stealth liposomal formulations were prepared with or without stearylamine encapsulating β-lap or β-lap:HPβ-CD named: positively charged conventional liposomes (SACL), neutral conventional liposomes (NCL), positively charged stealth liposomes (SASL) and neutral stealth liposomes (NSL) (Table 1). Unloaded and drug loaded liposomal formulations were prepared using the thin lipid film method (27,41). Briefly, lipids and β-lap were dissolved in a mixture of $CHCl_3$:MeOH (3:1 v/v) under magnetic stirring. The solvents were completely removed under pressure for 60 min (37 ± 1 °C, 80 rpm), resulting in a thin lipid film. This film was then hydrated with 10 mL of phosphate buffer solution (pH 7.4), obtaining multilamellar liposomes. The liposomal suspension was then sonicated (Vibra Cell, BRANSON, USA) at 200 W and 40 Hz for 300 s to form small unilamellar vesicles.

β-lap:HPβ-CD complexes were prepared using the *freeze-drying* technique and characterized as previously published (27) and the liposomes encapsulating the complexes were prepared as described above, but the aqueous phase was consisted of a phosphate buffer solution (pH 7.4) containing β-lap:HPβ-CD inclusion complex. After preparation liposomes were lyophilized at 4×10^6 Bars using 264 mM of trehalose as a cryoprotectant agent.

Table 1: Constituents of neutral and positively charged conventional and stealth liposomes encapsulating β-lap or β-lap:HPβ-CD inclusion complex.

Group of Formulations	Liposomes	Lipids (%mol)				Drugs (mg mL^{-1})	
		PC	DSPE-PEG	CH	SA	β-lap	β-lap:HPβ-CD
I	Unloaded-NCL	8	_	2	_	_	_
	β-lap-NCL	8	_	2	_	0.5	_
	β-lap:HPβ-CD-NCL	8	_	2	_	_	0.5
II	Unloaded-NSL	7.5	0.5	2	_	_	_
	β-lap-NSL	7.5	0.5	2	_	0.5	_
	β-lap:HPβ-CD-NSL	7.5	0.5	2	_	_	0.5
III	Unloaded-SASL	6.5	0.5	2	1	_	_
	β-lap-SASL	6.5	0.5	2	1	0.5	_
	β-lap:HPβ-CD-SASL	6.5	0.5	2	1	_	0.5

NCL= neutral conventional liposomes; NSL= neutral stealth liposomes; SASL= positively charged stealth liposomes; PC= phosphatidylcholine; DSPE-PEG= distearoyl phosphatidylethanolamine polyethylene glycol 2000; CH= cholesterol; SA= stearylamine.

2.2.2 Stability and characterization of conventional and stealth liposomes encapsulating β-lap or β-lap:HPβ-CD

Liposomes stability was evaluated using standard accelerated and long-term stability testing as described by Cavalcanti et al. (27). Liposomes were characterized in dispersion form and after redispersion of the lyophilized dosage form to verify the effect of lyophilization process on their stability after preparation and storage at 4 °C for one year. The analyzed parameters were: macroscopic appearance, pH variation, particle size (Ø), polydispersity index (PDI), zeta potential and drug encapsulation efficiency (%EE). The pH of liposome dispersions was measured in a digital pH meter (Bioblock Scientific 99622, Prolabo, Paris, France) at room temperature. The particle size and PDI were determined by photon correlation spectroscopy (Delsa™ Nano S Particle analyzer, Beckman-Coulter, CA, USA). The zeta potential was measured by dynamic light scattering method (Zetatrac NC-148, Microtrac, PA, USA). β-lap content was analyzed using UV spectroscopy (β-lap standard curve ranging from 0.5 to 3.0 μg

mL^{-1}). Drug encapsulation efficiency was determined after ultrafiltration/ultracentrifugation of liposomes using filtrate units (Amicon Ultra Centrifugal Filters, Millipore, MA, USA) and calculated as: % EE = $[(\beta\text{-lap}_{filtrate} - \beta\text{-lap}_{initial})/\beta\text{-lap}_{initial}] \times 100$.

2.2.2.3 In vitro release kinetics of β-lapachone from conventional and stealth liposomes

The *in vitro* release kinetics was performed to evaluate the release profile of β-lap from the liposomes developed in order to observed *in vitro* that are expected to ocuur *in vivo*, the maintenance of the plasma drug concentration constant. The release kinetics of β-lap or β-lap:HPβ-CD from conventional and stealth liposomes were analyzed using the dialysis technique against pH 7.4 phosphate buffer solution at 37 °C under sink conditions. Briefly, an aliquot of the liposomal formulation (1 mL) containing β-lap or β-lap:HPβ-CD was inserted into a dialysis membrane (MW cut-off 12,400), which was subsequently sealed and suspended in the release medium (150 mL). The system was maintained under magnetic stirring. In predetermined time intervals, 1 mL samples of the release medium were removed and the release medium was replaced. Samples were assayed for β-lap quantification at 256 nm using a standard curve of β-lap from 0.5 to 3 μg mL^{-1} concentrations. The kinetic data of β-lap from liposomal formulations were fitted according to an exponential model using the following equation: $M_t/M_\infty = (1 - k_1.e^{-k2t})$, where, M_t and M_∞ are the mass of the drug released at a determined time (t) and at an infinite time (t_∞) of the kinetic process, respectively; k_1 is a fitting constant, and k_2 is the kinetic rate constant.

2.3 In vitro antimicrobial activity

2.3.1 Microbial strains and culture media

The studied Gram-positive bacteria strains were: methicillin/oxacillin-resistant *Staphylococcus aureus* (MRSA - ATCC 33591), methicillin/oxacillin-susceptible *Staphylococcus aureus (*MSSA - ATCC 29213), hospital- and community-acquired MRSA. The microorganisms were cultured on Müeller-Hinton agar and allowed to grow for 24 h at 37 °C. Identification of *S. aureus* was performed by catalase and coagulase tests. MRSA strains were confirmed to be methicillin resistant by disk diffusion method with cefoxitin and oxacilin, as well as a screening using Müeller-Hinton agar supplemented with 4% NaCl and 6 μg/mL of oxacillin. Furthermore, PCR was performed for *mecA* gene detection.

2.3.2 Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) Determination

The *in vitro* antibacterial activity of β-lapachone, β-lap:HPβ-CD, conventional and stealth liposomes encapsulating β-lap or β-lap:HPβ-CD was evaluated by Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) determination. MICs and MBCs were determined by the standard broth microdilution method in Müeller-Hinton broth (MHB) according to Clinical and Laboratory Standard Institute (42). β-lap and β-lap:HPβ-CD were dissolved in dimethyl sulphoxide (DMSO)/MHB and vancomycin (VAN) and oxacillin (OXA) were dissolved in saline/MHB.

The 96-well microlitres plates were filled with 100 µL of MHB and then 100 µL of each sample by following a serial dilution to obtain different drug concentrations (0.25 to 256 mg/L). Ten microlitres of each bacterial suspension was then added, suitably diluted with the same broth to achieve a final bacterial concentration of 10^5 colony-forming units/mL in each well. Microplates were then incubated at 37 °C for 24 h. The positive control well was consisted of MHB with bacterial suspension and negative control well was consisted of MHB with the tested drug. DMSO, HPβ-CD and unloaded liposomes were also used to evaluate the possible effects of these constituents in the bacterial growth.

The MIC was defined as the lowest concentration of the drug that exhibited complete inhibition of microbial growth. The MBC was defined as the lowest concentration of the drug that resulted in more than 99.9% reduction of the initial bacterial inoculum. An aliquot of 0.02 mL of the well where the MIC results showed no bacterial growth was seeded in MHB without addition of drug, incubated at 37°C for 24 h and thus the MBC was determined evaluating no bacterial growth.

2.4 Statistical Analysis

The statistical analyses were performed by the one-way analysis of variance (ANOVA) followed by least significant difference test using GraphPad Prim 5 Demo ($p<0.05$).

3. Results

3.1 Stability and characterization of conventional and stealth liposomes encapsulating β-lap or β-lap:HPβ-CD

In this study conventional and stealth liposomes encapsulating β-lapachone or β-lap:HPβ-CD inclusion complex were prepared with or without the positively charged lipid stearylamine in order to evaluate the effect of the lipid constituents on the liposome properties (Table 1). All liposome formulations exhibited Tyndall effect characteristic of particles in the nanometer range. No statistically significant difference ($p<0.05$) in all evaluated parameters (encapsulation efficiency, particle size, polydispersity index, zeta potential and pH) was verified between formulations based on the same lipid constitution for each group (NCL, SASL and NSL), although there are statistically significant differences between the groups related to some parameters, such as particle size and zeta potential (Table 2).

The particle size and pH of neutral and positively charged conventional and stealth liposomes ranged from 88.7 ± 1.5 nm to 132.6 ± 3.3 nm and 7.4 to 7.7, respectively.

When compared the surface charge of SACL (+20 mV) (27) and SASL (0.23 mV) liposomes it was found that the stealth formulations presented own characteristics, since the PEG causes a conformational change on the surface of the liposomes neutralizing the positive charge of stearylamine (Table 2).

The polydispersity index of the formulations was less than 0.35, maintaining the same standard of the experiments with positively charged conventional liposomes encapsulating β-lap or β-lap:HPβ-CD, as previously reported (27). Related to the β-lap encapsulation efficiency, all liposomal formulations presented EE above 97%.

The liposomal formulations maintained stable after accelerated stability tests (horizontal mechanical stirring and centrifugation), maintaining its original micro and macroscopic features as well as pH, Ø and PDI, suggesting that the formulations would resist to stress conditions. After accelerated stability study, the Ø ranged from 82.3 ± 2.2 to 136.2 ± 2.4 nm, PDI ranged from 0.250 ± 0.01 to 0.390 ± 0.10 and pH ranged from 7.3 to 7.8.

Table 2: Characterization of neutral, positively charged, conventional and stealth liposomes encapsulating β-lap or β-lap:HPβ-CD inclusion complex.

Group of Formulations	Liposomes	EE (% ± S.D.)	Ø (nm ± S.D.)	PDI	Zeta potential (mV ± S.D.)	pH
I	Unloaded-NCL	_	133.3 ± 1.9[a]	0.282	+4.26 ± 0.27[c]	7.4
	β-lap-NCL	99.2 ± 0.2	130.8 ± 4.4[a]	0.311	+4.54 ± 0.62[c]	7.4
	β-lap:HPβ-CD-NCL	97.5 ± 1.1	132.6 ± 3.3[a]	0.274	+3.33 ± 0.54[c]	7.4
II	Unloaded-NSL	_	112.4 ± 1.9[b]	0.299	+0.25 ± 0.05[e]	7.4
	β-lap-NSL	98.8 ± 0.6	88.7 ± 1.5[b]	0.255	+0.23 ± 0.04[e]	7.4
	β-lap:HPβ-CD-NSL	98.1 ± 1.3	89.2 ± 6.4[b]	0.268	+0.22 ± 0.02[e]	7.4
III	Unloaded-SASL	_	100.3 ± 1.6[b]	0.286	-0.26 ± 0.01[e]	7.6
	β-lap-SASL	98.9 ± 0.4	105.7 ± 2.8[b]	0.308	+0.22 ± 0.03[e]	7.6
	β-lap:HPβ-CD-SASL	97.4 ± 0.3	105.7 ± 8.6[b]	0.340	+0.22 ± 0.02[e]	7.7

Liposome formulations after preparation dispersion form before lyophilization: NCL= neutral conventional liposomes; NSL= neutral stealth liposomes; SASL= positively charged stealth liposomes. EE= encapsulation efficiency; Ø= particle size; PDI= polydispersity index.

[a] and [b] represent statistically significant difference ($p<0.05$). [c] and [e] represent statistically significant difference ($p<0.01$). [d]and [c,e] represent statistically significant difference ($p<0.001$).

In the long term stability the neutral stealth liposomes encapsulating the β-lap or β-lap:HPβ-CD remained stable for 60 days in suspension form, i.e. showed no changes in its macro and microscopic appearance, or sudden change of pH, particle size, polydispersity index and encapsulation efficiency (Table 3). As already observed by our research group, positively charged conventional liposomes encapsulating β-lap or β-lap:HPβ-CD also mantained stable by 60 days in suspension form (27).

However, neutral conventional liposomes and positively charged stealth liposomes encapsulating the β-lap or β-lap:HPβ-CD were unstable after 8 days after storage in the colloidal dispersion form. The presence of β-lap crystals was observed by macroscopy and lipid agglomerate was visualized.

Table 3: Evaluation of long-term stability of neutral stealth liposomes encapsulating β-lap or β-lap:HPβ-CD inclusion complex.

	β-lap-NSL				β-lap:HPβ-CD-NSL			
Time (days)	EE (% ± S.D.)	Ø (nm ± S.D.)	PDI	pH	EE (% ± S.D.)	Ø (nm ± S.D.)	PDI	pH
0	98.8 ± 0.6	88.7 ± 1.5	0.255	7.4	98.1 ± 1.3	89.2 ± 6.4	0.268	7.4
15	98.7 ± 0.3	88.9 ± 4.2	0.334	7.5	98.0 ± 1.1	86.0 ± 1.2	0.268	7.4
30	97.1 ± 1.5	86.9 ± 3.6	0.250	7.5	97.6 ± 0.7	102.8 ± 1.9	0.278	7.4
60	98.6 ± 0.4	89.4 ± 5.1	0.223	7.5	97.5 ± 0.3	104.8 ± 2.6	0.304	7.5

Liposome formulations before lyophilization (dispersed form, storage at 4°C): SACL= positively charged conventional liposomes; NSL= neutral stealth liposomes. EE=encapsulation efficiency; Ø= particle size; PDI= polydispersity index.

Remarkable stability results were found for lyophilized SASL and NSL formulations stored at 4°C after 1 year. On the contrary, lyophilized NCL formulations were unstable presented high Ø and PDI values. The incorporation of SA in the stealth liposomes caused instability observed during the long term stability study; however the lyophilization process preserved the initial characteristics of the liposomes (Table 4).

Table 4: Characterization of neutral and positively charged conventional and stealth liposomes encapsulating β-lap or β-lap:HPβ-CD inclusion complex.

Group of Formulations	Liposomes	EE (% ± S.D.)	Ø (nm ± S.D.)	PDI	Zeta potential (mV ± S.D.)	pH
	Unloaded-NCL (t_0)	_	135.3 ± 2.4	0.382	+3.18 ± 0.7	7.6
	(t_{12})		139.0 ± 25.0	0.419	+3.76 ± 0.1	7.4
I	β-lap-NCL	96.1 ± 0.2	220.9 ± 4.4	0.311	+4.54 ± 0.62	7.6
		96.4 ± 0.7	274.9 ± 5.6	0.426	+3.00 ± 1.0	7.4
	β-lap:HPβ-CD-NCL	97.4 ± 0.3	142.9 ± 2.9	0.412	+3.12 ± 0.48	7.4
		97.6 ± 0.5	162.3 ± 5.6	0.521	+2.98 ± 0.86	7.3
	Unloaded-NSL	_	123.5 ± 2.4	0.310	+0.24 ± 0.01	7.4
			131.0 ± 2.5	0.312	+0.23 ± 0.02	7.4
II	β-lap-NSL	98.6 ± 0.7	98.1 ± 0.9	0.315	+0.22 ± 0.01	7.4
		98.1 ± 0.2	96.7 ± 0.4	0.305	+0.21 ± 0.01	7.4
	β-lap:HPβ-CD-NSL	98.2 ± 0.6	92.1 ± 3.4	0.299	+0.21 ± 0.02	7.4
		98.1 ± 0.3	96.4 ± 3.3	0.349	+0.21 ± 0.01	7.3
	Unloaded-SASL	_	110.6 ± 2.9	0.386	-0.20 ± 0.03	7.4
			116.8 ± 2.6	0.345	-0.17± 0.02	7.5
III	β-lap-SASL	98.1 ± 0.2	115.3 ± 11.1	0.310	+0.20 ± 0.04	7.4

	97.9 ± 0.4	110.3 ± 31.3	0.335	+0.20 ± 0.01	7.4
β-lap:HPβ-CD-SASL	97.9 ± 0.5	125.3 ± 9.3	0.350	+0.21 ± 0.03	7.5
	97.4 ± 0.2	111.4 ± 1.2	0.360	+0.20 ± 0.03	7.4

Redispersed lyophilized liposome formulations after preparation (t_0) and after storage at 4°C for 1 year (t_{12}): NCL= neutral conventional liposomes; NSL= neutral stealth liposomes SASL= positively charged stealth liposomes. EE= encapsulation efficiency; Ø= particle size; PDI= polydispersity index.

3.2 In vitro release kinetics of β-lapachone from conventional and stealth liposomes

In vitro release kinetics showed that the NSL encapsulating β-lap or β-lap:HPβ-CD presented a similar release profile of the SACL performed by Cavalcanti et al. (27), with burst effect around 40% in the first 4h that can be attribute to their smaller size (100 nm). It was observed a release rate of β-lap of approximately 60% to 80% in the first 24h (Fig. 1). The release kinetics of β-lap from the liposomes was fitted according to the exponential model $M_t/M_\infty = (1 - k_1.e^{-k2t})$. For the NSL encapsulating β-lap, M_∞ = 824.84 ± 19.0 μg, k_1 = 1.0034 ± 0.0355 and k_2 = 0.2186 ± 0.0161 was obtained and the release of β-lap from neutral stealth formulations encapsulating β-lap:HPβ-CD yielded M_∞ = 828.43 ± 19.64 μg, k_1 = 1.0797 ± 0.0586 and k_2 = 0.2165 ± 0.0203. The rate constants of 296.74 ± 6.6 μg/h and 253.09 ± 4.8 μg/h (Fig. 1, insert A) were calculated for NSL encapsulating β-lap and β-lap:HPβ-CD, respectively, considering the first 8 hours of the kinetics. Cavalcanti et al. (27) observed that the SACL encapsulating β-lap presented M_∞ = 583.43 ± 7.68 μg, k_1 = 0.9464 ± 0.0037 and k_2 = 0.2011 ± 0.0075 and the release of β-lap from SACL encapsulating β-lap:HPβ-CD yielded M_∞ = 609.53 ± 2.44 μg, k_1 = 1.0083 ± 0.0034 and k_2 = 0.2075 ± 0.0034. The rate constants of 183.95 ± 1.07 μg/h and 216.25 ± 7.5 μg/h (Fig. 1, insert B) were calculated for SACL encapsulating β-lap and β-lap:HPβ-CD, respectively.

