UNIVERSIDADE FEDERAL DE PERNAMBUCO

CENTRO DE CIÊNCIAS BIOLÓGICAS

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

PREPARAÇÃO E CARACTERIZAÇÃO DE NANOCÁPSULAS FURTIVAS E SÍTIO-ESPECÍFICAS PARA O TRATAMENTO DO CÂNCER

MILENA SALES FERRAZ

Fevereiro, 2013

Recife – PE

UNIVERSIDADE FEDERAL DE PERNAMBUCO

CENTRO DE CIÊNCIAS BIOLÓGICAS

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

PREPARAÇÃO E CARACTERIZAÇÃO DE NANOCÁPSULAS FURTIVAS E SÍTIO-ESPECÍFICAS PARA O TRATAMENTO DO CÂNCER

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.

Doutoranda: MILENA SALES FERRAZ

Orientadora: Profa. Dra. NEREIDE STELA SANTOS MAGALHÃES

Co-orientadora: Profa. Dra. ROSA MARIA SOUTO MAIOR

Fevereiro, 2013

Recife – PE

Catalogação na Fonte:
Bibliotecário Bruno Márcio Gouveia, CRB-4/1788

F381p Ferraz, Milena Sales
Preparação e caracterização de nanocápsulas furtivas e sítio-específicas para o tratamento do câncer / Milena Sales Ferraz. – Recife: O Autor, 2013.

106 f. : il., fig. tab.
Orientadora: Nereide Stela Santos Magalhães
Coorientadora: Rosa Maria Souto Maior
Tese (doutorado) – Universidade Federal de Pernambuco. Centro de Ciências Biológicas. Pós-graduação em Ciências Biológicas, 2013.
Inclui bibliografia e anexos

1. Farmacologia 2. Farmacocinética 3. Toxicologia 4. Câncer I. Magalhães, Stela Santos (orientadora) II. Souto Maior, Rosa Maria (coorientadora) III. Título.

615.3 CDD (22.ed.) UFPE/CCB-2013-097

MILENA SALES FERRAZ

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.

BANCA EXAMINADORA

PRESIDENTE: Profa. Dra. Nereide Stela Santos Magalhães

Departamento de Farmácia da Universidade Federal de Pernambuco

Assinatura:_____

EXAMINADOR INTERNO: Prof. Dr. Luiz Bezerra de Carvalho Junior

Departamento de Bioquímica da Universidade Federal de Pernambuco

Assinatura:_____

EXAMINADOR INTERNO: Profa. Dra. Adriana Fontes

Departamento de Biofísica da Universidade Federal de Pernambuco

Assinatura:_____

EXAMINADOR EXTERNO: Profa. Dra. Noemia Pereira da Silva Santos

Núcleo de Ciências Biológicas do Centro Acadêmico de Vitória – CAV/UFPE

Assinatura:_____

EXAMINADOR EXTERNO: Profa. Dra. Mariane Cajubá de Britto Lira

Núcleo de Nutrição do Centro Acadêmico de Vitória – CAV/UFPE

Assinatura:_____

SUPLENTE INTERNO: Profa Dra. Maria Tereza dos Santos Correia

Departamento de Bioquímica da Universidade Federal de Pernambuco (UFPE) -

Assinatura:_____

SUPLENTE EXTERNO: Profa. Dra. Isabella Macário Ferro Cavalcanti

Núcleo de Enfermagem do Centro Acadêmico de Vitória – CAV/UFPE

Assinatura:_____

Dedico esta tese a minha mãe, Lucia Sales, por ser sempre meu exemplo, meu alicerce, meu porto seguro, minha fortaleza.

AGRADECIMENTOS

À Deus, pela luz com a qual ilumina os caminhos de minha vida e sigo aprendendo, crescendo e realizando meus sonhos com a certeza que Ele está zelando por mim.

À minha mãe, Lucia Maria de Sales, pelo exemplo de vida, amor, dedicação e "garra", que me fez ser quem eu sou e ter chegado até aqui. Mainha, te amo!

Às minhas irmãs, Patrícia, Daniela e Sarita, pela compreensão nos momentos de impaciência e fraternidade demonstrada. Além de me presentearem com meus lindos sobrinhos.

Aos meus sobrinhos-filhos, Nicolle, Jaime Júnior, Rodrigo, Melissa, Antonio Neto, Renato e Maria Lúcia, meus pequenininhos, que com apenas um sorriso me trazem muita alegria e a certeza de que a vida continua.

Ao meu esposo, Marconi, pelo amor, carinho, respeito, compreensão e companheirismo, tão necessários na pessoa que desejamos estar ao nosso lado em todos os momentos. Amo você!

À minha orientadora Profa. Dra. Nereide Stela Santos Magalhães, pela orientação, confiança e por todos os conhecimentos dados em minha iniciação científica até o meu doutorado. Agradeço ainda pela oportunidade de ter contribuído com a evolução do Grupo de Sistemas de Liberação Controlada de fármacos. Muito Obrigada!

À minha co-orientadora Profa. Dra. Rosa Maria Souto Maior, por ter me recebido de "portas abertas" no seu laboratório e pela colaboração fundamental em todos os experimentos da síntese polimérica.

À Profa. Dra. Noemia Pereira de Silva Santos pela contribuição na realização deste trabalho e por sua paciência e boa vontade no esclarecimento de minhas dúvidas desde o meu mestrado.

Ao Prof. Dr. José Luiz de Lima Filho, Diretor do Laboratório de Imunopatologia Keizo-Asami – LIKA, por ceder às instalações físicas para realização deste trabalho.

Ao Prof. Dr. Luiz Bezerra de Carvalho Junior, responsável pelo setor de Bioquímica do LIKA, pelo incentivo e prover as condições necessárias ao bom funcionamento do setor.

A todos que fazem parte do grupo de sistema de liberação controlada de medicamentos, em especial: Rafaela, Isabella, Catarine, Larissa Morgana, Andressa, Larissa Chaves, Camilla, Thiers, Laércio, Sarah, Ana Lígia, Tiago, Rebecca, João Neto, Hanna, Ada, Marília, Patrícia e Pabyton, pela amizade e alegrias durante os momentos que passamos juntos.

Ao amigo e mestre Fábio Fidelis, pela amizade, companheirismo e pelos intermináveis trabalhos realizados nos também finais de semanas no laboratório.

Às minhas inesquecíveis amigas, Marcela, Mariane e Taciana, pelos vários anos de amizade, convivência e "desabafos". Beijos!

A todos os amigos e colegas do Laboratório de Bioquímica do LIKA-UFPE pela convivência em laboratório.

Aos funcionários do biotério do LIKA Maria Helena e Felipe pelos cuidados com os animais experimentais utilizados neste trabalho.

A todos os funcionários do LIKA, em especial ao Sr. Otaviano Costa, Rafael Padilha, Sr. Moisés Melo, Hilma, Conceição, Vera e Edson. Obrigada por cada aparelho consertado, por cada amostra congelada e liofilizada, por cada porta aberta, por sempre terem sido tão atenciosos comigo.

Ao Laboratório de Imunopatologia Keizo-Asami (LIKA), Departamento de Química Funcamental (DQF) e Centro de Tecnologias Estratégicas do Nordeste (CETENE-PE) pela disponibilidade da infraestrutura e suporte dado a este trabalho.

Ao Ministério de Ciência e Tecnologia e Inovação (MCTI), ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), à Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), e à Pró-Reitoria de Pesquisa e Pós-Graduação da Universidade Federal de Pernambuco (PROPESQ-UFPE) pelo suporte financeiro durante o curso deste trabalho.

A todos os colegas e amigos que de forma direta ou indireta contribuíram para a realização desse trabalho, meus sinceros agradecimentos.

RESUMO

O presente estudo objetivou sintetizar um polímero sítio-específico de ácido d,l-láctico e glicólico (PLGA) conjugado ao polietileno glicol (PEG) e ao folato (FOL), desenvolver nanocápsulas furtivas e sítio-específicas contendo β-lapachona (β-lap), validar um método analítico simples, rápido e específico para quantificação da β-lapachona em plasma de ratos através da cromatografia líquida de alta eficiência e analisar o perfil farmacocinético da β-lap encapsulada em nanocápsulas em comparação ao fármaco livre. Primeiramente, o PLGA-PEG-FOL foi sintetizado com um percentual de conjugação de 97% e caracterizado por espectroscopia ^{1}H RMN e FTIR. A β-lapachona foi encapsulada em nanocápsulas convencionais (NC/ βlap), PEGuiladas (NC-PEG/ βlap) e sítio-específicas (NC-FOL/ βlap), as quais foram preparadas pelo método de deposição interfacial de polímero pré-formado. As nanocápsulas foram caracterizadas por tamanho de partícula, índice de polidispersão, eficiência de encapsulação, potencial zeta e estudo de cinética de liberação *in vitro*. Os resultados demonstraram que o método de preparação permitiu a formação de nanocápsulas sítio-específicas nanométricas, monodispersas e com uma alta eficiência de encapsulação do fármaco. Uma redução do potencial zeta em módulo foi observada nas NC-PEG/ βlap e NC-FOL/ βlap em relação às NC/ βlap, provavelmente devido à presença do PEG e folato na superfície destes sistemas. Uma liberação de β-lapachona em torno de 80% a partir das nanocápsulas foi alcançada em 24 h. No estudo de citotoxicidade celular frente às linhagens celulares KG-1 e HeLa, as nanocápsulas sítio-específicas exibiram um efeito antiproliferativo relevante quanto comparados ao fármaco livre. Para a validação do método analítico, empregou-se cromatografia em fase reversa com fase móvel constituída por mistura de metanol e água (80:20, v/v), a um fluxo de 0,9 mL/min. Os analitos foram detectados por UV a 256 nm. A β-lapachona foi extraída das amostras de plasma após adição de metanol. O tempo de retenção foi de 4,5 min. Este método apresentou linearidade na faixa de concentração entre 0,5-16 μg/mL (r = 0,9996), com limite de quantificação de 0,2 μg/mL e exatidão de aproximadamente 100%. O método analítico desenvolvido neste trabalho demonstrou especificidade, linearidade, precisão e exatidão. O perfil farmacocinético das NC-FOL/β-lap foi avaliado e comparado ao da β-lap livre, após injeção intraperitoneal (i.p.) em ratos. Os parâmetros farmacocinéticos foram estimados por uma abordagem não-compartimental utilizando equações clássicas. Neste estudo, as nanopartículas mostraram alterações significativas no perfil farmacocinético das NC-FOL/β-lap. Diante dos resultados obtidos, sugere-se que as nanocápsulas PEGuiladas e sítio-específicas contendo β-lapachona poderão proporcionar um maior benefício terapêutico através de sistemas de longa duração e específico para células cancerosas.

Palavras-chave: PLGA-PEG, folato, nanocápsulas, β-lapachona, citotoxicidade, farmacocinética.

ABSTRACT

The aim of this study was to synthetize a site-specific polymer of lactic and glycolic acid (PLGA) conjugated to polyethylene glycol (PEG) and folate (FOL), to develop conventional, PEGylated and targeted nanocapsules encapsulating β-lapachone (β-lap), to validate a simple, fast and specific analytical method for quantification of β-lapachone in rats plasma by high-performance liquid chromatography and to analyze the pharmacokinetics profile of β-lap-loaded nanocapsules in comparison with free drug. Firstly, the PLGA-PEG-FOL was synthesized with conjugation percentage of 97% and characterized by ^{1}H NMR spectroscopy and FTIR. The β-lapachone was encapsulated into conventional (NC/ βlap), PEGylated (NC-PEG/ βlap) nanocapsules and targeted (NC-FOL/ βlap), which were prepared by the interfacial deposition of preformed polymer method. The nanocapsules were characterized for particle size, polidispersity index, encapsulation efficiency, zeta potential and *in vitro* release kinetic study of β-lapachone from nanocapsules. The results demonstrated that the preparation method permitted the formation of nanometers, monodisperse and with a high drug encapsulation efficiency. A reduction in the zeta potential was observed in NC-PEG/ βlap and NC-PEG/ βlap compared to NC/ βlap, probably due to the presence of folate and PEG on the surface of these systems. A β-lapachone release about 80% from the nanocapsules was reached in 24 h. In the cellular cytotoxicity study against KG-1 and HeLa targeted nanocapsules exhibited an antiproliferative effect as compared to free drug. For the validation of the analytical method, reverse phase chromatography was used with a mobile phase consisting of methanol-water (80:20, v / v) at a flow rate of 0.9 mL / min. Analytes were detected by UV at 256 nm. The β-lapachone was extracted from plasma samples after the addition of methanol. The retention time was 4.5 min. This method showed linearity in the concentration range between 0.5 to 16 μg / mL (r = 0.9996), with a quantification limit of 0.2 μg / mL and accuracy of approximately 100%. The analytical method developed in this study demonstrated specificity, linearity, precision and accuracy. The pharmacokinetic profile of NC-FOL/β-lap was evaluated and compared with the free β- lap after intraperitoneal (ip) in rats. Pharmacokinetic parameters were estimated by non-compartmental approach using classical equations. In this study, nanoparticles showed significant changes in NC-FOL/β-lap pharmacokinetic profile.

Based on these results, it is suggested that β-lap-loaded PEGylated and targeted nanocapsules may provide greater therapeutic benefit through of long duration and specific systems for cancer cells.

Keywords: PLGA-PEG, folate, nanocapsules, β-lapachone, citotoxicity, pharmacokinetics.

LISTA DE ILUSTRAÇÕES

REVISÃO BIBLIOGRÁFICA

Figura 1 - Distribuição percentual dos dez tipos de câncer mais incidentes estimados para 2012 por sexo, exceto câncer de pele não melanoma..... 16
Figura 2 - Estrutura química da β-lapachona..... 18
Figura 3 - Microscopia eletrônica de varredura da β-lapachona com um aumento de 1000× 19
Figura 4 - Mecanismo de ação proposto para a β-lapachona em células cancerígenas que conduzem a apoptose..... 21
Figura 5 - Comparação entre o tamanho de pequenas estruturas. A área destacada em vermelho mostra a área que corresponde aos "nanomateriais"..... 23
Figura 6 - Tipos de nanocarreadores de fármacos atualmente descritos em estudos pré-clínicos e clínicos..... 24
Figura 7 - Representação esquemática das nanopartículas poliméricas: a) fármaco dissolvido no núcleo oleoso das nanocápsulas; b) fármaco adsorvido à parede polimérica das nanocápsulas; c) fármaco retido na matriz polimérica das nanoesferas; d) fármaco adsorvido ou disperso molecularmente na matriz polimérica das nanoesferas..... 25
Figura 8 - Representação esquemática de uma nanopartícula PEGuilada..... 26
Figura 9 - Representação de conformações diferentes de PEG, formado através da sua incorporação sobre as superfícies das nanopartículas em diferentes densidades: A. Conformação tipo congumelo. B. Conformação tipo escova..... 27
Figura 10 - Diferenças entre o tecido normal e o tumoral que explicam o direcionamento passivo dos nanocarreadores através do efeito de permeabilidade e retenção aumentadas. A. Tecido normal contendo vasos sanguíneos lineares mantidos pelos pericitos. Os vasos linfáticos estão presentes. B. Tecido tumoral contendo vasos sanguíneos defeituosos com muitas fenestrações. Os vasos linfáticos estão ausentes..... 28
Figura 11 - Representação esquemática de uma nanopartícula sítio-específica PEGuilada... 29
Figura 12 - A. Direcionamento passivo de nanopartículas. (1) Nanocarreadores atingem tumores. (2) Fármacos livres difundem livremente dentro e fora dos vasos sanguíneos do tumor devido ao seu pequeno tamanho, ao contrário dos fármacos encapsulados, resultando no efeito EPR. B. Direcionamento ativo. (1) Ligantes acoplados na superfície de nanopartículas se ligam a receptores expressos por células cancerígenas ou (2) as células endoteliais angiogênicas..... 30
Figura 13 - Estrutura química do ácido fólico..... 32

Figura 14 - Endocitose de conjugados fármaco-folato. Os conjugados fármaco-folato exógenos ligam-se especificamente aos receptores de folato (FR) presentes na superfície das células alvo. A membrana plasmática sofre invaginação circundando o complexo conjugado/FR para formar uma vesícula intracelular (endossomo). Como o lúmem do endossomo acidifica. Os FR sofrem mudanças em sua conformação e liberam o conjugado no citosol. ,..... 33

CAPÍTULO 1

Fig. 1. Synthesis of PLGA–PEG–FOL conjugate..... 49

Fig. 2. ^{1}H NMR spectrum of PLGA-PEG-FOL……..... 53

Fig. 3. FT-IR spectrum of PLGA-PEG, folate and PLGA-PEG-FOL…….. 54

Fig. 4. Release profile of β-lapachone from the non-targeted (NC/β-lap) (■) and targeted (NC-FOL/β-lap) (●) nanocapsules under *sink* conditions. Each point represents the mean of three different experiments ± standard deviation……..... 57

Fig. 5. Cytotoxicity of free β-lapachone, non-targeted (NC/β-lap) and targeted (NC-FOL/β-lap) nanocapsules on KG-1 (A) and HeLa (B) cancer cells after 48 h of incubation. Results are mean values ± SD of cell viability percentage (n = 3)……..... 58

CAPÍTULO 2

Fig. 1. Chemical structure of β-lapachone ($C_{15}H_4O_3$)…….. 67

Fig. 2. Representative HPLC Chromatograms for (A) the blank plasma and (B) the plasma spiked with β-lap standard solutions (16 μg/mL)……. 71

Fig. 3. Mean plasma concentration–time profile of β-lap (μg/mL) following an intraperitonial dose of 40 mg/kg to three Wistar rats…… 74

CAPÍTULO 3

Fig. 1. Blood levels of intraperitoneally injected β-lap delivered in solution (■) and PLGA-PEG-FOL nanocapsules (●). The values plotted are mean ± SD…… 84

LISTA DE TABELAS

REVISÃO BIBLIOGRÁFICA

Tabela 1 - Atividade citotóxica da β-lapachona em diferentes linhagens de células tumorais..... 20

Tabela 2 - Exemplos de nanocarreadores usando a estratégia de direcionamento ativo sítio-específico..... 31

CAPÍTULO 1

Table 1. Physicochemical characterization of non-targeted (NC/β-lap) and targeted (NC-FOL/β-lap) nanocapsules containing β-lapachone…… 55

Table 2. IC_{50} values of free β-lapachone, non-targeted (NC/β-lap) and targeted (NC-FOL/β-lap) nanocapsules in KG-1 and HeLa cancer cells after 48 h of incubation. Results are mean values ± SD (n= 3)…….. 59

CAPÍTULO 2

Table 1. Linearity of the HPLC method for β-lap in rat plasma..... 72

Table 2. Accuracy for the quantitation of β-lap in rat plasma…… 72

Table 3. Precision evaluation of the HPLC method for quantifying β-lap in rat plasma….. 73

CAPÍTULO 3

Table 1. Comparative pharmacokinetic parameters of β-lap delivered in solution or through PLGA-PEG-FOL nanocapsules (NC-FOL/β-lap) after intraperitoneal injection….. 85

LISTA DE ABREVIATURAS E SIGLAS

ATP	Adenosina tri-fosfato
CIM	Concentração Inibitória Mínima
D.P.	Desvio padrão
DCC	Diciclohexilcarbonimida
DCU	Diciclohexil uréia
DMSO	Dimetilsulfóxido
DNA	Ácido desoxirribonucleico
EE	Eficiência de encapsulação
EMA	Agência de medicina européia
EPR	Efeito de permeabilidade e retenção aumentada
FDA	Administração de Alimentos e Medicamentos (*Food and Drug Administration)*
FR	Receptor de folato
FOL	Folato
HPβ-CD	2- hidroxipropil-β-ciclodextrina
IC_{50}	Concentração para inibir 50% do crescimento celular
INCA	Instituto Nacional do Câncer
NAD(P)H	Nicotinamida adenina dinucleotídeo fosfato
NADH	Nicotinamida adenina dinucleotídeo
NC	Nanocápsulas
NC/β-lap	Nanocápsulas convencionais contendo β-lapachona
NC-PEG/β-lap	Nanocápsulas PEGuiladas contendo β-lapachona
NC-FOL/β-lap	Nanocápsulas sítio-específicas contendo β-lapachona
NHS	N-hidroxisucinimida
NQO1	NAD(P)H: quinona oxidorreductase 1
OMS	Organização Mundial de Saúde
PDI	Índice de polidipersão
PEG	Polietilenoglicol
PLGA	Copolímero de ácido láctico e glicólico
PM	Peso molecular
RMN H^1/^{1}H-NMR	Ressonância magnética nuclear de prótons
ROS	Espécies reativas de oxigênio
SFM	Sistema fagocitário mononuclear
UV	Ultravioleta

SUMÁRIO

RESUMO..... vii
ABSTRACT..... viii
LISTA DE ILUSTRAÇÕES..... ix
LISTA DE TABELAS..... xi
LISTA DE ABREVIATURAS E SIGLAS..... xii
1. INTRODUÇÃO..... 14
2. REVISÃO DE LITERATURA..... 16
2.1 – Câncer..... 16
2.1.1 – Tratamentos do câncer..... 17
2.2 - β-lapachona..... 18
2.2.1 – Características gerais..... 18
2.2.2 – Atividades biológicas..... 18
2.2.3 – Mecanismo de ação..... 20
2.2.4 – Toxicologia..... 22
2.3 – Nanotecnologia..... 22
2.4 – Sistemas de Liberação Controlada de Fármacos..... 23
2.4.1 - Nanopatículas poliméricas..... 24
2.4.2 - Nanopatículas furtivas..... 26
2.4.3 - Nanopatículas sítio-específicas..... 29
2.4.3.1 – Liberação de fármacos mediada por receptores de folato..... 31
3. REFERÊNCIAS..... 34
4. OBJETIVOS..... 43
5. CAPÍTULO 1: Preparation and characterization of PEGylated and Folate targeted nanocapsules of β-lapachone..... 44
6. CAPÍTULO 2: Development and validation of HPLC method to determine β-lapachone in rat plasma: Application to pharmacokinetic studies..... 65
7. CAPÍTULO 3: Pharmacokinetics studies of β-lapachone loaded PLGA-PEG-FOL nanocapsules after intraperitoneal administration..... 77
8. CONCLUSÕES..... 89
9. PERSPECTIVAS..... 91
10. ANEXOS..... 92

1. INTRODUÇÃO

Câncer é um termo genérico dado a um conjunto de mais de 100 doenças que têm em comum o crescimento desordenado de células que invadem os tecidos e órgãos. Esta é uma das doenças que mais atinge a população mundial. A quimioterapia tornou-se um componente integrante do tratamento anticancerígeno. Atualmente, diferentes agentes quimioterápicos são utilizados de forma eficaz para a terapia anticancerígena, contudo, os agentes quimioterápicos convencionais ainda exibem baixa especificidade em atingir o tecido tumoral, além de frequentemente serem restritos por uma toxicidade dose-limitante. Desta forma, essa patologia tem estimulado os pesquisadores a buscar novos agentes anticancerígenos como uma estratégia importante para o estabelecimento de terapias alternativas (GU et al., 2007; PARHI; MOHANTY; SAHOO, 2012; PARHI; MOHANTY; SAHOO, 2012).

A β-lapachona é uma orto-naftoquinona que pode ser extraída da casca do Ipê-Roxo (*Tabebuia avellanedae*) ou obtida por semi-síntese a partir do lapachol (LI; AVERBOUK; PARDEE, 1993; ALVES et al., 2008). Estudos têm demonstrado que essa molécula apresenta diversas atividades biológicas, dentre elas, a atividade antineoplásica (WOO; CHOI, 2005). Apesar do seu potencial terapêutico, a β-lapachona apresenta limitações, assim como baixa solubilidade em água (0,038 mg/mL) (NASONGKLA et al., 2003) e hepatoxicidade (ALMEIDA et al., 2009). 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.

Atualmente define-se como sistemas de liberação controlada àqueles nos quais o agente ativo é liberado sob uma cinética bem estabelecida. Estes sistemas frequentemente descritos como “drug delivery systems”, oferecem diversas vantagens em relação às formas de dosagens convencionais, tais como: protegem certos princípios ativos lábeis da degradação e/ou inativação pelo suco gástrico, melhoram a biodisponibilidade dos mesmos, aumentam a penetração celular de fármacos, além de reduzir os efeitos toxicológicos (PANYAM; LABHASETWAR, 2003).

Existe uma variedade de nanocarreadores descrito na literatura que apresentam diferentes tipos de aplicações. Dentre estes sistemas estão as nanocápsulas, as quais são constituídas por um invólucro polimérico disposto ao redor de um núcleo oleoso, podendo o fármaco estar dissolvido neste núcleo e/ou adsorvido à parede polimérica (KUMARI; YADAV; YADAV, 2010).

O principal obstáculo para a utilização das nanocápsulas convencionais se deve a rápida remoção destes nanocarredores do organismo pelo sistema fagocitário mononuclear (SFM), contudo as nanocápsulas podem ser revestidas com moléculas que alterem sua hidrofobicidade, proporcionando uma camada hidrofílica na superfície. O método mais comum para a modificação

de superfície é a utilização de um polímero hidrofílico e não iônico, como o polietileno glicol (PEG), um processo chamado “PEGuilação” (MOSQUEIRA et al., 2001)

Na tentativa de aumentar a especificidade de interação dos nanossistemas com células alvo e elevar a quantidade do fármaco liberado nestas células, a pesquisa neste campo foi focada no desenvolvimento de nanopartículas sítio-específicas. Estes utilizam ligantes acoplados em sua superfície, que conferem seletividade para distribuir o fármaco encapsulado no sítio de ação desejado proporcionando uma maior seletividade e eficácia em comparação ao direcionamento passivo (SAPRA; ALLEN, 2003).

Uma das mais extensivamente estudada molécula alvo para entrega de fármacos sítio-específicos é o ácido fólico (vitamina B9 ou folato) (LEAMON; LOW, 2001). O receptor de folato (FR) está presente em praticamente todas as células, porém é expresso em níveis elevados principalmente em células cancerígenas, tais como, cânceres epiteliais de ovário, colo-retal, mama, próstata, pulmão, nariz, garganta, cérebro e leucemia (SUDIMACK; LEE, 2000; LEAMON; LOW, 2001; ALEXIS et al., 2008).

Vários tipos de carreadores de fármacos têm sido conjugados ao folato, incluindo lipossomas, micelas e nanopartículas poliméricas. Para o direcionamento dos lipossomas e nanopartículas, uma cadeia de PEG ligante é frequentemente requerida. Múltiplas moléculas de folato são conjugadas a cada partícula, que permite uma elevada afinidade de interação com os FR (ZHAO; LI; LEE, 2008). Além disso, sistemas de liberação de fármacos conjugados com folato demonstraram uma alta citotoxicidade e captura celular de células cancerígenas que expressam o FR (YOO et al., 2004; ESMAELLI et al., 2008; NIE et al., 2009; LIANG et al., 2011; SHMEEDA et al., 2010; SAXENA; NAGUIB; HUSSAIN, 2012).

Portanto, o desenvolvimento de sistemas de liberação controlada de fármacos, furtivos e/ou sítios específicos**,** capazes de viabilizar a administração da β-lapachona em uma formulação que melhore sua solubilidade, bem como, aperfeiçoe a dose terapêutica diminuindo os efeitos tóxicos e direcione o fármaco para o tumor aumentando a eficácia terapêutica, torna-se uma inovação útil no desenvolvimento de alternativas para o tratamento do câncer.

2. REVISÃO BIBLIOGRÁFICA

2.1 - Câncer

O câncer é uma das doenças mais devastadoras a qual envolve várias alterações genéticas e anormalidades celulares. Esta complexidade e heterogeneidade promovem o crescimento agressivo de células cancerígenas que levam à morbidade e mortalidade em diversos pacientes (PARHI; MOHANTY; SAHOO, 2012).

Mais de 11 milhões de pessoas são diagnosticadas com câncer a cada ano, e esta doença é responsável por cerca de 7 milhões de mortes por ano (12,5% das mortes em todo o mundo), tornando esta doença um problema de saúde pública mundial (PARCK et al., 2008). Segundo a Organização Mundial da Saúde (OMS) é estimado que, no ano 2030, ocorram 27 milhões de casos incidentes de câncer, 17 milhões de mortes por câncer e 75 milhões de pessoas vivas com câncer anualmente (INCA, 2011).

No Brasil, as estimativas para o ano de 2012 serão válidas também para o ano de 2013 e apontam a ocorrência de aproximadamente 518.510 casos novos de câncer. Os tipos mais incidentes serão próstata, pulmão, cólon e reto e estômago para o sexo masculino; e mama, colo do útero, cólon e reto e glândula tireóide para o sexo feminino (Figura 1) (INCA, 2011).

Figura 1 - Distribuição percentual dos dez tipos de câncer mais incidentes estimados para 2012 por sexo, exceto câncer de pele não melanoma.

Localização primária	casos novos	percentual			Localização primária	casos novos	percentual
Próstata	60.180	30,8%	Homens	Mulheres	Mama Feminina	52.680	27,9%
Traqueia, Brônquio e Pulmão	17.210	8,8%			Colo do Útero	17.540	9,3%
Cólon e Reto	14.180	7,3%			Cólon e Reto	15.960	8,4%
Estômago	12.670	6,5%			Glândula Tireoide	10.590	5,6%
Cavidade Oral	9.990	5,1%			Traqueia, Brônquio e Pulmão	10.110	5,3%
Esôfago	7.770	4,0%			Estômago	7.420	3,9%
Bexiga	6.210	3,2%			Ovário	6.190	3,3%
Laringe	6.110	3,1%			Corpo do Útero	4.520	2,4%
Linfoma não Hodgkin	5.190	2,7%			Linfoma não Hodgkin	4.450	2,4%
Sistema Nervoso Central	4.820	2,5%			Sistema Nervoso Central	4.450	2,4%

*Números arredondados para 10 ou múltiplos de 10

Fonte: INCA, 2011.