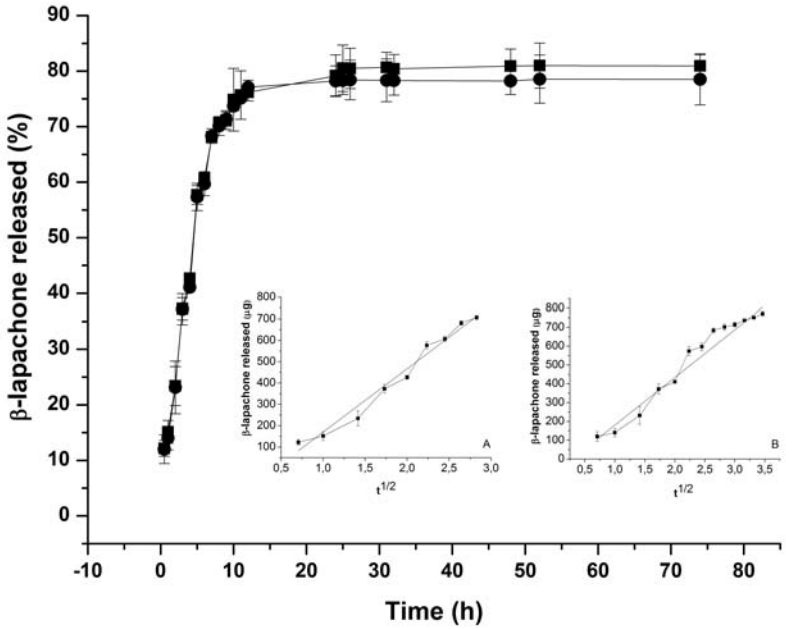

Fig. 1. Release kinetics of β-lapachone from liposomal formulations in pH 7.4 phosphate buffer solution at 37 °C: neutral stealth liposome encapsulating β-lap (■) and neutral stealth liposome encapsulating β-lap:HPβ-CD (●). Insert A: release of β-lap from neutral stealth liposome encapsulating β-lap (■). Insert B: release of β-lap from neutral stealth liposome encapsulating β-lap:HPβ-CD (■). Data are expressed as mean ± S.D. (n = 3).

The neutral conventional formulations and SASL encapsulating β-lap showed a similar release profile with burst effect around 40% in the first 2h and the release rate of β-lap were approximately 70% in the first 24h (Fig. 2). For the NCL encapsulating β-lap, M_∞ = 715.63 ± 3.61 μg, k_1 = 1.2673 ± 0.1706 and k_2 = 0.6004 ± 0.0874 was obtained and the release of β-lap from SASL encapsulating β-lap yielded M_∞ = 741.44 ± 6.57 μg, k_1 = 1.2349 ± 0.0556 and k_2 = 0.5574 ± 0.0319. The rate constants of 373.1 ± 12.65 μg/h (Fig. 2, insert A) and 503.00 ± 30.71 μg/h (Fig. 2, insert B) were calculated for NCL and SASL encapsulating β-lap, respectively.

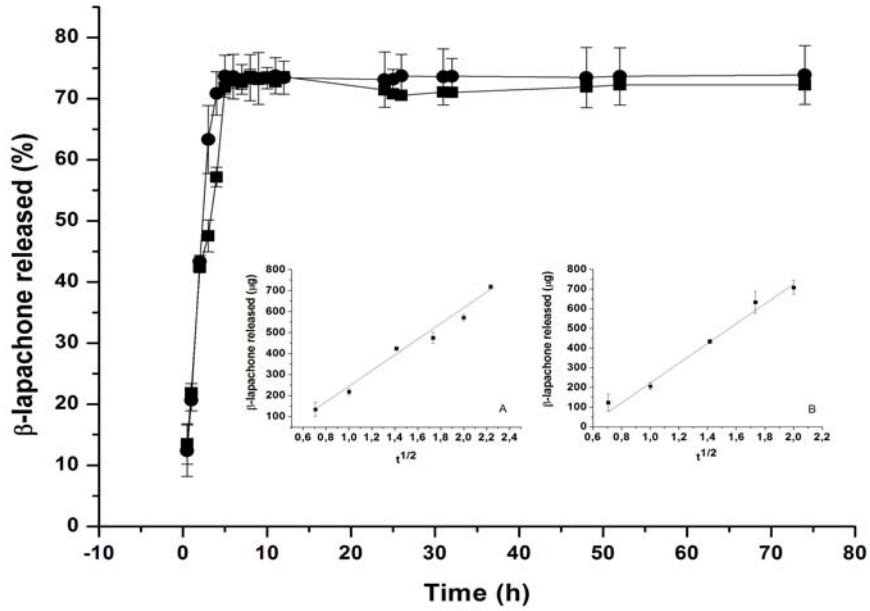

Fig. 2. Release kinetics of β-lapachone from liposomal formulations in pH 7.4 phosphate buffer solution at 37 °C: neutral conventional formulations encapsulating β-lap (■) and positively charged stealth liposome encapsulating β-lap (●). Inserts A and B: release of β-lap from neutral conventional formulations encapsulating β-lap and positively charged stealth liposome encapsulating β-lap, respectively. Data are expressed as mean ± S.D. (n = 3).

These same formulations encapsulating β-lap:HPβ-CD presented similar profile with burst effect around 40% in the first 5h and release rate of the release rate of β-lap:HPβ-CD were approximately 50% in the first 24h (Fig. 3). For the NCL encapsulating β-lap:HPβ-CD M_∞ = 515.74 ± 2.09 μg, k_1 = 1.0392 ± 0.0443 and k_2 = 0.3191 ± 0.0223 was obtained and the release of β-lap from SASL encapsulating β-lap:HPβ-CD yielded M_∞ = 486.63 ± 3.64 μg, k_1 = 1.0659 ± 0.0390 and k_2 = 0.3694 ± 0.0196. The rate constants of 216.33 ± 10.27 μg/h (Fig. 3, insert A) and 210.95 ± 21.15 μg/h (Fig. 3, insert B) were calculated for NCL and SASL encapsulating β-lap:HPβ-CD, respectively.

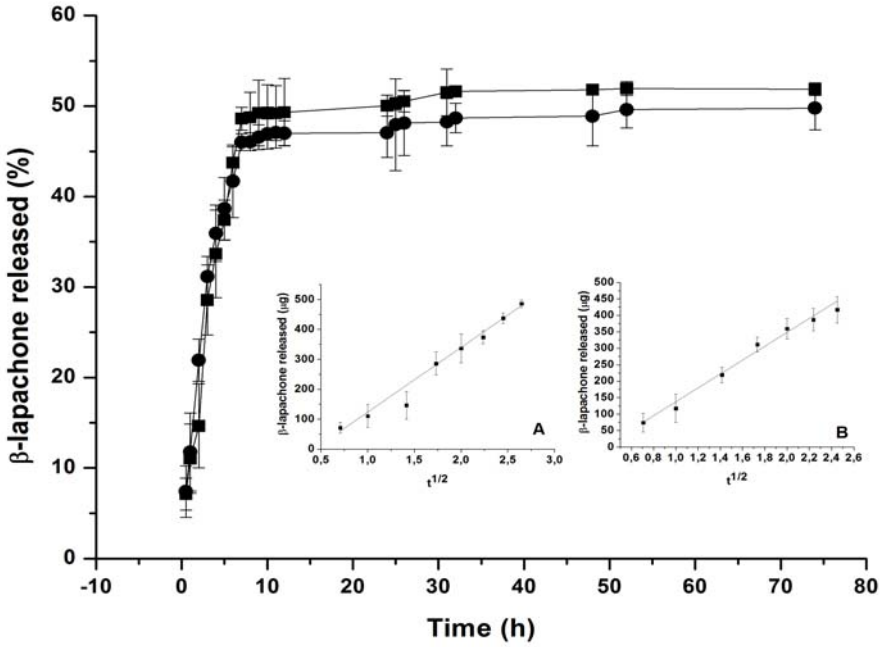

Fig. 3. Release kinetics of β-lapachone from liposomal formulations in pH 7.4 phosphate buffer solution at 37 °C: neutral conventional formulations encapsulating β-lap:HPβ-CD (■) and positively charged stealth liposome encapsulating β-lap:HPβ-CD (●). Inserts A and B: release of β-lap from neutral conventional formulations encapsulating β-lap:HPβ-CD and positively charged stealth liposome encapsulating β-lap:HPβ-CD, respectively. Data are expressed as mean ± S.D. (n = 3).

3.3. In vitro antimicrobial activity of liposome formulations containing β-lap or β-lap:HPβ-CD inclusion complex

The *in vitro* antibacterial activity assesses the resistance or susceptibility profile of each microorganism facing active compounds in relation to the standard drugs oxacillin (OXA) and vancomycin (VAN). As a result, MRSA and MSSA are resistant and susceptible to OXA (MIC >256 mg/L and MIC = 0.25 mg/L), respectively. β-lap and β-lap:HPβ-CD inclusion complex presented equal MIC (1-2 mg/L) and MBC (1-16 mg/L) values for microorganism strains, confirming that the complexation of β-lap with the cyclodextrin do not inhibited its antimicrobial activity (Table 5). It should be emphasized that the hospital-acquired MRSA strain showed a relative resistance to β-lap and β-lap:HPβ-CD (MBC=16 mg/L), but MRSA ATCC 33591 and community-acquired MRSA presented MBC of 2 mg/L.

It is important to mention that no bacteria growth inhibition occurred when exposed to negative controls, i.e. DMSO, HPβ-CD and unloaded liposomes, suggesting that none of these samples are able to induce bacterial death (data not shown).

As can be seen in Table 5, β-lap-NCL and β-lap:HPβ-CD-NCL proved to be inactive or less active compared to the other liposomal formulations against the microorganisms analyzed in this study. As expected, conventional and stealth β-lap:HPβ-CD-loaded liposomes exhibit MIC and MBC values higher than conventional and stealth β-lap-loaded liposomes.

Table 5: Antimicrobial activity of β-lapachone and β-lap:HPβ-CD inclusion complex encapsulated in conventional and stealth lipossomes against susceptible and multiresistant Gram-positive *S. aureus* strains.

Compound/ Liposome Formulations	MSSA (ATCC 29213)		MRSA (ATCC 33591)		MRSA (community strain)		MRSA (hospital strain)	
	MIC (mg/L)	MBC (mg/L)	MIC (mg/L)	MBC (mg/L)	MIC (mg/L)	MBC (mg/L)	MIC (mg/L)	MBC (mg/L)
β-lap	1	1	2	2	2	2	2	16
β-lap-SACL	2	2	4	4	4	8	4	16
β-lap-NCL	16	16	16	16	>256	>256	>256	>256
β-lap-SASL	2	4	4	4	4	4	4	16
β-lap-NSL	2	4	4	4	4	8	4	16
β-lap:HPβ-CD	1	1	2	2	2	2	2	16
β-lap:HPβ-CD-SACL	4	8	16	16	8	16	16	32
β-lap:HPβ-CD-NCL	64	64	64	64	>256	>256	>256	>256
β-lap:HPβ-CD-SASL	4	4	16	16	8	16	16	32
β-lap:HPβ-CD-NSL	4	8	16	16	8	16	16	32

MSSA = methicillin/oxacillin-susceptible *S. aureus*; MRSA = methicillin/oxacillin-resistant *S. aureus*; MIC= minimum inhibitory concentration; MBC= minimum bactericidal concentration; Liposome formulations in dispersed dosage form containing β-lap or β-lap:HPβ-CD inclusion complex: SACL= positively charged conventional liposomes; NCL= neutral conventional liposomes; SASL= positively charged stealth liposomes; NSL= neutral stealth liposomes.

It should be emphasized that in this study the MBC/MIC ratios were ≤ 4 showing that β-lap as well as the encapsulated β-lap or β-lap:HPβ-CD inclusion complex in stable liposomes, presented bacteriostatic and bactericidal activities.

4. Discussion

In the present study we investigated the stability, *in vitro* release kinetics and i*n vitro* antimicrobial activity of conventional and stealth liposomes encapsulating β-lap or β-lap:HPβ-CD inclusion complex. Firstly, it was founded that all formulations presented particle size in the nanometer range, favoring to tumor drug direction (43) or treatment of infection process (33). Moreover, stealth liposomes (NSL = 99.8 ± 11 nm and SASL = 103.9 ± 2.5 nm) showed particle size slightly smaller than conventional liposomes (NCL = 132.2 ± 1.05 nm and SACL = 106.9 ± 3.8 nm). These results corroborated previously experiments performed by Muppidi et al. (37) that encapsulated vancomycin into conventional and stealth liposomes (254 ± 147 nm and 245 ± 139 nm, respectively) as well as that carried out by Kim et al. (44) that encapsulated tissue plasminogen activator into conventional and stealth liposomes (172.8 ± 4.9 nm and 149.8 ± 4.1nm, respectively).

As already reported by Fatouros et al. (45) and confirmed in our early studies with positively charged conventional liposomes encapsulating β-lap or β-lap:HPβ-CD (27), the present results revealed no statistically significant difference (ANOVA, $p<0.05$) between the zeta potential values for formulations in the same group (Table 2), suggesting that whatever the drug is encapsulated (aqueous cavity or lipid bilayer) the zeta potential is not changed.

Rathore and Ghosh (46) encapsulated ricin, a heterodimeric cytotoxic protein that has been used as an anticancer agent, into stealth liposomes containing neutral, positively and negatively phospholipid and they observed that the presence of PEG on the surface of the particles became all these types of liposomes electrically neutral. Probably the PEG caused an electrostatic shield making the formulations neutral even in the presence of charged phospholipid. Furthermore, the presence of PEG on the surface of liposomes protects the polar head group of phospholipids and reduces the electrophoretic mobility of the colloidal vesicles (47).

The data of PDI (<0.35) of the liposomal formulations indicated that they are homogeneous in size, since PDI varies from 0 to monodispersed samples and around 1 for polydispersed samples (48).

The higher encapsulation efficiency (> 97%) of β-lap or β-lap:HPβ-CD into liposomes provided an advantage, since Blanco et al. (49) demonstrated that micelles of PEG-PLA-containing β-lap presented encapsulation efficiency below 50%. The neutral conventional, and neutral or positively charged stealth liposomes encapsulating β-lap or β-lap:HPβ-CD showed EE% similar to that observed in the experiments of Cavalcanti et al. (27), where the EE of β-lap into positively charged conventional liposomes encapsulating β-lap or β-lap:HPβ-CD was also above 97%.

As expected, the lyophilization process increased the shelf-life of liposomal formulations and preserved them in dried form to maintain the same particle size distribution, to avoid vesicle aggregation and to retain of encapsulated compound within the liposome after hydration. These results corroborate what has been already suggested by El-Nesr et al. (50) and supported by Cavalcanti et al. (27) that the concentration of the cryoprotectant agent (trehalose=264 mM) is appropriate to avoid vesicle aggregation or instability of the formulations developed.

When compared the release kinetics of the free molecule and β-lap encapsulated into liposomes, it was observed that all the formulations presented burst effect lower than the release of the free β-lap (approximately 88% in the first 3 h). The NSL and the SACL encapsulating β-lap or β-lap:HPβ-CD, NCL and SASL encapsulating β-lap:HPβ-CD presented the rate constants lower than that observed in the release kinetics of free β-lap (350.14 ± 60.59 μg/h). However, the NCL encapsulating β-lap or β-lap:HPβ-CD were not stable in the long term stability study, as well as after the redispersion of the lyophilized form. As a result, these formulations are not considered as suitable candidates for further studies.

Related to the *in vitro* antimicrobial activity, as reported in the literature, the MIC_{OXA} >256 mg/L for MRSA strains (4,51) is higher than that found for β-lap or β-lap:HPβ-CD (2 mg/L). MSSA ATCC 29213, MRSA ATCC 33591and community-acquired MRSA presented MIC_{VAN} equal to 0.5 mg/L. Emphasis should be done that hospital-acquired MRSA exhibit a reduced vancomycin susceptibility (MIC = 2 mg/L). Additionally, Howden et al. (52) had reported the reduced MRSA vancomycin susceptibility (MIC = 2-4 mg/L) and infections caused by this microorganism have been reported from a number of countries but the effective therapy is unknown.

Our results of β-lap and β-lap:HPβ-CD antimicrobial activities is comparable to linezolid, an antibiotic approved by the FDA and currently available in the market, which presents MIC values of 1-3 mg/L and 2 mg/L against MRSA and MSSA, respectively (9,53).

The activity of various analogues and derivatives of β-lap and lapachol against the same microorganisms used in our study had already been analyzed. Lapachol derivatives presented antimicrobial activity against MSSA ATCC 29213 with MICs of 128 mg/L (13), which are much higher than that of β-lap and β-lap:HPβ-CD (MIC = 2 mg/L).

Clinical MRSA strains were susceptible to naphthoquinone derivatives, however presented resistance to some commercially available antimicrobial agents, except vancomycin (55). Most of the naphthoquinone compounds showed MIC values between 30 and 125 mg/L while other derivates presented MICs higher than 500 mg/L. The MBC values were higher than 500 mg/L, showing that the naphthoquinones tested only exhibit bacteriostatic activity against clinical MRSA strains. Our results again proved the efficacy of the antimicrobial activity of free or encapsulated β-lap in liposomes.

The antimicrobial activity of β-lap and β-lap:HPβ-CD was still evident by comparing the MICs of the α-lapachone derivatives against MRSA ATCC 33591 and MSSA ATCC 29213 found in the experiments performed by Machado et al. (56). In that study, the MICs ranged from 31.2 to 62.5 mg/L, but the MICs observed in our experiments showed that β-lap and β-lap:HPβ-CD are at least 30-fold more active than those derivates against the same ATCC bacterial strains.