2.1.1 - Tratamentos para o câncer

A remoção cirúrgica de um tumor e dos tecidos circunvizinhos afetados é considerado o procedimento primário para tumores grandes o suficiente para serem manipulados. Entretanto, dificilmente a cirurgia é suficiente e, geralmente, é inevitável a permanência de células residuais afetadas. Além da cirurgia, outros recursos terapêuticos podem ser usados como a quimioterapia (WANG et al., 2008).

A quimioterapia tornou-se um componente integrante do tratamento anticancerígeno. Atualmente, diferentes agentes quimioterápicos são utilizados de forma eficaz para a terapia anticâncer, contudo, apesar dos últimos 30 anos de esforço da oncologia em descobrir novos fármacos, os agentes quimioterápicos convencionais ainda exibem baixa especificidade em atingir o tecido tumoral, além de frequentemente serem restritos por uma toxicidade dose-limitante (GU et al., 2007; PARHI; MOHANTY; SAHOO, 2012).

A quimioterapia depende de vários fatores como os fármacos e as doses utilizadas, a via de administração e a condição do paciente. Além disso, durante o percurso até o tumor, o fármaco pode interagir com outros tecidos e ser metabolizado pelo organismo, comprometendo a sua biodisponibilidade no tumor e, por conseguinte, sua eficácia (JAIN, 2001; OOYAMA et al., 2008).

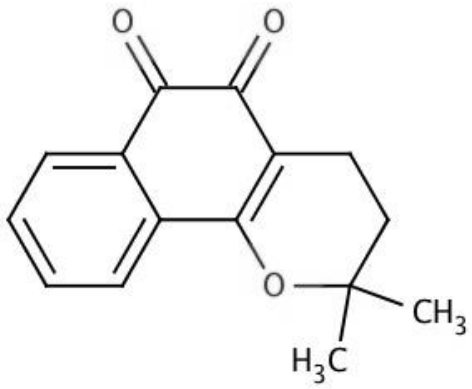
A fim de se obter uma terapia eficaz, é necessário aprimorar o conhecimento sobre a fisiopatologia do câncer, descobrir novos fármacos, e desenvolver novas tecnologias biomédicas. Atualmente, a terapia anticancerígena tornou-se uma abordagem multidisciplinar a qual requer uma estreita colaboração entre os clínicos, os pesquisadores e os engenheiros biomédicos (DANHIER et al., 2012).

Existem diversos medicamentos aprovados para uso na terapia anticancerígena, porém a resistência ao tratamento 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).

2.2 – β-lapachona

A β-lapachona (3,4-diidro-2,2-dimetil-2H-naftol (1,2-b)pirano-5,6-diona, β-lap) é uma *orto*-naftoquinona (Figura 2) que pode ser extraída da casca do Ipê-Roxo (*Tabebuia avellanedae*), árvore nativa da América do Sul com alta frequência no Brasil (ALVES et al., 2008). Porém, em larga escala, a β-lap é obtida por semi-síntese a partir do lapachol por aquecimento ou hidrólise a frio com ácido sulfúrico (LI; AVERBOUK; PARDEE, 1993).

Figura 2 - Estrutura química da β-lapachona.



2.2.1 - Características gerais

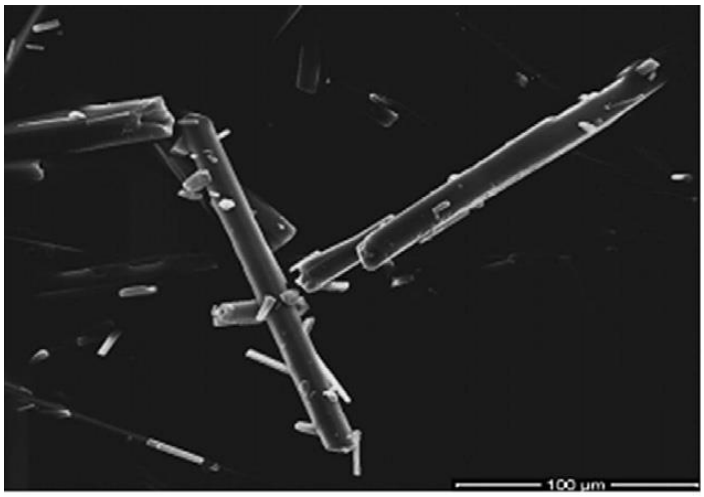
Dentre as suas propriedades químicas gerais, a β-lap caracteriza-se por ser uma substância de pigmentação laranja-avermelhada, de forma de cristais cilíndricos (Figura 3), com peso molecular de 242,3 Da e ponto de fusão entre 154,5 – 155,5 ºC. É estruturalmente estável em pH de 3 a 9 (SILVA; FERREIRA.; SOUZA, 2003). A β-lap é uma molécula hidrofóbica, com solubilidade em água igual a 0,038 mg/mL ou 0,16 mM, o que limita sua aplicação clínica (NASONGKLA et al., 2003).

2.2.2 - Atividades biológicas

A β-lapachona apresenta inúmeras ações farmacológicas, 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; LI et al., 1993), antiinflamatória (MOON et al., 2007), cicatrizante (KUNG et al., 2008) e antineoplásica (WOO; CHOI, 2005).

Figura 3 – Microscopia eletrônica de varredura da β-lapachona com um aumento de 1000×.



Fonte: CAVALCANTI et al., 2011.

Com relação à atividade antiproliferativa, a β-lap atua frente a várias linhagens de células cancerígenas, incluindo câncer de mama (SIEGEL; ROSS, 2000), câncer de próstata (LI et al., 1999; BLANCO et al., 2007; DONG et al., 2009), mieloma múltiplo (LI et al., 2000; GUPTA et al., 2002), hepatoma (LAI et al., 1998), pâncreas (OUGH et al., 2005) e leucemia humana (CHAU et al., 1998).

Desde a descoberta de sua excelente atividade antiproliferativa, a β-lap tem se destacado na linha de pesquisa de obtenção de novos quimioterápicos. Consequentemente, vários estudos foram realizados para determinar a concentração mínima deste fármaco necessária para inibir 50% do crescimento celular (IC_{50}). A tabela 1 demonstra a citotoxicidade da β-lapachona em diferentes linhagens de células tumorais e suas respectivas IC_{50}.

A utilização da β-lap em combinação com outros fármacos quimioterápicos tais como o taxol, tem sido reportada. Estudos *in vivo* demonstraram que a administração da β-lapachona por via intraperitoneal nas doses de 25 a 50 mg/Kg, reduziu a formação de tumores em camundongos inoculados com células de câncer de ovário em 75% utilizando uma dose de 50 mg/mL. No entanto, quando associada ao taxol, houve uma potencialização no efeito farmacológico, observando uma redução de quase 100% nos nódulos tumorais (LI et al., 1999).

Tabela 1 - Atividade citotóxica da β-lapachona em diferentes linhagens de células tumorais.

Linhagem celular	**IC_{50} (μM)**	**Referências**
MCF-7 (câncer de mama)	1,7 3 ~ 4 2,5	NASONGKLA et al., 2003; BENTLE et al., 2007; LI et al., 1999; WUERZBERGER et al., 1998
MDAMB-231 (câncer de mama)	6,6	REINICKE et al., 2005
21MT (células de câncer de mama humana)	~ 4	LI et al., 1999
HBL-100 (câncer de mama)	0,69	BONIFAZI et al., 2010
PC-3 (câncer de próstata)	3,5	REINICKE et al., 2005;
DU-145 (câncer de próstata)	7,4 4	EYONG et al., 2008; LI et al., 1999
HL-60 (leucemia promielocítica)	1,65	MOON et al., 2010
KB (tumor nasofaríngeo)	6,6	KRISHNAN et al., 2001
HCT-8 (câncer de colo-retal)	0,83	SILVA-JÚNIOR et al., 2007
Skmel-28 (Melanoma)	~ 4	LI et al., 1999
A2780DDP (Câncer de ovário)	~ 2 1,1	LI et al., 1999 BONIFAZI et al., 2010

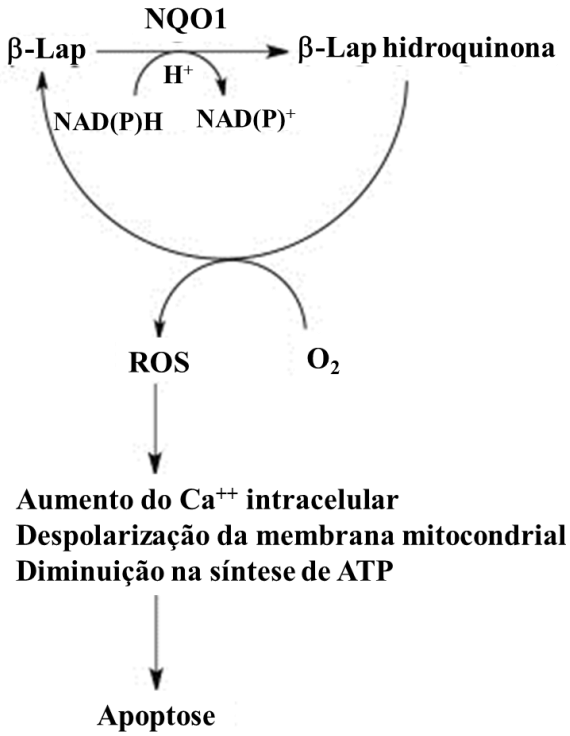
2.2.3 - Mecanismo de ação

Estudos reportam que a β-lapachona inibindo a enzima topoisomerases I, pois a incubação direta desta substância com a topoisomerase I, antes da adição de DNA como substrato, aumenta drasticamente o seu efeito inibitório, sugerindo a interação direta da β-lap com a topoisomerase I (PARDEE et al, 2002).

Além disso, há evidências da participação da enzima nicotinamida adenina dinucleotídeo fosfato: quinona oxirreductase-1 (NQO1), na ativação da β-lap no processo de apoptose. A NQO1 é expressa em maiores concentrações em vários tipos de tumores, incluindo os de mama, pulmão e colorretal, do que em tecidos normais, a qual 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) (Figura 4). Bey e colaboradores (2007) demonstraram que a β-lap é um agente terapêutico efetivo capaz de matar células de carcinoma de pulmão, principalmente as células do tipo NSCLC (câncer de pulmão de células não pequenas), por estas conterem maior concentração desta enzima. Esta observação abre perspectivas para novas estratégias para a quimioterapia seletiva de alguns tipos de câncer, como o de pulmão, próstata e de pâncreas.

Figura 4 – Mecanismo de ação proposto para a β-lapachona em células cancerígenas que conduzem a apoptose.



Fonte: Adaptado de SIEGEL; YAN; ROSS, 2012.

2.2.4 - Toxicologia

Almeida e colaboradores (2009) avaliaram a toxicidade da β-lap em ratos Wistar de ambos os sexos. No estudo realizado em ratas grávidas tratadas via intraperitoneal com doses de 40, 80 e 160 mg/Kg de β-lapachona foi possível observar alterações anatômicas em fetos. A β-lap demonstrou ações abortivas e teratogênicas nas doses utilizadas, bem como, alterações hematológicas no número total dos leucócitos, o que indicou a estimulação do sistema imunológico pela β-lapachona.

Na avaliação do efeito da β-lap ao longo de 21 dias, a análise bioquímica evidenciou um aumento significante nos níveis das enzimas gama glutamil transferase, fosfatase alcalina e glutamato piruvato transaminase. Estes resultaram indicaram um dano hepático, principalmente no ducto biliar, quando associado ao aumento da bilirrubinemia. Contudo, na análise histopatológica do fígado e rim, não foram observadas alterações. Ao contrário do baço, onde foram encontrados folículos aumentados na polpa branca (ALMEIDA et al, 2009).

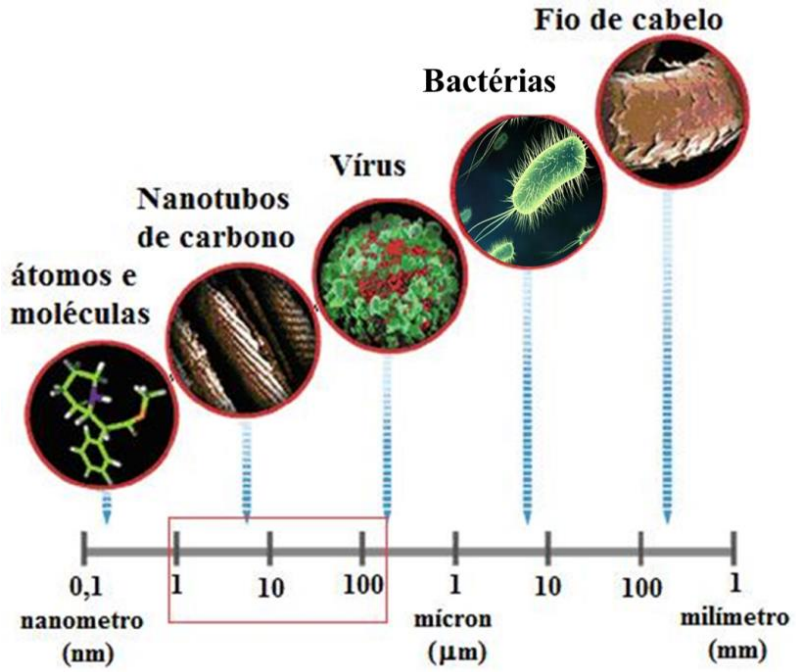
Diante do exposto, a β-lap é apontada como um novo e potente agente antitumoral, porém por apresentar baixa solubilidade em água e toxicidade, a sua aplicação terapêutica é limitada. Desta forma recentes pesquisas têm voltado à atenção para a nanotecnologia com o intuito de encontrar formas mais eficientes de direcionar o fármaco para o alvo tumoral, diminuindo a dose terapêutica e, em consequência, os efeitos adversos relacionados com a biodistribuição não específica do fármaco.

2.3 - Nanotecnologia

A nanotecnologia é uma área da ciência dedicada ao design, construção, e utilização de estruturas funcionais em escala nanométrica. Colocando este intervalo de tamanho em perspectiva, uma pequena molécula, um vírus, uma bactéria, e uma secção transversal de um cabelo humano são em torno de 1, 100, 100, e 100.000 nm, respectivamente (Figura 5) (ALEX et al., 2008, PARCK et al., 2008).

Existem numerosas aplicações para a nanotecnologia, dentre entre elas, o tratamento, diagnóstico, monitoramento e controle dos sistemas biológicos têm recentemente sido referidos como “nanomedicina” pelo Instituto Nacional de Saúde (PARK et al., 2008). A nanomedicina é um dos ramos mais promissores da medicina contemporânea, retendo boa parte dos esforços científicos na busca de novos tratamentos para doenças como o câncer e doenças infecciosas (PISON et al., 2006; KAGAN; BAYIR; SHVEDOVA, 2005).

Figura 5 - Comparação entre o tamanho de pequenas estruturas. A área destacada em vermelho mostra a área que corresponde aos “nanomateriais”.



Fonte: Adaptado de http://www.ofitexto.com.brconteudoimagens.gif.

A nanotecnologia do câncer é um novo campo da pesquisa interdisciplinar permeando através da biologia, química, engenharia e medicina objetivando levar maiores avanços para o diagnóstico e tratamento do câncer (WANG et al., 2010). Atualmente, existe um grande foco na utilização de sistemas de liberação controlada de fármacos como uma evolução da terapia tradicional anticancerígena (PARK et al., 2008).

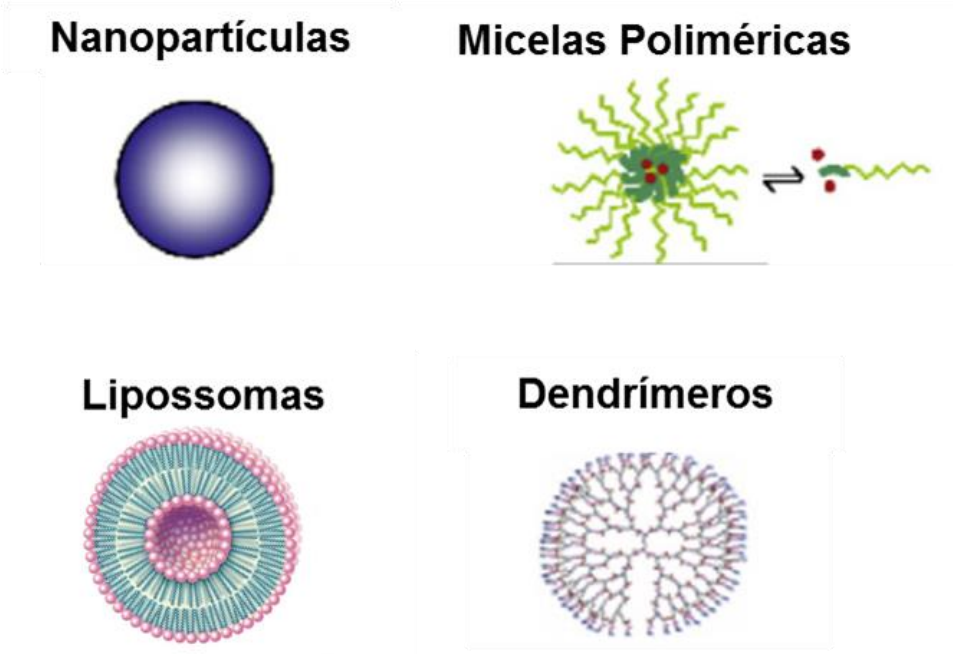
2.4 - Sistemas de Liberação Controlada de Fármacos

Atualmente define-se como sistemas de liberação controlada àqueles nos quais o agente ativo é liberado sob uma cinética bem estabelecida. Estes sistemas frequentemente descritos como “drug delivery systems”, oferecem diversas vantagens em relação às formas de dosagens convencionais, tais como: protegem certos princípios ativos lábeis da degradação e/ou inativação pelo suco gástrico, melhoram a biodisponibilidade dos mesmos, aumentam a penetração celular de fármacos, além de reduzir os efeitos toxicológicos (PANYAM; LABHASETWAR, 2003).

Existe uma variedade de nanocarreadores descrito na literatura que apresentam diferentes tipos de aplicações. Dentre os principais vetores utilizados como sistemas de liberação controlada,

destacam-se os sistemas poliméricos como nanopartículas (nanoesferas e nanocápsulas), micelas poliméricas constituídas de polímeros anfifílicos associados em solução aquosa, além de outros sistemas, como os lipossomas, que são vesículas com núcleo aquoso envolvido por uma bicamada lipídica, e dendrímeros que representam séries repetidas de compostos macromoleculares que formam uma cavidade em seu interior (Figura 6) (RAWAT, 2006; DANHIER; FERON; PRÉAT, 2010).

Figura 6 – Tipos de nanocarreadores de fármacos atualmente descritos em estudos pré-clínicos e clínicos.



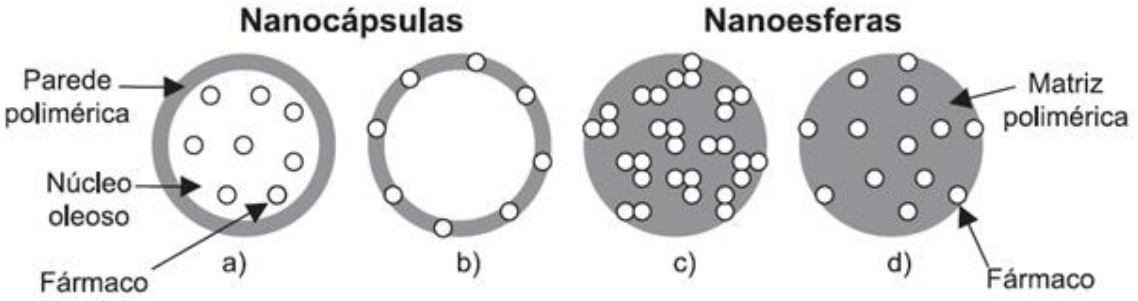
Fonte: Adaptado de DANHIER; FERON; PRÉAT, 2010.

2.4.1 - Nanopartículas poliméricas

Nanopartículas são estruturas esféricas e sólidas em uma escala de tamanho de 10 a 1000 nm de diâmetro, e refere-se a dois tipos de estruturas diferentes, nanoesferas e nanocápsulas, cada uma com suas características, as quais diferem entre si segundo a composição e organização estrutural. As nanocápsulas são constituídas por um invólucro polimérico disposto ao redor de um núcleo oleoso, podendo o fármaco estar dissolvido neste núcleo e/ou adsorvido à parede polimérica. Por outro lado, as nanoesferas, que não apresentam óleo em sua composição, são formadas por uma

matriz polimérica, onde o fármaco pode ficar retido ou adsorvido (Figura 7) (SOPPIMATH et al., 2001; KUMARI; YADAV; YADAV, 2010).

Figura 7 - Representação esquemática das nanopartículas poliméricas: a) fármaco dissolvido no núcleo oleoso das nanocápsulas; b) fármaco adsorvido à parede polimérica das nanocápsulas; c) fármaco retido na matriz polimérica das nanoesferas; d) fármaco adsorvido ou disperso molecularmente na matriz polimérica das nanoesferas.



Fonte: SCHAFFAZICK et al., 2003.

As nanopartículas poliméricas liberam o medicamento por meio do mecanismo de difusão ou erosão controlada, a partir de um núcleo através da membrana ou matriz polimérica. A membrana de revestimento atua como uma barreira para liberação, portanto, a solubilidade e difusividade do fármaco na membrana polimérica tornam-se fatores determinantes na liberação do mesmo. Além disso, a taxa de liberação também pode ser afetada pela interação iônica entre o fármaco e os excipientes da formulação. Havendo a interação do fármaco com um dos excipientes para formar um complexo menos solúvel em água, a liberação do mesmo pode ser muito lenta, e com um pequeno efeito de liberação rápida inicial (Efeito *burst*) (MUDSHINGE et al., 2011).

Para desenvolver um sistema nanopartículado eficaz, ambos, o fármaco liberado e a biodegradação polimérica, são fatores considerados importantes. Em geral, a taxa de liberação do fármaco depende (1) da solubilidade do fármaco, (2) da dessorção na superfície ligada ou fármaco adsorvido, (3) na difusão do fármaco através da matriz das nanopartículas, e (4) da combinação do processo de erosão/difusão (MOHANRAJ; CHEN, 2006).

O poli(ácido láctico-co-glicólico) (PLGA) é um dos polímeros biodegradáveis mais utilizados na preparação das nanopartículas, porque a sua hidrólise leva aos monômeros ácido láctico e ácido glicólico, os quais são facilmente metabolizados pelo corpo via ciclo de Krebs. Devido à mínima toxicidade associada ao PLGA, este polímero é aprovado pela Administração de Alimentos e Fármacos dos EUA (FDA/US) e pela Agência de Medicina Européia (EMA) para seu

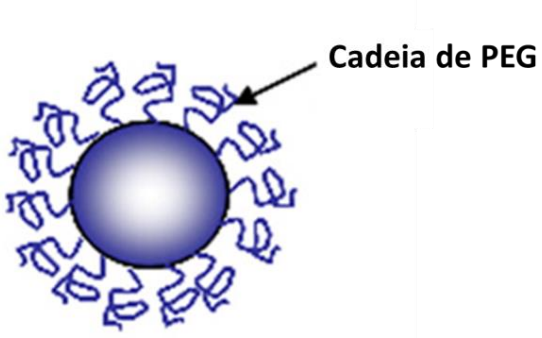
uso em vários sistemas de liberação de fármacos em humanos (KUMARI; YADAV; YADAV, 2010).

2.4.2 - Nanopartículas furtivas

O principal obstáculo ao uso das nanopartículas poliméricas é que são rapidamente removidas do organismo pelo sistema fagocitário mononuclear (SFM). Os macrófagos do SFM são capazes de remover nanopartículas desprotegidas da circulação sanguínea segundos após a administração intravenosa, tornando-as ineficazes como dispositivos de liberação de fármaco órgão-específico. Porém, esses macrófagos não podem identificar diretamente as nanopartículas, mas reconhecem opsoninas específicas ligadas a superfície dessas partículas. Com efeito, uma vez na corrente sanguínea, nanopartículas de superfície não modificadas (nanopartículas convencionais) são rapidamente opsonizadas e fagocitadas pelo SFM (SINGH, R.; LILLARD JR, 2009; KUMARI; YADAV; YADAV, 2010; DANHIER et al., 2012).

Vários métodos de modificações superficiais têm sido desenvolvidos para produzir nanopartículas que não sejam reconhecidos pelo SFM. As nanopartículas podem ser revestidas com moléculas que alterem sua hidrofobicidade, proporcionando uma camada hidrofílica na superfície. O método mais comum para a modificação de superfície é a utilização de um polímero hidrofílico e não-iônico, como o polietileno glicol (PEG), um processo chamado “PEGuilação” (MOSQUEIRA et al., 2001) (Figura 8).

Figura 8 - Representação esquemática de uma nanopartícula PEGuilada.



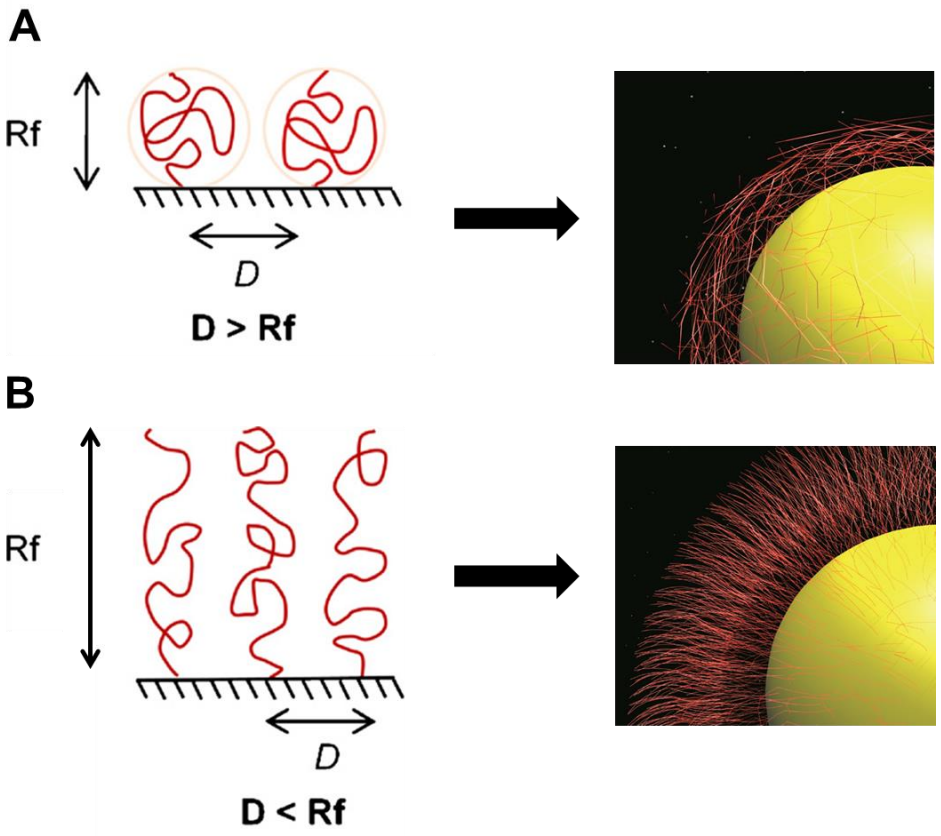
Fonte: Adaptado de DANHIER et al., 2012.

Para contribuir com as características “stealth” das nanopartículas PEGuiladas, existem três fatores importantes: (i) o peso molecular da cadeia do PEG, (ii) a densidade das cadeias de superfície e (iii) a conformação. Com a criação de uma camada hidrofílica protetora em torno das nanopartículas, as forças de repulsão estéricas repelir a absorção das proteínas opsoninas, desse modo bloqueando e atrasando o processo de opsonização. Amplamente, tem sido demonstrado que

a "PEGuilação" aumenta o tempo de meia-vida das nanopartículas na circulação sanguínea (OWENS; PEPPAS, 2006).

Quando apresentadas em uma superfície de nanopartícula, as cadeias de PEG individuais exibem um Raio de Flory (Rf) que representa o volume que cada cadeia de PEG flexível ocupa. Aumentando a densidade de PEG na superfície das nanopartículas, gera uma redução na distância, D, entre cada molécula de PEG na superfície do nanossitema. Quando D > Rf, as cadeias de PEG do polímero irão adquirir uma conformação em "cogumelo". Quando D < Rf, a pressão lateral entre as cadeias de PEG forçará a extensão do polímero resultando em uma conformação em "escova" (Figura 9). O arranjo das cadeias de PEG presente na superfície das nanopartículas na configuração de escova gera uma maior repulsão das proteínas (opsoninas), e geralmente aumenta o tempo de meia vida na circulação. Contudo, as nanopartículas são frequentemente revestidas com cadeias de PEG entre 3.400 a 10.000 Da, pois grandes aumentos no raio hidrodinâmico das partículas podem encurtar o tempo de meia vida. (WANG; THANOU, 2010; JOKERST et al., 2011).

Figura 9 - Representação de conformações diferentes de PEG, formado através da sua incorporação sobre as superfícies das nanopartículas em diferentes densidades: A. Conformação tipo cogumelo. B. Conformação tipo escova.

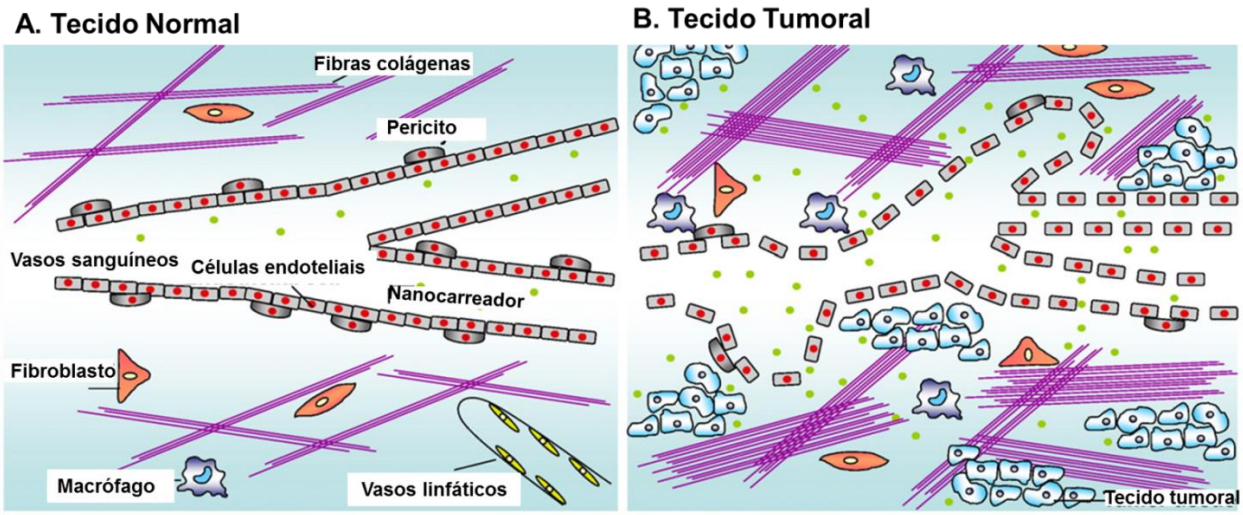


Fonte: Adaptado de WANG; THANOU, 2010; JOKERST et al., 2011.

Além disso, estudos relatam que as nanopartículas poliméricas que apresentam circulação prolongada nos vasos sanguíneos, acumulam-se passivamente nos tumores, sugerindo a existência de um mecanismo passivo de retenção e a potencialidade desses sistemas nanoestruturados em tratamentos para o câncer (PARK et al.,2008).

A vascularização normal é caracterizada por vasos intactos e contínuos. Por outro lado, os vasos sanguíneos tumorais são geralmente caracterizados por anormalidades como alta proporção de células endoteliais proliferativas e formação anormal da membrana, devido à rápida vascularização necessária para prover oxigênio e nutrientes para o tumor crescer rapidamente. A estrutura dos vasos sanguíneos de um tumor, composta por fenestras e lacunas, associada à ausência de um sistema linfático intacto, torna os vasos sanguíneos neste local permeáveis a macromoléculas e nanopartículas. Dessa forma, as nanopartículas tendem a se acumular nos tecidos tumorais passivamente. Este fenômeno de atingir o alvo (tumor) passivamente foi chamado de efeito de permeabilidade e retenção aumentadas (do inglês *Enhanced Permeability and Retention*, ou EPR) (Figura 10) (ALEXIS et al., 2008; PARK et al., 2008; TORCHILIN, 2011).

Figura 10 – Diferenças entre o tecido normal e o tumoral que explicam o direcionamento passivo dos nanocarreadores através do efeito de permeabilidade e retenção aumentadas. A. Tecido normal contendo vasos sanguíneos lineares mantidos pelos pericitos. Os vasos linfáticos estão presentes. B. Tecido tumoral contendo vasos sanguíneos defeituosos com muitas fenestrações. Os vasos linfáticos estão ausentes.



Fonte: Adaptado de DANHIER et al., 2010.

O efeito EPR será aumentado se o nanocarreador tiver a capacidade de evadir-se do sistema imune e circular durante um longo período. Altas concentrações de fármacos nanoencapsulados podem ser encontradas no sítio do tumor, quando comparadas ao tecido normal. Para este fim, pelo menos três propriedades são particularmente importantes: (i) o nanocarreador ideal deve ter um

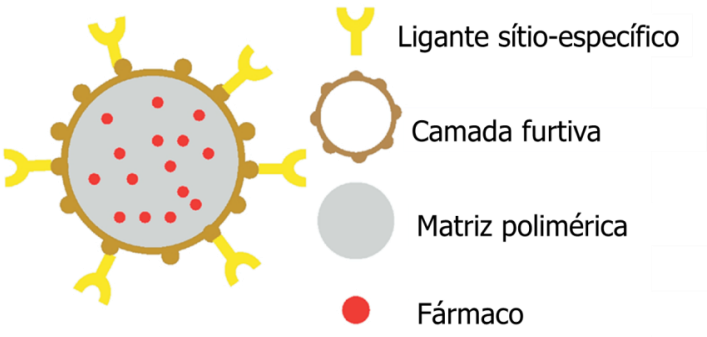
tamanho entre 10 e 100 nm. De fato, para um extravasamento eficiente das fenestrações na vasculatura, as nanopartículas devem ser muito menores que 400 nm. Por outro lado, para evitar a filtração através dos rins, os nanocarreadores precisam ser maiores que 10 nm, e para evitar a captura de uma determinada pelo fígado precisam ser menores que 100 nm; (ii) A carga das partículas deve ser neutra ou aniônica para uma evasão eficiente da eliminação renal; (iii) As nanopartículas devem ser mascaradas, visando o não reconhecimento pelo sistema retículo-endotelial, o qual destrói qualquer material estranho através de opsonização seguido por fagocitose (IYER et al., 2006; GULLOTTI; YEO, 2009; DANHIER et al., 2010; RUOSLAHTI; BHATIA; SAILOR, 2010).

2.4.3 - Nanopartículas sítio-específicas

Na tentativa de aumentar a especificidade de interação dos nanossistemas com células alvo e elevar a quantidade do fármaco liberado nestas células, a pesquisa neste campo foi focada no desenvolvimento de lipossomas e nanopartículas sítio-específicas. Estes utilizam ligantes acoplados em sua superfície, que conferem seletividade para distribuir o fármaco encapsulado no sítio de ação desejado proporcionando uma maior seletividade e eficácia em comparação com o direcionamento passivo (SAPRA; ALLEN, 2003).

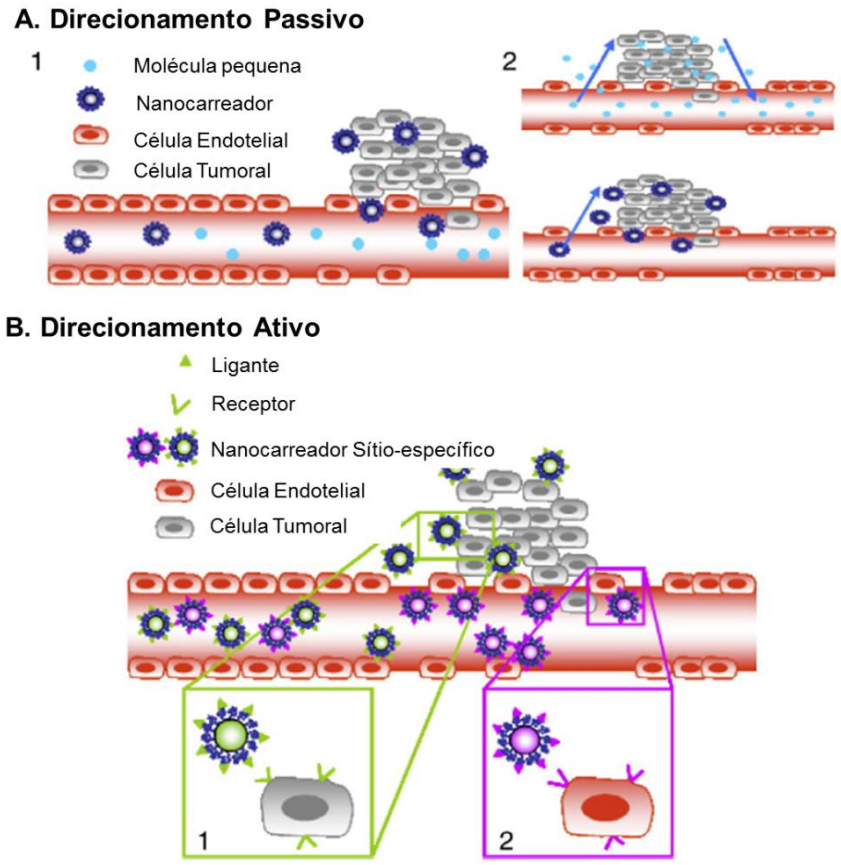
No direcionamento ativo, os ligantes sitio-específicos são acoplados à superfície do nanocarreador (Figura 11) para a ligação a receptores adequados expressos no local alvo (Figura 12). O ligante é escolhido para se ligar a um receptor super expresso por células tumorais e não expresso por células normais. Além disso, os receptores-alvo devem ser expressos de forma homogênea em todas as células-alvo (DANHIER et al., 2010).

Figura 11 – Representação esquemática de uma nanopartícula sítio-específica PEGuilada.



Fonte: ALEXIS et al., 2008.

Figura 12 – A. Direcionamento passivo de nanopartículas. (1) Nanocarreadores atingem tumores. (2) Fármacos livres difundem livremente dentro e fora dos vasos sanguíneos do tumor devido ao seu pequeno tamanho, ao contrário dos fármacos encapsulados, resultando no efeito EPR. B. Direcionamento ativo. (1) Ligantes acoplados na superfície de nanopartículas se ligam a receptores expressos por células cancerígenas ou células endoteliais angiogênicas. (2) as



Fonte: Adaptado de DANHIER et al., 2012.

Alguns exemplos de ligantes de reconhecimento são os anticorpos, polissacarídeos, peptídeos, proteínas virais e lectinas, que são ligados covalentemente a superfície dos nanossistemas a fim de carrear os fármacos para o local específico de ação (BATISTA et al., 2007; EDWARDS; BAEUMNER, 2006). Atualmente, vários estudos pré-clínicos têm sido publicados, utilizando diferentes nanossitemas e ligantes sítio-específicos (Tabela 2).

Tabela 2 – Exemplos de nanocarreadores usando a estratégia de direcionamento ativo sítio-específico.

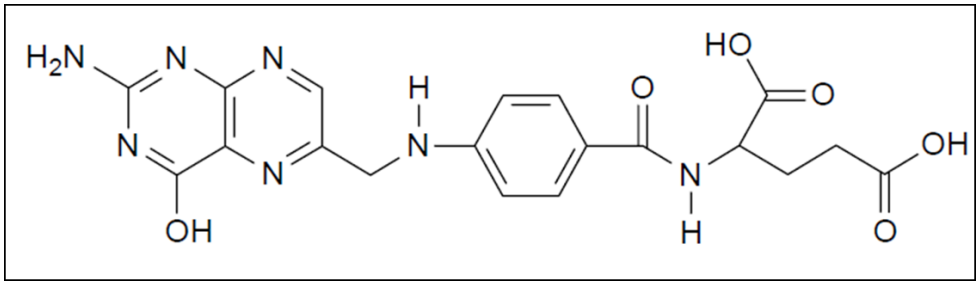
Ligante alvo	**Nanocarreadores**	**Indicação/Célula tumoral**	**Estado**	**Referência**
Transferrina	Lipossomas	Câncer de ovário A2780	*In vitro*	KRIEGER et al., 2010
	Lipossomas	Glioma C6	Pré-clínico	YING et al., 2010
	Lipossomas	Tumor metastático sólido	Fase I	SUZUKI et al., 2008
Folato	Lipossomas	KB Humano	Pré-clínico	GABIZON et al., 2003
	Nanopartículas	SKOV3	*In vitro*	ESMAELLI et al., 2008
	Micelas	KB Humano	Pré-clínico	YOO; PARK, 2004
EGF	Lipossomas	MCF-7	Pré-clínico	PARK et al., 2002
	Lipossomas	Câncer de mama MCF-7	Pré-clínico	KIRPOTIN et al., 2006
Lectinas	Micelas	HepG2	*In vitro*	WANG et al., 2008
	Lipossomas	Melanoma b16	*In vitro*	ELIAZ et al., 2004
Peptídeo RGD	Lipossomas	Melanoma b16	Pré-clínico	XIONG et al., 2005
	Nanopartículas	Tumor pancreático	Pré-clínico	MURPHY et al., 2008

2.4.3.1 - Liberação de fármacos mediada por receptores de folato

Uma das mais extensivamente estudadas molécula alvo para entrega de fármacos sítio-específicos é o ácido fólico (vitamina B9 ou folato). O ácido fólico é uma vitamina de baixo peso molecular (Mw = 441 Da) requerida pelas células eucarióticas como coenzima na transferência de carbono no metabolismo dos ácidos nucléicos e dos aminoácidos (LEAMON; LOW, 2001).

A estrutura química do ácido fólico está demonstrada na Figura 13. Devido a dois grupamentos ácidos carboxílicos posicionados nas extremidades distais da molécula, a permeabilidade passiva através da membrana é mínima. Para contornar este obstáculo, a natureza desenvolveu dois mecanismos para a internalização celular desta vitamina. O primeiro mecanismo envolve uma proteína de membrana de baixa afinidade (K_D ~1-5 μm) que transporta folato diretamente para o citosol celular. O segundo mecanismo utiliza um receptor glicoprotéico de alta afinidade (K_D ~100 pm), geralmente referido como receptor de folato (FR), que preferencialmente medeia a captura do folato para dentro das células por endocitose (LEAMON; LOW, 2001; PARK et al, 2008).

Figura 13 – Estrutura química do ácido fólico.



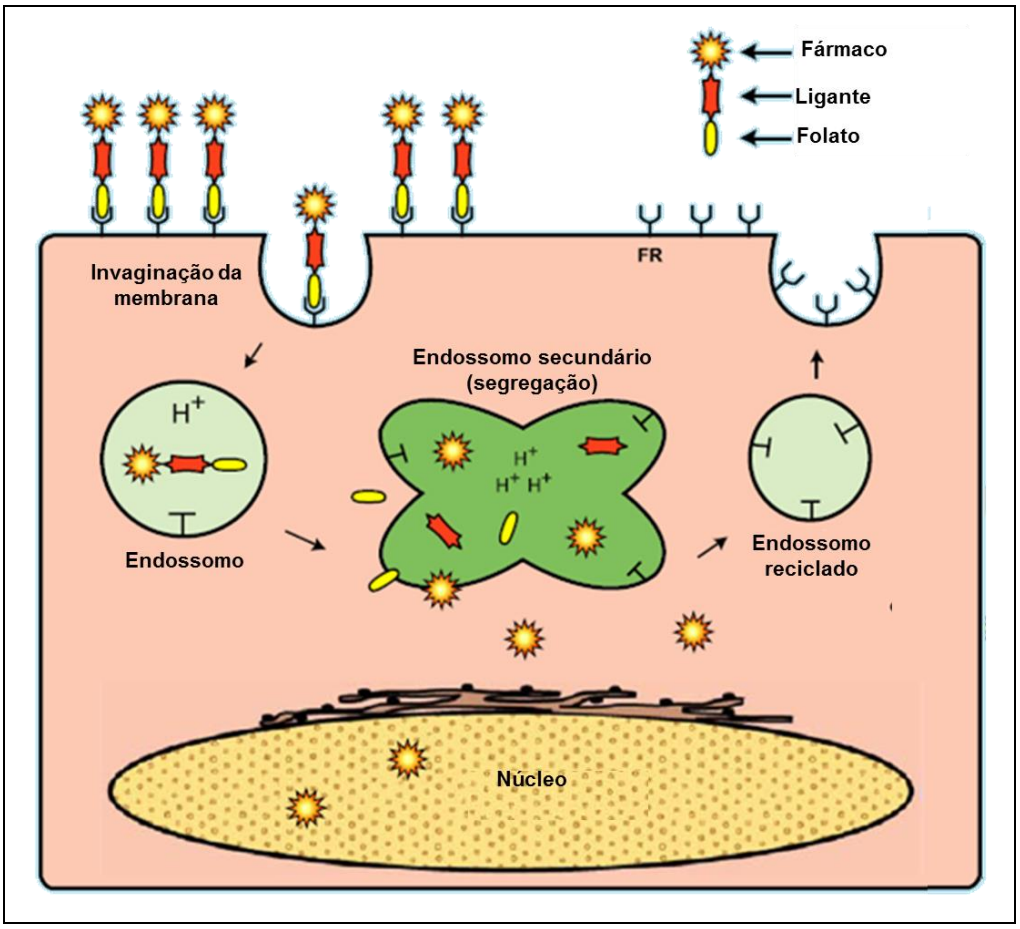
Fonte: LEAMON; LOW, 2001.

A proteína transportadora de folato está presente em praticamente todas as células, enquanto que o FR de alta afinidade é expresso em níveis elevados principalmente em células cancerígenas, tais como, cânceres epiteliais de ovário, colo-retal, mama, próstata, pulmão, nariz, garganta, cérebro e leucemia (SUDIMACK; LEE , 2000; LEAMON; LOW, 2001; ALEXIS et al., 2008).

O direcionamento de fármacos mediado por folato foi idealizado logo após Kamen e colaboradores (1986) relatarem que o folato entrava na célula via endocitose mediada por receptor. O processo fisiológico que visa à liberação sítio-específica de fármacos via folato é idêntica ao da vitamina livre. Como ilustrado na figura 14, o conjugado exógeno fármaco-folato liga-se ao FR localizado na membrana celular. Imediatamente após a ligação, a membrana plasmática circunda o complexo conjugado/FR começa a invaginar até a formação de uma vesícula interna, denominado endossomo primário. O pH do lúmen da vesícula diminui através da ação de bombas de prótons que estão localizadas na membrana endossomal. Esta acidificação presumivelmente irá protonar os grupos carboxil existentes na proteína FR promovendo uma mudança conformacional que permitirá a liberação do folato (LEAMON; REDDY, 2004).

Vários tipos de carreadores de fármacos têm sido conjugados ao folato, incluindo lipossomas, micelas e nanopartículas poliméricas. Para o direcionamento dos lipossomas e nanopartículas, uma cadeia de PEG ligante é frequentemente requerida. Múltiplas moléculas de folato são conjugadas a cada partícula, que permite uma elevada afinidade de interação com os FR (ZHAO; LI; LEE, 2008). Além disso, sistemas de liberação de fármacos conjugados com folato demonstraram uma alta citotoxicidade e captura celular de células cancerígenas que expressam o FR (YOO et al., 2004; ESMAELLI et al., 2008; NIE et al., 2009; LIANG et al., 2011; SHMEEDA et al., 2010; SAXENA; NAGUIB; HUSSAIN, 2012).

Figura 14 – Endocitose de conjugados fármaco-folato. Os conjugados fármaco-folato exógenos ligam-se especificamente aos receptores de folato (FR) presentes na superfície das células alvo. A membrana plasmática sofre invaginação circundando o complexo conjugado/FR para formar uma vesícula intracelular (endossomo). Como o lúmem do endossomo acidifica. Os FR sofrem mudanças em sua conformação e liberam o conjugado no citosol.



Fonte: LEAMON, LOW, 2001.

Portanto, o desenvolvimento de sistemas de liberação controlada de medicamento, furtivos e/ou sítios específicos**,** capazes de viabilizar a administração da β-lapachona em uma formulação que melhore sua solubilidade, bem como, aperfeiçoe a dose terapêutica diminuindo os efeitos tóxicos e direcione o fármaco para o tumor aumentando a eficácia terapêutica, torna-se uma inovação útil no desenvolvimento de alternativas para o tratamento do câncer.

3. REFERÊNCIAS

ALEXIS, F. et al. New frontiers in nanotechnology for cancer treatment. **Urologic Oncology: Seminars and Original Investigations**, v.26, p.74–85, 2008.

ALMEIDA, E.R. Toxicological Assessment of Beta-lapachone on Organs from Pregnant and Non-pregnant Rats. **Phytotherapy research**, 2009.

ALVES, G.M.C. et al. Purificação e caracterização da β-lapachona e estudo de estabilidade dos cristais em diferentes condições de armazenamento. **Química Nova**, v. 31, p. 413–416, 2008.

BATISTA, C.M.; CARVALHO, C.M.B.; SANTOS-MAGALHÃES, N.S. Lipossomas e suas aplicações terapêuticas: Estado da arte. **Revista Brasileira de Ciências Farmacêuticas**, v. 43, p 167-179, 2007.

BENTLE, M.S. et al. Nonhomologous end joining is essential for cellular resistance to the novel antitumor agent, β-lapachone. **Cancer Research**, v. 67, p.6936–6945, 2007.

BEY, E.A. et al. An NQO1- and PARP-1-mediated cell death pathway induced in non-small-cell lung cancer cells by β-lapachone. **Proceedings of the National Academy of Sciences**. v.104, p.11832–118372007.

BLANCO, E. et al. β-Lapachone-containing PEG–PLA polymer micelles as novel nanotherapeutics against NQO1-overexpressing tumor cells. **Journal of Controlled Release**, v. 122, p. 365–374, 2007.

BONIFAZI, E. L.; RÍOS-LUCI, C.; LEÓN, L. G; BURTON, G., PADRÓN, J. M. Antiproliferative activity of synthetic naphthoquinones related to lapachol. First synthesis of 5-hydroxylapachol. **Bioorganic & Medicinal Chemistry**, v.18, p. 2621-2630, 2010.

CAVALCANTI, I.M. et al. 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 Sciences**, v. 44, p. 332–340, 2011.

CHAU, Y. P.; SHIAH, S. G.; DON, M. J.; KUO, M. L. Involvement of hydrogen peroxide in topoisomerase inhibitor β-lapachone-induced apoptosis and differentiation in human leukemia cells. **Free Radical Biology and Medicine**, v. 24, p. 660-670, 1998.

CUNHA-FILHO, M.S.S.; MARTÍNEZ-PACHECO, R.; LANDÍN, M. Dissolution rate

enhancement of the novel antitumoral β-lapachone by solvent change precipitation of microparticles. **European Journal of Pharmaceutics and Biopharmaceutics,** v. 69, p. 871–877, 2008.

DANHIER, F. et al. PLGA-based nanoparticles: An overview of biomedical applications. **Journal Controlled Release**. v.161, p.505-522, 2012.

DANHIER, F.; FERON. O.; PRÉAT, V. To exploit the tumor microenvironment: Passive and active tumor targeting of nanocarriers for anti-cancer drug delivery. **Journal of Controlled Release**, v. 148, p.135–146, 2010.

DONG, Y. et al. Intratumoral delivery of β-lapachone via polymer implants for prostate cancer therapy. **Clinical Cancer Research,** v. 15, p. 131–139, 2009.

EDWARDS, K. A.; BAEUMNER, A. J. Liposomes in analyses. **Talanta**, v. 68, p.1432-1441, 2006.

ELIAZ, R.E. et al. Determination and modeling of kinetics of cancer cell killing by doxorubicin and doxorubicin encapsulated in targeted liposomes. **Cancer Research**. v.64, p.711–718, 2004.

ESMAEILI, F., et al. Folate-receptor-targeted delivery of docetaxel nanoparticles prepared by PLGA-PEG-folate conjugate. **Journal of Drug Target**, v.16, p.415–423, 2008.

EYONG, K. O. et al. Semisynthesis and antitumoral activity of 2-acetylfuranonaphthoquinone and other naphthoquinone derivatives from lapachol. **Bioorganic & Medicinal Chemistry Letters**, v. 18, p. 5387-5390, 2008.

GABIZON, A., et al. In vivo fate of folate-targeted polyethylene-glycol liposomes in tumor-bearing mice. **Clinical Cancer Research**. v.9, p.6551–6559, 2003.

GOULART, M.O.F. et al. Trypanocidal activity and redox potential of heterocyclic- and 2-hydroxy-naphthoquinones. **Bioorganic Medicinal Chemistry Letters,** v. 7, p. 2043–2048, 1997.

GU, F.X. et al. Targeted nanoparticles for cancer therapy. **Nanotoday**. v. 2, p. 14-21, 2007.

GUIRAUD, P. et al. Comparison of antibacterial and antifungal activities of lapachol and β-lapachone. **Planta Médica**, v. 60, p. 373–374, 1994.

GULLOTTI, E., YEO,Y. Extracellularly activated nanocarriers: a new paradigm of tumor targeted drug delivery. **Molecular Pharmaceutics**, v.6 , p.1041–1051,2009.

GUPTA, D. et al. β-lapachone, a novel plant product, overcomes drug resistance in human multiple myeloma cells. **Experimental Hematology**, v.30, p.711–720, 2002.

IYER, A.K. et al. Exploiting the enhanced permeability and retention effect for tumor targeting. **Drug Discovery Toda**, v.11, p.812–818, 2006.

INSTITUTO NACIONAL DE CÂNCER José Alencar Gomes da Silva. Coordenação Geral de Ações Estratégicas. Coordenação de Prevenção e Vigilância. Estimativa 2012: incidência de câncer no Brasil. Rio de Janeiro: Inca, 2011.118 p.

JAIN, R.K. Delivery of molecular medicine to solid tumors: lessons from in vivo imaging of gene expression and function. **Journal Controlled Release**, v.74, p.7-25, 2001.

JOKERST, J.V. et al. Nanoparticle PEGylation for imaging and therapy. **Nanomedicine**, v.6, p. 715–728, 2011.

JULIANO, R.L.; DAOUD, S.S. Liposomes as a delivery system for membrane-active antitumor drugs. **Journal of Controlled Release**, v. 11, p.225–232, 1990.

KAGAN, V. E.; BAYIR, H.; SHVEDOVA, A. A. Nanomedicine and nanotoxicology*:* two sides of the same coin. **Nanomedicine**, v.1, p. 313-316, 2005.

KIRPOTIN, D.B. et al. Antibody targeting of long-circulating lipidic nanoparticles does not increase tumor localization but does increase internalization in animal models, **Cancer Research**. v.66, p.6732–6740, 2006.

KRIEGER, M.L., et al. Overcoming cisplatin resistance of ovarian cancer cells by targeted liposomes *in vitro*. **International Journal Pharmaceutics**. v.389, p.10–17, 2010.

KRISHMAN, P.; BASTOW, K.F. Novel mechanism of cellular DNA topoisomerase II inhibition by the pyranophthoquinone derivates α-lapachone and β-lapachona. **Cancer Chemotherapy Pharmacology,** v. 47, p. 187-198, 2001.

KUNG, H.N. et al. *In vitro* and *in vivo* wound healing-promoting activities of β-lapachone. **American Journal of Physiology. Cell Physiology,** v. 295, p. C931–C943, 2008.

KUMARI, A.; YADAV, S.K.; YADAV, S.C. Biodegradable polymeric nanoparticles based drug delivery systems. **Colloids and Surfaces B: Biointerfaces**, v.75, p.1–18, 2010.

LAI, C.C. et al. Beta-Lapachone induced cell death in human hepatoma (HepA2) cells. **Histology and Histopathology,** v. 13, p. 89–97, 1998.

LEAMON, C.P., LOW, P.S. Folate-mediated targeting: From diagnostics to drug and gene delivery. **Drug Discovered**. v. 6, p.44–51, 2001.

LEAMON, C.P., REDDY, J.A. Folate-targeted chemotherapy. **Advanced Drug Delivery Reviews**. v.56, p.1127– 1141, 2004.

LI, C.J. et al. Potent inhibition of tumor survival *in vivo* by β-lapachone plus taxol: Combining drugs imposes different artificial checkpoints. **Proceedings of the National Academy of Sciences**, v. 96, p. 13369–13374, 1999.

LI, C.J.; AVERBOUK, L.; PARDEE, A.B. β-lapachone, a novel DNA topoisomerase I inhibitor with a mode of action different from camptothecin. **Journal of Biological Chemistry**, v. 268, p. 22463–22468, 1993.

LI, Y. et al. Potent induction of apoptosis by ß-lapachone in human multiple myeloma cell lines and patient cells. **Molecular Medicine**, v. 6, p. 1008–1015, 2000.

LIANG, C., et al. Improved therapeutic effect of folate-decorated PLGA–PEG nanoparticles for endometrial carcinoma. **Bioorganic & Medicinal Chemistry**. v.19, p.4057-4066, 2011.

LOPES, J.N. et al. *In vitro* and *in vivo* evaluation of the toxicity of 1,4-naphthoquinone and 1,2-naphthoquinone derivatives against *Trypanosoma cruzi*. **Annals of Tropical Medicine and Parasitology,** v. 72, p. 523–531, 1978.

MEDEIROS C.S. et al. Antifungal activity of the naphthoquinone beta-lapachone against disseminated infection with *Cryptococcus neoformans* var. *neoformans* in dexamethasone-immunosuppressed swiss mice. **Brazilian Journal of Medical and Biological Research,** v. 43, p. 345–349, 2010.

MOHANRAJ, V.J.; CHEN, Y. Nanoparticles – A Review. **Tropical Journal of Pharmaceutical Research**, v.5, p.561-573, 2006.

MOON, D. et al. Anti-inflammatory effects of β-lapachone in lipopolysaccharide-stimulated BV2 microglia. **International Immunopharmacology**, v. 7, p. 506–514, 2007.

MOON, D, O. et al. Beta-lapachone (LAPA) decreases cell viability and telomerase activity in leukemia cells: suppression of telomerase activity by LAPA. **Journal of Medicinal Food**, 13, p. 481-8, 2010.

MOSQUEIRA, V. C. F., et al. Relationship between complement activation, cellular uptake and surface physicochemical aspects of novel PEG-modified nanocapsules **Pharmacological Research.** v. 18, p. 1411-1419, 2001.

MOURA, K.C.G. et al. Trypanocidal activity of isolated naphthoquinones from *Tabebuia* and some heterocyclic derivatives: a review from an interdisciplinary study. **Journal of Brazilian Chemical Society,** v. 12, p. 325–338, 2001.

MUDSHINGE, S. R., A., et al. Nanoparticles: Emerging carriers for drug delivery. **Saudi Pharmaceutical Journal**, v.19, p.129-141, 2011.

MURPHY, E.A. et al. Nanoparticle-mediated drug delivery to tumor vasculature suppresses metastasis. **Proceedings of the National Academy of Sciences USA** . v.105, p.9343–9348, 2008.