The inactivity of the β-lap encapsulated in NCL can be explained by the fact that this formulation is unstable in the presence of Müeller-Hinton broth medium, presenting lipid precipitation. This NCL instability may lead to the drug-phospholipid micelle or assembly occurrence, which could interfere with the drug-bacteria contact, consequently hinding the drug activity. The β-lap encapsulated in stable liposomes (SACL, NSL, SASL) showed MIC ranging from 2 to 8 mg/L for MSSA, 4 to 16 mg/L for MRSA, 4 to 16 mg/L for community-acquired MRSA and 4 to 32 mg/L for hospital-acquired MRSA. Furthermore, the lower activity of β-lap:HPβ-CD-loaded liposomes compared to β-lap-loaded liposomes can be explained by the fact that in the first formulations the β-lap:HPβ-CD is encapsulated in the inner aqueous phase of liposomes. Thus, the drug-cyclodextrin complex should be broken and the drug must cross the lipid membrane of liposomes to act.

An important factor to be considered in liposome drug carriers is the ability of these systems to interact with the bacterial cell wall. In this way, some liposome constituents such as SA and PEG may influence the activity profile of drugs encapsulated in these formulations. SA is a positively charged lipid that could interact with the negative charged surface of Gram-positive bacteria, which is conferred by teichoic acid molecules (57-60). Consequently, the liposomes containing SA are electrostatically attracted by bacteria and thus these formulations may act more efficiently. The attractiveness of bacteria for surfaces coated with PEG is not fully understood. Most studies affirm that the PEG coating of surfaces can prevent the bacteria adhesion on these surfaces (61,62). However, other studies have been reported that PEG is not completely effective for preventing the adhesion of bacteria in PEG-coated surfaces (63,64). In fact, rubber-PEG coated do not inhibited *S. aureus* biofilm formation on its surface, differently of the same surface coated with PEG copolymers, evidencing that PEG has a medium antifouling activity (64).

Although the encapsulation of β-lap or β-lap:HPβ-CD in liposomes do not reduce MIC values compared with the free molecule, β-lap or β-lap:HPβ-CD-loaded liposomes were more active than the naphthoquinone and lapachol (MIC= 30-125 and 128 mg/L, respectively) against MSSA ATCC 25922 and ATCC 29213 (13,56), respectively, and lapachol (MIC= 256 mg/L) against MRSA strains (13).

Our results also corroborates with that reported by Omria et al. (65) on the evaluation of the antimicrobial activity of free and encapsulated tobramycin and netilmicin in liposomes against MSSA ATCC 29213. The MICs of free drugs were lower than that of encapsulated in liposomes. However, the liposomes have the *in vivo* advantages such as the enhancement of the drug biodistribution, targeting to infected cells while protecting surrounding healthy tissue, the reduction of side effects, thus providing a greater patient complience for the therapy (36). In this study, we developed conventional liposomes that are designed to act more effectively against pathogens internalized by the macrophages (33-35), and stealth liposomes that are maintained for a longer period of time in the circulation and thus can affect other infected organs/tissues (36).

Finally, the bacteriostatic and bactericidal activity of the free and encapsulated β-lap in liposomes is a remarkable finding. Taken into accounting, nowadays, only few drugs are available in the market for the treatment of multiresistant microorganisms, β-lap and our newly developed liposomal formulations encapsulating this compound can contribute significantly to the treatment of the infections caused by multiresistant bacteria, especially MRSA.

5. Conclusion

The conventional and stealth β-lap or β-lap:HPβ-CD-loaded liposomes were prepared and characterized as nanometer vesicles. The both free and encapsulated β-lap in liposomes had bacteriostatic and bactericidal activity against community- and hospital-acquired MRSA strains. β-lap and their newly developed liposomal formulations presented antimicrobial activity comparable to the antibiotics currently available in the market to the treatment of infectious diseases, especially against MRSA strains.

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6. CAPÍTULO 2

A ser submetido ao International Journal of Antimicrobial Agents

In vitro antifungal activity of β-lapachone and β-lapachone:2-hydroxypropyl-β-cyclodextrin-loaded conventional and stealth liposomes against *Cryptococcus neoformans*

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Abstract

Invasive infections especially caused by *Cryptococcus neoformans* are frequently in imunocompromised patients, and the treatment is not always easily established. Drugs encapsulated into drug delivery systems such as liposomes could provide an option for treating cryptococcosis. The aim of this study was thus to evaluate the *in vitro* antifungal activity of β-lap or β-lap:HPβ-CD encapsulated into conventional and stealth liposomes against clinical isolates of *C. neoformans* using the microdilution method according to the *Clinical and Laboratory Standards Institute* (CLSI). β-lap and β-lap:HPβ-CD showed the ability to inhibit the fungi growth (MIC = 3.91 mg/L and MFC = 7.81 mg/L), but the stable liposomes were more efficient since they presented MIC and MFC values lower than the free drugs (MIC < 2 mg/L and MFC < 4 mg/L). Furthermore, free and encapsulated β-lap exhibit fungistatic and fungicidal activity. This is a remarkable result, taken into account that fluconazole, an antifungal currently available in the market, has only fungistatic activity. Transmission electron microscopy showed the adhesion of liposomes on the capsule of fungal cells, possibly facilitating the drug release and allowing it to exert its fungicidal activity. In agreement with other reports, the encapsulation of antimicrobial and antifungal drugs into liposomes can improve the antimicrobial activity of these drugs. Thus, the findings of this study suggest that β-lap-loaded liposomes may be a prospective strategy for treating invasive cryptococcosis.

***Keywords*:** β-lapachone; 2-hydroxypropyl-β-cyclodextrin; liposomes; *Cryptococcus neoformans*; *in vitro* antifungal activity.

1. Introduction

In the last years, many infectious diseases emerged and caused concern in the community, especially the opportunistic invasive fungal infections [1], such as cryptococcosis [2]. This infection is caused by *Cryptococcus* species mainly *C. neoformans* and its major neurological manifestation is the meningitis. Currently, this mycosis has assumed an important role in the cause of morbidity and mortality among immunocompromised patients with the acquired immunodeficiency syndrome (AIDS) or receiving cancer chemotherapy [3-6]. However, cryptococcosis has acquired additional concern to the community in general, since cases of cryptococcosis in immunocompetent patients have been already reported [7-9].

Unfortunately, there are few drugs available for the treatment of cryptococcosis infections and some of them still have limitations, such as toxicity and unfavorable pharmacokinetic profile, which restrict their therapeutic use. Considering these limiting factors, it is imperative to develop new antimicrobials or therapeutic strategies for treating these infections [10].

β-lapachone (β-lap) is a naturally compound extracted from the bark of *Tabebuia avellanedae* [11,12] or produced by a semi-synthesis of lapachol, also obtained from the same plant species [13]. β-lap presents several pharmacological activities with therapeutic potential [14-18], including antifungal activity [19,20], but it is a poorly water soluble (0.038 mg mL^{-1} or 0.16 mM) compound [21] and causes toxicycity [22]. However, the β-lap solubility has been overcomed by forming inclusion complexes of β-lap with 2-hydroxypropyl-β-cyclodextrin (β-lap:HPβ-CD), as well as its encapsulation in liposomes [23] was proposed for improving its biological activity.

Liposomes are aqueous vesicles surrounded by one or more lipid bilayers that can encapsulate drugs allowing their administration by reducing toxicity and improving bioavailability. The succesuful liposomal encapsulation of an antifungal agent is the case of amphotericin B (AmBisome®) that is used in therapy of systemic fungal infections in immunocompromised patients with improved efficace and reduced nephotoxicity [24,25].

Depending on the therapeutic purpose, we can use conventional liposomes that can interact with the cells of the mononuclear phagocytic system (MPS), such as macrophages, once these drug delivery systems are more rapidly recognized by them [26]. On the other hand, if there is a necessity to prolonged the permanence of liposomes in the blood circulation, stealth liposomes

are a promising alternative. In this case the surface of these systems is modified by coating with hydrophilic polymers such as polyethylene glycol (PEG) [26,27].

Based on these findings, β-lapachone (β-lap) or β-lap:2-hydroxypropyl-β-CD inclusion complex (β-lap:HPβ-CD) encapsulated in liposomes are offered as a new strategy for the treatement of cryptococcosis. In the present study, we propose therefore the evaluation of the *in vitro* antifungal activity of β-lap or β-lap:HPβ-CD encapsulated in liposomes against *C. neoformans* strains obtained from the cerebrospinal fluid (CSF) of immunocompromised patients.

2. Materials and Methods

2.1 Materials

β-lapachone, obtained from lapachol by a semi-synthetic route was supplied by Dr. Alexandre Góes from the Federal University of Pernambuco (Brazil). The liposomes were prepared as described by Cavalcanti et al. [23]. The following formulations were tested: neutral conventional liposomes (NCL), positively charged conventional liposomes (SACL), neutral stealth liposomes (NSL) and positively charged stealth liposomes (SASL) containing β-lap or β-lapachone:2-hydroxypropyl-β-cyclodextrin (β-lap:HPβ-CD) inclusion complex. Roswell Park Memorial Institute (RPMI) medium and morpholine-propanesulfonic acid (MOPS) were purchased from Sigma-Aldrich (St Louis, USA). Sabouraud dextrose agar (SDA) was purchased from Difco Laboratories (Detroit, Michigan, USA). Microtiter plates were obtained from TPP (Trasadingen, Switzerland) and 0.22 μm membranes were purchased from Millipore (Darmstadt, Germany). Solvents and other chemicals were supplied by Merck (Darmstadt, Germany).

2.2 Methodology

2.2.1 Fungal strains

Fifteen *C. neoformans* strains obtained from the cerebrospinal fluid (CSF) of immunocompromised patients were analyzed in this study. The samples were supplied by Pernambuco Neurological Diagnosis Service and clinically processed for mycological diagnosis using standard methods. The biologic of the fungal species as already performed by Dra. Neves research group (data not shown). Cultures were prepared using Sabouraud Dextrose Agar-SDA (Difco) with chloramphenicol (50 mg mL^{-1}) and incubated at 35°C in an aerobic atmosphere for

72 h. *Candida parapsilosis* (ATTC 22019) and *C. krusei* (ATCC 6528) strains were used as quality controls.

2.2.2 Antifungal susceptibility testing

2.2.2.1 Minimal Inhibitory Concentration (MIC) and Minimal Fungicide Concentration (MFC) Determination

Antifungal activities of β-lap, β-lap:HPβ-CD, conventional and stealth liposomes encapsulating β-lap or β-lap:HPβ-CD against *Cryptococcus neoformans* strains were determined by the standard broth microdilution method [28]. The *Cryptococcus neoformans* strains were cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) buffered to pH 7.0 with 0.165 M MOPS (Gibco BRL). β-lap and β-lap:HPβ-CD were dissolved in dimethyl sulphoxide (DMSO)/RPMI.

The *C. neoformans* strains were maintained on SDA medium and incubated at 35°C. Suspensions of the isolates were prepared, and their density was adjusted according to the 0.5 MacFarland standards for 90% transmission using a spectrophotometer. The volume of the inoculums was adjusted to 5.0 mL of sterilized saline and was further diluted in RPMI 1640 to a concentration of 2–5 x 10^3 cells/mL.

The 96-well microlitres plates were filled with 100 µL of RPMI 1640 broth and then 100 µL of each sample by following a serial dilution to obtain different drug concentrations (0.24 to 125 mg/L). One hundred microlitres of each fungi suspension was then added and the microplates were then incubated at 35 °C for 74 h to determine the Minimal Inhibitory Concentration (MIC). The growth inhibition was demonstrated by visual observation and the MICs were determined as the lower concentration able to inhibit fungal growth in 100% [28].

The positive control well consisted of RPMI 1640 broth with fungal suspension and negative control well consisted of RPMI broth with the tested drugs. DMSO, HPβ-CD and unloaded liposomes were also used to evaluate the possible effects of these constituents in the fungal growth. Amphotericin B (AmB) and Fluconazole (FLZ) were used as reference drugs.

To determine the minimal fungicide concentrations (MFCs) the content of the wells that showed 100% growth inhibition was transferred onto SDA in Petri dishes. The dishes were then incubated at 35°C for 3 days to determine the fungal viability. The MFC was confirmed by the absence of fungal growth.

2.3 Statistical Analysis

The statistical analyses were performed by the one-way analysis of variance (ANOVA) followed by least significant difference test using GraphPad Prim 5 Demo ($p<0.05$).

2.4 Transmission electron microscopy

The transmission electron microscopy (TEM) was performed following the methodology adapted from Nicolosi et al. [29]. Briefly, a drop of β-lap or β-lap-loaded liposomes was mixed with *C. neoformans* for 1 h at 37°C and fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer solution (pH 7.2) at 4°C for 1 h. A drop of the above suspension was layered on a Holley Carbon-coated copper grids and they were then negatively stained by dipping in 1% (w/v) PTA (pH 6.8) for 30 s. Observations were carried out using a FEI Morgagni 268(D) transmission electron microscope (FEI Company, Eindhoven, The Netherlands).

3. Results

3.1 Antifungal susceptibility testing

The antifungal activity of β-lap and β-lap:HPβ-CD encapsulated into liposomes (MIC and MFC) is exhibit in Table 1. β-lapachone and β-lap:HPβ-CD showed fungistatic activity against C. neoformans strains in concentrations ranged from 1.95 to 3.91 mg/L and 1.95 to 7.81 mg/L, respectively, and fungicidal activity ranged from 3.91 to 7.81 mg/L.When β-lap or β-lap:HP-β-CD were loaded in conventional (SACL) and stealth liposomes (NSL and SASL), remarkable MIC and MFC values were measured (2 mg/L for MIC and 4 mg/L for MFC). The amphotericin B and fluconazole showed fungistatic activity in concentrations ranging from 0.03 to 1 mg/L and 2 to 32 mg/L, respectively. On the other hand, fluconazole showed only fungistatic activity, while amphotericin B presented fungicidal activity in concentrations from 0.03 to 2 mg/L against *C. neoformans* isolates.

Table 1: Antifungal activity of β-lap-loaded conventional and stealth liposomes against *Cryptococcus neoformans*.

Cryptococcus neoformans strains	β-lap		β-lap:HPβ-CD		β-lap-SACL		β-lap:HPβ-CD-SACL		β-lap-NCL		β-lap:HPβ-CD-NCL		β-lap-SASL		β-lap:HPβ-CD-SASL		β-lap-NSL		β-lap:HPβ-CD-NSL	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
	mg/L		mg/L		mg/L		mg/L		mg/L		mg/L		mg/L		mg/L		mg/L		mg/L	
155	1.95	3.91	1.95	3.91	0.49	0.98	0.49	0.98	0.98	3.91	0.98	0.98	0.49	0.98	0.49	0.98	0.49	1.95	0.49	1.95
123	3.91	3.91	3.91	7.81	0.98	1.95	0.98	1.95	1.95	7.81	1.95	7.81	0.98	3.91	0.98	0.98	1.95	1.95	1.95	1.95
5811	3.91	7.81	3.91	3.91	1.95	1.95	0.98	1.95	1.95	15.63	0.98	1.95	0.98	3.91	0.98	3.91	0.98	3.91	0.98	3.91
126	3.91	7.81	3.91	7.81	0.98	1.95	1.95	1.95	1.95	7.81	1.95	1.95	0.98	1.95	0.98	1.95	0.98	3.91	0.98	3.91
5813	1.95	7.81	3.91	7.81	0.98	1.95	0.98	1.95	0.98	3.91	1.95	7.81	0.98	3.91	0.98	1.95	0.98	3.91	0.98	3.91
5814	3.91	7.81	3.91	7.81	0.98	1.95	0.98	1.95	1.95	15.63	1.95	7.81	0.98	3.91	0.98	3.91	1.95	3.91	0.98	3.91
151	3.91	7.81	3.91	7.81	1.95	1.95	0.98	1.95	1.95	7.81	1.95	7.81	0.98	1.95	0.98	3.91	0.98	3.91	0.98	3.91
124	3.91	7.81	3.91	7.81	0.98	1.95	1.95	1.95	1.95	3.91	1.95	7.81	0.98	0.98	3.91	3.91	0.98	3.91	0.98	3.91
186	1.95	7.81	1.95	3.91	0.98	1.95	0.98	0.98	0.98	3.91	0.98	0.98	0.98	0.98	0.98	0.98	0.49	1.95	0.49	1.95
132	3.91	3.91	7.81	7.81	0.98	1.95	0.98	1.95	3.91	15.63	3.91	7.81	3.91	3.91	0.98	3.91	0.98	3.91	0.98	3.91
5822	3.91	7.81	3.91	7.81	0.98	0.98	0.98	1.95	0.98	3.91	0.98	1.95	0.98	3.91	0.98	1.95	0.98	3.91	1.95	3.91
5809	3.91	7.81	3.91	7.81	0.98	1.95	0.98	1.95	1.95	15.63	1.95	7.81	0.98	3.91	0.98	1.95	0.98	3.91	1.95	3.91
5810	3.91	7.81	3.91	7.81	0.98	1.95	0.98	1.95	1.95	15.63	1.95	1.95	0.98	1.95	0.98	3.91	0.98	3.91	0.98	1.95
5820	3.91	7.81	3.91	7.81	0.98	1.95	0.98	1.95	1.95	15.63	1.95	7.81	0.98	3.91	0.98	3.91	0.98	3.91	0.98	3.91
5819	1.95	7.81	7.81	7.81	0.98	1.95	0.98	1.95	3.91	15.63	3.91	7.81	3.91	3.91	3.91	3.91	1.95	1.95	1.95	1.95
MIC/MFC	3.91^{a}/7.81^{d}		3.91^{a}/7.81^{d}		0.98^{b}/1.95^{e}		0.98^{b}/1.95^{e}		1.95^{c}/15.63^{f}		1.95^{c}/7.81^{d}		0.98^{b}/3.91^{g}		0.98^{b}/3.91^{g}		0.98^{b}/3.91^{g}		0.98^{b}/3.91^{g}	
%MIC/MFC	73%/80%		73%/ 80%		80%/87%		80%/87%		60%/47%		60%/60%		80%/60%		80%/57%		67%/ 73%		67%/67%	

SACL= positively charged conventional liposomes; NCL= neutral conventional liposomes; SASL= positively charged stealth liposomes; NSL= neutral stealth liposomes; β-lap:HPβ-CD= β-lapachone:2-hydroxypropyl-β-cyclodextrin . [a] and [b,c] represent statistically significant difference ($p<0.001$). [c] and [b] represent statistically significant difference ($p<0.05$). [d] and [e,f] represent statistically significant difference ($p<0.001$). [e] and [f] represent statistically significant difference ($p<0.001$). [g] and [d,e] represent statistically significant difference ($p<0.01$).