NASONGKLA, N. et al. Enhancement of solubility and bioavailability of beta-lapachone using cyclodextrin inclusion complexes. **Pharmaceutical Research,** v. 20, p. 1626–1633, 2003.

NIE, Y., et al. Synthesis, characterization and transfection of a novel folate - targeted multipolymeric nanoparticles for gene delivery. **Journal of Materials Science: Materials in Medicine**. v. 20, p. 1849-57, 2009.

OLIVEIRA, C.G.T. et al. Synthesis and antimicrobial evaluation of 3-hydrazino-naphthoquinones as analogs of lapachol. **Journal of Brazilian Chemistry Society**, v. 12, p. 339–345, 2001.

OOYAMA, A. et al. Anti-angiogenic effect of 5-Fluorouracil-based drugs against human colon cancer xenografts.**Cancer letters.** v. 267, p. 26-36, 2008.

OUGH, M. et al. Efficacy of β-lapachone in pancreatic cancer treatment: exploiting the novel, therapeutic target NQO1. **Cancer Biotherapy**, v. 4, p. 95–102, 2005.

OWENS, D.E., PEPPAS, N.A. Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. **International Journal Pharmaceutics**. v.307, p.93–102, 2006.

PANYAM, J.; LABHASETWAR, V. Biodegradable nanoparticles for drug and gene delivery to cells and tissue**. Advanced Drug Delivery Reviews**, v.55, p. 329-347, 2003.

PARDEE, A.B.; LI, Y.Z.; LI, C.J. Cancer therapy with β-lapachone. **Current Cancer Drug Targets**, v. 2, p. 227–242, 2002.

PARHI, P., MOHANTY, C., SAHOO, S.K. Nanotechnology-based combinational drug delivery: an emerging approach for cancer therapy. **Drug Discovery Today**. v.17, p.1044-1042, 2012.

PARK, J.H. et al. Polymeric nanomedicine for cancer therapy. **Progress in Polymer Science**. v.33, p.113–137, 2008.

PARK, J.W. et al. Anti-HER2 immunoliposomes: enhanced efficacy attributable to targeted delivery. **Clinical Cancer Reseach**. v.8, p.1172–1181, 2002.

PEREIRA, E.M. et al. *Tabebuia avellanedae* naphthoquinones: activity against methicillin-resistant staphylococcal strains, cytotoxic activity and *in vivo* dermal irritability analysis. **Annals of Clinical Microbiology and Antimicrobials**, v, 5, p. 1–7, 2006.

PETRO, R.A.; DE SIMONE, J.M. Strategies in the design of nanoparticles for therapeutic applications. **Nature Reviews Drug Discovery**, v. 9, p. 615–627, 2010.

PINK, J.J. et al. NAD(P)H:quinone oxidoreductase (NQO1) activity is the principal determinant of β-lapachone cytotoxicity. **The Journal of Biological Chemistry**, v. 275, p. 5416–5424, 2000.

PISON, U. U.; WELTE, T. T.; GIERSIG, M, M.; GRONEBERG, D. A. Nanomedicine for respiratory diseases. **European Journal of Pharmacology**, v. 533, p. 341–350, 2006.

PLANCHON, S.M. et al. β-Lapachone-Induced Apoptosis in Human Prostate Cancer Cells: Involvement of NQO1/xip3. **Experimental Cell Research,** v. 267, p. 95–106, 2001.

RAWAT, M. et al.. Nanocarriers: Promising Vehicle for Bioactive Drugs. **Biological & Pharmaceutical Bulletin**, v. 29, p. 1790-1798, 2006.

REINICKE, K. E. et al. Development of β-Lapachone Prodrugs for Therapy Against Human Cancer Cells with Elevated NAD(P)H:Quinone Oxidoreductase 1Levels. **Cancer Therapy: Preclinical**, v. 11, p. 3055-3064, 2005.

RUOSLAHTI, E., BHATIA, S.N., SAILOR, M.J. Targeting of drugs and nanoparticles to tumors. **Journal of Cell Biology**. vol. 188, p.759–768, 2010.

SAPRA, P.; ALLEN, T.M. Ligant-target lipossomal anticancer drugs. **Progress in lipid Research**, v. 42, p. 439–462, 2003.

SAXENA, V., NAGUIB, Y., HUSSAIN, M. D. Folate receptor targeted 17-allylamino-17-demethoxygeldanamycin (17-AAG) loaded polymeric nanoparticles for breast cancer. **Colloids and Surfaces B: Biointerfaces**. v. 94, p.274– 280, 2012.

SCHAFFAZICK, S. R. et al. Physicochemical characterization and stability of the polymeric nanoparticle systems for drug administration. **Química Nova,** v. 26, n. 5, p. 726-737, 2003.

SCHAFFNER-SABBA, K. et al. β-Lapachone: synthesis of serivatives and sctivities in tumor models. **Journal of Medical Chemistry**, v. 27, p. 990–994, 1984.

SCHUERCH, A.R.; WEHRLI, W. β-Lapachone, an Inhibitor of Oncornavirus Reverse Transcriptase and Eukaryotic DNA Polymerase-α. **Journal of Biochemistry,** v. 84, p. 197–205, 1978.

SHMEEDA, H. et al. Delivery of zoledronic acid encapsulated in folate-targeted liposome results in potent in vitro cytotoxic activity on tumor cells. **Journal Controled Release.** v.146, p 76–83, 2010.

SIEGEL, D.; ROSS D. Immunodetection of NAD(P)H:quinone oxidoreductase 1 (NQO1) in human tissues. **Free Radical Biology & Medicine**, v. 29, p. 246–253, 2000.

SIEGEL, D., YAN, C.,ROSS, D. NAD(P)H:quinone oxidoreductase 1 (NQO1) in the sensitivity and resistance to antitumor quinones. **Biochemical Pharmacology**, v.83, p. 1033–1040, 2007.

SILVA-JÚNIOR, E.N. et al. Synthesis and potent antitumor activity of new arylamino derivatives of nor-β-lapachone and nor-α-lapachone. **Bioorganic & Medicinal Chemistry,** v. 15, p. 7035–7041, 2007.

SILVA, M.N.; FERREIRA, V.F.; SOUZA, M.C.B.V. Um panorama atual da química e da farmacologia de naftoquinonas, com ênfase na β-lapachona e derivados. **Química Nova**, v. 26, p. 407–416, 2003.

SINGH, R.; LILLARD JR, J.W. Nanoparticle-based targeted drug delivery. **Experimental and Molecular Pathology**, v. 86, p. 215-223, 2009.

SOPPIMATH, K.S., et al. Biodegradable polymeric nanoparticles as drug delivery devices. **Journal of Controlled Release**, v.70, p.1-20, 2001.

SUDIMACK, J., LEE, R.J.Targeted drug delivery via the folate receptor. Advanced **Drug Delivery Reviews**, v. 41, p. 147–162, 2000.

SUZUKI, M. et al. Synergistic effects of radiation and b-lapachone in du-145 human prostate cancer cells in vitro. **Radiation Research**, v. 165, p. 525–531, 2006.

SUZUKI, R. Effective anti-tumor activity of oxaliplatin encapsulated in transferrin-PEG-liposome. **International Journal Pharmaceutics**. v.346, p.143–150, 2008.

TORCHILIN, V. Tumor delivery of macromolecular drugs based on the EPR effect. **Advanced Drug Delivery Reviews**, v.63, p.131–135, 2011.

YANG, R.Y. et al. Synthetic methods for the preparation of ARQ 501 (β-Lapachone) human blood metabolites. **Bioorganic & Medicinal Chemistry**. v.16, p.5635–5643, 2008.

YING, X. et al. Dual-targeting daunorubicin liposomes improve the therapeutic efficacy of brain glioma in animals. **Journal Controled Release**. v.141, p.183–192,2010.

YOO, H. S., PARK, T.G. Folate receptor targeted biodegradable polymeric doxorubicin micelles. **Journal of Controlled Release.** v. 96, p. 273– 283, 2004.

WANG, M.; THANOU, M. Targeting nanoparticles to cancer. **Pharmacological Research**, v.62, p. 90–99, 2010.

WANG, X.X. et al. The use of mitochondrial targeting resveratrol liposomes modified with a dequalinium polyethylene glycol-distearoylphosphatidyl ethanolamine conjugate to induce apoptosis in resistant lung cancer cells. **Biomaterials,** v. 32, p. 5673–87, 2011.

WANG, Y.C. et al. Functionalized micelles from block copolymer of polyphosphoester and poly(epsilon-caprolactone) for receptor-mediated drug delivery. **Journal Controled Release.** v.128, p.32–40, 2008.

WOO, H.J.; CHOI, Y.H. Growth inhibition of A549 human lung carcinoma cells by β-lapachone through induction of apoptosis and inhibition of telomerase activity. **International Journal of Oncology**, v. 26, p. 1017–1023, 2005.

WUERZBERGER, S.M.; PINK, J.J.; PLANCHON, S.M., BYERS, K.L., BORNMANN, W.G.; BOOTHMAN, D.A. Induction of apoptosis in MCF-7:WS8 breast cancer cells by β-Lapachone. **Cancer Research**, v.58, p.1876-1885, 1998.

XIONG, X.B. et al. Intracellular delivery of doxorubicin with RGD-modified sterically stabilized liposomes for an improved antitumor efficacy: in vitro and in vivo. **Journal Pharmacetical Science**. v.94, p.1782–1793, 2005.

ZHAO, X., LI, H., LEE, R.J. Targeted drug delivery via folate receptors. **Expert Opinion on Drug Delivery**. v.5, p.309-319, 2008.

4. OBJETIVOS

4.1 - Objetivo Geral

Preparar e caracterizar nanocápsulas furtivas e sítio-específicas contendo β-lapachona, assim como, avaliar a atividade altiproliferativa e a farmacocinética da β-lapachona encapsulada nesses sistemas para o tratamento do câncer.

4.2 - Objetivos Específicos

- Sintetizar e caracterizar derivados do copolímero PLGA-PEG conjugado ao folato;
- Obter nanocápsulas furtivas e sítio-específicas (com PEG e folato em suas superfícies) contendo o β-lapachona;
- Determinar as características físico-químicas das nanocápsulas;
- Estudar a cinética de liberação *in vitro* da β-lapachona, a partir das nanocápsulas;
- Desenvolver e validar uma metodologia analítica para quantificação da β-lapachona em plasma de ratos Wistar;
- Analisar a citotoxidade das nanocápsulas furtivas e sítio-específicas frente a várias linhagens de células cancerígenas;
- Avaliar a farmacocinética da β-lapachona a partir das nanocápsulas.

5. CAPÍTULO 1

A ser submetido ao *International Journal of Pharmaceutics*

Preparation and characterization of PEGylated and Folate targeted nanocapsules of β-lapachone

Milena S. Ferraz [a], Fábio J. F. Almeida [a], Noemia P. S. Santos [a,b], Rosa M. Souto-Maior[c], Nereide S. Santos-Magalhães[a,*]

[a]Laboratório de ImunopatologiaKeizo-Asami, Universidade Federal de Pernambuco, Recife, PE, Brazil

[b]Laboratório de Nanotecnologia, Biotecnologia e Cultivo de células - Centro Acadêmico de Vitória/UFPE,Vitória de Santo Antão, PE, Brazil

[c]Departamento de Química Fundamental, Universidade Federal de Pernambuco, Recife, PE, Brazil

*Corresponding author

Prof. Nereide Stela Santos-Magalhães

Universidade Federal de Pernambuco

Laboratório de Imunopatologia Keizo-Asami (LIKA)

Av. Prof. Moraes Rego, 1235, Cidade Universitária, 50670-901, Recife-PE, Brazil.

Phone: + 55 81 21268587; Fax: +55 81 21268485

E-mail: nereide.magalhaes@gmail.com

ABSTRACT

In the present study PEGylated and folate conjugated-PEG-PLGA nanocapsules were developed as an alternative carrier of β-lapachone. Firstly, the copolymer was synthesized with presenting conjugation percentage of 98 % and characterized by ^{1}H NMR and FT-IR spectroscopy. Conventional (NC/β-lap), PEGylated (NC-PEG/β-lap) and targeted (NC-FOL/β-lap) nanocapsules containing β-lapachone were prepared by the interfacial deposition technique and characterized for particle size, polydispersity index, zeta potential, encapsulation efficiency. The *in vitro* drug release from nanocapsules was evaluated using dialysis method under *sink* conditions. The effects of polyetilenoglycol and folate on the nanocapsules surface were evaluated by cytotoxicity studies using KG-1 and HeLa cell lines. Nanocapsules containing β-lapachone were obtained with a low polydispersity index, negative zeta potential and high encapsulation efficiency. Through *in vitro* release study, it was possible to evaluate the release profile of β–lapachone from nanocapsules with a maximum drug release in 24 h. In the cytotoxicity study, targeted nanocapsules exhibited relevant cytotoxicity than the free drug, conventional and PEGylated nanocapsules. The results of the formulation characteristics and cytotoxicity study suggest that the surface modified nanocapsules formulation could be a promising carrier for β-lapachone.

Keywords: Polyethyleneglycol, β-lapachone, folate, nanocapsules, cytotoxicity.

1. Introduction

β-lapachone (3,4-dihydro-2,2-dimethyl-2*H*-naphtho[1,2-*b*]pyran-5,6-dione, β-lap, molecular weight 242 Da) is an investigational anticancer agent that consists of a small molecule naturally obtained from the bark of plants of the species *Tabebuia avellanedae*, or by a semi-synthesis from lapachol (Li et., 1993). Its cytotoxicity at micromolar (μM) concentrations against a variety of cancer cells in culture, in particular in prostate cells (Kumi-Diaka, 2002; Dong et al., 2009; Eyong et al., 2008; Yamashita et al., 2009), lung cells (Yamashita et al., 2009; Blanco et al., 2007), pancreas cells (Ough et al., 2005; Li et al., 2011), breast cells (Reinicke et al., 2005; Silva-Júnior et al., 2011) and ovaries cells (Li et al., 1999), indicates its potential against tumor growth. However, the therapeutic use of β-lap still presents limitations, mainly due to its low water solubility (16 mM) (Nasongkla et al., 2003) and hepatotoxicity (Almeida et al., 2009). Some approaches have been proposed drug delivery system, including polymer micelles (Blanco et al., 2007) and liposomes (Cavalcanti et al., 2011), in order to improve the therapeutic effects of β-lapachone and reduce its side effects.

Drug delivery systems have advantages over conventional therapeutic systems for cancer treatment. They are capable to maintain constant and within to the therapeutic range drug concentration in plasma, ensuring a higher bioavailability and reduced collateral effects, decreasing the doses number required, enhancing, thus the patients adherence to the treatment (Mora-Huertas et al., 2010). Thus, it becomes essential to develop nanocarriers systems, such as nanoparticles, which are multifunctional devices, able to diagnose and treat, effectively and safely, diseases with high mortality such as cancer (Sing and Lillard Jr, 2009).

Conventional nanocarriers are rapidly cleared from the bloodstream. Proteins adsorbed or attached on the surface, promoting the recognition, capture and removal of carriers from the bloodstream by phagocytic cells of the mononuclear phagocyte system (MPS) (Mosqueira et al., 2001a). Structural changes in the surface of these nanoparticulate systems have been proposed in order to decrease the capture by the monophagocyte system. One of these modifications is the addition of the polymer chains of polyethylene glycol (PEG) on the surface of the nanoparticles. The PEG is a hydrophilic polymer, biodegradable and biocompatible, which is used in pharmaceuticals to reduce the interaction of nanocarriers with blood proteins, reducing opsonization and increasing the circulation time and, consequently, therapeutic efficacy (Mosqueira et al., 2001b; Gref, 2000).

Nowadays, PEGylated and targeted drug delivery systems have been extensively exploited to improve the therapeutic efficiency and reduce severe side effects of anticancer drugs. Nanoparticles have been widely studied due to its advantages including small size, acceptable

biocompatibility, high drug encapsulation efficiency especially for hydrophobic drugs, controlled drug release manner, high cellular internalization efficiency, desired pharmacokinetics, and long circulation half-life (Moghimi et al., 2001; Zhang et al., 2008; Wang et al., 2010).

To achieve active targeting, nanoparticles should be equipped with functional molecules which can recognize and adhere to biomarkers on the surface of targeting and delivery of drugs into tumor cells. The receptor-mediated endocytic delivery is a widely utilized approach and the folate receptor on the cell membrane is a potential molecular target for tumor-selective drug delivery (Garcia-Bennett et al., 2011).

Folate has been used as a targeting moiety for enhancing the therapeutic efficacy of many anticancer drugs. Folate receptor (FR) is a membrane glycoprotein, over-expressed on the surfaces of many cancer cells, while it is almost absent in most normal tissues (Sudimack et al., 2000; Hilgenbrink et al., 2005). Various drug carries such as liposomes (Xiang et al., 2008; Tyagi and Ghosh, 2011), polymers conjugates (Yoo and Park, 2004a), polymeric micelles (Yoo and Park, 2004b; Zhao and Yung, 2008) and nanoparticles (Garcia-Bennett et al., 2011) have been successfully linked to folic acid for targeted delivery of drug to cancer cells. Recently, it has been reported that folate-mediated delivery of drug-loaded nanoparticles can enable binding, promote uptake, and have increased cytotoxicity to cancer cells *in vitro* (Valencia et al., 2011; Boddu et al., 2012; Saxena et al., 2012; Zhao et al., 2012).

In the present study, poly (d,l-lactide-*co*-glycolide)-poly(ethylene glycol)-folate (PLGA-PEG-FOL) was synthesized to form nanocapsules for encapsulating β-lapachone. Characterization of nanocapsules, including size, zeta potential, drug encapsulation efficiency and *in vitro* release, was carried out and their cytotoxic effect against cancer cells were investigated *in vitro*.

2. Materials and methods

2.1. Materials

β-lapachone (β-lap), obtained from lapachol by a semi-synthetic route was supplied by Dr. Alexandre Góes (UFPE, Brazil). Poly (d,l-lactide-co-glycolide) (PLGA 50:50, RG502H, Mw:9000) with terminal carboxylic acid group was purchased from Boehringer Ingelheim (Ingelheim am Rhein, Germany). Copolymer diblock of PLGA and polyethyleneglycol attached covalently (PLGA-PEG, Resomer® RGP d50105; PLGA 50/50 45 kDa, containing approximately 10% PEG 5kDa) was supplied by Boehringer Ingelheim (Ingelheim am Rhein, Germany). Poly (ethylene glycol)-bis-amine (PEG-bis-amine, Mw: 3400), N-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), folate (FOL), Poloxamer 407, soybean oil and 3-(4,5-

dimethylthiaol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St. Louis, USA). Soybean phosphatidylcholine (PC) (Lipoid S 100®) was purchased from Lipoid GMBH (Ludwigshafen, Germany). Dulbecco's modified Eagle's medium (DMEM) and penicillin/streptomycin solution were obtained from Invitrogen (Gaithersburg, USA). Solvents and other chemicals were supplied by Merck (Darmstadt, Germany).

2.2. Synthesis of the PLGA–PEG–FOL conjugate

The synthesis of folate conjugates followed a four-step reaction: 1) PLGA activation, 2) conjugation of activated PLGA with PEG-bis-amine, 3) activation of folate, and 4) conjugation of folate to PLGA–PEG (Fig. 1).

2.2.1. PLGA activation

Briefly, PLGA (0.56 mmol) dissolved in methylene chloride was activated by DCC (1.12 mmol) and NHS (1.12 mmol) at room temperature under nitrogen atmosphere for 24 h (Yoo and Park, 2004b). The resultant solution was centrifuged (3000 rpm for 30 min) to remove the by-product dicyclohexylurea (DCU). The activated PLGA (PLGA-NHS) was precipitated by ice-cold diethyl ether and dried under vacuum. The yield of this reaction was 76 %.

2.2.2. Conjugation of activated PLGA with PEG-bis-amine

Activated PLGA (0.11 mmol) and PEG-bis-amine (0.55 mmol) was dissolved in methylene chloride in separate flask. After complete solubilization of the polymers, the PLGA solution was added to PEG solution in a drop-wise manner gentle stirring. The reaction mixture was stirred under inert nitrogen atmosphere for 6 h. For purification, the reaction solution was added into vigorously stirred ethanol at room temperature. The precipitated polymer was washed three times with ethanol and dried under vacuum. A yield of 67 % was obtained. The structure of the product was confirmed by ^{1}H-NMR.

The ethanol solution resulting from the precipitation and washing of PLGA-PEG was evaporated under reduced pressure to recover the PEG-bis-amine unreacted. After rotaevaporation of the solvent, the PEG was dried under vacuum, which was recovered 69 % of excess PEG-bis-amine added in the reaction.

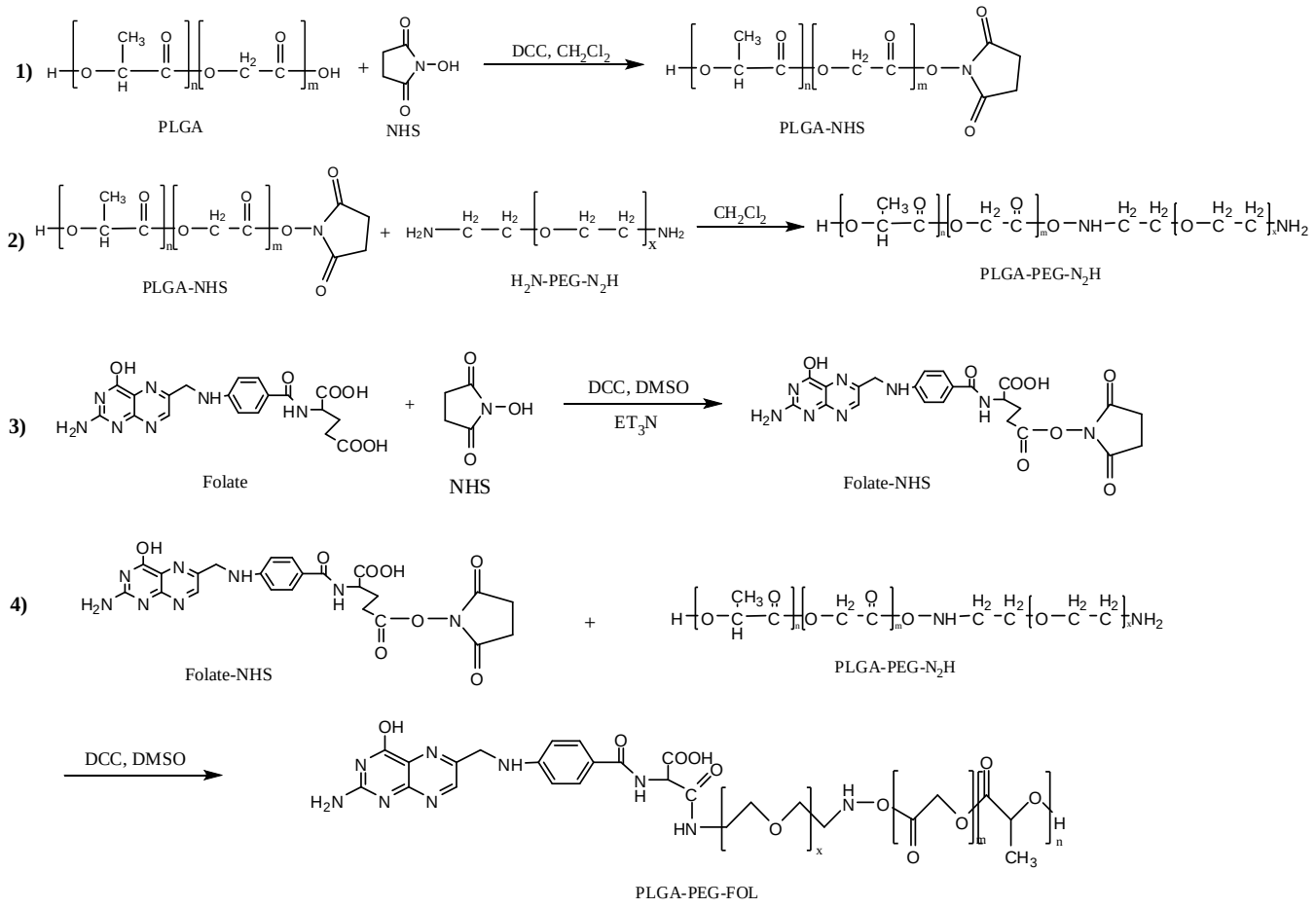


Fig.1. Synthesis of PLGA–PEG–FOL conjugate.

2.2.3. Activation of folate (formation of folate-NHS)

Folate (1.1 mmol) was first activated with DCC (2.2 mmol) and NHS (2.2 mmol) in anhydrous DMSO in presence of 0.25 mL triethylamine as a catalyst, under light protected and nitrogen atmosphere for 24 h. The solution was filtered to remove the dicyclohexylurea by-product, and then precipitated in cold anhydrous ether. The product was obtained in the dry state following several steps of ether washing, decantation and vacuum drying.

2.2.4. Conjugation of folate to PLGA-PEG

PLGA-PEG (0.04 mmol) was dissolved in anhydrous DMSO and was mixed with folate-NHS (0.1 mmol) and DCC (0.1 mmol). The reaction was performed under inert nitrogen atmosphere at room temperature for 12 h and then mixed with 50 mL of distilled water and centrifuged at 3000 rpm for 1 h. After discarding the pellet, the supernatant was dialyzed (MWCO= 10 kDa) against distilled water for 72 h and freeze dried. The yield of this reaction was 74 %.

2.3. Characterization of PLGA-PEG-FOL

The chemical structure of the synthesized PLGA-PEG-FOL was characterized by ^{1}H NMR spectroscopy (Varian Unity Plus, Varian, California, USA) in d_6-DMSO at 400 MHz and FT-IR spectroscopy (Bruker IFS66, Bruker Optics, Karlsruhe, Germany).

The conjugation percentage was calculated by determining the amount of folate conjugated in PLGA-PEG-FOL. A known amount of dried PLGA-PEG-FOL was dissolved in DMSO and an UV absorbance value at 285 nm was measured to determine the concentration of conjugated folic acid. Serially diluted concentrations of folate in DMSO were used to construct a calibration curve in the range 1-10 μg/mL.

2.4. Preparation and characterization of β-lap-loaded nanocapsules

Conventional (NC/β-lap), PEGylated (NC-PEG/β-lap) and targeted (NC-FOL/β-lap) nanocapsules containing β-lapachone were prepared by the interfacial deposition technique proposed by Fessi et al. (1989). Briefly, PLGA (75 mg), PLGA-PEG (75 mg) or mixing the polymers PLGA/PLGA-PEG-FOL (67.5/7.5 mg), soybean oil, soybean phosphatidylcholine and β-lap were dissolved in acetone. Organic phase was added slowly and under magnetic stirring in aqueous phase consisted of poloxamer dissolved in phosphate buffer (pH 7.4). Subsequently, the colloidal dispersion was concentrated under reduced pressure to obtain a final concentration of 1 mg/mL of β-lap.

2.5. Physicochemical characterization of nanocapsules

The particle size and polydispersity index of nanocapsules was measured by photon autocorrelation spectroscopy using laser particle analyzer DelsaTMNano-S (Beckman Coulter, California, USA). Zeta potential of the nanocapsules was determined by electrophoretic mobility using a ZetasizerNano-ZS9 (Malvern, Worcestershire, UK).

β-lap concentration encapsulated into nanocapsules was determined for HPLC. The chromatographic analysis was performed in equipment Aliance 2695 (Waters, Milford Massachusetts, USA) coupled to a photodiode array (PDA) 2998 (Waters, Milford Massachusetts, USA), operated at 256 nm. Reversed phase column C18 (250 mm × 4,6 mm, 5 mm, XBridge™ Waters) was used with mobile phase composed of methanol and water (80:20, v/v), at a flow rate of 1 mL/min at 37 ºC and injection volume of 50 μL. The calibration curve β-lap was prepared from dilutions of a stock solution (0.4 mg/mL) and varying concentrations 1-80 μg/mL.

Drug content was determined after dissolution of nanocapsules (40 μL) in methanol (960 μL). The solution was stirred for 30 s, centrifuged at 10,000 rpm for 5 minutes, filtered (0.22 μm filters, Millex®, Millipore, Massachusetts, USA) and injected into the HPLC system.

The encapsulation efficiency of β-lap into nanocapsules was determined after submitting samples to ultrafiltration associated with centrifugation at 10,000 rpm for 1 h using Microcon® filter units (Millipore, Billerica, USA). The concentration of β-lapachone in the ultrafiltrate was determined by HPLC and the encapsulation efficiency (%) was calculated by the difference between the total drug concentrations in the preparation and the unencapsulated (free in the ultrafiltrate) drug.

2.6. In vitro release kinetics

The *in vitro* release profile of β-lap from nanocapsules was evaluated using dialysis method under *sink* conditions (Cavalcanti et al., 2011). An aliquot of the suspension of NC was placed to a dialysis bag (cellulose membrane, MWCO = 10 kDa, Sigma-Aldrich, St. Louis USA) which was subsequently sealed and inserted into a release medium (phosphate buffer pH 7.4). The system was maintained at 37 ºC ± 1 ºC under agitation. Aliquots of the release medium were collected at intervals of predetermined time. Each aliquot taken from the release medium was replenished with the same volume of buffer solution. The concentration of β-lap released was quantified by HPLC at 256 nm. The kinetic assays were performed in triplicate.

2.7. Cell culture studies

2.7.1. Cell culture

Cervical carcinomas cell line (HeLa) and human myeloid leukemia cell line (KG-1) obtained from Rio de Janeiro Cell Bank (Rio de Janeiro, Brazil) were cultivated in DMEM medium supplemented with 10% fetal bovine serum and 1 % penicillin–streptomycin at 37 ºC in a humidified incubator with 5% CO_2. The cells were maintained in an exponential growth phase by periodic subcultivation.

2.7.2. Cytotoxicity studies

The cancer cells were seeded in 96-well plates at a density of 1×10^4 viable cells per well and incubated for 24 h to allow cell attachment. The cells were incubated with the free β-lap drug, and β-lap-loaded targeted or non-targeted nanocapsules suspension at concentrations from 1.0 to 8.0 μM for 48 h. The diluent for preparing the working solution was the DMEM culture medium.

At designated time interval, 25 μL MTT (5 mg/mL in PBS) was added to each well and the culture medium containing MTT solution was removed after 3 h. The formazan crystals were dissolved in 200 μL DMSO and read at 630 nm by a microplate reader (BioTek Instruments, Winooski, USA). Cell viability was calculated using the following equation: % Cell viability = (Abs_{sample} / $Abs_{control}$) × 100, where Abs_{sample} was the absorbance of cells incubated with the samples and $Abs_{control}$ is the absorbance of control cells incubated with cell culture medium only. The cytotoxicity of samples was expressed as the drud concentration required inhibiting growth of the cells by 50 % relative to control (IC_{50}).

3. Results and discussion

3.1. Characterization of PLGA-PEG-FOL

Initially, the carboxylic group end PLGA was activated with NHS in the presence of DCC, which was then conjugated to primary amine group of PEG-bis-amine. An excess amount of PEG was used to prevent the formation of PLGA-PEG-PLGA triblock copolymers (Murugesan et al, 2008).

The choice of solvent for the precipitation of PLGA-PEG was of great importance to avoid co-precipitation of unreacted PEG, which could interfere in the subsequent reaction. Therefore, some solvents have been tested and the ethanol was chosen for the precipitation and purification of the PEG-PLGA due to PEG is very soluble in this solvent unlike the PLGA.

After activation, the folate was coupled to PLGA-PEG via amine linkage, where the ^{1}H NMR spectrum of PLGA-PEG-FOL (Fig. 2) confirmed the synthesis of the conjugate copolymer. The peak at 3.64 ppm corresponded to the -CH_2 protons of PEG. The multiplets at 1.53-1.58 ppm and 5.15-5.23 ppm are attributed to -CH3 and -CH protons of the d, l-lactic acid repeats, respectively. The multiplets at 4.66-4.90 ppm correspond to the glycolic acid -CH_2 protons. The high complexity of the peaks resulted from mixed d-lactic, l-lactic and glycolic acid sequences in the polymer backbone (Kasperczyk, 1996). Small peaks at 6.6 ppm, 7.8 ppm and 8.7 ppm were attributed to aromatic protons associated with folate (Liang et al, 2011; Boddu et al., 2012).

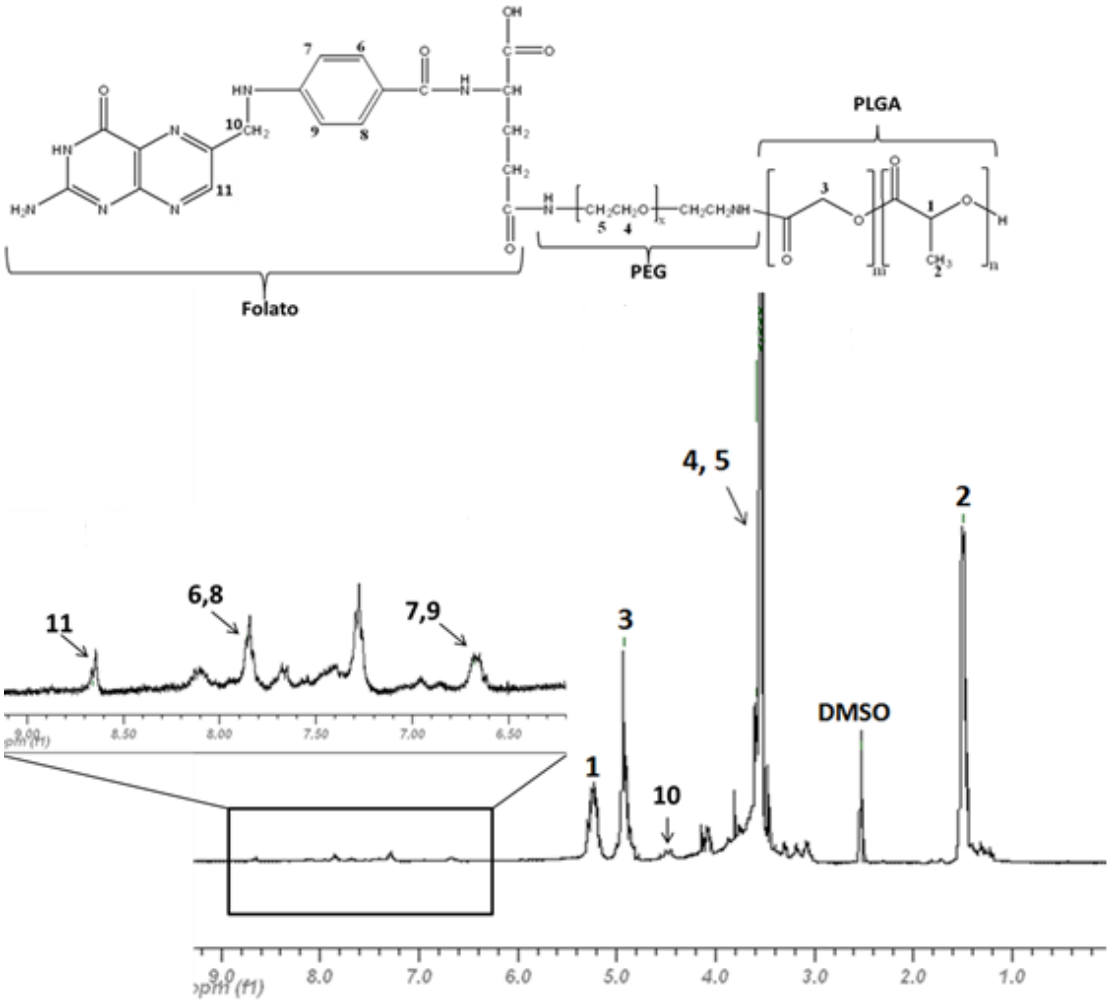


Fig. 2. ^{1}H NMR spectrum of PLGA-PEG-FOL.

The synthesized copolymer PLGA-PEG-FOL was futher confirmed by FT-IR spectrum as displayed in Fig. 3. In this spectrum are observed the following absorption bands (ν, cm^{-1}): 2945-2868 (CH_2, CH_3), 1760 (C=O), 1455-1358 (CH_3), which characterize the PLGA. A strong band in 1096 cm^{-1} attributed to the stretching vibrations of C-O of the PEG was observed. The bands in 1691 cm^{-1} and 1608 cm^{-1} can be attributed to stretching vibrations C=O and N-H of the folate, respectively (Stevanovic et al, 2008). All these results demonstrated successful introduction of folate into the backbone of diblock copolymer PLGA–PEG to form a conjugate.

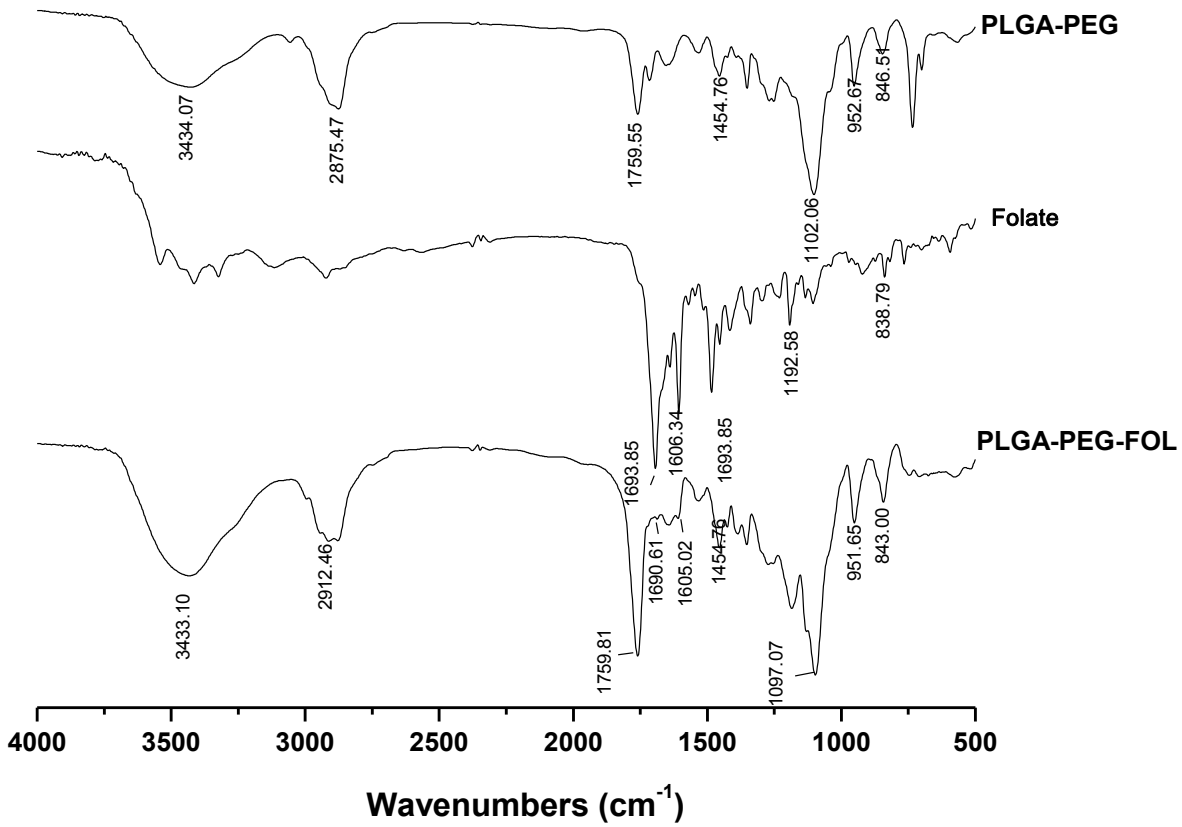


Fig. 3. FT-IR spectrum of PLGA-PEG, folate and PLGA-PEG-FOL.

The conjugation percentage of folate on the PLGA-PEG-FOL was determined by ultraviolet (UV) spectrophotometric analysis of the lyophilized product at 285 nm. The calibration curve was obtained: Absorption= 0.0705 × [folate] (μg/mL) + 0.0122, R^2=0.9999. Based on the theoretical calculation, the conjugation percentage of folate to PLGA-PEG was 98 %. This percentage of conjugation was higher than any found in the literature (Yoo and Park, 2004b; Esmaelli et al., 2008; Nie et al., 2009; Ebrahimnejad et al., 2010).

3.2. Physicochemical characterization of nanocapsules

The nanocapsules containing β-lapachone were prepared by the interfacial deposition technique, where the physicochemical characteristics are summarized in Table 1.

Particle size and polydispersity index of nanocapsules are an important characteristics to be evaluated during the development of pharmaceutical formulations, because they indicate the uniformity of size and dispersion of a formulation. In addition, they can influence drug loading, drug release, *in vivo* distribution, and targeting ability of these delivery systems (Fukumori and Chikawa, 2006; Singh and Lillard Jr., 2009). The nanocapsules samples exhibited narrow size

distribution and monodisperse unimodal pattern with polydispersity index below 0.3, besides presenting appropriate sizes for nanoscale colloidal dispersions. As it has been shown in table 1, the PEGylated and targeted nanocapsules (NC-FOL/β-lap) had a larger size (151.2 and 149.9 nm, respectively) whereas conventional (NC/β-lap) nanocapsules (137.8 nm). The increased size of NC-FOL/β-lap may be due to the presence of PEG and folate at the surface of nanocapsules (Mosqueira et al., 2001b; Esmaeli et al., 2008; Murugesan et al., 2008).

Table 1

Physicochemical characterization of conventional (NC/β-lap) PEGylated (NC-PEG/β-lap) and targeted (NC-FOL/β-lap) nanocapsules containing β-lapachone.

Nanocapsules	**Particle Size (nm)**	**PDI**	**EE (% ± S.D.)**	**Zeta potential (mV ± S.D.)**
NC/β-lap	137.8	0.265	98.9 ± 1.7	-13.0 ± 0.6
NC-PEG/β-lap	151.2	0.241	98.2 ± 0.9	-6.5 ± 0.2
NC-FOL/β-lap	149.9	0.283	98.5 ± 1.5	-5.4 ± 0.5

PDI = polydispersity index; EE= encapsulation efficiency.

Zeta potential is another important index for the stability of the nanoparticles suspension. The high absolute value of zeta potential indicates a high electric charge on the surface of the drug-loaded nanoparticles, which can cause strong repulson forces among particles to prevent aggregation (Singh and Lillard Jr., 2009). The zeta potencial value for conventional, PEGylateg and targeted nanocapsules was –13.0 ± 0.6 mV, -6.5 ± 0.2 mV and –5.4 ± 0.5 mV, respectively. Since PEG is non-ionic, this zeta potential decrease demonstrated the presence of PEG layer on the surface (Gref et al., 1995). The smaller and negative zeta potential of PLGA–PEG was also reported previously (Park et al., 2005b; Zhao and Yung, 2008).

For the PLGA–PEG–FOL nanocapsules, the protonated amino acid groups of folate may affect the surface charge present on the surface of PLGA nanocapsules. The small and negative zeta potential of PLGA-PEG-FOL was also reported in other studies (Esmaeli et al., 2008; Ebrahimnejad et al., 2010).

The encapsulation efficiency of β-lap into nanocapsules was determined by HPLC. The calibration curve was obtained: Area = 332127 × [β-lap] (μg/mL) - 137974, r = 0.9998. The β-lap chromatogram solubilized in methanol showed a defined peak with a retention time of 4.1 min.

In this study, high values of β-lap encapsulation efficiency in NC/β-lap (98.9 ± 1.7 %) NC-PEG/β-lap (98.2 ± 0.9 %) and NC-FOL/β-lap (98.5 ± 1.5 %) nanocapsules were obtained. When using the interfacial deposition technique, nanocapsules are obtained with high encapsulation capacity for lipophilic drugs in a reproducible way and effective, due to its central oily cavity (Cauchetier et al., 2003; Mora-Huerta et al., 2010). Results of the lipophilic drugs encapsulation efficiency in nanocapsules suspensions around 100 % were observed in several studies (Cauchetier et al., 2003; Santos et al., 2005).

3.3. In vitro release study

The formulations studied showed a biphasic drug release pattern characterized by an initial *burst* phase followed by a release phase. The *in vitro* release profile of β-lapachone from the conventional, PEGylated and targeted nanocapsules is showed in Fig. 4. *Burst* effect of 34 ± 1.62 %, 30 ± 0.89 % and 36 ± 0.44 % were observed in the first 2 h of β-lap release from NC/β-lap, NC-PEG/β-lap and NC-FOL/β-lap, respectively. The *burst* release is probably due to the release of drug poorly entrapped or associated to the particles surface that is prone to diffuse to the medium faster (Klose et al., 2008). The maximum drug release was achieved within 24 h around 79 ± 0.29 %, 78 ± 0.76 % and 80 ± 0.42 % of its initial content, for conventional, PEGylated and targeted nanocapsules, respectively. The data reflect the ability of nanocapsules to control drug release in the phosphate buffer solution under *in vitro sink* conditions. It is a well-known fact that several factors have an effect on the release of entrapped drugs in nanocarrier systems. Particle size, solubility or affinity of the drug to the oily phase and the polymer, and drug loading are common parameters that govern the kinetic profile of drugs. Thus, *in vitro* kinetics of nanoparticulated systems, especially in the case of nanocapsules, remains a challenge in the evaluation of drug release and some lipophilic drugs presented different profiles when encapsulated into nanocapsules. The usnic acid was completely released from PLGA nanocapsules within 48 hours (Santos et al., 2005). In opposed, the release of atovaquone from PLGA nanocapsules was slow, just 18.9 % in three months (Cauchetier et al., 2003). Comparing the kinetic release of β-lap-loaded conventional, PEGylated and targeted nanocapsules, the presence of PEG and folate on the surface of nanocapsules did not affect the drug release rate.

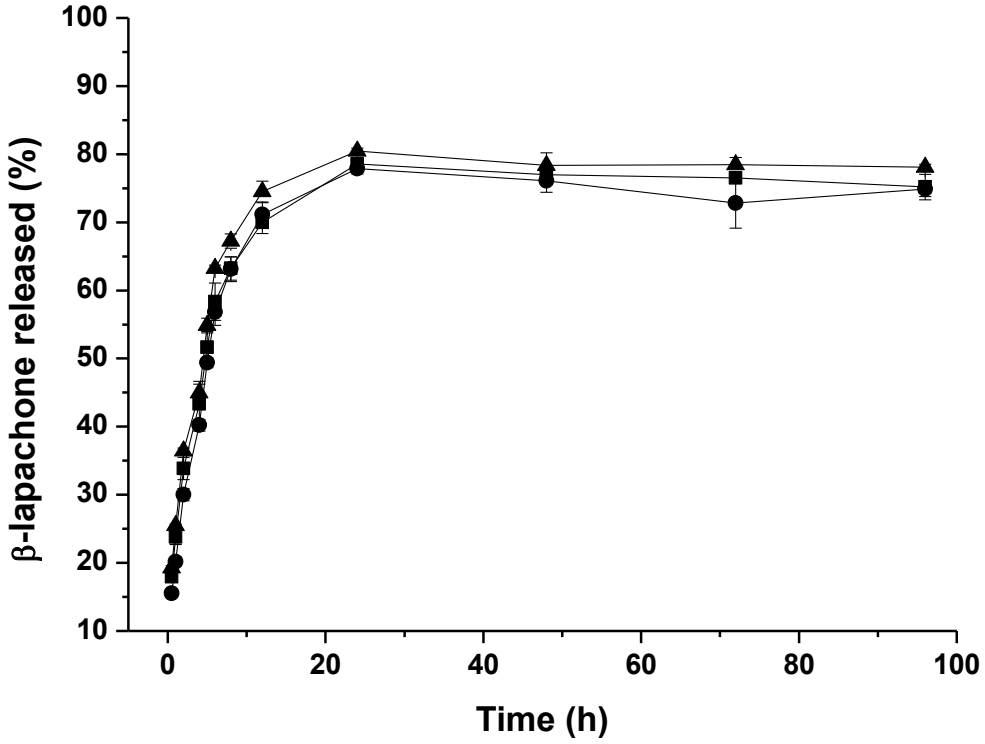


Fig. 4. Release profile of β-lapachone from the conventional (NC/β-lap) (■) PEGylated (NC-PEG/β-lap) (●) and targeted (NC-FOL/β-lap) (▲) nanocapsules under *sink* conditions. Each point represents the mean of three different experiments ± standard deviation.

3.4. Cytotoxicity studies

To evaluate the cell cytotoxicity of the free β-lap and the drug formulated in the nanocapsules were realized using the KG-1 and HeLa cell lines. Fig. 5 shows the KG-1and HeLa cell viability of the free β-lap and the drug formulated in the nanocapsules, which were determined by MTT assay. As the β-lap concentration increased, the cell viability of cancer cells was decreased. The cells lines exhibit a significant dose-dependent reduction in their viability after treatment with free β-lap and the drug formulated in the nanocapsules.

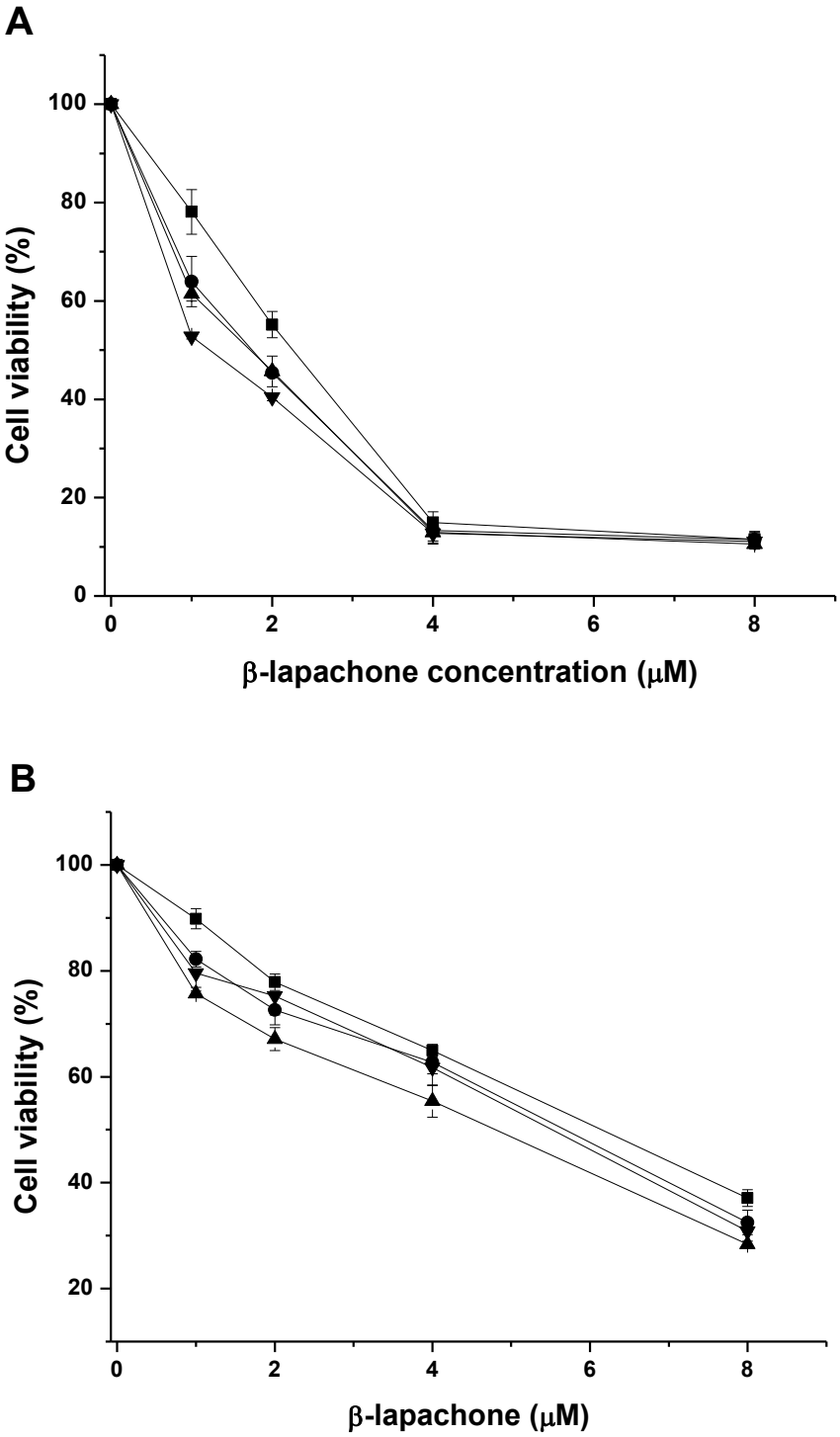


Fig. 5. Cytotoxicity of free β-lapachone (■), conventional (●), PEGylated (▲) and targeted (▼) nanocapsules on KG-1 (A) and HeLa (B) cancer cells after 48 h of incubation. Results are mean values ± SD of cell viability percentage (n = 3).

The cytotoxicity of free and encapsulated β-lap against HeLa cells was less than of KG-1 cells (Fig. 5B). However, similar to results obtained with KG-1cells, NC-FOL/β-lap (IC_{50} = 4.8 ± 0.48 μM) exhibited higher cytotoxicity that the free β-lap (IC_{50} = 6.2 ± 0.46 μM), NC/β-lap (IC_{50} = 5.7 ± 0.32 μM) and NC-PEG/β-lap (IC_{50} = 5.5 ± 0.81 μM) against the KG-1 and HeLa cells. Thus, the

enhanced cytotoxic result for targeted nanocapsules can be attributed to the increased cellular uptake of β-lap, which is also reported in the literature (Zhao and Yung, 2010; Liang et al., 2011; Saxena et al., 2012).

Table 2

IC_{50} values of free β-lapachone, conventional (NC/β-lap), PEGylated (NC-PEG/β-lap), and targeted (NC-FOL/β-lap) nanocapsules in KG-1 and HeLa cancer cells after 48 h of incubation. Results are mean values ± SD (n= 3).

Formulation	IC_{50} Values (μM)	
	KG-1 Cells	HeLa Cells
β-lap free	2.3 ± 0.03	6.2 ± 0.46
NC/β-lap	1.9 ± 0.26	5.7 ± 0.32
NC-PEG/β-lap	1.8 ± 0.21	5.5 ± 0.81
NC-FOL/β-lap	1.1 ± 0.16	4.8 ± 0.48

4. Conclusion

In this study, was synthesized and characterized PLGA-PEG-FOL conjugated polymer. Conventional, PEGylated and targeted nanocapsules containing β-lapachone were obtained with a low polydispersity index, negative Zeta potential and high encapsulation efficiency of drug into nanocapsules. Through *in vitro* release study, it was possible to plot a profile of β-lapachone release from nanocapsules with a maximum drug release in 24 h. The cell cytotoxicity of the free β-lap and encapsulated in nanocapsules was evaluated against the KG-1 and HeLa cells, which the targeted nanocapsules exhibited relevant cytotoxicity than the free drug, conventional and PEGylated nanocapsules. These results imply that β-lapachone-loaded surface modified nanocapsules could have high potentials to be used for chemotherapy.

Acknowledgements

The authors thank the Brazilian Council for Scientific and Technological Development (CNPq) and the Science and Technology Development of the Pernambuco State of Brazil (FACEPE) for financial support.

References

Almeida, E.R., Lucena, F.R.L., Silva, C.V. N. S., Costa-Junior, W.S., Cavalcanti, J.B., Couto, G. L., Silva, L. L. S., Mota, D. L., Silveira, A.B., Filho, S. D. S., Silva, A. C. P., 2009. Toxicological assessment of beta-lapachone on organs from pregnant and non-pregnant rats. Phytother. Res. 23, 1276-1280.

Blanco, E., Bey, E.A., Dong, Y., Weinberg, B.D., Sutton, D.M., Boothman, D.A., Gao, J., 2007. β-lapachone-containing PEG–PLA polymer micelles as novel nanotherapeutics against NQO1-overexpressing tumor cells. J. Control Release 122, 365–374.

Boddu, S.H.S., Vaishya, R., Jwala, J., Vadlapudi, A., Pal, D., Mitra, A.K., 2012. Preparation and Characterization of folate conjugated nanoparticles of doxorubicin using PLGA-PEG-FOL Polymer. Med. Chem. 2, 068-075.

Cauchetier, E., Deniau, M., Fessi, H., Astier, A., Paul, M., 2003. Atovaquone-loaded nanocapsules: influence of the nature of the polymer on their *in vitro* characteristics. Int J. Pharm. 250, 273-281.

Cavalcanti, I.M.F., Mendonça, E.A.M., Lira, M.C.B., Honrato, S.B., Camara, C.A., Amorim, R.V.S., Filho, J.M., Rabello, M.M.F., Hernandes, M.Z., Ayala, A.P., Santos-Magalhães, N.S., 2011. The encapsulation of β-lapachone in 2-hydroxypropyl-β-cyclodextrin inclusion complex into liposomes: A physicochemical evaluation and molecular modeling approach. Eur. J. Pharm. Sci. 4, 332–340.

Dong, Y., Chin, SF., Blanco, E., Bey, E.A., Kabbani, W., Xie, X.J., Bornmann, W.G., Boothman, D.A., Gao, J., 2009. Intratumoral delivery of β-lapachone via polymer implants for prostate cancer therapy. Clin. Cancer Res. 15, 131–139.

Dong, Y., Feng, S.S., 2004. Methoxy poly(ethylene glycol)–poly(lactide) (MPEG–PLA) nanoparticles for controlled delivery of anticancer drugs. Biomaterials 25, 2843–2849.

Ebrahimnejad, P., Dinarvand, R., Sajadi, A., Jaafari, M.R., Nomani, A.R., Azizi, E., Rad-Malekshahi, M., Atyabi, F., 2011. Characterization, blood profile and biodistribution properties of surface modified PLGA nanoparticles of SN-38. Int J. Pharm. 406, 122–127.

Esmaeili, F., Ghahremani, M.H., Ostad, S.N., Atyabi, F., Seyedabadi, M., Malekshahi, M.R., Amini, M., Dinarvand, R., 2008. Folate-receptor-targeted delivery of docetaxel nanoparticles prepared by PLGA–PEG–folate conjugate. J. Drug Target. 16, 415-423.

Eyong, K.O., Kumar, P.S., Kuete, V., Folefoc, G.N., Nkengfack, E.A., Baskaran, S., 2008. Semisynthesis and antitumoral activity of 2-acetylfuranonaphthoquinone and other naphthoquinone derivatives from lapachol. Bioorg. Med. Chem. Lett. 18, 5387–5390.

Fukumori, Y., Chikawa, H. Nanoparticles for cancer therapy and diagnosis., 2006. Adv Powder Tech. 17, 1-28.

Garcia-Bennett, A.; Nees, M.; Fadeel, B., 2011. In search of the holy grail: Folate-targeted nanoparticles for cancer therapy. Biochem Pharmacol. 81, 976-984.

Gref, R., Domb, A., Quellec, P., Blunk, T., Muller, R.H., Verbavatz, J.M., Langer, R., 1995. The controlled intravenous delivery of drugs using PEGcoated sterically stabilized nanospheres. Adv. Drug Deliv. Rev. 16, 215–233.

Gref, R.; Lück, M.; Quellec, P.; Marchand, M.; Dellacherie, E.; Harnisch, S.; Blunk, T.; Müller, R. H. 2000. 'Stealth' corona-core nanoparticles surface modified by polyethylene glycol (PEG): influences of the corona (PEG chain length and surface density) and of the core composition on phagocytic uptake and plasma protein adsorption. Colloids and Surfaces B: Biointerfaces 18, 301–313.