4. Discussion

Cryptococcosis is a fungal infection that exhibit very serious consequences, especially in immunocompromised patients. However there are few therapeutic options for the treatment of invasive fungal infections, in parallel the resistance is another fact that contributes to the failures in the treatment of cryptococcoosis [31,32]. Several articles proposed the encapsulation of antimicrobial and antifungal agents in drug delivery systems such as liposomes, due to their hability to reduce drug toxicity and improve therapeutic efficacy [29,33,34]. In this study it was possible to evaluate the antifungal activity of β-lap or β-lap:HPβ-CD encapsulated in conventional and stealth liposomes.

The routinely used drugs for the treatment of cryptococcosis are amphotericin B and fluconazole, which exhibit MIC values lower than the MICs of β-lap, but these reference drugs showed some drawbacks related to their side effects, such as fever, chills, tachycardia, hypertension, nausea, vomiting, tachypnea, nephrotoxicity, anemia, gastrointestinal intolerance, hepatotoxicity, teratogenicity and hypersensitivity [39,40]. Furthermore, fluconazole has only fungistatic activity while free or encapsulated β-lap in liposomes showed both fungistatic and fungicidal activity (MFC/MIC values were ≤ 4).

There was no statistically significant difference between the MIC values of β-lap or β-lap:HPβ-CD encapsulated in SACL, NSL and SASL. However, β-lap-NCL showed a statistically significant difference compared to all other preparations, as well as the free molecule. β-lap or β-lap:HPβ-CD encapsulated in SACL, NSL and SASL showed remarkable antifungal activity compared to β-lap and β-lap:HPβ-CD-NCL as well as the free molecule (Table 1).

The particle size (Ø), index polydispersity (PDI), the surface charge and drug encapsulation efficiency, as well as the lipid constituents of liposomes are important factors that can influence the drug release and biological effectiveness of these drug delivery systems [35,36]. As expected, conventional and stealth liposomes without stearylamine exhibit neutral surface charge. As already reported, positively charged pegylated liposomes exhibit neutral surface because the positive charges of stearylamine are covered by PEG molecules [37,38].

If we observed the lipid constituents of these liposomes, the formulations that have SA, PEG or SA/PEG are associated with a high antifungal activity among these systems. *C. neoformans* have capsular polysaccharide and melanin that conferred a negative charge for the fungi cell wall [41-44]. Probably for this reason the positive charges of the formulations

containing SA were attracted by the negative of the fungi, thus can occur the adhesion of liposomes in fungi wall and consequently drug release. It is important emphasize that this electrostatic attraction of positively charged constituents by the negative charges of microorganims has been reported in the literature. Liu et al. [45] and Wang et al. [46] described a potential activity of cationic antimicrobial peptide (CAMP) for the treatment of microbial infections that can be explained by the capacity of CAMP interact with the negatively charged outer leaflet of the cytoplasmic membranes of microbes. Furthermore the presence of PEG can also have provided a better activity due to a possible attraction of PEG with the fungi wall constituents, but there are no reports about this fact.

Remarkable results found herein should be emphasized in comparison with amphotericin B encapsulated into liposomes (Ambisome®), which has been used for the treatment of various fungal infections [47,48]. In fact, MIC and MFC values found for β-lap or β-lap:HPβ-CD encapsulated in SACL, NSL and SASL (0.98 mg/L and 1.95 mg/L, 0.98 mg/L and 3.91 mg/L, 0.98 mg/L and 3.91 mg/L, respectively) were lower than that of Ambisome® (MIC= 8 mg/L and MFC >8 mg/L) as reported [49].

Its described here promising data, since we developed conventional (SACL) and stealth liposomes (NSL and SASL) with high capacity to eliminate *C. neoformans in vitro*. These two options of treatment are interesting because *C. neoformans* can be located inside macrophages/monocytes [50-52], thus conventional formulations can act more effectively, but these fungi may be located outside of macrophages [53-56], then the stealth formulations would be a better therapeutic choice. Further these liposome formulations may also be used prophylactically in patients with suspect of cryptococcosis, but does not yet have a confirmed diagnosis since *C. neoformans* may remain dormant for many years in macrophages [57].

It is important to mention that the *C. neoformans* do not exhibit growth inhibition when exposed to DMSO, HPβ-CD and unloaded liposomes (data not shown), proving that none of them is able to induce fungi death, although some lipid constituents seem to be a possible determinant in binding/adhesion of the vesicles in the fungi wall.

TEM analysis was carried out to confirm the interaction of β-lap-loaded liposomes with the fungi cell wall. Initially, it is important to emphasize that β-lap presenta acicular characteristic crystals in the powder form [23]. TEM negative staining analysis (Fig. 1a and b) shows morphological alterations of *C. neoformans* treated with β-lap. As shown in Fig 1c and d, fungus

are surrounded by liposomes and it is evident some modifications related to the interaction of the vesicles with the fungal cell, indicating that cell wall alterations can be the possible antifungal mechanism of action of β-lap in *C. neoformans*, however more studies are needed to be performed to confirm this hypothesis.

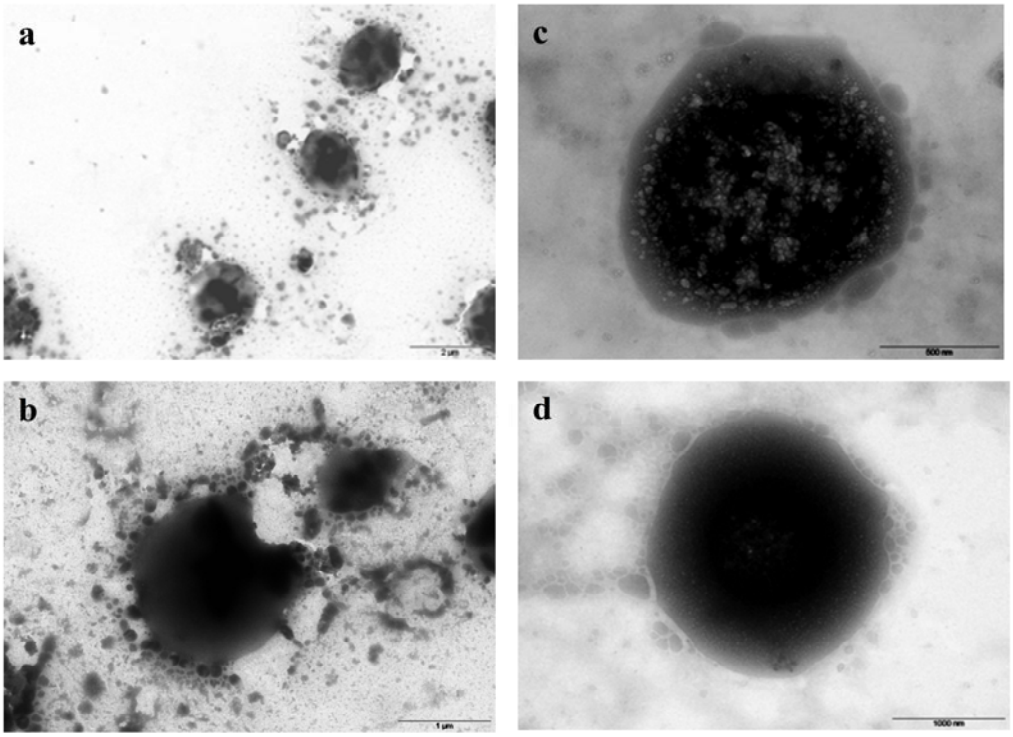

Fig. 1. Transmission electron microscopy of β-lapachone-*Cryptococcus neoformans* interaction (a and b) and fusion of the liposomes with *C. neoformans* (c and d). Bars: 2 μm (a), 1 μm (b), 500 nm (c) and 1000 nm (d).

Evidences of liposome-microorganisms interaction had already been reported by Nicolosi et al. [29]. These authors performed TEM and SEM of Gram-negative bacteria exposed to liposomes containing vancomycin, where they could observe that liposomes fuse with the outer membrane of these bacteria and can release the drug into the periplasmic space.

The fungal capsule is a cellular component that has many biological properties, but the most important function is the inhibition of phagocytosis, thereby conferring resistance to this microorganism to the host immune system action [58-60]. Thus the liposomes could be an

alternative to overcome this limitation since they can act in the fungal cell, releasing the drug directly into the fungal cell or near to its surface.

Thus, the present results offered the liposomes encapsulating β-lap an important candidate to the anticryptococcosis therapy. In fact, *in vivo* studies previous performed revealed that β-lap can be used in the treatment of mice with disseminated cryptococcosis, since this drug reduced the *C. neoformans* population 10,000 times in vitals such as lungs and brain, although β-lap presented localized toxic effects [20]. Further, the liposomes may overcome this limitation of the β-lap toxicity and improve its antifungal activity.

5. Conclusion

The present findings suggest that β-lap or β-lap:HPβ-CD encapsulated in liposomal formulations exhibit *in vitro* antifungal activity against *C. neoformans* and they can be offered as an alternative to the treatment of cryptococcosis. However, further *in vivo* studies should be carried out.

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7. CAPÍTULO 3

A ser submetido ao International Journal of Oncology

Antiproliferative activity of β-lapachone-loaded liposomes against human prostate carcinoma cells

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Abstract

The aim of this study was to evaluate the *in vitro* activity of conventional and stealth liposomes encapsulating β-lap or β-lap:HPβ-CD inclusion complex against human prostate carcinoma cells (DU-145). Liposomes were prepared using the hydration of lipid film and the citotoxicity was performed by MTT assay. Morphological alterations in DU-145 cells caused by the treatment with β-lap or the liposomes were evaluated by confocal microscopy. The β-lap or β-lap:HPβ-CD encapsulated into liposomes presented equal or slightly higher IC_{70} when comparing to free free molecule (2.5 μM), evidencing that β-lap presented so active that the liposomes or 1.3 to1.8-fold more active that the systems developed. In our experiments it was found that low concentrations of β-lap (<1 μM) enhanced the proliferation in DU-145 with peaked at 0.5 μM at 121.2 ± 2.1%. The cytotoxic effects of all liposomes formulations were found to be time- and concentration dependent. The confocal microscopy revealed that the DU-145 cells presented the first evidence of the decrease in their viability and morphological alterations such as mitotic figures, condensation of chromatin, nucleus fragmentation, apoptotic bodies and giant cells with the increase of the β-lap concentration in solution or encapsulated into liposomes. The cell line DU-145 appears to have low internalization efficiency of conventional and stealth liposomes, therefore our formulations showed equal or slightly less activity than the free drug. It is know that, *in vivo*, conventional and stealth liposomes can enhance biodistribution, while protecting surrounding healthy tissue. In that way the encapsulation of β-lap or β-lap:HPβ-CD into conventional or stealth liposomes described in this work could optimize the therapeutic application of this promising drug. Furthermore, this study encourages future research related to pharmacokinetic, biodistribution and the *in vivo* antitumor activity of the liposomes developed.

***Keywords*:** β-lapachone; 2-hydroxypropyl-β-cyclodextrin; liposomes; cytotoxicity, DU-145 cells.

1. Introduction

Cancer is already included as the second cause of death in the population, preceded only by cardiovascular diseases. In recent decades, prostate and breast cancer have emerged as one of the most common diseases and are considered the leading cause of cancer death in men and women, respectively (1). Despite improvements in cancer treatment, the actual chemotherapy regimens have limitations, such as a narrow therapeutic index which does not allow the administration of an appropriate amount of a drug aimed pharmacological effectiveness. In addition, conventional treatment (radiotherapy and chemotherapy) do not ensure that normal cells are not affected by non-selective drugs (2). Therefore, the development of new effective therapeutics with minimal side effects is the objective study of the scientific community in the area of oncology.

β-lapachone (β-lap) is a semi-synthetic compound, obtained from lapachol, a natural *ortho*-naphthoquinone extracted from the bark of *Tabebuia avellanedae*, an indigenous tree originated in South America, especially in Brazil. It has been shown that β-lap has a variety of pharmacological effects (3,4), which the main highlight is its significant cytotoxic effect by inducing apoptosis in human cancer cells, in particular in prostate cells (5-8), lung cells (8,9), pancreas cells (10,11), breast cells (12,13) and ovaries cells (14). Several studies have suggested that β-lap could directly target topoisomerases and inhibit its activity (15,16) without causing any damage to DNA (11,17). The mechanism of action is independent of caspases and p53 status (12), as well as this molecule has a higher selectivity against cancer cells with high expression of the enzyme NAD(P)H:quinone oxidoreductase-1 (NQO1) (6).

Some patents reported the antitumor activity of β-lap (18,19) and indicated the molecule as a potential candidate for anti-cancer therapy (19,20). However, the therapeutic use of β-lap still presents limitations, mainly due to its low water solubility (0.16 mM) and toxicity (10). Consequently, it is then necessary devices or systems to increase their solubility as well as an efficient strategy for β-lap delivery. Some studies have suggested the use of systems that can enhance the solubility and the bioavailability of β-lap, in this context some authors have been developed inclusion complex of β-lap:HPβ-CD (21-23), PEG–PLA polymer micelles encapsulating β-lap (9) and conventional liposomes entrapped β-lap and β-lap:HPβ-CD (23). The results related to inclusion complex indicate that the complexation may offer an advantage in improving the bioavailability of the antitumor agent. Nasongkla *et al* (21) showed that the

formation of β-lap inclusion complex with β-CD or HPβ-CD significantly increased the drug solubility, besides showing similar activity compared to the pure molecule. Cunha-Filho *et al* (22) evaluated the compatibility of β-lap with solid excipients, such as a variety of cyclodextrins, and the physico-chemical properties showing that this molecule may interact with HPβ-CD, but they did not evaluate the antiproliferative activity of the complex developed. In 2007, Blanco *et al* (9) evaluated the activity of free β-lap and encapsulated in PEG-PLA micelles against lung cancer cells (H596), breast cancer cells (MDA-MB-231) and human prostate carcinoma cells (DU-145). In DU-145, there was less than 10% loss in cell survival after administration of β-lap micelles, thus showing a certain resistance of this cell line to β-lap micelles. Therefore, the encapsulation of inclusion complex in controlled release systems such as liposomes (24), able to modulate *in vitro* and *in vivo* cleavage of the drug:CD, contributing to improvement of the pharmacokinetic profile of the drugs (25,26). In this context, Cavalcanti *et al* (23) previously developed inclusion complex of β-lap:HPβ-CD and encapsulated into liposomes, but the antiproliferative activity of these systems had not yet been performed. Thus, the aim of this study was to evaluate the i*n vitro* activity of conventional and stealth liposomes encapsulation β-lap or β-lap:HPβ-CD against human DU-145 prostate carcinoma cells.

2. Materials and Methods

2.1 Materials

Pure β-lapachone, obtained from lapachol by a semi-synthetic route was supplied by Dr. Alexandre Góes (UFPE, Brazil). Cholesterol (CH), trehalose, stearylamine (SA), 2-hydroxypropyl-β-cyclodextrin (HPβ-CD) and RPMI 1640 medium were purchased from Sigma-Aldrich (St. Louis, USA); soybean phosphatidylcholine (PC) (Lipoid S 100®) and distearoylphosphatidylethanolamine-polyethylene glycol 2000 (DSPE-PEG2000) were obtained from Lipoid GMBH (Ludwigshafen, Germany). Human DU-145 prostate carcinoma cells were obtained from the American Type Culture Collection (ATCC). Fetal bovine serum (FBS), Vybrant® MTT Cell Proliferation Assay Kit (V-13154), 4'6-diamidino-2-phen-ylindole (DAPI) and acridine orange were purchased from Invitrogen Life Technologies (Gaithersburg, USA). Solvents and other chemicals were supplied by Merck (Darmstadt, Germany).

2.2 Methodology

2.2.1 Preparation and characterization of conventional and stealth liposomes encapsulating β-lap or β-lap:HPβ-CD

Preliminary studies conducted by our research group (23) developed positively charged conventional liposomes encapsulating β-lap or β-lap:HPβ-CD with drug/lipid ratio of 1:28.48. In this report we prepared neutral conventional liposomes, positively charged stealth liposomes and neutral stealth liposomes without drug, encapsulating β-lap or β-lap:HPβ-CD (Table 1) and analyze the *in vitro* activity of these liposomes against DU-145. The liposomes were prepared using the thin lipid film method (27). Briefly, lipids and β-lap were dissolved in a mixture of $CHCl_3$:MeOH (3:1 v/v) under magnetic stirring. The solvents were completly removed under pressure for 60 min (37 ± 1 °C, 80 rpm), resulting in a thin lipid film formation. This film was then hydrated with 10 ml of phosphate buffer solution (pH 7.4), obtaining multilamellar liposomes. The liposomal suspension was then sonicated (Vibra Cell, BRANSON, USA) at 200W and 40 Hz for 300 s to form small unilamellar liposomes.

β-lap:HPβ-CD complex were prepared using the *freeze-drying* technique according to Cavalcanti *et al* (23) and the liposomes encapsulating β-lap:HPβ-CD were prepared as described above, but the aqueous phase was consisted of a phosphate buffer solution (pH 7.4) containing β-lap:HPβ-CD inclusion complex.

The liposomes were evaluated using parameters such as, particle size, pH, zeta potential and β-lap encapsulation efficiency, as previously described (23).

2.2.2 Cell Culture

DU-145 cells were obtained from the American Type Culture Collection (ATCC) supplied by Dra. M. Aparecida Nagai. DU-145 were maintained in RPMI 1640 medium (Sigma Chemical Corporation) supplemented with 100 $U.ml^{-1}$ penicillin, 100 μg/ml streptomycin, 0.25 $\mu g.ml^{-1}$ fungizon, and 10% fetal bovine serum (FBS). The cells were plated into 25 cm^2 plastic tissue culture flasks and maintained at 37 °C in a humidified 95% air and 5% CO_2 atmosphere until a confluence of 70%–80% was reached and they were then trypsinized to obtain a suspension containing single cells. After, the cell suspensions were then seeded with 5% FBS at a density of 1×10^4 cells/well in 96-well microtiter plates.

2.2.3 Cytotoxicity assay

For all experiments, cells were plated, allowed at least 24 h to initiate log-phase growth, and then exposed to β-lap, β-lap or β lap:HPβ-CD-loaded liposomes at 0, 0.5, 1.5, 2.5, 3.5 and 5 μM concentrations for 24 h, reaching a final volume of 200 μl. HPβ-CD and unloaded liposomes were used as negative control. The cell viability was measured using the MTT assay. In this test, after 24 h of treatment, the medium was removed, new fresh medium (100 μl) was added to each well as well as 10 μl of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution. The cells were then incubated at 37 °C for 4h. After incubation time, 100 μl of 0.01 M SDS-HCl solution was added to each well and incubated at 37 °C for 16 h in a humidified chamber. Finally, the absorption was measured at 570 nm in a microplate reader (Biotrack II, Amersham Biosciences, United Kingdom). The citotoxicity was expressed as the concentration required to inhibit 70% of cell proliferation (IC_{70}).

A time-dependent cytotoxicity of β-lap, β-lap or β lap:HPβ-CD-loaded liposomes against DU-145 cells was also performed. The cells were plated, allowed at least 24 h to initiate log-phase growth, and then exposed to β-lap, β-lap or β-lap:HPβ-CD-loaded liposomes at 1.5 μM concentrations for 24 h, 48 h, 72 h and 96 h. After every 24 h of exposure, drug-containing media were removed and the treatment was repeated. The cell viability was measured using the MTT assay as described above.

2.2.4 Statistical Analysis

The statistical analyses were performed by the one-way analysis of variance (ANOVA) followed by least significant difference test using GraphPad Prim 5 Demo ($p<0.05$).

2.2.5 Confocal microscopy

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In these studies, the cell suspensions were seeded in eight-well chamber slides (Thermo Fisher Scientific, USA) as described above and exposed to β-lap, positively charged conventional liposomes encapsulating β-lap and positively charged conventional liposomes encapsulating β-lap:HPβ-CD at doses of 0.25 and 1.5 μM for 24 h. The cells were also exposed to negative controls, to be exact DMSO, buffer phosphate and liposomes without drug. After 24 h of treatment, the cells were washed with PBS and add 4% paraformaldehyde for 20 min.

Subsequently, the cells were incubated with acridine orange to a final concentration of 8.5 μg.ml^{-1} and DAPI to a final concentration of 5 μg ml^{-1} for 10 minutes. Then, they were washed twice with PBS and then 20 μl of glycerol was placed in each well, finally the coverslip was placed over the blade. A Zeiss LSM Meta 510 scanning confocal microscope was used for immunofluorescence analysis and image capture. The cells were seeded in 5% FBS or 5% charcoal-dextran FBS, which is a steroid hormone and growth factors-free serum.

3. Results and Discussion

3.1 Conventional and stealth liposomes encapsulating β-lap or β-lap:HPβ-CD

Both conventional and stealth liposome encapsulating β-lap or β-lap:HPβ-CD were successfully prepared by thin lipid film hydration followed by sonication. The choice of lipid composition and lipid/cholesterol ratio used in this study was based on our previous studies (23). The pH of all formulations ranged from 7.4 to 7.7 and the systems developed exhibited particle size in the nanometer scale, below 150 nm, ideal for tumor therapy since these systems will be administered intravenously (28). The encapsulation efficiency of conventional and stealth liposomes encapsulating β-lap or β-lap:HPβ-CD was above 96%, thus showing high encapsulation of the molecule in the systems developed, to be precise a proper drug/lipid ratio. Regarding the zeta potential, the conventional and stealth formulations without SA presented neutral surface charge as expected. The conventional formulations containing SA had a positive surface charge that was conferred by the presence of the SA, a positively charged lipid, however stealth formulations containing SA exhibited neutral surface charge. This event has already been observed and can be explained by the attachment of PEG on the surface of liposomes neutralizes the electrical properties of all types of liposomes (neutral, positively charged, negatively charged) (29). Vono *et al* (30) also observed that the presence of PEG on the surface of liposomes protects the polar head group of phospholipids and reduces the electrophoretic mobility of the colloidal vesicles, neutralizing the charge of liposomes. Moreover, stealth liposomes encapsulating β-lap or β-lap:HPβ-CD had no demonstrable significant impact on principal characteristics of the conventional liposomes, such as encapsulation efficiency, particle size, zeta potential or pH.

3.2 Antiproliferative activity of β-lap-loaded liposomes against DU-145 cells

Most studies of *in vitro* activity of β-lap against several cancer lines showed an IC_{50} equal to or above 2 μM, where this molecule has apoptotic activity, but few reports were acknowledged about the activity of this molecule at low doses. In our experiments it was found that low concentrations of β-lap (<1 μM) enhanced the proliferation in DU-145 with peaked at 0.5 μM at 121.2 ± 2.1%. Kung *et al* (31) evaluating the cytotoxicity of low doses of β-lap (0.01-1 μM) against different normal human cells, such as keratinocytes (HEKn and XB-2), fibroblasts (3T3 and HS68) and endothelial cells (HUVEC and EAhy926), also observed that β-lapachone promote cell proliferation up 110% for all cell lines tested (133% for 3T3 cells, 145% for EAhy926 cells and HUVEC cells, 196% for HEK cell, 125% for XB-2 cells and 113% for HS68 cells). It is remarkable that regardless whether the cell is normal or carcinogen the β-lap at low doses stimulates cell proliferation, thereby β-lap should be carrefully used in therapy to avoid cancer cell proliferation. These data emphasize the β-lap encapsulation into drug delivery systems, since they maintaining the drug plasmatic concentration can avoid toxic levels, as well as the subtherapeutic levels, which could stimulates cell proliferation.

In the present report, we compared the activity of conventional and stealth liposomes encapsulating β-lap or β-lap:HPβ-CD under identical *in vitro* conditions. The β-lap or β-lap:HPβ-CD-loaded liposomes presented equal or slightly higher IC_{70} compared to the free molecule, evidencing that β-lap presented so active that the liposomes or 1.3 to1.8-fold more active than the systems developed (Table 1). Roth *et al* (32) encapsulated doxorubicin (DOX) and topotecan (TPT) in stealth liposomes and evaluated the cytotoxicity of these systems against human prostate carcinoma cells used in our study. In their experiments it was possible to observed that DOX and TPT presented more *in vitro* activity than that of the stealth liposomes (TPT is 1.2-fold more active than the stealth liposomes and DOX is 9-fold more active than the same type of liposomes). As been already reported (32), our results also suggested that probably the level of uptake varied depending on some parameters, such as, the cell lines and specific liposomal drugs.

It was also possible also to observed that most liposomes showed equal antiproliferative activity with IC_{70} value of 3.3 μM, indicating that there was no statistical difference significant (ANOVA) in the liposomes cytotoxicity activity ($p<0.05$). It is interesting emphasize that the cells cancer membranes have a negative charge due to the higher expression of the anionic molecules with the mucins *0*- glycosides which acid character is due to the presence of the

carboxylic grups of the sialic acid residues and the sulfate groups (33,34). Thus, the presence of SA in the formulations probably influence the interaction between liposomes and cells membranes and thus the nanosystems could have been inserted in the lipid bilayer of the cell, destabilizing it and causing lysis or cell death. Moreover, it should be noted that, as expected, the unloaded liposome formulations do not showed *in vitro* toxicity (data not shown).

Table 1: IC_{70} values of free β-lap and β-lap or β-lap:HPβ-CD-loaded liposomes.

Liposome formulations	IC_{70} values (μM)
β-lap	2.5[a]
β-lap-SACL	3.3[b]
β-lap:HPβ-CD-SACL	2.5 [a]
β-lap-NCL	3.3[b]
β-lap:HPβ-CD-NCL	3.3[b]
β-lap-SASL	3.3[b]
β-lap:HPβ-CD-SACL	4.6[c]
β-lap- NSL	3.3[b]
β-lap:HPβ-CD-NSL	3.3[b]

[a] and [b,c] represent statistically significant difference ($p<0.05$). [b] and [c] represent statistically significant difference ($p<0.01$).

In Figs. 1 and 2 is possible observed that free β-lap, conventional or stealth formulations encapsulating β-lap or β-lap:HPβ-CD exhibit time-dependent cytotoxicity against DU-145, with growth inhibition around 35% in the first 48h and 80% in 96h of drugs exposure.

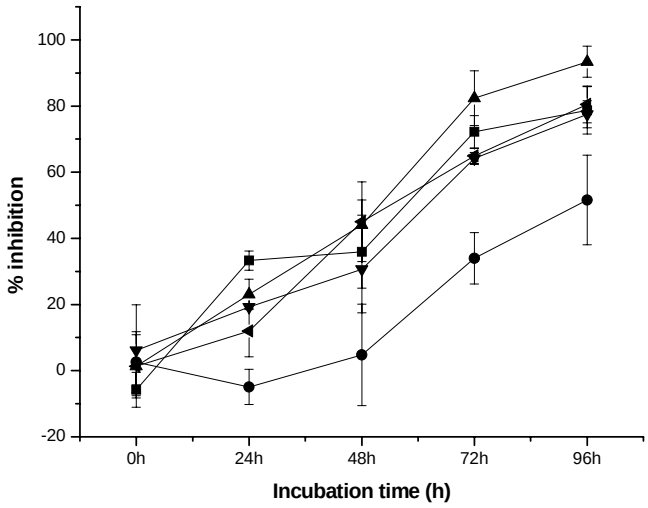

Fig. 1. Time-dependent cytotoxicity of free β-lap, β-lap or β-lap:HPβ-CD-loaded conventional liposomes against DU-145 cells. Free β-lap (■), neutral conventional encapsulating β-lap (▼) and neutral conventional encapsulating β-lap:HPβ-CD (◄), positively charged conventional encapsulating β-lap (●), positively charged conventional encapsulating β-lap:HPβ-CD (▲). β-lap concentration was 1.5 μM. Data are expressed as mean ± S.D. (n = 3).

Its important emphasize that in general the stealth liposomes exhibit cytotoxicity slightly higher when compared to the conventional formulations, although this diference was not statistically significant. Moraes *et al* (35) entrapped a boronated compound *o*-carboranylpropylamine (CPA) into conventional and stealth liposomes and evaluated the cytotoxicity of the free CPA as well as these formulations against glioblastoma multiforme cell line (SK-MG-1). They also observed that in the first exposure periods equivalent cell growth inhibition was obtained with the use of conventional and stealth liposomes.

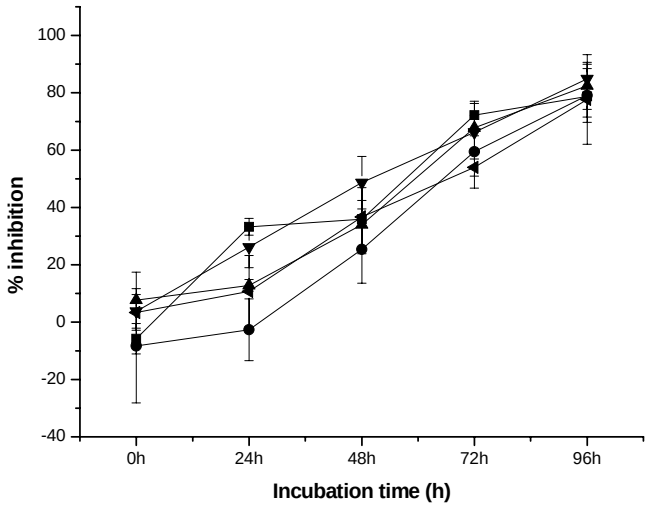

Fig. 2. Time-dependent cytotoxicity of free β-lap, β-lap or β-lap:HPβ-CD-loaded stealth liposomes against DU-145 cells. Free β-lap (■), neutral stealth encapsulating β-lap (▼) and neutral stealth encapsulating β-lap:HPβ-CD (◄), positively charged stealth encapsulating β-lap (•), positively charged stealth encapsulating β-lap:HPβ-CD (▲). β-lap concentration was 1.5 μM. Data are expressed as mean ± S.D. (n = 3).

3.3 Confocal microscopy analysis

The confocal microscopy was carried out to investigate the possible alterations in cell morphology caused by the treatment with β-lap or positively charged conventional liposomes encapsulating β-lap or β-lap:HPβ-CD. In this study the cells were cultured in 5% FBS (Fig. 3) or 5% charcoal-dextran FBS (Fig. 4). In both cases it was observed that while the concentration of β-lap or β-lap encapsulated into liposomes was increased the cells presented the first evidence of the decrease in their viability and morphological alterations such as mitotic figures, condensation of chromatin, fragmentation of nuclei, membrane blebbing, apoptotic bodies and giant cell (Fig. 3 and Fig. 4).

In Figs. 3a and 4a as well as Figs. 3b and 4b the DU-145 cells were treated with free β-lap in the concentrations of 0.25 and 1.5 μM, respectively. In these cases it was possible to observed nucleus fragmentation (Fig. 3a), pronounced membrane blebbings (Fig. 3b), nucleus

fragmentation, mitotic figures and giant cells (Fig. 4a) furthermore in Fig. 4b the giant cell becomes stronger.

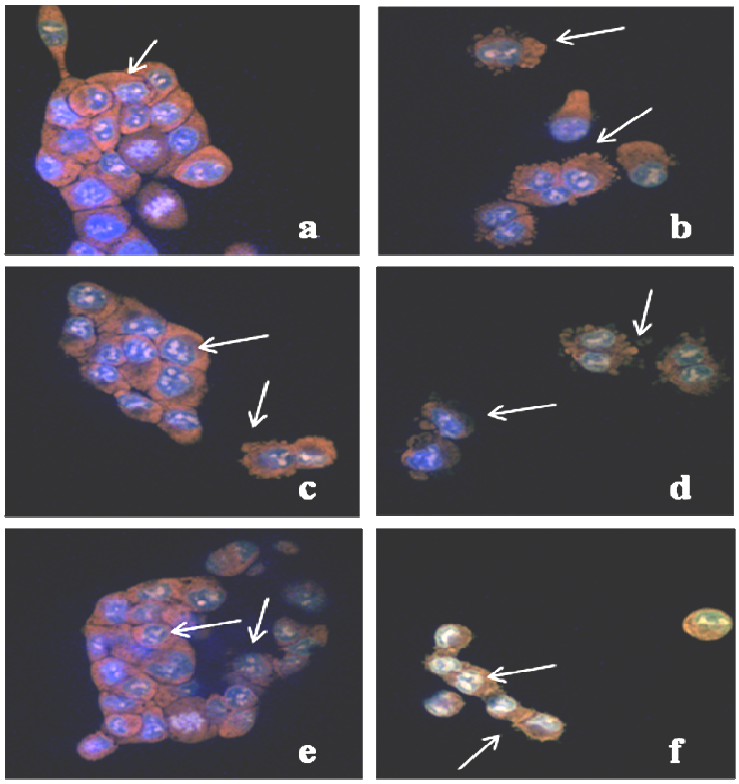

Fig. 3. Confocal microscopy of DU-145 cells seeded in 5% FBS treated with β-lap, positively charged conventional liposomes encapsulating β-lap or β-lap:HPβ-CD: a (cells treated with 0.25 μM of β-lap), b (cells treated with 1.5 μM of β-lap), c (cells treated with 0.25 μM of positively charged conventional liposomes encapsulating β-lap), d (cells treated with 1.5 μM of positively charged conventional liposomes encapsulating β-lap), e (cells treated with 0.25 μM of positively charged conventional liposomes encapsulating β-lap:HPβ-CD), f (cells treated with 1.5 μM of positively charged conventional liposomes encapsulating β-lap:HPβ-CD).

In Figs. 3c and 4c as well as Figs. 3d and 4d the DU-145 cells were treated with positively charged conventional liposomes encapsulating β-lap in the concentrations of 0.25 μM and 1.5 μM, respectively. In these cases it was evidenced nucleus fragmentation and the early formation of membrane blebbings (Fig. 3c), pronounced membrane blebbings and apoptotic bodies (Fig. 3d), nucleus fragmentation (Fig. 4c) and nucleus condensation and fragmentation (Fig. 4d).

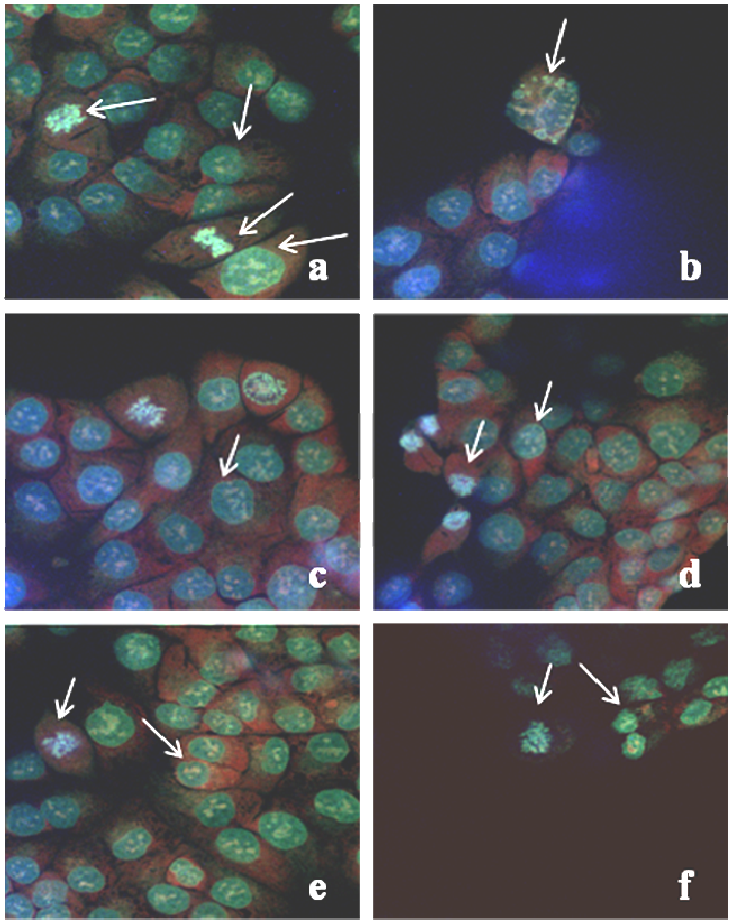

Fig. 4. Confocal microscopy of DU-145 cells seeded in 5% charcoal-dextran FBS treated with β-lap, positively charged conventional liposomes encapsulating β-lap or β-lap:HPβ-CD: a (cells treated with 0.25 µM of β-lap), b (cells treated with 1.5 µM of β-lap), c (cells treated with 0.25 µM of positively charged conventional liposomes encapsulating β-lap), d (cells treated with 1.5 µM of positively charged conventional liposomes encapsulating β-lap), e (cells treated with 0.25 µM of positively charged conventional liposomes encapsulating β-lap:HPβ-CD), f (cells treated with 1.5 µM of positively charged conventional liposomes encapsulating β-lap:HPβ-CD).