Hilgenbrink, A.R.; Low, P.S., 2005. Folate receptor-mediated drug targeting: From therapeutics to diagnostics. J. Pharm. Sci. 94, 2135-2146.

Kasperczyk, J. Microstructural Analysis of poly(l-lactide)-co-(glycolide) by ^{1}H and ^{13}C n.m.r. Spectroscopy., 1996. Polymer 37, 201-203.

Klose, D., Siepmann, F., Elkharraz, K., Siepmann, J., 2008. PLGA-based drug delivery systems: Importance of the type of drug and device geometry. Int J. Pharm. 354, 95–103.

Kumi-Diaka, J., 2002. Chemosensitivity of human prostate cancer cells PC3 and LNCaP to genisteinisoflavone and β-lapachone. Biol. Cell 94, 37–44.

Li, C.J., Averbouk, L., Pardee, A.B., 1993. β-lapachone, a novel DNA topoisomerase I inhibitor with a mode of action different from camptothecin. J. Biol. Chem. 268, 22463–22468.

Li, C., Li, Y.Z., Pinto, A.V., Pardee, A.B., 1999. Potent inhibition of tumor survival *in vivo* by β-lapachone plus taxol: Combining drugs imposes different artificial checkpoints. Proc. Natl. Acad. Sci. 96, 13369–13374.

Li, L.S., Bey, E.A., Dong, Y., Meng, J., Patra, B., Yan, J., Xie, X.J., Brekken, R.A., Barnett, C.C., Bornmann, W.G., Gao, J., Boothman, D.A., 2011. Modulating endogenous NQO1 levels identifies key regulatory mechanisms of action of β-lapachone for pancreatic cancer therapy. Clin. Cancer Res. 17, 275–285.

Liang, C., Yang, Y., Ling, Y., Huang, Y., Li, T., Li, X., 2011. Improved therapeutic effect of folate-decorated PLGA–PEG nanoparticles for endometrial carcinoma. Bioorg. Med. Chem. 19, 4057-4066.

Moghimi, S. M., Hunter, A.C., Clifford, M.J., 2001. Long-circulating and target-specific nanoparticles: Theory to practice. Pharmacol. Rev. 53, 283–318.

Mora-Huertas, C. E.; Fessi, H.; Elaissari, A., 2010. Polymer-based nanocapsules for drug delivery. Int J. Pharm. 385, 113-142.

Mosqueira, V. C. F.; Legrand, P.; Gulik A, Bourdon O, Gref R, Labarre D; Barratt, G. 2001b. Relationship between complement activation, cellular uptake and surface physicochemical aspects of novel PEG-modified nanocapsules. Biomaterials 22, 2967–2979.

Mosqueira, V. C. F.; Legrand, P.; Morgat, J. L.; Vert, M.; Mysiakine, E.; Gref, R.; Devissaguet, J. P.; Barratt, G. 2001a. Biodistribution of long-circulating PEG-grafted nanocapsules in mice: effects of PEG chain length and density. Pharm. Res. 18, 1411-1419.

Murugesan, S., Mishra, P., Jain, N.K., 2008. Development of Folate-Conjugated PEGylated Poly (d,l-lactide-co-glycolide) Nanoparticulate Carrier for Docetaxel. Cur. Nanosci. 4, 402-408.

Nasongkla N, Wiedmann AF, Bruening A, Beman M, Ray D, Bommann WG, Boothman DA, Gao J. 2003. Enhancement of solubility and bioavailability of β-lapachone using cyclodextrin inclusion complexes. Pharm. Res. 20, 1626-1633.

Nie, Y., Zhag, Z., Li, L., Luo, K., Ding, H., Gu,.Z., 2009. Synthesis, characterization and transfection of a novel folate - targeted multipolymericnanoparticles for gene delivery. J Mater Sci: Mater Med. 20, 1849-1857.

Ough, M., Lewis, A., Bey, E.A., Gao, J., Ritchie, J.M., Bornmann, W., Boothman, D.A., Oberley, L.W., Culle, N.J.J, 2005. Efficacy of β-lapachone in pancreatic cancer treatment: exploiting the novel, therapeutic target NQO1. Cancer Biol. Ther. 4, 95–102.

Park, E.K., Lee, S.B., Lee, Y.M., 2005. Preparation and characterization of methoxy poly(ethylene glycol)/poly(caprolactone) amphiphilic block copolymeric nanospheres for tunor-specific folate-mediated targeting of anticancer drugs. Biomaterials 26, 1053–1061.

Reinicke, K.E., Bey, E.A., Bentle, M.S., Pink, J.J., Ingalls, S.T., 2005. Development of β-lapachone prodrugs for therapy against human cancer cells with elevated NAD(P)H:quinineoxidoreductase 1 levels. Clin. Cancer Res. 11, 3055–3064.

Santos, N.P., Nascimento, S.C., Silva, J.F., Pereira, E.C.G., Silva, N.H., Honda, N.K., Santos-Magalhães, N.S, 2005. Usnic acid-loaded nanocapsules: an evaluation of cytotoxicity. J. Drug Del. Sci. Tech. 15, 355-361.

Saxena, V., Naguib, Y., Hussain, M. D., 2012. Folate receptor targeted 17-allylamino-17-demethoxygeldanamycin (17-AAG) loaded polymeric nanoparticles for breast cancer. Colloids Surf. B: Biointer. 94, 274– 280.

Silva-Júnior, E.N., Cavalcanti, B.C., Guimarães, T.T., Pinto, M.C.F.R., Cabral, I.O., Pessoa, C., Costa-Lotufo, L.V., Moraes, M.O., Andrade, C.K.Z., Santos, M.R., Simone, C.A., Goulart, M.O.F., Pinto, A.V., 2011. Synthesis and evaluation of quinonoid compounds against tumor cell lines. Eur. J. Med. Chem. 46, 399-410.

Sing, R., Lillard Jr, J.W., 2009. Nanoparticle-based targeted drug delivery. Exp. Mol. Pathol. 86, 215-223.

Stevanovic, M., Radulovic, A., Jordovic, B., Uskokovic, D., 2008. Poly (DL-lactide-co-glycolide) Nanospheres for the Sustained Release of Folic Acid. J. Biomed. Nanotech. 4, 1–10.

Sudimack, J., Lee, R.J., 2000. Targeted drug delivery via the folate receptor. Adv. Drug Deliv. Rev. 41, 147–162.

Tyagi, N, Ghosh, P. C., 2011. Folate receptor mediated targeted delivery of ricin entrapped into stericallystabilized liposomes to human epidermoid carcinoma (KB) cells: Effect of monensin intercalated into folate-tagged liposomes. Eur. J. Pharm. Sci. 43, 343–353.

Valencia, P.M., Hanewich-Hollatz, M.H., Gao, W., Karim, F., Langer, R., Karnik, R., Farokhzad, O.C., 2011. Effects of ligands with different water solubilities on self-assembly and properties of targeted nanoparticles. Biomaterials, 32, 6226-6233.

Wang, M.; Thanou, M., 2010. Targeting nanoparticles to cancer. Pharmacol. Res. 62, 90-99.

Woo, H.J.; Choi, Y.H., 2005. Growth inhibition of A549 human lung carcinoma cells by β-lapachone through induction of apoptosis and inhibition of telomerase activity. Int. J. Oncol. 26, 1017–1023.

Yamashita, M., Kaneko, M., Tokuda, H., Nishimura, K., Kumeda, Y., Iida, A., 2009. Synthesis and evaluation of bioactive naphthoquinones from the Brazilian medicinal plant, Tabebuia avellanedae. Bioorg. Med. Chem. Lett. 17, 6286–6289.

Yoo, H. S., Park, T.G., 2004a. Folate-receptor-targeted delivery of doxorubicin nano-aggregatesstabilized by doxorubicin–PEG–folate conjugate. J. Control. Rel. 100, 247–256.

Yoo, H. S., Park, T.G., 2004b. Folate receptor targeted biodegradable polymeric doxorubicin micelles. J. Control. Rel. 96, 273– 283.

Xiang, G., Wu, J., Lu, Y., Liu, Z., Lee, R.J., 2008. Synthesis and evaluation of a novel ligand for folate-mediated targeting liposomes. Int. J. Pharm. 356, 29–36.

Zhang, G, L., Gu, F.X., Chan, J.M., Wang, A.Z., Langer, R.S., Farokhzad, O.C., 2008. Nanoparticles in medicine: therapeutic applications and developments. Clin. Pharmacol. Ther. 83, 761–769.

Zhao, H., Yung, L.Y.L., 2008. Selectivity of folate conjugated polymer micelles against different tumor cells. Int. J. Pharm. 349, 256–268.

Zhao, P., Wang, H., Yu, M., Liao, Z., Wang, X., Zhang, F., Ji, W., Wu, B., Han, J., Zhang, H., Wang, H., Chang, J., Niu, R., 2012. Paclitaxel loaded folic acid targeted nanoparticles of mixed lipid-shelland polymer-core: *In vitro* and *in vivo* evaluation. Eur. J. Pharm. Biopharm. 81, 248-56.

6. CAPÍTULO 2

A ser submetido ao J*ournal of Chromatography B*

Development and validation of HPLC method to determine β-lapachone in rat plasma: Application to pharmacokinetic studies

Milena S. Ferraz [a], Fábio J. F. Almeida [a], Noemia P. S. Santos [a,b], Nereide S. Santos-Magalhães[a,*]

[a]Laboratório de ImunopatologiaKeizo-Asami, Universidade Federal de Pernambuco, Recife, PE, Brazil

[b]Laboratório de Nanotecnologia, Biotecnologia e Cultura de Células - Centro Acadêmico de Vitória/UFPE,Vitória de Santo Antão, PE, Brazil

*Corresponding author

Prof. Nereide Stela Santos-Magalhães

Universidade Federal de Pernambuco

Laboratório de Imunopatologia Keizo-Asami (LIKA)

Av. Prof. Moraes Rego, 1235, Cidade Universitária, 50670-901, Recife-PE, Brazil.

Phone: + 55 81 21268587; Fax: +55 81 21268485

E-mail: nereide.magalhaes@gmail.com

ABSTRACT

A simple, specific and rapid high performance liquid chromatography (HPLC) method for the determination of β-lapachone in rat plasma was developed and validated. β-lapachone was quantified on a C_{18} reverse phase column (250 mm × 4.6 mm, 5 mm,) at 40 ºC, using a mobile phase composed of methanol and water (80:20, v/v), at a flow rate of 0.9 mL/min, and with UV detection at 256 nm. β-lapachone was extracted from plasma samples after the addition of methanol at 4 °C. The method was proven to be linear over a plasma β-lapachone concentration range of 0.5 to 16 μg/mL with a mean correlation coefficient of 0.9996. The intermediate precision indicated that the difference between the means was statistically insignificant ($p < 0.05$). The recovery of β-lapachone from rat plasma was around 100 %. The limit of detection and the limit of quantification of β-lapachone were determined to be 0.07 μg/mL and 0.22 μg/mL, respectively. The developed method was successfully applied to assess the pharmacokinetics of β-lapachone in rat plasma.

Keywords: β-lapachone, plasma, HPLC , validation, pharmacokinetics.

1. Introduction

β-lapachone (3,4-dihydro-2,2-dimenthyl-2H-napthol [1,2‑b] pyran-5,6-dione, β-lap) is a *ortho*-naphthoquinone (Fig. 1) which can be extracted from the bark of *Tabebuia avellanedae*, a native tree originating in South America and found particularly in Brazil [1]. However, in large scale, β-lap is obtained by semi-synthesis from lapachol by heating or cold hydrolysis with sulfuric acid [2].

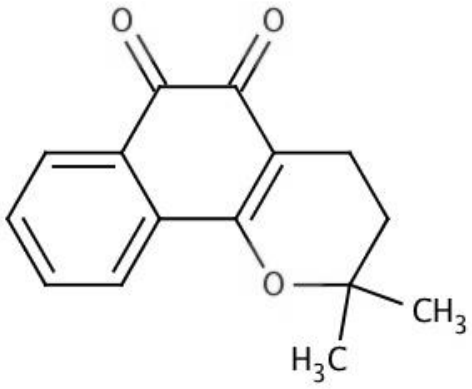


Fig. 1. Chemical structure of β-lapachone ($C_{15}H_4O_3$).

β-lapachone (β-lap) has been shown to have a variety of pharmacological effects, such as, antibacterial [3, 4], antifungal [5, 6], trypanocidal [7-10], antiviral [2, 11, 12], anti-inflammatory [13], antineoplastic [14].

Although many studies involving β-lap, relatively few publications describing methodologies for its quantification in biological fluids has been proposed. Glen et al. [15] published a high performance liquid chromatography (HPLC) reverse phase method for determination of β-lap in human plasma samples. In this study, the mobile phase was a mixture (v/v) of 45 % acetonitrile and 55 % K_2HPO_4 containing 0.003 % triethylamine, and the chloroform, as the extraction liquid, where a recovery of only 67 % of the drug was obtained. More recently, Savage et al. [16] developed a method for the quantification of β-lap in a stable hydroxypropyl-β-cyclodextrin formulation (ARQ 501) in mouse plasma using liquid chromatography/tandem mass spectrometry (LC-MS/MS). The mobile phase consisted of 0.1 % formic acid in water and 0.1 % formic acid in acetonitrile with a concentration gradient by varying the percentage of acetonitrile (40-95 %). The samples were processed using protein precipitation with acetonitrile and the recovery was more than 100 % due to a small interference of the plasma matrix.

In this scenario, the objective of this study was to develop and validate a new and simple analytical method by HPLC for quantification of β-lapachone in rats plasma with sensitivity and in extraction procedure, to explore the *in vivo* pharmacological properties of drug.

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). HPLC-grade metanol were supplied by Merck (Darmstadt, Germany). Purified water was generated by a Milli-Q® ultra-pure water purification system (Millipore, Massachusetts, USA).

2.2. Chromatographic Conditions

Chromatographic analysis were performed using Aliance 2695 equipment (Waters, Miliford, EUA) coupled to diode array detector (PDA) 2998 (Waters, Miliford, EUA) under isocratic elution at 40 °C. Reversed phase column C18 (250 mm × 4,6 mm, 5 mm, XBridge™ Waters) was used with mobile phase composed of methanol and water (80:20, v/v), at a flow rate of 0.9 mL/min. Injection volume was 50 μL and the wavelength for UV detection was set to 236 nm.

2.3. Preparation of standards and plasma samples

A stock solution of β-lap (1 mg/mL) was prepared in methanol. Six working standard solutions containing 6, 12, 24, 48, 96 and 192 μg/mL of β-lap were prepared by further dilution of β-lap stock solution with appropriate volumes of methanol.

Control plasma was obtained from untreated rats and spiked with β-lap standard solutions to yield calibration standards in plasma at concentrations of 0.5, 1, 2, 4, 8 and 16 μg/mL. Each β-lapachone-containing rat plasma sample was extracted and deproteinized by mixing it with methanol at 4 ºC. The mixture was briefly vortex-mixed for 30 s and centrifuged at 20.000 *g* for 10 min at 25 ºC. The supernatant was filtered (Millex® filters, 0.22 μm, Millipore, Massachusetts, USA) and injected into the HPLC system.

2.4. Method validation

The HPLC method to quantify β-lap in plasma was validated by determining its linearity, recovery, accuracy, precision, specificity, quantification and detection limits, according to international guidelines of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use [17].

2.4.1. Linearity

The linearity of the method was verified by the average of three authentic analytical curves at six different concentration levels of β-lap (0.5, 1, 2, 4, 8 e 16 μg/mL). The linearity of the analytical curve was established by linear correlation between concentration, independent variable (x), and ratio between the areas of the chromatographic peaks of the drug and the internal standard, dependent variable (y). The correlation parameters were estimated through the least square regression method.

2.4.2. Recovery

The extraction recovery was determined by comparing the results of plasma samples added β-lap subjected to the extraction process at analyzes of β-lap samples in methanol not subjected to this process, in three different concentrations (1, 4 and 16 μg/mL) and three replications.

2.4.3. Accuracy

The accuracy was assessed by analysis of plasma samples for quality control at three different concentration levels: low (1 μg/mL), medium (4 μg/mL) and high (16 μg/mL). All samples were prepared in triplicate (n = 9). The coefficient of variation and percentage of recovery were used to assess the accuracy defined as: Accuracy = (found drug concentration / theoretical drug concentration) × 100.

2.4.4. Precision

The precision assays were determined using three samples at different concentrations (1, 4 and 16 μg/mL) performed at the same day (intraday) and in two consecutive days by two analysts (inter-day). Precision was expressed as RSD %. Statistical analyses were performed using Student's t-test.

2.4.5. Limits of detection (LOD) and quantification (LOQ)

Limits of detection (LOD) and quantification (LOQ) were estimated, mathematically, from the average of three authentic analytical curves. The calculation to determine the corresponding values based on the standard deviation of the residual of the regression line and its relation to the slope in the analytical curve. To determine the limit of detection and quantification were utilized equations: LOQ = (SD / I) × 3.3 and LOQ = (SD / I) × 10, where LOD is the limit of detection; LOQ is the limit of quantification, SD is the standard deviation of the intercept with respect to the y axis and I is the slope of the calibration curve.

2.5. Pharmacokinetic Studies

The experiments were approved by the Ethics in Research Committee of the Federal University of Pernambuco, Recife, Brazil (Protocol 23076.033910/2012-32). The applicability of the developed HPLC method for β-lap in rat plasma was demonstrated by the results obtained from pharmacokinetic studies conducted in three male Wistar rats (250 g - 350 g). The drug was dissolved in 10 % DMSO plus 3 % Tween 80 (v/v in PBS) to produce stock solutions of 4 mg/mL and each rat received an intraperitoneal dose of 40 mg/kg of β-lap. A blood sample was collected from the tail vein into microtubes containing EDTA-K_3 (VACUETTE®, Greiner Bio-One, Kremsmuenster, Austria) at 0.5, 1, 2, 4, 8 and 12 h following drug administration. Plasma was separated by centrifugation and stored at –20 °C until analysis that was processed as described in sample preparation.

3. Results and discussion

3.1. Method validation

The developed method was specific for β-lap and good separation was obtained with the plasma components, with retention time of 4.5 minutes, allowing total analysis time for each sample about 6 minutes. Moreover, the chromatographic peaks obtained were symmetrical with excellent baseline resolution (Fig. 2). Pharmacokinetic assays generate many samples, so it is interesting that the analytical methods for these assays show short time analysis.

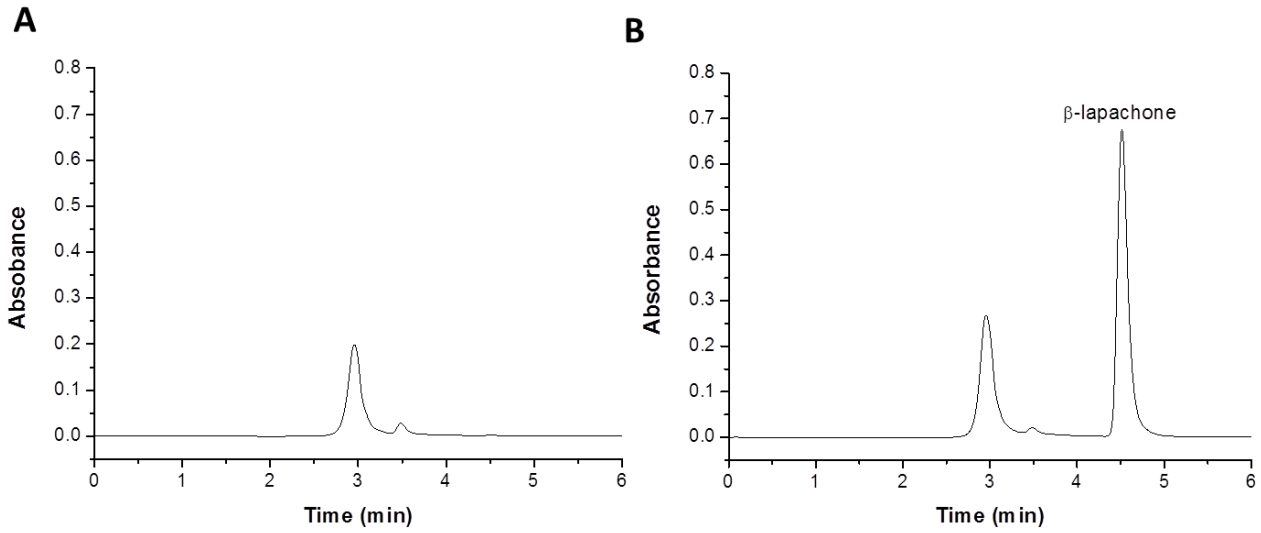


Fig. 2. Representative HPLC Chromatograms for (A) the control plasma and (B) the plasma spiked with β-lap standard solutions (16 μg/mL).

The method was linear between 0.5 to 16 μg/mL. The mean linear regression equation obtained from three calibration curve was y = 379042 x – 26626. The correlation coefficient obtained was 0.99996, meaning that 99.99 % of the total variation around the mean is explained by the linear regression, confirming the suitability of the method to the range evaluated. The data relative standard deviations (RSD) of the method range are shown in Table 1. The maximum relative standard deviation was 4.73 %, thus lower than the maximum value of 5 % required by ICH. Therefore, the analytical method developed has linearity range between β-lap concentrations of 0.5 to 16 μg/mL.

The extraction recovery of three different concentrations, 1, 4 and 16 μg/mL, was 99.58 ± 1.99 %, 99.40 % ± 1.07 and 99.46 ± 2.31 %, respectively. The organic solvent used in this study was methanol at 4 °C unlike previous studies, which used chloroform [15] and acetonitrile [16].

Table 1

Linearity of the HPLC method for β-lap determination in rat plasma.

$[\beta\text{-lap}]_{theorical}$ (μg/mL)	Area ± SD	$[\beta\text{-lap}]_{found}$ ± SD	RSD (%)
0.5	178207 ± 3146	0.54 ± 0.01	1.77
1	349283 ± 16555	0.99 ± 0.04	4.73
2	724186 ± 30563	1.98 ± 0.08	4.22
4	1471627 ± 20455	3.95 ± 0.05	1.39
8	3021511 ± 112399	8.04 ± 0.29	3.72
16	6035251 ± 144891	15.99 ± 0.382	2.40

SD= Standard deviation; RSD (%) = Relative standard deviation.

The accuracy was verified for three concentration levels: low, medium and high. The experimental data obtained showed an average accuracy of 99.3 % (98.8 to 99.9 %) and the maximum standard deviation was about 4 % (Table 2). The ICH regulates the accuracy results should not be less than 95 % and the relative standard deviation does not exceed 5 %, so the results of this study demonstrate that small variations in the concentration of β-lap can be quantified by the method developed.

Table 2

Accuracy for the quantification of β-lap in rat plasma.

[β-lap] (μg/ml)				Statistics			
Theorical		Found		Mean	SD	RSD (%)	A (%)
1	0.94	1.04	0.99	0.99	0.04	4.40	99.2
4	3.97	3.89	3.99	3.95	0.05	1.37	98.8
16	15.81	15.73	16.43	15.99	0.38	2.39	99.9

SD= Standard deviation; RSD (%) = Relative standard deviation.

The precision evaluates the variation of tests performed on different days and analysts. In this study, using the Student t test was evaluated the possible existence of a statistically significant difference between the mean values of assays performed on different days and with different analysts. As can be seen in Table 3 in all cases t calculated was always less than t critical, showing a lack of statistically significant difference between the means.

The values related to the limits of detection (LOD) and quantitation (LOQ) estimates were 0.07 and 0.22 μg/mL respectively. With these results, it appears that the method has sensitivity to detect and quantify the β-lap.

Table 3

Precision evaluation of the HPLC method for quantifying β-lap in rat plasma.

Theorical concentration (μg/mL)	t values		
	$t_{calculate}$		$t_{critical}$
	Interday	Intraday	
1.0	2.32	0.81	
4.0	0.41	1.09	4.30*
16.0	0.77	0.86	

*Theoretical value t critical = 4.30 based on the t-test comparing average with standard value at p = 0.05 level

3.2. Application of the HPLC method to pharmacokinetic studies

The proposed HPLC method was successfully applied to monitor quantitatively the time course of plasma β-lap concentrations after intraperitoneal administration of a single 40 mg/kg dose of the drug to three adult male Wistar rats. The mean plasma drug concentration-time profile observed in these pharmacokinetics studies is shown in Fig. 3. The following mean (± SD; n = 3) non compartmental pharmacokinetic parameters were derived from these data: maximum β-lap plasma concentration (C_{max}) = 1.74 ± 0.20 μg/mL; total area under the β-lap plasma concentration-time curve (AUC) = 421.1 ± 9.9 μg.min/mL; terminal phase elimination half-life ($T_{1/2}$) = 2.4 ± 0.18 h; apparent volume of distribution divided by the fraction of the intraperitoneal dose absorbed (Vd/F) = 24.5 ± 1.5 L; total body clearance divided by the fraction of the dose absorbed (CL/F) = 79.8 ± 2.1 mL/kg/min; and the mean residence time of β-lap in the body (MRT) = 306.9 ± 19.7 min.

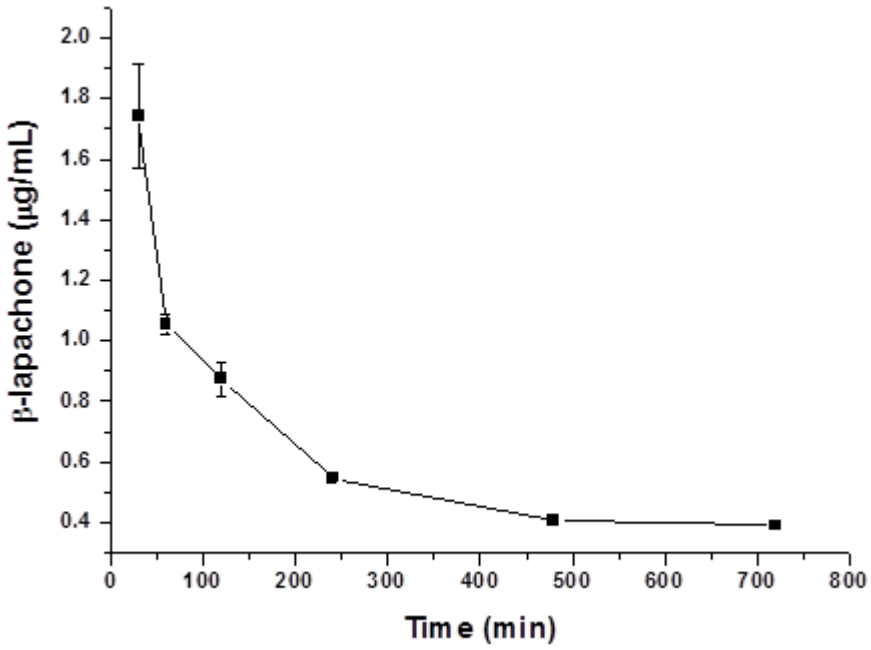


Fig. 3. Mean plasma concentration–time profile of β-lap (μg/mL) following an intraperitonial dose of 40 mg/kg administred to three Wistar rats (n=3).

4. Conclusion

A simple, rapid, specific, sensitive and reproducible HPLC method for the quantitative determination of β-lapachone in rat plasma has been developed and validated. The method is suitable for studying the pharmacokinetics of β-lapachone using rat as an animal model.

Acknowledgements

The authors thank the Brazilian Council for Scientific and Technological Development (CNPq) and the Science and Technology Development of the Pernambuco State of Brazil (FACEPE) for financial support.

References

[1] G.M.C. Alves, L.A. Rolim, P.J. Rolim-Neto, A.C.L. Leite, D.J. Brondani, F.P.M. Medeiros, L.W. Bieber, F.J.B. Mendonça-Júnior. Quím. Nova. 31 (2008) 413.

[2] C.J. Li, L. Averbouk, A.B. Pardee. J. Biol. Chem. 268 (1993) 22463.

[3] C.G.T. Oliveira, F.F. Miranda, V.F. Ferreira, C.C. Freitas, R.F. Rabello, J.M. Carballido, L.C.D. Corrêa. J. Braz. Chem. Soc. 12 (2001) 339.

[4] E.M. Pereira, T.B. Machado, I.C.R. Leal, D.M. Jesus, C.R.A. Damaso, A.V. Pinto, M. Giambiagi-deMarval, R.M. Kuster, K.R.N. Santos. An. Clin. Microb. Antimicrob. 5 (2006) 1.

[5] P. Guiraud, R. Steiman, G.M. Campos-Takaki, F. Seigle-Murandi, M. Simeon de Buochberg. Plant. Med. 60 (1994) 373.

[6] C.S. Medeiros, N.T. Pontes-Filho, C.A. Camara, J.V. Lima-Filho, P.C. Oliveira, S.A. Lemos, A.F.G. Leal, J.O.C. Brandão, R.P. Neves. Braz. J. Med. Biol. Res. 43 (2010) 345.

[7] J.N. Lopes, F.S. Cruz, R. Docampo, M.E. Vasconcellos, M.C. Sampaio, A.V. Pinto, B. Gilbert. Ann. Trop. Med. Parasitol. 72 (1978) 523.

[8] M.O.F. Goulart, C.L. Zani, J. Tonholo, L.R. Freitas, F.C. Abreu, A.B. Oliveira, D.S. Raslan, S. Starling, E. Chiari. Bioorg. Med. Chem. Lett. 7 (1997) 2043.

[9] K.C.G. Moura, F.S. Emery, C. Neves-Pinto, M.C. R. Pinto, A.P. Dantas, K. Salomão, S.L. Castro, A.V. Pinto. J. Braz. Chem. Soc. 12 (2001) 325.

[10] M.N. Silva, V.F. Ferreira, M.C.B.V. Souza. Quím. Nova. 26 (2003) 407.

[11] A.R. Schuerch, W. Wehrli. J. Biochem. 84 (1978) 197.

[12] K. Schaffner-Sabba, K.H. Schmidt-Ruppin, W. Wehrli, A.R. Schuerch, J.W. Wasley. J. Med. Chem. 27 (1984) 990.

[13] D.O. Moon, Y.H. Choi, N.D. Kim, Y.M. Park, G.Y. Kim. Int Immunopharmacol. 7 (2007) 506.

[14] H.J. Woo, Y.H. Choi. Int J Oncol. 26 (2005) 1017.

[15] V.L. Glen, P.R. Hutson, N.J. Kehli, D.A. Boothman, G. Wilding, J. Chromatogr. B. 692 (1997) 181.

[16] R.E. Savage, T. Hall, K. Bresciano, J. Bailey, M. Starace, T.C.K. Chan. J. Chromatogr. B. 872 (2008) 148.

[17] International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), Q2B: Guideline on Validation of Analytical Procedure-Methodology, E-Publishing, London, (1996) p. 10.

7. CAPÍTULO 3

A ser submetido ao *International Journal of Pharmaceutics*

Pharmacokinetics studies of β-lapachone-loaded PLGA-PEG-FOL nanocapsules after intraperitoneal administration

Milena S. Ferraz[a], Fábio J. F. Almeida[a], Noemia P. S. Santos[a,b], Nereide S. Santos-Magalhães[a,*]

[a]Laboratório de Imunopatologia Keizo-Asami, Universidade Federal de Pernambuco, Recife, PE, Brazil

[b]Laboratório de Nanotecnologia, Biotecnologia e Cultura de Células - Centro Acadêmico de Vitória /UFPE, Vitória de Santo Antão, PE, Brazil

*Corresponding author

Prof. Nereide Stela Santos-Magalhães

Universidade Federal de Pernambuco

Laboratório de Imunopatologia Keizo-Asami (LIKA)

Av. Prof. Moraes Rego, 1235, Cidade Universitária, 50670-901, Recife-PE, Brazil.

Phone: + 55 81 21268587; Fax: +55 81 21268485

E-mail: nereide.magalhaes@gmail.com

ABSTRACT

The aim of the study was to evaluate the pharmacokinetics profile of β-lapachone (β-lap) in solution and through PLGA-PEG-FOL nanocapsules in Wistar rats after intraperitoneal administration. The nanocapsules containing β-lapachone (NC-FOL/β-lap) were prepared by the interfacial deposition technique and characterized by particle size, zeta potential and encapsulation efficiency. Pharmacokinetic parameters were estimated by a non-compartmental approach using classic equations. After preparation, the NC-FOL/β-lap formulations showed appropriate particle size, encapsulation efficiency and surface charges. In the pharmacokinetics study, the nanoparticles showed significant changes in the β-lap pharmacokinetic profile compared to free drug. Overall, the use of folate-targeted nanocapsules containing β-lapachone in cancer therapy can be promising strategic, but further studies are warranted.

Keywords: PLGA-PEG-FOL, β-lapachone, folate, targeted nanocapsules, pharmacokinetics.

1. Introduction

Nanoparticles, by using active targeting strategies, can enhance the intracellular concentration of drugs in cancer cells while avoiding toxicity in normal cells. Furthermore, when nanoparticles bind to specific receptors and then enter the cell, they are usually enveloped by endosomes via receptor-mediated endocytosis (Leamon and Reddy, 2004). Some ligands can substantially increase site-specific targeting. In particular, folate has been used as a targeting moiety for enhancing the therapeutic efficacy and improved pharmacokinetic of many anticancer drugs encapsulated in nanoparticles (Garcia-Bennett et al., 2011). Folate receptor (FR) is a membrane glycoprotein, over-expressed on the surfaces of many cancer cells, while it is almost absent in most normal tissues (Sudimack et al., 2000; Hilgenbrink et al., 2005). In the literature, folate decorated nanoparticles of biodegradable polymers have been found to increase the cellular uptake and cell cytotoxicity of the formulated anticancer drugs (Valencia et al., 2011, Boddu et al., 2012, Saxena et al., 2012, Zhao et al., 2012).

In this study, poly (lactide-co-glycolide)-polyetilenoglycol-folate (PLGA-PEG-FOL) polymer conjugate were synthesized, and folate-targeted nanocapsules were prepared for targeted drug delivery with β-lapachone (β-lap). The β-lap is an ortho-naphthoquinone that can be extracted from the bark of Ipe-Roxo (*Tabebuia avellanedae*) or obtained by semi-synthesis from lapachol (Li et al., 1993). It has been shown that β-lap has a variety of pharmacological effects, among them the antineoplastic activity (Woo and Choi, 2005). Despite its therapeutic potential, the β-lap has limitations such as low solubility in water, hepatotoxicity and nonspecific drug distribution of β-lap limit its clinical potential (Nasongkla et al. 2003; Almeida et al. 2009). Some studies have suggested the use of drug delivery systems that can enhance bioavailability of β-lap, in this context some authors have been developed β-lap-loaded micelles (Blanco et al., 2007) and liposomes (Cavalcanti et al., 2011).

The aim of this work is to study the pharmacokinetics of β-lap in rats after single dose intraperitoneal administration of folate-targeted nanocapsules in comparison with free drug.

2. Materials and methods

2.1. Materials

β-lapachone (β-lap), obtained from lapachol by a semi-synthetic route was supplied by Dr. Alexandre Góes (UFPE, Brazil). Poly (d,l-lactide-co-glycolide) (PLGA 50:50, RG502H, Mw: 9000) with terminal carboxylic acid group was purchased from Boehringer Ingelheim (Ingelheim am Rhein, Germany). Poly(ethylene glycol)-bis-amine (PEG-bis-amine, Mw: 3400), N-

hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), folate (FOL), Poloxamer 407 and soybean oil were obtained from Sigma (St. Louis, USA). Soybean phosphatidylcholine (PC) (Lipoid S 100®) was purchased from Lipoid GMBH (Ludwigshafen, Germany). Solvents and other chemicals were supplied by Merck (Darmstadt, Germany).

2.2. Synthesis of the PLGA–PEG–FOL conjugate

PLGA–PEG-FOL conjugate was prepared according to the method developed by Yoo and Park (2004), but with minor modifications. PLGA dissolved in methylene chloride was activated by DCC and NHS at room temperature under nitrogen atmosphere for 24 h (PLGA/NHS/DCC stoichiometric molar ratio: 1/2/2). The resultant solution was centrifuged and precipitated by dropping into ice-cold diethyl ether and the activated PLGA was completely dried under vacuum. The activated PLGA dissolved in methylene chloride was added slowly to PEG–bis-amine dissolved in methylene chloride in a drop-wise manner with gentle stirring. The reaction was carried out for 6 h under nitrogen atmosphere (PLGA/PEG–bis-amine stoichiometric molar ratio: 1/5) and the resultant solution was precipitated by addition of ethanol. The precipitated product, amine-terminated di-block copolymer, PLGA–PEG–NH_2 was centrifuged and dried under vacuum. Folate-conjugated di-block copolymer was synthesized by coupling the PLGA–PEG–NH_2 di-block copolymer to an activated folate. PLGA–PEG–NH_2 was dissolved in anhydrous DMSO and was mixed with folate-NHS and DCC (PLGA–PEG–NH_2/folate stoichiometric molar ratio: 1/2). The reaction was performed under inert nitrogen atmosphere at room temperature for 12 h and then mixed with 50 ml of distilled water and centrifuged at 3000 rpm for 1h. After discarding the pellet, the supernatant was dialyzed (MWCO= 10 kDa) against distilled water for 72 h and freeze dried. Based on the molar ratio, the conjugation percentage of PLGA-PEG-FOL was found to be 98%. The conjugation percentage was calculated by determining the amount of folic acid conjugated in PLGA–PEG–FOL. A known amount of dried PLGA–PEG-FOL was dissolved in DMSO, and the UV absorbance value at 285 nm was measured to determine the concentration of conjugated folic acid. Serially diluted concentrations of folate in DMSO were used to construct a calibration curve.

2.3. Preparation of β-lap-loaded PLGA-PEG-FOL nanocapsules

Targeted nanocapsules containing β-lapachone (NC-FOL/ β-lap) were prepared by the interfacial deposition technique proposed by Santos et al., 2005. Briefly, mixing the polymers PLGA/PLGA-PEG-FOL, soybean oil, soybean phosphatidylcholine and β-lap were dissolved in acetone. Organic phase was added slowly and under magnetic stirring in aqueous phase consisted of poloxamer dissolved in phosphate buffer (pH 7.4). Subsequently, the colloidal dispersion was concentrated under reduced pressure to obtain a final concentration of 1mg/mL of β-lap.

The particle size of nanocapsules was measured by photon autocorrelation spectroscopy (DelsaTMNano-S, Beckman Coulter, California, USA) and zeta potential of the nanocapsules was determined by electrophoretic mobility (Zetasizer Nano-ZS9, Malvern, Worcestershire, UK).

The encapsulation efficiency of β-lap into nanocapsules was determined after submitting samples to ultrafiltration associated with centrifugation at 10,000 rpm for 1h using Microcon® filter units (Millipore, Billerica, USA). The concentration of β-lapachone in the ultrafiltrate was determined by a HPLC validated method and the encapsulation efficiency (%) was calculated by the difference between the total drug concentrations in the preparation and the unencapsulated (free in the ultrafiltrate) drug.

2.4. Pharmacokinetic Studies

2.4.1. Animals

The protocol for animal studies was approved by the Ethics in Research Committee of the Federal University of Pernambuco, Recife, Brazil (Protocol 23076.033910/2012-32). The experiments were carried out on male Wistar rats (250-350 g). The rats were housed under standard conditions at room temperature 22 ± 2 ºC, with a humidity roughly 65% and a 12-h light to 12-h dark cycles. Water and food *ad libitum* were supplied.

2.4.2. Study design

The animals were divided into two groups (n = 42). Groups I and II were administered 40 mg/kg of β-lap solutions (group I) or NC-FOL/ β-lap (group II) intraperitoneally. The drug was dissolved in 1 % DMSO plus 3 % Tween 80 (v/v in PBS) to produce stock β-lap solutions. Blood sample of three rats was collected from via cardiac puncture into microtubes containing EDTA-K3 (VACUETTE®, Greiner Bio-One, Kremsmuenster, Austria) for each time 0.5, 1, 2, 4, 8, 12 and 24 h following drug administration. Plasma was separated by centrifugation (1500 g for 10 min) and stored at –20 °C until analysis.

2.4.3. Plasma sample treatment

Each plasma sample was extracted and deproteinized by mixing it with methanol at 4 ºC. The mixture was briefly vortex-mixed for 30 s and centrifuged at 20,000 *g* for 10 min. The supernatant was filtrated (Millex® filters, 0.22 μm, Millipore, Massachusetts, USA) and injected into the HPLC system.

2.4.4. HPLC analysis

Chromatographic analysis were performed using Aliance 2695 equipment (Waters, Miliford, EUA) coupled to diode array detector (PDA) 2998 (Waters, Miliford, EUA) under isocratic elution at 40 °C. Reversed phase column C18 (250 mm × 4,6 mm, 5 mm, XBridge™ Waters) was used with mobile phase composed of methanol and water (80:20, v/v), at a flow rate of 0.9 mL/min. Injection volume was 50 μL and the wavelength for UV detection was set to 236 nm. The calibration curve β-lap in plasma at concentrations of 0.5, 1, 2, 4 and 8 μg/mL was prepared from dilutions of a β-lap stock solution (1 mg/ml) in methanol.

2.4.5. Pharmacokinetic data analysis

Pharmacokinetic parameters were estimated by a non-compartmental approach using classic equations. The maximum peak β-lap in plasma (C_{max}) and the time to reach maximum peak concentration (T_{max}) were obtained directly from the individual plasma concentration–time profiles. The elimination rate constant (k_e) was computed from a log-linear regression of the β-lap concentration data during the elimination phase and the half-life ($t_{1/2}$) was calculated as $\ln(2)/k_e$. The area under the concentration–time curve from time zero to time t (AUC_t) was calculated using the trapezoidal method. The area under the plasma concentration-time curve from time zero to infinity (AUC_∞) was obtained by the addition of AUC_t and the extrapolated area determined by last measured concentration (C_{last}) / ke. The apparent total body clearance of the drug from plasma (CL) was calculated from the quotient of the dose and AUC_∞. The mean residence time (MRT) was calculated by the ratio of the area under the first moment concentration versus time curve ($AUMC_\infty$) and AUC_∞. The apparent volume of distribution at steady state after non-intravenous administration (Vdss/F) was estimated by the product between MRT and CL.

The data were presented as mean ± SD, and the Student's t test was used to analyze differences between both groups. A *p*-value < 0.05 was considered significant.

3. Results and discussion

3.1. Preparation of β-lap-loaded PLGA-PEG-FOL nanocapsules

The nanocapsules containing β-lapachone (NC-FOL/ β-lap) were prepared by the interfacial deposition technique. NC-FOL/ β-lap presented a particle mean diameter of 150 ± 5.6 nm, being this size considered appropriate for *in vivo* administration of nanocapsules by intraperitoneal. Measurement of the zeta potential, which was – 5.4 ± 0.5 mV, assessed the surface charge of β-lap-loaded nanocapsules. The low zeta potencial is due the presence of folate on the surface of PLGA-

PEG-FOL nanocapsules which minimize carboxylic acid groups of the polymer PLGA (Esmaeli et al., 2008; Ebrahimnejad et al., 2010). In addition, high values of β-lap encapsulation efficiency in NC-FOL/ β-lap (99 ± 1.5 %) were obtained. Nanocapsules were obtained using the interfacial deposition technique presented high encapsulation capacity for lipophilic drugs due to its central oily cavity (Cauchetie et al., 2003; Mora-Huertas, 2010).

3.2. Pharmacokinetic Studies

Pharmacokinetic profile was performed in male rats via a single i.p. administration of free β-lap and encapsulated nanocapsules at the same dose of 40 mg/kg. The mean plasma concentration–time profiles for the free β-lap or β-lap-loaded nanocapsules (NC-FOL/ β-lap) are presented in Fig. 1. Pharmacokinetic parameters of β-lap were determined using non-compartmental analysis, and the pharmacokinetic parameters are summarized in Table 1.

As shown at all-time points in Fig. 1, the β-lap plasma concentrations were higher in rats administered with β-lap solution than those administered with NC-FOL/ β-lap. Therefore, the C_{max} value of β-lap in the nanocapsules (1.66 ± 0.06 μg/mL) was significantly ($p < 0.05$) lower than that obtained with β-lap solution (2.20 ± 0.21 μg/mL) and the T_{max} in animals treated with NC-FOL/ β-lap was equal to those treated with β-lap solution. However, the MRT and AUC_{∞} of β-lap delivered through nanocapsules were higher than for the β-lap solution.

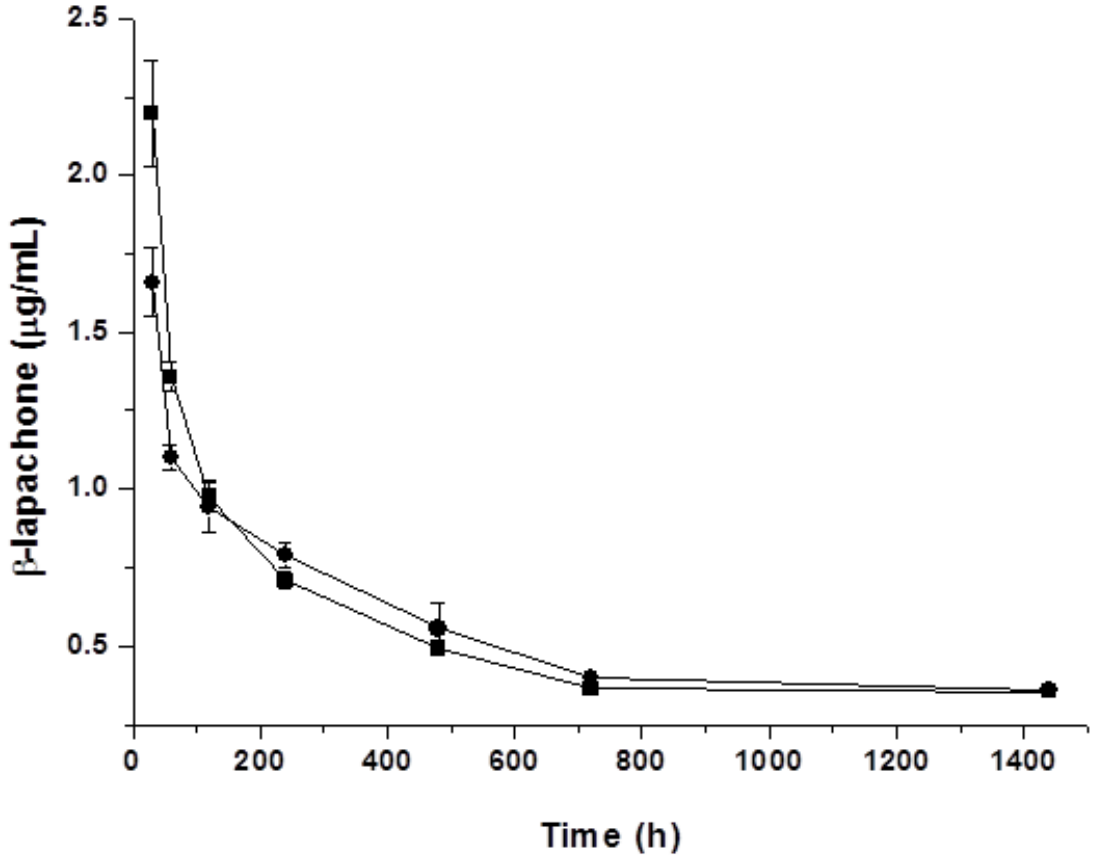


Fig. 1. Blood levels of intraperitoneally injected β-lap delivered in solution (■) and PLGA-PEG-FOL nanocapsules (●) after i.p. administration. The values plotted are mean ± SD.

In addition, the half-life ($t_{1/2}$) of β-lap was ($P < 0.05$) increased 2.4-fold by nanocapsules. The elimination rate constant of β-lap delivered through nanocapsules (0.12 h^{-1}) was lower than for β-lap solution (0.29 h^{-1}). The lower elimination constant (k_e) of β-lap delivered through nanocapsules compared to β-lap solution indicates their slow clearance from the body, which is supported by the enhanced half-life and AUC.

Compared with the β-lap solution, CL was decreased with NC-FOL/β-lap. These results showed that the incorporation of β-lap into nanocapsules decreases the clearance of drug from peritoneal cavity into blood can be useful in local chemotherapy of peritoneal tumors, where in the tumor cells are exposed to the nanocapsules containing β-lap for longer time and are expected to result in greater antitumor activity.

Table 1

Comparative pharmacokinetic parameters of β-lap delivered in solution or through PLGA-PEG-FOL nanocapsules (NC-FOL/β-lap) after intraperitoneal injection.

Pharmacokinetic parameters	**β-lap solution**	**NC-FOL/β-lap**
k_e (h^{-1})	0.29 ± 0.0	0.12 ± 0.0
AUC_∞ (μg/min/L)	580.9 ± 14.5	702.5 ± 60.9
C_{max} (μg/mL)	2.20 ± 0.2	1.66 ± 0.1
t_{max} (min)	30 ± 0.0	30 ± 0.0
$t_{1/2}$ (h)	2.4 ± 0.6	5.8 ± 1.3
MRT (h)	5.3 ± 0.2	9.1 ± 1.4
CL (mL/kg/min)	68.9 ± 1.7	57.2 ± 4.7
Vdss/F (L/kg)	21.8 ± 1.1	30.7 ± 5.3

Physico-chemical properties of nanoparticles such as size, surface charge, hydrophobicity, polymer composition and modifying the surface all contribute toward protein binding, biodistribution, cellular uptake and immune response. It has been reported that use of nanoparticles modifies the drug pharmacokinetic parameters (Couvreur and Vauthier, 2006). Other advantages include improved bioavailability and decreased toxicity due to high loading efficiency which results in lower doses administered. These factors all culminate in an increase in patient permanence to treatment (Kingsley et al., 2006). In this study, PLGA-PEG-FOL nanoparticles showed significant changes in the β-lap pharmacokinetic profile compared to free drug. Similar results can be observed in others pharmacokinetic studies using PLGA nanoparticles as drug delivery system (Averineni et al., 2012; Booysen et al., 2013).

4. Conclusion

Folate-targeted nanocapsules were prepared by the interfacial deposition technique with appropriate particle size, encapsulation efficiency and surface charges. Through the pharmacokinetics study, it was found that the encapsulation of β-lapachone into nanocapsules changes the pharmacokinetic profile after i.p. administration to rats. These results obtained in this study suggested that the use of folate-target nanocapsules in cancer therapy can be promising strategic to enhanced anticancer activity, but further studies are warranted.

Acknowledgements

The authors thank the Brazilian Council for Scientific and Technological Development (CNPq) and the Science and Technology Development of the Pernambuco State of Brazil (FACEPE) for financial support.

References

Almeida, E.R., Lucena, F.R.L., Silva, C.V. N. S., Costa-Junior, W.S., Cavalcanti, J.B., Couto, G. L., Silva, L. L. S., Mota, D. L., Silveira, A.B., Filho, S. D. S., Silva, A. C. P., 2009. Toxicological assessment of beta-lapachone on organs from pregnant and non-pregnant rats. Phytother. Res. 23, 1276-1280.

Averineni, R.K., Shavi, G.V., Gurram, A.K., Deshpande, P.B., Arumugam, K., Maliyakkal, N., Meka, S.R., Nayanabhirama, U., 2012. PLGA 50:50 nanoparticles of paclitaxel: Development, in vitro anti-tumor activity in BT-549 cells and in vivo evaluation. Bull. Mater. Sci., 35, 319–326.

Blanco, E., Bey, E.A., Dong, Y., Weinberg, B.D., Sutton, D.M., Boothman, D.A., Gao, J., 2007. β-Lapachone-containing PEG–PLA polymer micelles as novel nanotherapeutics against NQO1-overexpressing tumor cells. J. Control Release 122, 365–374.

Boddu, S.H.S., Vaishya, R., Jwala, J., Vadlapudi, A., Pal, D., Mitra, A.K., 2012. Preparation and Characterization of Folate Conjugated Nanoparticles of doxorubicin using PLGA-PEG-FOL Polymer. Med. Chem. 2, 068-075.

Booysen, L.L.I.J., Kalombo, L., Brooks, Ehansend, R., Gilliland, J., Gruppo, V., Lungenhofer, Semete-Makokotlela, P.B., Swai, H.S., Kotze, A.F., Lenaerts, A., Plessis, L.H., 2013. *In vivo/in vitro* pharmacokinetic and pharmacodynamic study of spray-dried poly-(dl-lactic-co-glycolic) acid nanoparticles encapsulating rifampicin and isoniazid. Int J. Pharm., 444, 10– 17.

Cavalcanti, I.M.F., Mendonça, E.A.M., Lira, M.C.B., Honrato, S.B., Camara, C.A., Amorim, R.V.S., Filho, J.M., Rabello, M.M.F., Hernandes, M.Z., Ayala, A.P., Santos-Magalhães, N.S., 2011. The encapsulation of β-lapachone in 2-hydroxypropyl-β-cyclodextrin inclusion complex into liposomes: A physicochemical evaluation and molecular modeling approach. Eur. J. Pharm. Sci. 4, 332–340.

Couvreur, P., Vauthier, C. Nanotechnology: intelligent design to treat complex disease. Pharm. Res**.**, v.23, 1417–1450, 2006.

Garcia-Bennett, A., Nees, M., Fadeel, B., 2011. In search of the holy grail: Folate-targeted nanoparticles for cancer therapy. Biochem Pharmacol. 81, 976-984.

Kingsley, J., Dou, H., Morehead, J., Rabinow, B., Gendelman, H., Destache, C., 2006. Nanotechnology: a focus on nanoparticles as a drug delivery system. J. Neuroim. Pharmacol. 1, 340–350.

Leamon, C.P., Reddy, J.A., 2004 Folate-targeted chemotherapy. Adv. Drug Deliv. Rev. 56, 1127–1141.

Li, C.J., Averbouk, L., Pardee, A.B., 1993. β-lapachone, a novel DNA topoisomerase I inhibitor with a mode of action different from camptothecin. J. Biol. Chem. 268, 22463–22468.

Mora-Huertas, C. E.; Fessi, H.; Elaissari, A., 2010. Polymer-based nanocapsules for drug delivery. Int J. Pharm. 385, 113-142.

Nasongkla N, Wiedmann AF, Bruening A, Beman M, Ray D, Bommann WG, Boothman DA, Gao J. 2003. Enhancement of solubility and bioavailability of β-lapachone using cyclodextrin inclusion complexes. Pharm. Res. 20, 1626-1633.

Nishiyama, M., Eguchi, H., 2009. Recent advances in cancer chemotherapy: current strategies, pharmacokinetics, and pharmacogenomics. Adv. Drug Deliv. Rev. 61, 367–368.

Santos, N.P., Nascimento, S.C., Silva, J.F., Pereira, E.C.G., Silva, N.H., Honda, N.K., Santos-Magalhães, N.S, 2005. Usnic acid-loaded nanocapsules: an evaluation of cytotoxicity. J. Drug Del. Sci. Tech. 15, 355-361.

Saxena, V., Naguib, Y., Hussain, M. D., 2012. Folate receptor targeted 17-allylamino-17-demethoxygeldanamycin (17-AAG)loaded polymeric nanoparticles for breast cancer. Colloids Surf. B: Biointer. 94, 274– 280.

Siegel, R., Ward, E., Brawley, O., Jemal, A., 2011. The impact of eliminating socioeconomic and racial disparities on premature cancer deaths. Cancer J. Clin. 61, 212–236.

Sudimack, J., Lee, R.J., 2000. Targeted drug delivery via the folate receptor. Adv. Drug Deliv. Rev. 41, 147–162.

Valencia, P.M., Hanewich-Hollatz, M.H., Gao, W., Karim, F., Langer, R., Karnik, R., Farokhzad, O.C., 2011. Effects of ligands with different water solubilities on self-assembly and properties of targeted nanoparticles. Biomaterials, 32, 6226-6233.

Woo, H.J.; Choi, Y.H., 2005. Growth inhibition of A549 human lung carcinoma cells by β-lapachone through induction of apoptosis and inhibition of telomerase activity. Int. J. Oncol. 26, 1017–1023.

Zhao, P., Wang, H., Yu, M., Liao, Z., Wang, X., Zhang, F., Ji, W., Wu, B., Han, J., Zhang, H., Wang, H., Chang, J., Niu, R., 2012. Paclitaxel loaded folic acid targeted nanoparticles of mixed lipid-shelland polymer-core: In vitro and in vivo evaluation. Eur. J. Pharm. Biopharm. 81, 248-56.

8. CONCLUSÕES

- O copolímero dibloco conjugado PLGA-PEG-FOL foi sintetizado e caracterizado através das técnicas de FTIR e RMN H^1;
- A determinação do percentual de conjugação do folato no PLGA-PEG-FOL foi realizada por espectrofotometria UV, onde foi observado um percentual de conjugação de aproximadamente 100%;
- Nanocápsulas convencionais, peguiladas e sítio-específicas contendo β-lapachona foram obtidas com tamanhos nanométricos, monodispersas e com elevadas eficiências de encapsulação;
- Uma redução do potencial zeta foi observada nas nanocápsulas peguiladas e sítio-específicas em relação às nanocápsulas convencionais, provavelmente devido à presença do PEG e folato na superfície destes sistemas;
- O perfil cinético de liberação da β-lapachona a partir das nanocápsulas de PLGA, PLGA-PEG e PLGA-PEG-FOL demonstrou uma liberação rápida do fármaco nas primeiras duas horas do processo cinético, seguida de uma liberação gradual e controlada da β-lapachona até as primeiras 24 h da cinética, atingindo um máximo de fármaco liberado;
- No estudo de citotoxicidade, a β-lapachona encapsulada em nanocápsulas sitio-específicas demonstrou um maior efeito citotóxico quando comparado ao fármaco livre, nanocápsulas convencionais e peguiladas;
- O método analítico para quantificação da β-lapachona em plasma de ratos demonstrou-se específico para o fármaco e, obtendo-se boa separação desta com os componentes do plasma, com tempo de retenção de 4,5 minutos;
- Os picos cromatográficos obtidos apresentaram-se simétricos e com excelente resolução da linha de base;
- O método mostrou-se linear entre as concentrações de 0,5 a 16 μg/mL, com um coeficiente de correlação de 0,99996;
- Os valores dos limites de detecção (LD) e quantificação (LQ) estimados foram 0,07 e 0,22 μg/mL, respectivamente;

- O método validado foi aplicado com sucesso para monitorização quantitativa das concentrações de β-lapachona após administração de uma dose única de 40 mg/kg do fármaco em ratos Wistar;

- Através do estudo de farmacocinética das nanocápsulas sítio-específicas contendo β-lapachona, verificou-se que a nanoencapsulação modifica os parâmetros farmacocinéticos do fármaco após administração intraperitoneal em ratos Wistar;

- A incorporação de β-lapachona, um potente agente antineoplásico, em nanocápsulas sítio-específicas pode ser uma nova estratégia para viabilizar a sua utilização na terapêutica do câncer.

9. PERSPECTIVAS

Como continuação deste trabalho, as nanocápsulas sítio-específicas contendo β-lapachona serão utilizadas em estudos de biodistribuição tecidual e atividade antitumoral.