In Fig. 3e and 4e as well as Fig. 3f and 4f the DU-145 cells were treated with positively charged conventional liposomes encapsulating β-lap:HPβ-CD in the concentrations of 0.25 µM and 1.5 µM, respectively. In these cases the cells exhibit nucleus fragmentation and the early formation of membrane blebbing (Fig. 3e), pronounced membrane blebbings and nucleus

condensation (Fig. 3f), nucleus fragmentation and condensation (Fig. 4e) as well as nucleus fragmentation and membrane blebbings (Fig 4f).

The morphological and biochemical alterations caused by apoptotic processes are easy observed in cells (36) such as cell shrinkage, membranes blebbing, nuclear condensation and apoptotic bodies (37,38). Although necrosis and apoptosis are processes that occur distinctly, morphological alterations due to apoptotic and necrotic can coexist after exposure to toxic chemicals, for example (39).

It is important emphasize that, as already described in the literature (40,41), in our experiments free β-lap as well as the drug encapsulated into liposomes presented evidence to corroborate that this molecule actually is able to induce apoptosis or necrosis (cell giants). In fact, the confocal microscopy revealed that the cytotoxicity of β-lap on DU-145 cells was drug concentration dependent.

DU-145 cells exposed to negative controls, to be exact DMSO, buffer phosphate and unloaded liposomes showed intact nuclei and nucleoli visible, indicating that none of these samples were able to induce cell death (Fig. 5).

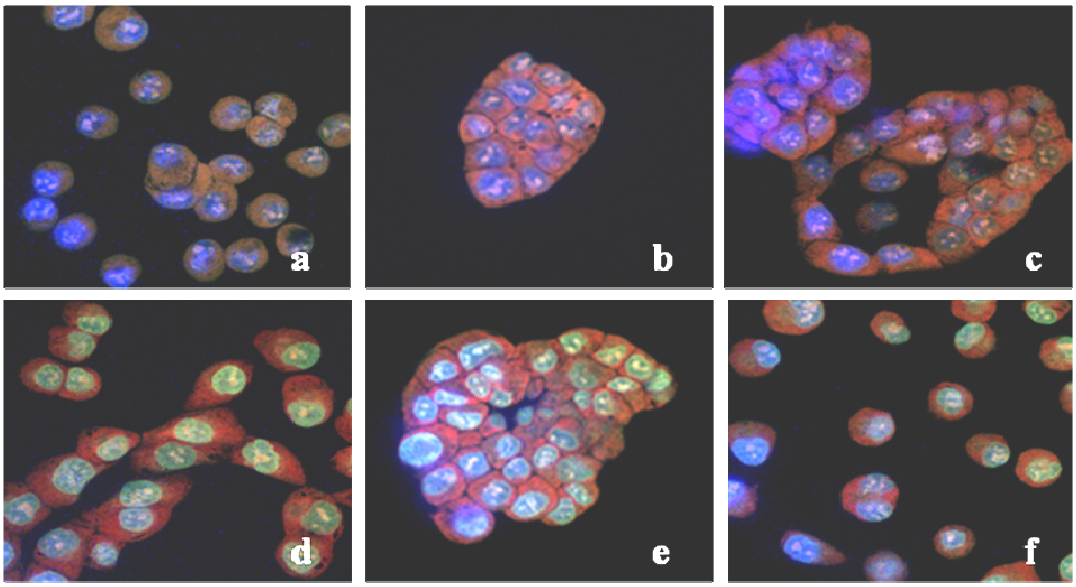

Fig. 5. Confocal microscopy of DU-145 cells seeded in 5% FBS or 5% charcoal-dextran FBS exposed to negative controls: a and d (DMSO), b and e (phosphate buffer) and c and f (unloaded conventional liposomes), respectively.

As previously reported (32), the cell line DU-145 appears to have low efficiency of internalization of conventional and stealth liposomes, therefore our formulations showed equal or slightly less activity than the free drug. However, conventional and stealth liposomes, in general *in vivo* applications, can enhance the biodistribution of the drug, while protecting surrounding healthy tissue, thus providing a greater acceptance of therapy by the patient (42,43). For all the advantages described above, the encapsulation of β-lap into conventional or stealth liposomes optimizes the therapeutic application of this antiproliferative molecule.

4. Conclusion

In conclusion, we demonstrated that a low dose of β-lapachone enhanced the proliferation of DU-145 cancer cells, but doses of β-lap above 1 μM presented cytotoxicity. Moreover, the results suggested that the conventional and stealth liposomes developed can further allow the use of the β-lap in the anticancer therapy, molecule that present a promising therapeutic potential, especially for human prostate carcinoma. Furthermore, this study encourages future research related to pharmacokinetic, biodistribution and the *in vivo* antitumor activity of the sthealth β-lap-loaded liposomes.

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8. CONCLUSÕES

- Lipossomas convencionais e furtivos neutros ou com carga de superfície positiva contendo β-lapachona ou complexo de inclusão β-lap:HPβ-CD foram preparados pelo método da hidratação do filme lipídico seguida de sonicação;
- Os lipossomas convencionais contendo estearilamina (SACL) e os furtivos neutros (NSL) encapsulando β-lapachona ou β-lap:HPβ-CD permaneceram estáveis ao armazenamento na forma de suspensão por aproximadamente 60 dias a 4 ºC;
- Os lipossomas convencionais neutros (NCL) e os furtivos contendo estearilamina (SASL) encapsulando β-lapachona ou β-lap:HPβ-CD permaneceram estáveis ao armazenamento na forma de suspensão por aproximadamente 7 dias a 4 ºC;
- Todas as formulações lipossomais permaneceram estáveis aos testes de estabilidade acelerada;
- A utilização de trealose como agente crioprotetor foi eficiente em conservar as características iniciais de algumas formulações lipossomais (SACL, NSL, SASL) após liofilização por 1 ano, porém não foi capaz de proporcionar o mesmo efeito na formulação NCL;
- Na cinética de liberação *in vitro*, todos os lipossomas apresentaram efeito *burst* menor comparado à molécula livre, assim como um perfil de liberação mais controlado do fármaco;
- NSL e SACL encapsulando β-lap ou β-lap:HPβ-CD, NCL e SASL encapsulando β-lap:HPβ-CD apresentaram menor velocidade de liberação quando comparada a molécula livre, porém NCL encapsulando β-lap ou β-lap:HPβ-CD não foram formulações estáveis;
- β-lapachona e β-lap:HPβ-CD apresentaram o mesmo perfil de atividade antimicrobiana frente a *Staphylococcus aureus* resistente à meticilina/oxacilina (MRSA ATCC 33591), *Staphylococcus aureus* sensível à meticilina/oxacilina (MSSA ATCC 29213), cepa hospitalar de *Staphylococcus aureus* resistente à meticilina/oxacilina (MRSA) e cepa comunitária de *Staphylococcus aureus* resistente à meticilina/oxacilina (MRSA), assim como ao *Cryptococcus neoformans*;

- Os lipossomas foram menos ativos que a molécula livre frente às bactérias (MSSA e MRSA), porém foram mais ativos que a molécula livre frente ao *Cryptococcus neoformans*;
- Na microscopia eletrônica de transmissão foi possível observar que os lipossomas ficam ao redor da célula fúngica;
- A β-lap livre ou encapsulada em lipossomas apresentaram atividade bacteriostática/fungistática, assim como atividade bactericida/fungicida;
- Os lipossomas convencionais e furtivos encapsulando β-lapachona ou β-lap:HPβ-CD apresentaram IC_{70} maior ou igual ao da molécula livre, evidenciando que a β-lap é tão ativa ou mais ativa que a molécula encapsulada nos lipossomas frente à linhagem celular de carcinoma de próstata (DU-145);
- Baixas concentrações de β-lap estimularam a proliferação de DU-145;
- A citotoxicidade da β-lap livre ou encapsulada em lipossomas frente à DU-145 é dose-tempo dependente;
- A microscopia confocal revelou que a linhagem celular DU-145 apresentou as primeiras evidências de diminuição da viabilidade celular e alterações morfológicas, como, por exemplo, figuras de mitose, condensação de cromatina, fragmentação de núcleo, corpos apoptóticos e células gigantes na presença de β-lapachona livre ou encapsulada nos lipossomas;
- A β-lap encapsulada nos lipossomas apresentou menor atividade quando comparada a moléculo livre frente às bactérias e a linhagem celular DU-145, porém, em aplicações *in vivo*, os lipossomas convencionais e furtivos podem melhorar a biodisponibilidade da β-lap, protegendo os tecidos sadios, assim como promovendo uma melhor aceitação do paciente à terapia;
- A encapsulação de β-lap em lipossomas representa uma nova estratégia para viabilizar a sua utilização na terapêutica do câncer e de infecções microbianas.

9. PERSPECTIVAS

Como perspectivas deste trabalho, estes sistemas serão utilizados em estudos *in vivo* para a avaliação da farmacocinética, biodisponibilidade e atividade antitumoral.

10. ANEXOS

ANEXO A

APROVAÇÃO EM CONCURSO

3ª Colocada do concurso para Professor Adjunto da Disciplina de Microbiologia e Imunologia do Departamento de Medicina Tropical no Centro de Ciências da Saúde da Universidade Federal de Pernambuco. A ser nomeada em Setembro de 2012.

ANEXO B

DOCÊNCIA EM DISCIPLINA DE GRADUAÇÃO

PROFESSORA SUBSTITUTA: Professora substituta da Disciplina de Microbiologia e Imunologia do Departamento de Medicina Tropical no Centro de Ciências da Saúde da Universidade Federal de Pernambuco. Aulas ministradas para os cursos de nutrição, medicina, biomedicina, odontologia diurno, odontologia noturno, farmácia e enfermagem. Contrato de 2 anos. Período: 2011.1, 2011.2, 2012.1 e 2012.2

ANEXO C

ARTIGO COMPLETO PUBLICADO EM REVISTA

Isabella M.F. Cavalcanti, Elisângela A.M. Mendonça, Mariane C.B. Lira, Sara B. Honorato, Celso, A. Camara, Rosa V. S. Amorim, Josué Mendes Filho, Marcelo M. Rabello, Marcelo Z. Hernandes, Alejandro P. Ayala, Nereide S. Santos-Magalhães. The encapsulation of β-lapachone in 2-hydroxypropyl-β-cyclodextrin inclusion complex into liposomes: a physicochemical evaluation and molecular modeling approach. European Journal of Pharmaceutical Science, v. 44, p. 332-340, 2011.

Elisângela A.M. Mendonça, Mariane C.B. Lira, Marcelo M. Rabello, Isabella M.F. Cavalcanti, Suely L Galdino, Ivan R. Pitta, Maria C.A. Lima, Maira G.R. Pitta, Marcelo Z. Hernandes, Nereide Santos-Magalhães. Molecular modeling and antiproliferative evaluation of the new anticancer candidate LPSF/AC04 in cyclodextrin inclusion complexes and encapsulated into liposomes. AAPS PharmSciTech, 2012.

ANEXO D

PROPRIEDADE INTELECTUAL EM PROCESSO DE DEPÓSITO

Título: "Processo de obtenção, uso e aplicações farmacêuticas da formulação lipossomal convencional contendo nimodipina direcionada ao tratamento da ansiedade e depressão e convulsão".

ANEXO E

ARTIGOS COMPLETOS SUBMETIDOS EM REVISTA

Pabyton G. Cadena, Marcela A. Pereira, Rafaela B. S. Cordeiro, Isabella M. F. Cavalcanti, Benício B. Neto, Maria do Carmo C. B. Pimentel, José Luiz Lima Filho, Valdinete L. Silva, Nereide S. Santos-Magalhães. Nanoencapsulation of quercetin and resveratrol into elastic liposomes. Submetido a Biochimica et Biophysica Acta BBA – Biomembranes, 2012.

ANEXO F

RESUMOS PUBLICADOS EM ANAIS DE CONGRESSO NACIONAIS E INTERNACIONAL

CAVALCANTI, I.M.F.; PONTES-NETO, J.G.; FERRAZ, R.S.; BELO, B.T.; SANTOS-MAGALHÃES, N.S. Lipossomas convencionais e furtivos contendo β-lapachona e complexos de inclusão β-lapachona:2-hidroxipropil-β-ciclodextrina: um estudo de cinética de liberação *in vitro*. III Congresso de Biomedicina e Farmácia da Faculdade ASCES, Caruaru, PE, 2012.

PARENTE, A.L.M.; FERRAZ, R.S.; **CAVALCANTI, I.M.F.;** LIRA, M.C.B.; SANTO-MAGALHÃES, N.S. Preparação e Cinética de Liberação *In Vitro* de Lipossomas Furtivos Contendo Ácido Úsnico para o Tratamento da Tuberculose. III Congresso de Biomedicina e Farmácia da Faculdade ASCES, Caruaru, PE, 2012.

CAVALCANTI, I.M.F.; BELO, B.T.; MENDONÇA, E.A.M.; SANTO-MAGALHÃES, N.S. Characterization Study of Conventional and Stealth Liposomes containing β-lapachone and β-lapachone:2-hydroxypropyl-β-cyclodextrin. III Simpósio Internacional em Diagnóstico e Terapêutica e VI Jornada Científica do LIKA, Recife, PE, 2011.

CAVALCANTI, I.M.F.; OLIVEIRA, P.C.; NEVES, R.P.; CADENA, P.G.; SANTO-MAGALHÃES, N.S. Encapsulation of ciclopirox olamine into liposomes for cryptococcosis treatment. III Simpósio Internacional em Diagnóstico e Terapêutica e VI Jornada Científica do LIKA, Recife, PE, 2011.

P.G. CADENA; M.A. PEREIRA; R.B.S. CORDEIRO; **I.M.F. CAVALCANTI**; B. BARROS NETO; M.C.B. PIMENTEL; J.L. LIMA-FILHO; V.L. SILVA; N.S.SANTOS-MAGALHÃES. Encapsulation of quercetin and resveratrol in liposomes: A fractional factorial design study. 3rd PharmSci Fair, Praga, República Tcheca, 2011.

FERRAZ, R.S., **CAVALCANTI, I.M.F**., SILVA, J.P.A., GALDINO, S.L., PITTA, I.R., SANTOS-MAGALHÃES, N.S. Validation of a UV Spectrophotometric Method for Determining LPSF/AC-04 Acridine Derivate Encapsulated into Liposomes. XL Annual Meeting of the Brazilian Biochemistry and Molecular Biology Society (SBBq), Foz do Iguaçu, PR, 2011.

CAVALCANTI, I.M.F., BARBOSA, I.A.. MENDONÇA, E.A.M., FERRAZ, M.S., FERRAZ, R.S., SANTOS-MAGALHÃES, N.S. β-lapachone Encapsulated into Liposomes and Nanocapsules: a Characterization Study. XL Annual Meeting of the Brazilian Biochemistry and Molecular Biology Society (SBBq), Foz do Iguaçu, PR, 2011.

FERRAZ, M.S.; **CAVALCANTI, I.M.F**.; BARBOSA, I.A.; MENDONÇA, E.A.M.; SANTOS-MAGALHÃES, N.S. Estudo comparativo da cinética de liberação *in vitro* da β-lapachona a partir de lipossomas e nanocápsulas. III Simpósio de Inovação em Ciências Biológicas, Recife, PE, 2010.

FERRAZ, M.S.; RODRIGUES-FONSECA, A.V.; **CAVALCANTI, I.M.F**.; BARBOSA, I.A.; LIRA, M.C.B.; SANTOS-MAGALHÃES, N.S. Estudo da solubilidade do ácido úsnico pela complexação com ciclodextrinas. III Simpósio de Inovação em Ciências Biológicas, Recife, PE, 2010.

SILVA, J.P.A.; FERRAZ, R.S.; **CAVALCANTI**, **I.M.F.;** GALDINO, S.L.; SANTOS-MAGALHÃES, N.S. Development and characterization of liposomes containing the acridine LPSF/AC-04. X Reunião Regional Nordeste Sociedade Brasileira de Bioquímica e Biologia Molecular-SBBq, Salvador, BA, 2010.

ANEXO G

RESUMOS EXPANDIDOS PUBLICADOS EM ANAIS DE CONGRESSO INTERNACIONAL

LIMA, R.G., **CAVALCANTI, I.M.F**., FERRAZ, M.S., BELO, B.T., ANJOS, J.V, NEVES, R.P., SANTOS-MAGALHÃES, N.S. Development of New Triazole-loaded Liposomes: *In Vitro* Evaluation Against *Candida Albicans.* 23rd International Symposium on Pharmaceutical & Biomedical Analysis, João Pessoa, PB, 2011.

MENDONÇA, E.A.M., PARENTE, A.L.M., **CAVALCANTI, I.M.F**., SANTOS-MAGALHÃES, N.S. Evaluation and characterization of LPSFAC4 cyclodextrin inclusion complexes. 23rd International Symposium on Pharmaceutical & Biomedical Analysis, João Pessoa, PB, 2011.

CADENA, P.G., PEREIRA, M.A., CORDEIRO, R.B.S., **CAVALCANTI, I.M.F**., BARROS NETO, B., PIMENTEL, M.C.B., LIMA-FILHO, J.L., SILVA, V.L. SANTOS-MAGALHÃES, N.S. Experimental design for coencapsulation of quercetin and resveratrol into sodium deoxycholate liposomes. 8th International Congress of Pharmaceutical Science, Ribeirão Preto, SP, 2011.

OLIVEIRA, P.C., NEVES, R.P., **CAVALCANTI, I.M.F**., CADENA, P.G., SANTOS-MAGALHÃES, N.S. Ciclopirox olamine-loaded liposomes: a physicochemical characterization. 8th International Congress of Pharmaceutical Science, Ribeirão Preto, SP, 2011.

ANEXO H

PRÊMIOS POR ATIVIDADES CIENTÍFICAS

2º lugar na categoria Terapêutica na VI Jornada Científica e III Simpósio Internacional em Diagnóstico e Terapêutica do Laboratório de Imunopatologia Keizo Asami, 2011.

1º lugar no IV Simpósio de Inovações em Ciências Biológicas (SICBIO-2011). Resumo intitulado "Desenvolvimento e caracterização de nanopartículas contendo ácido úsnico".

1º lugar no III Simpósio de Inovações em Ciências Biológicas (SICBIO-2010). Resumo intitulado "Estudo comparativo da cinética de liberação in vitro da β-lapachona a partir de lipossomas e nanocápsulas".

ANEXO I

ORGANIZAÇÃO DE EVENTOS CIENTÍFICOS

Vice-presidente do III Simpósio de Inovação em Ciências Biológicas (CCB-UFPE).
Local: Recife – PE.
Período: 17/11/2010 a 19/11/2010.

ANEXO J

AUXILIO EM AULAS PRÁTICAS EM PÓS-GRADUAÇÃO *STRICTO SENSU*

1. Disciplina do Programa de Pós-Graduação em Ciências Farmacêuticas intitulada "**CF908 TECNOLOGIA FARMACÊUTICA".**
Período: 2011.2 – Carga Horária: 20h

2. Disciplina do Programa de Pós-Graduação em Ciências Biológicas intitulada "**DCB922 NANOBIOTECNOLOGIA E SISTEMAS DE LIBERAÇÃO CONTROLADA DE FÁRMACOS".**
Período: 2010.2 – Carga Horária: 20h

3. Disciplina do Programa de Pós-Graduação em Inovação Terapêutica intitulada "**PIT-924 NANOSSISTEMAS TERAPEÛTICOS E DE DIAGNÓSTICO: UMA INOVAÇÃO FÁRMACO-TECNOLÓGICA".**

Período: 2009.2 – Carga Horária: 20h

ANEXO K

ORIENTAÇÃO DE TRABALHO DE CONCLUSÃO DE CURSO DE GRADUAÇÃO

ORIENTAÇÃO:

Aluna: Janessa Domingos da Silva

Título da Monografia: Avaliação do perfil de prescrições médicas para o tratamento de pacientes depressivos.

Curso: Farmácia-UFPE

Data: 16/12/2010

ORIENTAÇÃO:

Aluna: Tarcyla de Andrade Gomes

Título da Monografia: Avaliação do perfil de protocolos terapêuticos utilizados na quimioterapia neodjuvante para o câncer de mama.

Curso: Farmácia-UFPE

Data: 07/12/2011

ANEXO L

PARTICIPAÇÃO COMO MEMBRO EFETIVO DE BANCA EXAMINADORA DE MONOGRAFIA/HABILITAÇÃO

PARTICIPAÇÃO EM BANCA EXAMINADORA DE MONOGRAFIA DE HABILITAÇÃO:

Aluna: Natália Fernandes Antas Florentino

Título da Monografia: *Diabetes mellitus* tipo 2 na infância: fatores de risco e diagnóstico laboratorial.

Curso: Farmácia-UFPE

Data: 27/06/2012

PARTICIPAÇÃO EM BANCA EXAMINADORA DE MONOGRAFIA:

Aluna: Rafaela Batista da Silva Cordeiro

Título da Monografia: Desenvolvimento de uma formulação lipossomal para a encapsulação do resveratrol.

Curso: Biomedicina-UFPE

Data: 05/01/2012

PARTICIPAÇÃO EM BANCA EXAMINADORA DE MONOGRAFIA:

Aluna: Marcela Araújo Pereira

Título da Monografia: Nanoencapsulação do flavonóide quercetina em lipossomas.

Curso: Biomedicina-UFPE

Data: 04/01/2012

PARTICIPAÇÃO EM BANCA EXAMINADORA DE MONOGRAFIA DE HABILITAÇÃO:

Aluna: Adriana Maria Costa Marques da Silva

Título da Monografia: Fisiopatologia e análise laboratorial da pielonefrite aguda.

Curso: Farmácia-UFPE

Data: 12/12/2011

PARTICIPAÇÃO EM BANCA EXAMINADORA DE MONOGRAFIA:

Aluna: Adélia da Costa Nóbrega Leite

Título da Monografia: Análise microbiológica em latas de refrigerantes no município de Passagem-PB.

Curso: Biomedicina-FIP

Data: 05/12/2011

PARTICIPAÇÃO EM BANCA EXAMINADORA DE MONOGRAFIA:

Aluna: Daniel de Mélo Carvalho

Título da Monografia: Avaliação epidemiológica de pacientes com câncer em um hospital público de referência do Recife (2008-2010).

Curso: Farmácia-UFPE

Data: 14/07/2011

PARTICIPAÇÃO EM BANCA EXAMINADORA DE MONOGRAFIA:

Aluna: Élica Rodrigues da Silva

Título da Monografia: Atenção farmacêutica: valorização do profissional e uma necessidade social.

Curso: Farmácia-UFPE

Data: 20/12/2010

PARTICIPAÇÃO EM BANCA EXAMINADORA DE MONOGRAFIA:

Aluna: Jamilka Leopoldina da Silva

Título da Monografia: Perfil do uso de anti-hipertensivos Inibidores da Enzima Conversora de Angiotensina na Farmácia Comunitária da Policlínica José Carneiro Lins.

Curso: Farmácia-UFPE

Data: 20/12/2010

PARTICIPAÇÃO EM BANCA EXAMINADORA DE MONOGRAFIA:

Aluna: Jéssica Priscila Avelino Silva

Título da Monografia: Identificação de reações adversas à aminoglicosídios injetáveis em um hospital de grande porte em Pernambuco. Curso: Farmácia-UFPE

Curso: Farmácia-UFPE

Data: 16/12/2010

PARTICIPAÇÃO EM BANCA EXAMINADORA DE MONOGRAFIA:

Aluna: Marcileide de Holanda Santos

Título da Monografia: Desenvolvimento e caracterização físico-química de lipossomas contendo a *trans*-desidrocrotonina.

Curso: Biomedicina-UFPE

Data: 16/12/2010

PARTICIPAÇÃO EM BANCA EXAMINADORA DE MONOGRAFIA:

Aluna: Rebeca Cavalcanti Silveira

Título da Monografia: Avaliação da adesão ao tratamento antirretroviral das pessoas vivendo com HIV/AIDS assistidas na farmácia ambulatorial do Hospital Barão de Lucena.

Curso: Farmácia-UFPE

Data: 20/06/2010

ANEXO M

AVALIAÇÃO DE PÔSTER

III Simpósio de Inovação em Ciências Biológicas (SICBIO-2010).

ANEXO N

PARTICIPAÇÃO EM CONGRESSOS

Evento: VI Jornada Científica e III Simpósio Internacional em Diagnóstico e Terapêutica do Laboratório de Imunopatologia Keizo-Asami.

Local: Recife – PE

Ano: 2011.

Evento: 8th International Congress of Pharmaceutical Science.

Local: Ribeirão Preto – SP.

Ano: 2011.

Evento: XL Annual Meeting of the Brazilian Biochemistry and Molecular Biology Society (SBBq).

Local: Foz do Iguaçu – PR.

Ano: 2011.

Evento: II Simpósio de Inovação em Ciências Biológicas (CCB-UFPE).

Local: Recife – PE.

Ano: 2010.

ANEXO O

NORMAS DAS REVISTAS

Antimicrobial Agents and Chemoterapy

Guide for Authors

ORGANIZATION AND FORMAT

Editorial Style

The editorial style of ASM journals conforms to the *ASM Style Manual for Journals* (American Society for Microbiology, 2012, in-house document) and *How To Write and Publish a Scientific Paper*, 6th ed. (Greenwood Press, Westport, CT, 2006), as interpreted and modified by the editors and the ASM Journals Department.

The editors and the Journals Department reserve the privilege of editing manuscripts to conform with the stylistic conventions set forth in the aforesaid publications and in these Instructions.

On receipt at ASM, an accepted manuscript undergoes an automated preediting, cleanup, and tagging process specific to the particular article type. To optimize this process, manuscripts must be supplied in the correct format and with the appropriate sections and headings.

Type every portion of the manuscript double-spaced (a minimum of 6 mm between lines), including figure legends, table footnotes, and references, and number all pages in sequence, including the abstract, figure legends, and tables. Place the last two items after the References section. (On initial submission, to assist review, the legend should be incorporated into the image file and appear beneath the figure. At the modification stage, figure legends must be provided as text files separate from the image file.) Manuscript pages must have continuous line numbers; manuscripts without line numbers may be editorially rejected by the editor, with a suggestion of resubmission after line numbers are added. The font size should be no smaller than 12 points. IIt is recommended that the following sets of characters be easily distinguishable in the manuscript: the numeral zero (0) and the letter “oh” (O); the numeral one (1), the letter “el” (l), and the letter “eye” (I); and a multiplication sign (×) and the letter “ex.” (x). Do not create symbols as graphics or use special fonts that are external to your word processing program; use the "insert symbol" function. Set the page size to 8.5 by 11 inches (ca. 21.6 by 28 cm). Italicize any words that should appear in italics, and indicate paragraph lead-ins in boldface type.

Manuscripts may be editorially rejected, without review, on the basis of poor English or lack of conformity to the standards set forth in these Instructions.

Authors who are unsure of proper English usage should have their manuscripts checked by someone proficient in the English language or engage a professional language editing service for help.

Full-Length Papers

Full-length papers should include the elements described in this section.

Title, running title, and byline. Each manuscript should present the results of an independent, cohesive study; thus, numbered series titles are not permitted. Exercise care in composing a title. Avoid the main title/subtitle arrangement, complete sentences, and unnecessary articles. On the title page, include the title, the running title (not to exceed 54 characters and spaces), the name of each author, the address(es) of the institution(s) at which the work was performed, each author's affiliation, and a footnote indicating the present address of any author no longer at the institution where the work was performed. Place a number sign (#) after the name of the author to whom inquiries regarding the paper should be directed (see "Correspondent footnote" below).

Study group in byline. A study group, surveillance team, working group, consortium, or the like (e.g., the Active Bacterial Core Surveillance Team) may be listed as a coauthor in the byline if its contributing members satisfy the requirements for authorship and accountability as described in these Instructions. The names (and institutional affiliations if desired) of the contributing members may be given as a separate paragraph in Acknowledgments.

If the contributing members of the group associated with the work do not fulfill the criteria of substantial contribution to and responsibility for the paper, the group may not be listed in the author byline. Instead, it and the names of its contributing members may be listed in the Acknowledgments section.

Correspondent footnote. The e-mail address for the corresponding author should be included on the title page of the manuscript. This information will be published in the article as a footnote to facilitate communication and will be used to notify the corresponding author of the availability of proofs and, later, of the PDF file of the published article. No more than two authors may be designated corresponding authors.

Abstract. Limit the abstract to 250 words or fewer and concisely summarize the basic content of the paper without presenting extensive experimental details. Avoid abbreviations and references, and do not include diagrams. When it is essential to include a reference, use the same format as shown for the References section but omit the article title. Conclude the abstract with a summary statement. Because the abstract will be published separately by abstracting services, it must be complete and understandable without reference to the text.

Introduction. The introduction should supply sufficient background information to allow the reader to understand and evaluate the results of the present study without referring to previous publications on the topic. The introduction should also provide the hypothesis that was addressed or the rationale for the study. References should be chosen carefully to provide the most salient background rather than an exhaustive review of the topic.

Case Report. The Case Report section, placed after the introduction and before Materials and Methods, is optional and gives relevant clinical information about one or more patients.

Materials and Methods. The Materials and Methods section should include sufficient technical information to allow the experiments to be repeated. When centrifugation conditions are critical, give enough information to enable another investigator to repeat the procedure: make of centrifuge, model of rotor, temperature, time at maximum speed, and centrifugal force (x g rather than revolutions per minute). For commonly used materials and methods (e.g., media and protein concentration determinations), a simple reference is sufficient. If several alternative methods are commonly used, it is helpful to identify the method briefly as well as to cite the reference. For example, it is preferable to state “cells were broken by ultrasonic treatment as previously described (9)” rather than “cells were broken as previously described (9).” This allows the reader to assess the method without constant reference to previous publications. Describe new methods completely, and give sources of unusual chemicals, equipment, or microbial strains. When large numbers of microbial strains or mutants are used in a study, include tables identifying the immediate sources (i.e., sources from whom the strains were obtained) and properties of the strains, mutants, bacteriophages, and plasmids, etc.

A method or strain, etc., used in only one of several experiments reported in the paper may be described in the Results section or very briefly (one or two sentences) in a table footnote or figure legend. It is expected that the sources from whom the strains were obtained will be identified.

Results. In the Results section, include the rationale or design of the experiments as well as the results; reserve extensive interpretation of the results for the Discussion section. Present the results as concisely as possible in one of the following: text, table(s), or figure(s). Avoid extensive use of graphs to present data that might be more concisely or more quantitatively presented in the text or tables. Limit photographs (particularly photomicrographs and electron micrographs) to those that are absolutely necessary to show the experimental findings. Number figures and tables in the order in which they are cited in the text, and be sure that all figures and tables are cited.

Discussion. The Discussion should provide an interpretation of the results in relation to previously published work and to the experimental system at hand and should not contain extensive repetition of the Results section or reiteration of the introduction. In short papers, the Results and Discussion sections may be combined.

Acknowledgments. The source of any financial support received for the work being published must be indicated in the Acknowledgments section. (It will be assumed that the absence of such an acknowledgment is a statement by the authors that no support was received.) The usual format is as follows: "This work was supported by Public Health Service grant CA-01234 from the National Cancer Institute."

Recognition of personal assistance should be given as a separate paragraph, as should any statements disclaiming endorsement or approval of the views reflected in the paper or of a product mentioned therein.

Appendixes. Appendixes that contain additional material to aid the reader are permitted. Titles, authors, and reference sections that are distinct from those of the primary article are not allowed. If it is not feasible to list the author(s) of the appendix in the byline or the Acknowledgments section of the primary article, rewrite the appendix so that it can be considered for publication as an independent article, either full-length or Short-Form style. Equations, tables, and figures should be labeled with the letter "A" preceding the numeral to distinguish them from those cited in the main body of the text.

References

Beginning with the January 2013 issue, ASM will change the way in which references are numbered throughout articles. Citations will be numbered in the order in which they appear in the article (citation-sequence reference system); ASM will no longer use the citation-name system with an alphabetized reference list. Also beginning with January 2013 issues, entries in References will include all authors' names; "et al." will not be used in author lines. The following describes the style that will be effective in January 2013.

(i) References listed in the References section. The References section must include all journal articles (both print and online), books and book chapters (both print and online), patents, theses and dissertations, published conference proceedings, meeting abstracts from published abstract books or journal supplements, letters (to the editor), and company publications, as well as in-press journal articles, book chapters, and books (publication title must be given). References should be cited in numerical order as they appear in the text (citation-sequence system). Since figures and tables will be inserted in the text where first cited, references first mentioned in figure legends and tables should be numbered so that the numbers are in sequence with those in the text. To ensure the correct order of reference numbers, the parts of the manuscript should be in the correct order before ordering the citations. **Provide the names of all the authors for each reference, as the author line will not be abbreviated and "et al." will not be used**. All listed references must be cited parenthetically by number in the text. Since title and byline information that is downloaded from PubMed does not always show accents, italics, or special characters, authors should refer to the PDF files or hard-copy versions of the articles and incorporate the necessary corrections in the submitted manuscript. Abbreviate journal names according to the PubMed Journals Database (National Library of Medicine, National Institutes of Health), the primary source for ASM style, but use periods on abbreviated words.

Follow the styles shown in the examples below for print references.

Caserta E, Haemig HAH, Manias DA, Tomsic J, Grundy FJ, Henkin TM, Dunny GM. 2012. *In vivo* and *in vitro* analyses of regulation of the pheromone-responsive prgQ promoter by the PrgX pheromone receptor protein. J. Bacteriol. **194**:3386-3394.

Falagas ME, Kasiakou SK. 2006. Use of international units when dosing colistin will help decrease confusion related to various formulations of the drug around the world. Antimicrob. Agents Chemother. **50:**2274-2275. (Letter.) {*"Letter" or "Letter to the editor" is allowed but not required at the end of such an entry.*}

Cox CS, Brown BR, Smith JC. J. Gen. Genet., in press.* {*Article title is optional; journal title is mandatory.*}

da Costa MS, Nobre MF, Rainey FA. 2001. Genus I. Thermus Brock and Freeze 1969, 295AL, emend. Nobre, Trüper and da Costa 1996b, 605, p. 404-414. *In* In Boone DR, Castenholz RW, Garrity GM (ed), Bergey's manual of systematic bacteriology, 2nd ed, vol 1. Springer, New York, NY.

Stratagene. 2006. Yeast DNA isolation system: instruction manual. Stratagene, La Jolla, CA. {*Use the company name as the author if none is provided for a company publication.*}

Forman MS, Valsamakis A. 2003. Specimen collection, transport, and processing: virology, p 1227-1241. *In* Murray PR, Baron EJ, Pfaller MA, Jorgensen JH, Yolken RH (ed), Manual of clinical microbiology, 8th ed, Washington, DC.

Fitzgerald G, Shaw D. *In* A. E. Waters (ed), Clinical microbiology, in press. EFH Publishing Co., Boston, MA.* {*Chapter title is optional.*}

García CO, Paira S, Burgos R, Molina J, Molina JF, Calvo C, Vega L, Jara LJ, García-Kutzbach A, Cuellar ML, Espinoza LR. 1996. Detection of *Salmonella* DNA in synovial membrane and synovial fluid from Latin American patients using the polymerase chain reaction. Arthritis Rheum. **39**(Suppl)**:**S185. {*Meeting abstract published in journal supplement.*}

Smith D, Johnson C, Maier M, Maurer JJ. 2005. Distribution of fimbrial, phage and plasmid associated virulence genes among poultry *Salmonella enterica* serovars, abstr P-038, p 445. Abstr. 105th Gen. Meet. Am. Soc. Microbiol. American Society for Microbiology, Washington, DC. {*Abstract title is optional.*}

Rotimi VO, Salako NO, Mohaddas EM, Philip LP. 2005. Abstr 45th Intersci Conf Antimicrob. Agents Chemother., abstr D-1658. {*Abstract title is optional.*}

Green PN, Hood D, Dow CS. 1984. Taxonomic status of some methylotrophic bacteria, p 251-254. *In* Crawford RL, Hanson RS (ed), Microbial growth on C1 compounds. Proceedings of the 4th International Symposium. American Society for Microbiology, Washington, DC.