10. ANEXOS

ANEXO A

NORMAS DAS REVISTAS

International Journal of Pharmaceutics

Guide for authors

INTRODUCTION

The *International Journal of Pharmaceutics* publishes innovative papers, reviews, mini-reviews, rapid communications and notes dealing with physical, chemical, biological, microbiological and engineering studies related to the conception, design, production, characterisation and evaluation of drug delivery systems *in vitro* and *in vivo*. "Drug" is defined as any therapeutic or diagnostic entity, including oligonucleotides, gene constructs and radiopharmaceuticals. Areas of particular interest include: pharmaceutical nanotechnology; physical pharmacy; polymer chemistry and physical chemistry as applied to pharmaceutics; excipient function and characterisation; biopharmaceutics; absorption mechanisms; membrane function and transport; novel routes and modes of delivery; responsive delivery systems, feedback and control mechanisms including biosensors; applications of cell and molecular biology to drug delivery; prodrug design; bioadhesion (carrier-ligand interactions); and biotechnology (protein and peptide formulation and delivery).
Note: For details on pharmaceutical nanotechnology, see Editorials in 279/1-2 281/1, and 288/1.

Types of paper

(1) **Full Length Manuscripts**
(2) **Rapid Communications**
(a) These articles should not exceed 1500 words or equivalent space.
(b) Figures should not be included otherwise delay in publication will be incurred.
(c) Do not subdivide the text into sections. An Abstract should be included as well as a full reference list.
(3) **Notes**
Should be prepared as described for full length manuscripts, except for the following:
(a) The maximum length should be 1500 words, including figures and tables.
(b) Do not subdivide the text into sections. An Abstract and reference list should be included.
(4) **Reviews and Mini-Reviews**
Suggestions for review articles will be considered by the Review-Editor. "Mini-reviews" of a topic are especially welcome.

Page charges

This journal has no page charges.

BEFORE YOU BEGIN

Ethics in publishing

For information on Ethics in publishing and Ethical guidelines for journal publication see http://www.elsevier.com/publishingethics and http://www.elsevier.com/ethicalguidelines.

Policy and ethics

The work described in your article must have been carried out in accordance with *The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans* http://www.wma.net/en/30publications/10policies/b3/index.html; *EU Directive 2010/63/EU for animal experiments* http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm; *Uniform Requirements for manuscripts submitted to Biomedical journals* http://www.icmje.org. This must be stated at an appropriate point in the article.
All authors are requested to disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could nappropriately influence, or be perceived to influence, their work. See also http://www.elsevier.com/conflictsofinterest.
Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Submission declaration and verification

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis or as an electronic preprint, see http://www.elsevier.com/postingpolicy), that it is not under consideration for publication elsewhere, that its publication

is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder. To verify originality, your article may be checked by the originality detection service CrossCheck http://www.elsevier.com/editors/plagdetect.

Contributors

Each author is required to declare his or her individual contribution to the article: all authors must have materially participated in the research and/or article preparation, so roles for all authors should be described. The statement that all authors have approved the final article should be true and included in the disclosure.

Authorship

All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

Changes to authorship

This policy concerns the addition, deletion, or rearrangement of author names in the authorship of accepted manuscripts:

Before the accepted manuscript is published in an online issue: Requests to add or remove an author, or to rearrange the author names, must be sent to the Journal Manager from the corresponding author of the accepted manuscript and must include: (a) the reason the name should be added or removed, or the author names rearranged and (b) written confirmation (e-mail, fax, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed. Requests that are not sent by the corresponding author will be forwarded by the Journal Manager to the corresponding author, who must follow the procedure as described above. Note that: (1) Journal Managers will inform the Journal Editors of any such requests and (2) publication of the accepted manuscript in an online issue is suspended until authorship has been agreed. *After the accepted manuscript is published in an online issue*: Any requests to add, delete, or rearrange author names in an article published in an online issue will follow the same policies as noted above and result in a corrigendum.

Copyright

Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement' (for more information on this and copyright see http://www.elsevier.com/copyright). Acceptance of the agreement will ensure the widest possible dissemination of information. An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.

Authors whose articles are published in the International Journal for Parasitology will be asked to transfer copyright for that article to the Australian Society for Parasitology, Inc. If there are any issues or conflicts of interest which might prevent the author transferring copyright, they should inform the Editor when submitting the manuscript. Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations (please consult http://www.elsevier.com/permissions). If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases: please consult http://www.elsevier.com/permissions.

Retained author rights

As an author you (or your employer or institution) retain certain rights; for details you are referred to: http://www.elsevier.com/authorsrights.

Role of the funding source

You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source(s) had no such involvement then this should be stated. Please see http://www.elsevier.com/funding.

Funding body agreements and policies

Elsevier has established agreements and developed policies to allow authors whose articles appear in journals published by Elsevier, to comply with potential manuscript archiving requirements as specified as conditions of their grant awards. To learn more about existing agreements and policies please visit http://www.elsevier.com/fundingbodies.

Open access

This journal does not ordinarily have publication charges; however, authors can now opt to make their articles available to all (including non-subscribers) via the ScienceDirect platform, for which a fee of $3000 applies (for further information on open access see http://www.elsevier.com/about/open-access/open-access-options). Please note that you can only make this choice after receiving notification that your article has been accepted for publication, to avoid any perception of conflict of interest. The fee excludes taxes and other potential costs such as color charges. In some cases, institutions and funding bodies have entered into agreement with Elsevier to meet these fees on behalf of their authors. Details of these agreements are available at http://www.elsevier.com/fundingbodies. Authors of accepted articles, who wish to take advantage of this option, should complete and submit the order form (available at http://www.elsevier.com/locate/openaccessform.pdf). Whatever access option you choose, you retain many rights as an

author, including the right to post a revised personal version of your article on your own website. More information can be found here: http://www.elsevier.com/authorsrights.

Language (usage and editing services)

Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's WebShop http://webshop.elsevier.com/languageediting/ or visit our customer support site http://support.elsevier.com for more information.

Submission

Submission to this journal proceeds totally online and you will be guided stepwise through the creation and uploading of your files. The system automatically converts source files to a single PDF file of the article, which is used in the peer-review process. Please note that even though manuscript source files are converted to PDF files at submission for the review process, these source files are needed for further processing after acceptance. All correspondence, including notification of the Editor's decision and requests for revision, takes place by e-mail removing the need for a paper trail.

Authors must state in a covering letter when submitting papers for publication the novelty embodied in their work or in the approach taken in their research. Routine bioequivalence studies are unlikely to find favour. No paper will be published which does not disclose fully the nature of the formulation used or details of materials which are key to the performance of a product, drug or excipient. Work which is predictable in outcome, for example the inclusion of another drug in a cyclodextrin to yield enhanced dissolution, will not be published unless it provides new insight into fundamental principles.

Referees

Please submit, with the manuscript, the names, addresses and e-mail addresses of three potential referees. Note that the editor retains the sole right to decide whether or not the suggested reviewers are used.

PREPARATION

Use of wordprocessing software

It is important that the file be saved in the native format of the wordprocessor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the wordprocessor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: http://www.elsevier.com/guidepublication). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork. To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your wordprocessor.

Article structure

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

• ***Author names and affiliations.*** Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that phone numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.**
• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.
A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself. The abstract must not exceed 200 words.

Graphical abstract

A Graphical abstract is mandatory for this journal. It should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership online. Authors must provide images that clearly represent the work described in the article. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more, but should be readable on screen at a size of 200 × 500 pixels (at 96 dpi this corresponds to 5 × 13 cm). Bear in mind readability after reduction, especially if using one of the figures from the article itself. Preferred file types: TIFF, EPS, PDF or MS Office files. See http://www.elsevier.com/graphicalabstracts for examples.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Units

Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

Database linking

Elsevier encourages authors to connect articles with external databases, giving their readers oneclick access to relevant databases that help to build a better understanding of the described research. Please refer to relevant database identifiers using the following format in your article: Database: xxxx (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN). See http://www.elsevier.com/databaselinking for more information and a full list of supported databases.

Math formulae

Present simple formulae in the line of normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article, using superscript Arabic numbers. Many wordprocessors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Table footnotes

Indicate each footnote in a table with a superscript lowercase letter.

Whilst it is accepted that authors sometimes need to manipulate images for clarity, manipulation for purposes of deception or fraud will be seen as scientific ethical abuse and will be dealt with accordingly. For graphical images, this journal is applying the following policy: no specific feature within an image may be enhanced, obscured, moved, removed, or introduced. Adjustments of brightness, contrast, or color balance are acceptable if and as long as they do

not obscure or eliminate any information present in the original. Nonlinear adjustments (e.g. changes to gamma settings) must be disclosed in the figure legend.

Electronic artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.
• Embed the used fonts if the application provides that option.
• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.
• Size the illustrations close to the desired dimensions of the printed version.
• Submit each illustration as a separate file.

A detailed guide on electronic artwork is available on our website:http://www.elsevier.com/artworkinstructions

You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format. Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

EPS (or PDF): Vector drawings, embed all used fonts.
TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.
TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi.
TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.

Please do not:

• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;
• Supply files that are too low in resolution;
• Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF, EPS or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article.** Please indicate your preference for color: in print or on the Web only. For further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions.

Please note: Because of technical complications which can arise by converting color figures to 'gray scale' (for the printed version should you not opt for color in print) please submit in addition usable black and white versions of all the color illustrations.

Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication and a copy of the title page of the relevant article must be submitted.

Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

References in a special issue

Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference management software

This journal has standard templates available in key reference management packages EndNote (http://www.endnote.com/support/enstyles.asp) and Reference Manager (http://refman.com/support/rmstyles.asp). Using plug-ins to wordprocessing packages, authors only need to select the appropriate journal template when preparing their article and the list of references and citations to these will be formatted according to the journal style which is described below.

Reference style

Text: All citations in the text should refer to:

1. *Single author:* the author's name (without initials, unless there is ambiguity) and the year of publication;
2. *Two authors:* both authors' names and the year of publication;
3. *Three or more authors:* first author's name followed by 'et al.' and the year of publication.Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.

Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al. (2010) have recently shown ....'

List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

Examples:

Reference to a journal publication:

Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci. Commun. 163, 51–59.

Reference to a book:

Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed. Longman, New York.

Reference to a chapter in an edited book:

Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.

Journal abbreviations source

Journal names should be abbreviated according to

Index Medicus journal abbreviations: http://www.nlm.nih.gov/tsd/serials/lji.html;

List of title word abbreviations: http://www.issn.org/2-22661-LTWA-online.php;

CAS (Chemical Abstracts Service): http://www.cas.org/content/references/corejournals.

Video data

Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include links to these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the files in one of our recommended file formats with a preferred maximum size of 50 MB. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages at http://www.elsevier.com/artworkinstructions. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

Supplementary data

Elsevier accepts electronic supplementary material to support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, highresolution images, background datasets, sound clips and more. Supplementary files supplied will be published online alongside the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. In order to ensure that your submitted material is directly usable, please provide the data in one of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file. For more detailed instructions please visit our artwork instruction pages at http://www.elsevier.com/artworkinstructions.

Submission checklist

It is hoped that this list will be useful during the final checking of an article prior to sending it to the journal's Editor for review. Please consult this Guide for Authors for further details of any item.

Ensure that the following items are present:

One Author designated as corresponding Author:

• E-mail address

• Full postal address
• Telephone and fax numbers
All necessary files have been uploaded
• Keywords
• All figure captions
• All tables (including title, description, footnotes)
Further considerations:
• Use continuous line numbering (every 5 lines) to facilitate reviewing of the manuscript.
• Manuscript has been "spellchecked" and "grammar-checked"
• References are in the correct format for this journal
• All references mentioned in the Reference list are cited in the text, and vice versa
• Permission has been obtained for use of copyrighted material from other sources (including the Web)
• Color figures are clearly marked as being intended for color reproduction on the Web (free of charge) and in print or to be reproduced in color on the Web (free of charge) and in black-and-white in print
• If only color on the Web is required, black and white versions of the figures are also supplied for printing purposes
For any further information please visit our customer support site at http://support.elsevier.com.

AFTER ACCEPTANCE

Use of the Digital Object Identifier

The Digital Object Identifier (DOI) may be used to cite and link to electronic documents. The DOI consists of a unique alpha-numeric character string which is assigned to a document by the publisher upon the initial electronic publication. The assigned DOI never changes. Therefore, it is an ideal medium for citing a document, particularly 'Articles in press' because they have not yet received their full bibliographic information. Example of a correctly given DOI (in URL format; here an article in the
journal *Physics Letters B*):
http://dx.doi.org/10.1016/j.physletb.2010.09.059
When you use a DOI to create links to documents on the web, the DOIs are guaranteed never to change.

Proofs

One set of page proofs (as PDF files) will be sent by e-mail to the corresponding author (if we do not have an e-mail address then paper proofs will be sent by post) or, a link will be provided in the e-mail so that authors can download the files themselves. Elsevier now provides authors with PDF proofs which can be annotated; for this you will need to download Adobe Reader version 7 (or higher) available free from http://get.adobe.com/reader. Instructions on how to annotate PDF files will accompany the proofs (also given online). The exact system requirements are given at the Adobe site: http://www.adobe.com/products/reader/tech-specs.html.
If you do not wish to use the PDF annotations function, you may list the corrections (including replies to the Query Form) and return them to Elsevier in an e-mail. Please list your corrections quoting line number. If, for any reason, this is not possible, then mark the corrections and any other comments (including replies to the Query Form) on a printout of your proof and return by fax, or scan the pages and e-mail, or by post. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor. We will do everything possible to get your article published quickly and accurately – please let us have all your corrections within 48 hours. It is important to ensure that all corrections are sent back to us in one communication: please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility. Note that Elsevier may proceed with the publication of your article if no response is received.

Offprints

The corresponding author, at no cost, will be provided with a PDF file of the article via e-mail or, alternatively, 25 free paper offprints. The PDF file is a watermarked version of the published article and includes a cover sheet with the journal cover image and a disclaimer outlining the terms and conditions of use. For an extra charge, more paper offprints can be ordered via the offprint order form which is sent once the article is accepted for publication. Both corresponding and co-authors may order offprints at any time via Elsevier's WebShop (http://webshop.elsevier.com/myarticleservices/offprints).
Authors requiring printed copies of multiple articles may use Elsevier WebShop's 'Create Your Own Book' service to collate multiple articles within a single cover (http://webshop.elsevier.com/myarticleservices/offprints/myarticlesservices/booklets).

AUTHOR INQUIRIES

For inquiries relating to the submission of articles (including electronic submission) please visit this journal's homepage. For detailed instructions on the preparation of electronic artwork, please visit http://www.elsevier.com/artworkinstructions. Contact details for questions arising after acceptance of an article, especially those relating to proofs, will be provided by the publisher. You can track accepted articles at http://www.elsevier.com/trackarticle. You can also check our Author FAQs at http://www.elsevier.com/authorFAQ and/or contact Customer Support via http://support.elsevier.com.

Journal of Chromatography B

GUIDE FOR AUTHORS

INTRODUCTION

The Journal of Chromatography B publishes papers on developments in separation science relevant to biology and biomedical research including both fundamental advances and applications. Analytical techniques which may be considered include the various facets of chromatography, electrophoresis and related methods, affinity and immunoaffinity-based methodologies, hyphenated and other multi-dimensional techniques, and microanalytical approaches. The journal also considers articles reporting developments in sample preparation, detection techniques including mass spectrometry, and data handling and analysis.

Developments related to preparative separations for the isolation and purification of components of biological systems may be published, including chromatographic and electrophoretic methods, affinity separations, field flow fractionation and other preparative approaches. Applications to the analysis of biological systems and samples will be considered when the analytical science contains a significant element of novelty, e.g. a new approach to the separation of a compound, novel combination of analytical techniques, or significantly improved analytical performance. Areas to be considered include:

• the qualitative and quantitative analysis of biopolymers including proteins, peptides and their post-translational modifications as well as nucleic acids and glycans • the comparative analysis of biological systems using proteomics, genomics, metabonomics and other "omics" approaches • clinical analysis, pharmacokinetics, metabolism, therapeutic drug monitoring, toxicological analysis, doping analysis, veterinary applications, analysis of environmental contaminants in biological systems • the screening and profiling of body fluids, tissues, cells, biological matrices and systems, analysis of endogenous compounds, biomarkers • identification of new bioactive compounds Applications which utilize published or commercial analytical or preparative protocols with little or no modification or where the results of the application rather than the analytical methodology comprise the major element of novelty of the manuscript should be directed to more specialized journals. Modifications to a previously published method may be considered for a short communication in cases where the improvement in performance is significant. Reports of analytical methods for compounds in early pharmaceutical development often lack general interest and will not be published unless the authors can demonstrate the broader significance of the methodology involved. Quality control analyses of bulk drugs, natural products or pharmaceutical formulations are not within scope.

Types of Paper

The following types of papers are published in the Journal of Chromatography B: Regular research papers (full-length papers), Review articles, Short Communications, Discussions, and Letters to the Editor. Review articles are invited or proposed in writing to the Editors, who welcome suggestions for subjects. An outline of the proposed Review should first be forwarded to the Editors for preliminary discussion prior to preparation. Short Communications are usually descriptions of short investigations, or they can report technical improvements of previously published procedures: they reflect the same quality of research as full-length papers, but should preferably not exceed five printed pages (typically no more than 2850 words (including title, authors and references), with no more than five figures and tables combined).

BEFORE YOU BEGIN

Ethics in publishing

For information on Ethics in publishing and Ethical guidelines for journal publication see http://www.elsevier.com/publishingethics and http://www.elsevier.com/ethicalguidelines.

Conflict of interest

All authors are requested to disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work. See also http://www.elsevier.com/conflictsofinterest.

Submission declaration and verification

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis or as an electronic preprint, see http://www.elsevier.com/postingpolicy), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder. To verify originality, your article may be checked by the originality detection service CrossCheck http://www.elsevier.com/editors/plagdetect.

Changes to authorship

This policy concerns the addition, deletion, or rearrangement of author names in the authorship of accepted manuscripts:

Before the accepted manuscript is published in an online issue: Requests to add or remove an author, or to rearrange the author names, must be sent to the Journal Manager from the corresponding author of the accepted manuscript and must include: (a) the reason the name should be added or removed, or the author names rearranged and (b) written confirmation (e-mail, fax, letter) from all authors that they agree with the addition, removal or rearrangement. In the

case of addition or removal of authors, this includes confirmation from the author being added or removed. Requests that are not sent by the corresponding author will be forwarded by the Journal Manager to the corresponding author, who must follow the procedure as described above. Note that: (1) Journal Managers will inform the Journal Editors of any such requests and (2) publication of the accepted manuscript in an online issue is suspended until authorship has been agreed. *After the accepted manuscript is published in an online issue*: Any requests to add, delete, or rearrange author names in an article published in an online issue will follow the same policies as noted above and result in a corrigendum.

Copyright

Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement' (for more information on this and copyright see http://www.elsevier.com/copyright). Acceptance of the agreement will ensure the widest possible dissemination of information. An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.

Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations (please consult http://www.elsevier.com/permissions). If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases: please consult http://www.elsevier.com/permissions.

Retained author rights

As an author you (or your employer or institution) retain certain rights; for details you are referred to: http://www.elsevier.com/authorsrights.

Role of the funding source

You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source(s) had no such involvement then this should be stated. Please see http://www.elsevier.com/funding.

Funding body agreements and policies

Elsevier has established agreements and developed policies to allow authors whose articles appear in journals published by Elsevier, to comply with potential manuscript archiving requirements as specified as conditions of their grant awards. To learn more about existing agreements and policies please visit http://www.elsevier.com/fundingbodies.

Open access

This journal does not ordinarily have publication charges; however, authors can now opt to make their articles available to all (including non-subscribers) via the ScienceDirect platform, for which a fee of $3000 applies (for further information on open access see http://www.elsevier.com/about/open-access/open-access-options). Please note that you can only make this choice after receiving notification that your article has been accepted for publication, to avoid any perception of conflict of interest. The fee excludes taxes and other potential costs such as color charges. In some cases, institutions and funding bodies have entered into agreement with Elsevier to meet these fees on behalf of their authors. Details of these agreements are available at http://www.elsevier.com/fundingbodies. Authors of accepted articles, who wish to take advantage of this option, should complete and submit the order form (available at http://www.elsevier.com/locate/openaccessform.pdf). Whatever access option you choose, you retain many rights as an author, including the right to post a revised personal version of your article on your own website. More information can be found here: http://www.elsevier.com/authorsrights.

Language (usage and editing services)

Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's WebShop http://webshop.elsevier.com/languageediting/ or visit our customer support site http://support.elsevier.com for more information.

Submission

Submission to this journal proceeds totally online and you will be guided stepwise through the creation and uploading of your files. The system automatically converts source files to a single PDF file of the article, which is used in the peer-review process. Please note that even though manuscript source files are converted to PDF files at submission for the review process, these source files are needed for further processing after acceptance. All correspondence, including notification of the Editor's decision and requests for revision, takes place by e-mail removing the need for a paper trail.

Every paper must be accompanied by a letter from the senior author, stating that he/she is submitting the paper for publication in the Journal of Chromatography B. Please submit your article via http://www.elsevier.com/locate/jchromb

Referees

Please submit, with the manuscript, the names, addresses and e-mail addresses of three potential referees. Note that the editor retains the sole right to decide whether or not the suggested reviewers are used.

PREPARATION

Use of wordprocessing software

It is important that the file be saved in the native format of the wordprocessor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the wordprocessor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: http://www.elsevier.com/guidepublication). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork. To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your wordprocessor.

Article structure

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Theory/calculation

A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

• ***Author names and affiliations.*** Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that phone numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.**

• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Highlights

Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). See http://www.elsevier.com/highlights for examples.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Nomenclature

Widely accepted symbols, abbreviations and units (SI) should be used. If there is any doubt about a particular symbol or abbreviation, the full expression followed by the abbreviation should be given the first time it appears in the text. Abbreviations used in tables and figures should be explained in the captions. In general, the recommendations of the International Union of Pure and Applied Chemistry (IUPAC) should be followed and attention should be given to the recommendations of the Analytical Chemistry Division in the journal Pure and Applied Chemistry: Nomenclature for Chromatography, Pure Appl. Chem., 65 (1993) 819-872. Decimal points should be indicated by full stops. All decimal numbers smaller than unity should include a leading zero (e.g. 0.11).

Math formulae

Present simple formulae in the line of normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article, using superscript Arabic numbers. Many wordprocessors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Table footnotes

Indicate each footnote in a table with a superscript lowercase letter.

Artwork

Electronic artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.
• Embed the used fonts if the application provides that option.
• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.
• Size the illustrations close to the desired dimensions of the printed version.
• Submit each illustration as a separate file.

A detailed guide on electronic artwork is available on our website: http://www.elsevier.com/artworkinstructions

You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format. Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

EPS (or PDF): Vector drawings, embed all used fonts.
TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.
TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi.
TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.

Please do not:

• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;

• Supply files that are too low in resolution;
• Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF, EPS or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article.** Please indicate your preference for color: in print or on the Web only. For further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions.

Please note: Because of technical complications which can arise by converting color figures to 'gray scale' (for the printed version should you not opt for color in print) please submit in addition usable black and white versions of all the color illustrations.

Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

References in a special issue

Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference management software

This journal has standard templates available in key reference management packages EndNote (http://www.endnote.com/support/enstyles.asp) and Reference Manager (http://refman.com/support/rmstyles.asp). Using plug-ins to wordprocessing packages, authors only need to select the appropriate journal template when preparing their article and the list of references and citations to these will be formatted according to the journal style which is described below.

Reference Style

Reference Style

Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given. Example: "..... as demonstrated [3,6]. Barnaby and Jones [8] obtained a different result ...."

List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.

Examples:

Reference to a journal publication:

[1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, J. Sci. Commun. 163 (2000) 51.

Reference to a book:

[2] W. Strunk Jr., E.B. White, The Elements of Style, Macmillan, New York, 3rd ed., 1979.

Reference to a chapter in an edited book:

[3] G.R. Mettam, L.B. Adams, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing, New York, 1994, p. 281.

Journal abbreviations source

Journal names should be abbreviated according to Index Medicus journal abbreviations: http://www.nlm.nih.gov/tsd/serials/lji.html; List of title word abbreviations: http://www.issn.org/2-22661-LTWA-online.php; CAS (Chemical Abstracts Service): http://www.cas.org/content/references/corejournals.

Video data

Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include links to these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the files in one of our recommended file formats with a preferred maximum size of 50 MB. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages at http://www.elsevier.com/artworkinstructions. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

Supplementary data

Elsevier accepts electronic supplementary material to support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, highresolution images, background datasets, sound clips and more. Supplementary files supplied will be published online alongside the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. In order to ensure that your submitted material is directly usable, please provide the data in one of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file. For more detailed instructions please visit our artwork instruction pages at http://www.elsevier.com/ artworkinstructions.

Submission checklist

The following list will be useful during the final checking of an article prior to sending it to the journal for review. Please consult this Guide for Authors for further details of any item.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:

• E-mail address
• Full postal address
• Phone numbers

All necessary files have been uploaded, and contain:

• Keywords
• All figure captions
• All tables (including title, description, footnotes)

Further considerations

• Manuscript has been 'spell-checked' and 'grammar-checked'
• References are in the correct format for this journal
• All references mentioned in the Reference list are cited in the text, and vice versa
• Permission has been obtained for use of copyrighted material from other sources (including the Web)
• Color figures are clearly marked as being intended for color reproduction on the Web (free of charge) and in print, or to be reproduced in color on the Web (free of charge) and in black-and-white in print • If only color on the Web is required, black-and-white versions of the figures are also supplied for printing purposes For any further information please visit our customer support site at http://support.elsevier.com.

AFTER ACCEPTANCE

Use of the Digital Object Identifier

The Digital Object Identifier (DOI) may be used to cite and link to electronic documents. The DOI consists of a unique alpha-numeric character string which is assigned to a document by the publisher upon the initial electronic publication. The assigned DOI never changes. Therefore, it is an ideal medium for citing a document, particularly 'Articles in press' because they have not yet received their full bibliographic information. Example of a correctly given DOI (in URL format; here an article in the journal *Physics Letters B*): http://dx.doi.org/10.1016/j.physletb.2010.09.059.

When you use a DOI to create links to documents on the web, the DOIs are guaranteed never to change.

Proofs

One set of page proofs (as PDF files) will be sent by e-mail to the corresponding author (if we do not have an e-mail address then paper proofs will be sent by post) or, a link will be provided in the e-mail so that authors can download the files themselves. Elsevier now provides authors with PDF proofs which can be annotated; for this you will need to download Adobe Reader version 7 (or higher) available free from http://get.adobe.com/reader. Instructions on how to annotate PDF files will accompany the proofs (also given online). The exact system requirements are given at the Adobe site: http://www.adobe.com/products/reader/tech-specs.html. If you do not wish to use the PDF annotations function, you may list the corrections (including replies to the Query Form) and return them to Elsevier in an e-mail. Please list your corrections quoting line number. If, for any reason, this is not possible, then mark the corrections and any other comments (including replies to the Query Form) on a printout of your proof and return by fax, or scan the

pages and e-mail, or by post. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor. We will do everything possible to get your article published quickly and accurately – please let us have all your corrections within 48 hours. It is important to ensure that all corrections are sent back to us in onecommunication: please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility. Note that Elsevier may proceed with the publication of your article if no response is received.

Offprints

The corresponding author, at no cost, will be provided with a PDF file of the article via email (the PDF file is a watermarked version of the published article and includes a cover sheet with the journal cover image and a disclaimer outlining the terms and conditions of use). For an extra charge, paper offprints can be ordered via the offprint order form which is sent once the article is accepted for publication. Both corresponding and co-authors may order offprints at any time via Elsevier's WebShop (http://webshop.elsevier.com/myarticleservices/offprints). Authors requiring printed copies of multiple articles may use Elsevier WebShop's 'Create Your Own Book' service to collate multiple articles within a single cover (http://webshop.elsevier.com/myarticleservices/offprints/myarticlesservices/booklets).

AUTHOR INQUIRIES

For inquiries relating to the submission of articles (including electronic submission) please visit this journal's homepage. For detailed instructions on the preparation of electronic artwork, please visit http://www.elsevier.com/artworkinstructions. Contact details for questions arising after acceptance of an article, especially those relating to proofs, will be provided by the publisher. You can track accepted articles at http://www.elsevier.com/trackarticle. You can also check our Author FAQs at http://www.elsevier.com/authorFAQ and/or contact Customer Support via http://support.elsevier.com.

ANEXO B

APROVAÇÃO DO COMITÊ DE ÉTICA

Universidade Federal de Pernambuco
Centro de Ciências Biológicas
Av. Prof. Nelson Chaves, s/n
50670-420 / Recife - PE - Brasil
fones: (55 81) 2126 8840 | 2126 8351
fax: (55 81) 2126 8350
www.ccb.ufpe.br

Recife, 10 de dezembro de 2012.

Ofício nº 532/12

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Profª. Noemia Pereira da Silva Santos**
Laboratório de Imunopatologia Keiso Asami- UFPE
Universidade Federal de Pernambuco
Processo nº 23076.033910/2012-32

Os membros da Comissão de Ética no Uso de Animais do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEUA-UFPE) avaliaram seu projeto de pesquisa intitulado, **"Preparação, caracterização, toxicidade, biodisponibilidade e atividade biológica de nanopartículas furtivas e sítio-específicas para o tratamento do câncer ".**

Concluímos que os procedimentos descritos para a utilização experimental dos animais encontram-se de acordo com as normas sugeridas pelo Colégio Brasileiro para Experimentação Animal e com as normas internacionais estabelecidas pelo National Institute of Health Guide for Care and Use of Laboratory Animals as quais são adotadas como critérios de avaliação e julgamento pela CEUA-UFPE.

Encontra-se de acordo com as normas vigentes no Brasil, especialmente a Lei 11.794 de 08 de outubro de 2008, que trata da questão do uso de animais para fins científicos e didáticos.

Diante do exposto, emitimos **parecer favorável** aos protocolos experimentais a serem realizados.

Origem dos animais: Biotério do Departamento de Nutrição-UFPE; Animais: ratos; Linhagem: Wistar; Sexo: Machos; Número de animais previsto no protocolo: 136 ratos; Peso: 200-300g; Idade: 50 a 60 dias.

Atenciosamente,

Profa. Maria Teresa Jansen
Presidente do CEEA