O'Malley DR. 1998. Ph.D. thesis. University of California, Los Angeles, CA. {*Title is optional.*}

Odell JC. April 1970. Process for batch culturing. U.S. patent 484,363,770. {*Include the name of the patented item/process if possible; the patent number is mandatory.*}

Elder BL, Sharp SE. 2003. Cumitech 39, Competency assessment in the clinical laboratory. Coordinating ed, Sharp SE. ASM Press, Washington, DC.

*A reference to an in-press ASM publication should state the control number (e.g., AAC00577-12) if it is a journal article or the name of the publication if it is a book.

Online-only references must provide essentially the same information that print references do. For online journal articles, posting or revision dates may replace the year of publication; a DOI (preferred) or URL is required for articles with nontraditional page numbers or electronic article identifiers. Some examples follow.

Winnick S, Lucas DO, Hartman AL, Toll D. 2005. How do you improve compliance? Pediatrics **115:**e718-e724.

Smith FX, Merianos HJ, Brunger AT, Engelman DM. 2001. Polar residues drive association of polyleucine transmembrane helices. Proc. Natl. Acad. Sci. U. S. A. **98:**2250-2255. doi:10.1073/pnas.041593698.

Dionne MS, Schneider DS. 2002. Screening the fruitfly immune system. Genome Biol. **3:**REVIEWS1010. http://genomebiology.com/2002/3/4/reviews/1010.

Gregory ST. 2 September 2009, posting date. Chapter 2.5.4, Structural basis for the decoding mechanism. *In* Böck A, et al (ed), EcoSal—*Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, DC. http://www.ecosal.org/. {*Note that each chapter has its own posting date*.}

Note: a posting or accession date is required for any online reference that is periodically updated or changed.

(ii) References cited in the text. References to unpublished data, manuscripts submitted for publication, unpublished conference presentations (e.g., a report or poster that has not appeared in published conference proceedings), personal communications, patent applications and patents pending, computer software, databases, and websites should be made parenthetically in the text as follows.

... similar results (R. B. Layton and C. C. Weathers, unpublished data).

... system was used (J. L. McInerney, A. F. Holden, and P. N. Brighton, submitted for publication).

... as described previously (M. G. Gordon and F. L. Rattner, presented at the Fourth Symposium on Food Microbiology, Overton, IL, 13 to 15 June 1989). {For nonpublished abstracts and posters, etc.}

... this new process (V. R. Smoll, 20 June 1999, Australian Patent Office). {For non-U.S. patent applications, give the date of publication of the application.}

... available in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/index.html).

... using ABC software (version 2.2; Department of Microbiology, State University [http://www.state.micro.edu]).

URLs for companies that produce any of the products mentioned in your study or for products being sold may not be included in the article. However, company URLs that permit access to scientific data related to the study or to shareware used in the study are permitted.

(iii) References related to supplemental material. References that are related only to supplemental material hosted by ASM or posted on a personal/institutional website should not be listed in the References section of an article; include them with the supplemental material itself.

(iv) Referencing ASM Accepts (publish-ahead-of-print) manuscripts. Citations of ASM Accepts manuscripts should look like the following example.

Wang GG, Pasillas MP, Kamps MP. 15 May 2006. Persistent transactivation by Meis1 replaces Hox function in myeloid leukemogenesis models: evidence for co-occupancy of Meis1-Pbx and Hox-Pbx complexes on promoters of leukemia-associated genes. Mol. Cell. Biol. doi:10.1128/MCB.00586-06.

Other journals may use different styles for their publish-ahead-of-print manuscripts, but citation entries must include

the following information: author name(s), posting date, title, journal title, and volume and page numbers and/or DOI. The following is an example:

Zhou FX, Merianos HJ, Brunger AT, Engelman DM. 13 February 2001, posting date. Polar residues drive association of polyleucine transmembrane helices. Proc. Natl. Acad. Sci. U. S. A. doi:10.1073/pnas.041593698.

Letters to the Editor

Two types of Letters to the Editor may be submitted. The first type (Comment Letter) is intended for comments on final, typeset articles published in the journal (not on publish-ahead-of-print manuscripts) and must cite published references to support the writer's argument. The second type (New-Data Letter) may report new, concise findings that are not appropriate for publication as full-length papers or Short-Form papers.

Letters may be **no more than 500 words long and must be typed double-spaced**. Refer to a recently published Letter for correct formatting. Note that authors and affiliations are listed at the foot of the Letter. Provide only the primary affiliation for each author.

All Letters to the Editor must be submitted electronically, and the type of Letter (New Data or Comment) must be selected from the drop-down list in the submission form. For Letters commenting on published articles, the cover letter should state the volume and issue in which the article was published, the title of the article, and the last name of the first author. In the Abstract section of the submission form, put "Not Applicable." Letters to the Editor do not have abstracts. Both types of Letter must have a title, which must appear on the manuscript and on the submission form. Figures and tables should be kept to a minimum.

If the Letter is related to a published article, it will be sent to the editor who handled the article in question. If the editor believes that publication is warranted, he/she will solicit a reply from the corresponding author of the article and give approval for publication.

New-Data Letters will be assigned to an editor according to subject matter and will be reviewed by that editor and/or a reviewer.

Please note that some indexing/abstracting services do not include Letters to the Editor in their databases.

ILLUSTRATIONS AND TABLES

Image Manipulation

Computer-generated images may be processed only minimally. Processing (e.g., changing contrast, brightness, or color balance) is acceptable only if applied to all parts of the image, as well as to the controls, equally, and descriptions of all such adjustments and the tools used (both hardware and software) must be provided in the manuscript. Unprocessed data and files must be retained by the authors and be provided to the editor on request.

Illustrations

File types and formats. Illustrations may be continuous-tone images, line drawings, or composites. Color graphics may be submitted, but the cost of printing in color must be borne by the author. Suggestions about how to reduce costs and ensure accurate color reproduction are given below.

On initial submission, illustrations should be supplied as PDF files, with the legend on the same page, to assist review. At the modification stage, production quality digital files must be provided, along with text files for the legends. The legends are copyedited and typeset for final publication, not included as part of the figure itself. All graphics submitted with modified manuscripts must be bitmap, grayscale, or in the RGB (preferred) or CMYK color mode. See “Color illustrations.” Halftone images (those with various densities or shades) must be grayscale, not bitmap. AAC accepts TIFF or EPS files but discourages PowerPoint for either black-and-white or color images.

For instructions on creating acceptable EPS and TIFF files, refer to the Cadmus digital art website, http://art.cadmus.com/da/index.jsp. PowerPoint requires users to pay close attention to the fonts used in their images (see the section on fonts below). If instructions for fonts are not followed exactly, images prepared for publication are subject to missing characters, improperly converted characters, or shifting/obscuring of elements or text in the

figure. For proper font use in PowerPoint images, refer to the Cadmus digital art website, http://art.cadmus.com/da/instructions/ppt_disclaimer.jsp.

We strongly recommend that before returning their modified manuscripts, authors check the acceptability of their digital images for production by running their files through Rapid Inspector, a tool provided at the following URL: http://rapidinspector.cadmus.com/RapidInspector/zmw/index.jsp. Rapid Inspector is an easy-to-use, Web-based application that identifies file characteristics that may render the image unusable for production.

If you require additional information, please send an e-mail inquiry to digitalart@cadmus.com.

Minimum resolution. It is extremely important that a high enough file resolution is used. All separate images that you import into a figure file must be at the correct resolution before they are placed. (For instance, placing a 72-dpi image in a 300-dpi EPS file will not result in the placed image meeting the minimum requirements for file resolution.) Note, however, that the higher the resolution, the larger the file and the longer the upload time. Publication quality will not be improved by using a resolution higher than the minimum. Minimum resolutions are as follows:

300 dpi for grayscale and color

600 dpi for combination art (lettering and images)

1,200 dpi for line art

Size. **All graphics should be submitted at their intended publication size**; that is, the image uploaded should be 100% of its print dimensions so that no reduction or enlargement is necessary. Resolution must be at the required level at the submitted size. Include only the significant portion of an illustration. White space must be cropped from the image, and excess space between panel labels and the image must be eliminated.

Maximum width for a 1-column figure: 20.6 picas (ca. 8.7 cm)

Maximum width for a 2-column figure: 42 picas (ca. 17.8 cm)

Minimum width for a 2-column figure: 26 picas (ca. 11.1 cm)

Maximum height for a standard figure: 54.7 picas (ca. 23.2 cm)

Maximum height for an oversized figure (no running title): 57.4 picas (ca. 24.3 cm)

Contrast. Illustrations must contain sufficient contrast to be viewed easily on a monitor or on the printed page.

Labeling and assembly. All final lettering and labeling must be incorporated into the figures.On initial submission, illustrations should be provided as PDF files, with the legend beneath each image, to assist review. At the modification stage, production quality digital figure files must be provided, along with text files for the legends. Put the figure number well outside the boundaries of the image itself. (Numbering may need to be changed at the copyediting stage.) Each figure must be uploaded as a separate file, and any multipanel figures must be assembled into one file; i.e., rather than uploading a separate file for each panel in a figure, assemble all panels in one piece and supply them as one file.

Fonts. To avoid font problems, set all type in one of the following fonts: Arial, Helvetica, Times Roman, European PI, Mathematical PI, or Symbol. Courier may be used but should be limited to nucleotide or amino acid sequences in which a nonproportional (monospace) font is required. All fonts other than these must be converted to paths (or outlines) in the application with which they were created.

Color illustrations. **Color costs must be borne by the author. See "Publication Charges." All figures submitted in color will be processed as color**. Adherence to the following guidelines will help to minimize costs and to ensure color reproduction that is as accurate as possible.

The final online version is considered the version of record for AAC and all other ASM journals. To maximize online reproduction, color illustrations should be supplied in the RGB color mode as either (i) RGB TIFF images with a resolution of at least 300 pixels per inch (raster files, consisting of pixels) or (ii) Illustrator-compatible EPS files with RGB color elements (vector files, consisting of lines, fonts, fills, and images). CMYK files are also accepted. Other than in color space, CMYK files must meet the same production criteria as RGB files.The RGB color space is the native color space of computer monitors and of most of the equipment and software used to capture scientific data, and it can display a wider range of colors (especially bright fluorescent hues) than the CMYK (cyan, magenta, yellow, black) color space used by print devices that put ink (or toner) on paper. For the print version (and reprints), ASM's print provider will automatically create CMYK versions of color illustrations from the supplied RGB versions. Color in the print journal may not match that in the online journal of record because of the smaller range of colors capable of being reproduced by CMYK inks on a printing press. For additional information on RGB versus CMYK color, refer to the Cadmus digital art site.

Drawings

Submit graphs, charts, complicated chemical or mathematical formulas, diagrams, and other drawings as finished products not requiring additional artwork or typesetting. All elements, including letters, numbers, and symbols, must be easily readable, and both axes of a graph must be labeled. Keep in mind that the journal is published both in print and online and that the same electronic files submitted by the authors are used to produce both formats.

When creating line art, please use the following guidelines:

All art must be submitted at its intended publication size. For acceptable dimensions, see "Size" above.

Avoid using screens (i.e., shading) in line art. It can be difficult and time-consuming to reproduce these images without moiré patterns. Various pattern backgrounds are preferable to screens, as long as the patterns are not imported from another application. If you must use images containing screens,

Generate the image at line screens of 85 lines per inch or less.

When applying multiple shades of gray, differentiate the gray levels by at least 20%.

Never use levels of gray below 5% or above 95% as they are likely to fade out or become totally black when output.

Use thick, solid lines that are no finer than 1 point in thickness.

No type should be smaller than 6 points at the final publication size.

Avoid layering type directly over shaded or textured areas.

Avoid the use of reversed type (white lettering on a black background).

Avoid heavy letters, which tend to close up, and unusual symbols, which the printer may not be able to reproduce in the legend.

If colors are used, avoid using similar shades of the same color and avoid very light colors.

In figure ordinate and abscissa scales (as well as table column headings), avoid the ambiguous use of numbers with exponents. Usually, it is preferable to use the Système International d'Unités (SI) symbols (μ for 10–6, m for 10–3, k

for 103, and M for 106, etc.). A complete listing of SI symbols can be found in the International Union of Pure and Applied Chemistry (IUPAC) publication *Quantities, Units and Symbols in Physical Chemistry* (RSC Publishing, Cambridge, United Kingdom, 2007); an abbreviated list is available at http://old.iupac.org/reports/1993/homann/index.html. Thus, a representation of 20,000 cpm on a figure ordinate should be made by the number 20 accompanied by the label kcpm.

When powers of 10 must be used, the journal requires that the exponent power be associated with the number shown. In representing 20,000 cells per ml, the numeral on the ordinate should be "2" and the label should be "104 cells per ml" (not "cells per ml x 10–4"). Likewise, an enzyme activity of 0.06 U/ml might be shown as 6 accompanied by the label "10–2 U/ml." The preferred designation is 60 mU/ml (milliunits per milliliter).

Figure Legends

On initial submission, to assist review, the legend should be incorporated in the image file and appear beneath the figure. At the modification stage, figure legends must be provided as text files separate from the image file.

Legends should provide enough information so that the figure is understandable without frequent reference to the text. However, detailed experimental methods must be described in the Materials and Methods section, not in a figure legend. A method that is unique to one of several experiments may be set forth in a legend only if the description is very brief (one or two sentences). Define all symbols used in the figure and define all abbreviations that are not used in the text.

Tables

Tables that contain artwork, chemical structures, or shading must be submitted as illustrations in an acceptable format at the modification stage. The preferred format for regular tables is Microsoft Word; however, WordPerfect and Acrobat PDF are also acceptable. Note that a straight Excel file is not currently an acceptable format. Excel files must be either embedded in a Word or WordPerfect document or converted to PDF before being uploaded.

Tables should be formatted as follows. Arrange the data so that **columns of like material read down, not across.** The headings should be sufficiently clear so that the meaning of the data is understandable without reference to the text. See the “Abbreviations” section of these Instructions for those that should be used in tables. Explanatory footnotes are acceptable, but more-extensive table "legends" are not. Footnotes should not include detailed descriptions of the experiment. Tables must include enough information to warrant table format; those with fewer than six pieces of data will be incorporated into the text by the copy editor. Table 2, on page 106, is an example of a well-constructed table.

Avoid tables (or figures) of raw data on drug susceptibility, therapeutic activity, or toxicity. Such data should be analyzed by an approved procedure, and the results should be presented in tabular form.

In Vitro Susceptibility Tests

Tabulate results of determinations of minimal inhibitory and bactericidal concentrations according to the range of concentrations of each antimicrobial agent required to inhibit or kill the members of a species or of each group of microorganisms tested, as well as the corresponding concentrations required to inhibit 50 and 90% of the strains (MIC_{50} and MIC_{90}, respectively) and those required to kill 50 and 90% of the strains (MBC_{50} and MBC_{90}, respectively). The MIC50 and MIC90 reported should be the actual concentrations tested that inhibited 50 and 90%, respectively, of the strains. They should not be values calculated from the actual data obtained. When only six to nine isolates of a species are tested, tabulate only the MIC range of each antimicrobial agent tested.

If more than a single drug is studied, insert a column labeled "Test agent" between the columns listing the organisms and the columns containing the numerical data and record data for each agent in the same isolate order. Cumulative displays of MICs or MBCs in tables or figures are acceptable only under unusual circumstances.

The percentage of strains susceptible and/or resistant to an antibiotic at its breakpoint concentration may be given only if an appropriate breakpoint has been approved, as by the Clinical and Laboratory Standards Institute (http://www.clsi.org). In the absence of approved breakpoints, authors cannot assign breakpoints or use breakpoints from related antibiotics. An exploratory analysis tabulating the percentage of strains inhibited over a range of concentrations is acceptable.

Bactericidal tests must be performed with a sufficient inoculum (>5 x 10^5 CFU/ml) and subculture volume (0.01 ml) to ensure accurate determination of the 99.9% killing endpoint, as described by Pearson et al. (Antimicrob. Agents Chemother. **18:**699-708, 1980) and Taylor et al. (Antimicrob. Agents Chemother. **23:**142-150, 1983). Inoculum size and subculture volume are also critical to studies of combinations of antimicrobial agents.

Synergy is defined in two-dimensional or checkerboard tests when the fractional inhibitory concentration (FIC) or fractional bactericidal concentration (FBC) index (Σ) is ≤0.5. In killing curves, synergy is defined as a ≥2-$\log^{10}$ decrease in CFU per milliliter between the combination and its most active constituent after 24 h, and the number of surviving organisms in the presence of the combination must be ≥2 $\log^{10}$ CFU/ml below the starting inoculum. At least one of the drugs must be present in a concentration which does not affect the growth curve of the test organism when used alone. Antagonism is defined by a ΣFIC or ΣFBC of >4.0.

When standard twofold-dilution schemes are used to determine checkerboard interactions, the inherent variability of the method casts doubt on the significance of interactions represented by ΣFICs or ΣFBCs of >0.5 but ≤4. Therefore, such interactions, if labeled at all, should be termed "indifferent." Alternatively, indices in this range may be described as "nonsynergistic" or "nonantagonistic," as appropriate. The technically imprecise term "additive" should be avoided, as it is too easily misunderstood. See reports by W. R. Greco et al. (Pharmacol. Rev. **47:**331-385, 1995), F. C. Odds (J. Antimicrob. Chemother. **52:**1, 2003), and M. D. Johnson et al. (Antimicrob. Agents Chemother. **48:**693-715, 2004) for further discussion of these issues.

For killing curve tests, the minimal, accurately countable number of CFU per milliliter must be stated and the method used for determining this number must be described. In the absence of any drug and with a sample size of 1 ml, this number is 30 (1.5 in log10) CFU. If procedures for drug inactivation or removal have not been performed, the author must state how drug carryover effects were eliminated or quantified. For drugs showing an inoculum effect, mere dilution below the MIC obtained in standard tests is not sufficient.

International Journal of Antimicrobial Agents

Guide for Authors

These guidelines generally follow the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals". The complete document appears at http://www.icmje.org.

Submission

The Journal now accepts online submissions only. Manuscripts can be submitted at http://ees.elsevier.com/ijaa/. Once the manuscript has been uploaded, our system automatically generates an electronic pdf proof, which is then used for reviewing. All correspondence, including notification of the Editor's decision and requests for revisions, will be managed via this system. Authors may also track the progress of their paper using this system to final decision.

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