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DANIEL CRÍSTIAN FERREIRA SOARES

ESTUDOS *IN VITRO* E *IN VIVO* DE LIPOSSOMAS CONTENDO GADOLÍNIO-159 PARA O TRATAMENTO DO CÂNCER

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ESTUDOS *IN VITRO* E *IN VIVO* DE LIPOSSOMAS CONTENDO GADOLÍNIO-159 PARA O TRATAMENTO DO CÂNCER

Tese apresentada como requisito parcial, para obter o grau de doutor em Ciências Farmacêuticas, submetida ao Programa de Pós-Graduação em Ciências Farmacêuticas da Faculdade de Farmácia da Universidade Federal de Minas Gerais.

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Belo Horizonte - MG

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“A maravilhosa disposição e harmonia do universo só pode ter tido origem segundo o plano de um Ser que tudo sabe e tudo pode. Isto fica sendo a minha última e mais elevada descoberta”.

(Isaac Newton)

RESUMO

No Brasil, as estimativas de novos casos de câncer válidas para o ano de 2010 e 2011 mostram que a doença será responsável pela morte de cerca de 500.000 pessoas. Como alternativa terapêutica, a técnica de radioterapia, amplamente empregada no tratamento de diversos tipos de tumores, atua indiscriminando células tumorais de células sadias. Buscando minimizar estes efeitos, sistemas nanoestruturados carreadores de radioisótopos como os lipossomas têm sido estudados com o objetivo de melhorar a especificidade de ação da radiação ionizante, entregando e retendo quantidades adequadas de material radioativo nas células tumorais, conduzindo-as à morte. Neste contexto, no presente trabalho, preparamos lipossomas furtivos pH-sensíveis contendo o complexo metálico radioativo ^{159}Gd-DTPA-BMA (^{159}Gd-SpHL) objetivando estudar *in vitro* e *in vivo* o tratamento do câncer. As vesículas apresentaram taxa de encapsulação de cerca de 20%, diâmetro médio de 100 nm e baixa cinética de liberação do radioisótopo em meios biológicos. A formulação foi caracterizada em termos físico-químicos e morfológicos e os resultados revelaram um baixo índice de polidispersividade e potencial Zeta negativo. Estudamos *in vitro* e *in vivo* sua ação contra células dos modelos tumorais de Ehrlich e RT2 (glioma murino). Os resultados dos estudos *in vitro* revelaram que o complexo radioativo apresentou importante citotoxicidade contra as células de dois dos três modelos estudados e que, estando encapsulado nos lipossomas, a citotoxicidade foi ampliada significativamente. Adicionalmente, investigamos a participação da proteína caspase-3 na morte das células de Ehrlich e RT2. Os resultados sugerem que o principal mecanismo envolvido na ação citotóxica do complexo radioativo está relacionado a apoptose. Os resultados dos estudos *in vivo* revelaram que os lipossomas contendo ^{159}Gd-DTPA-BMA acumularam-se significativamente no tumor sólido de Ehrlich de camundongos Swiss. Objetivando a melhoria dessa captação, confeccionamos lipossomas pH-sensíveis recobertos com folato contendo o mesmo complexo radioativo (^{159}Gd-FTSpHL). Os resultados revelaram um aumento de cerca de três vezes na captação tumoral. Entretanto, observamos que as duas formulações também foram significativamente acumuladas no fígado e no baço. Neste ínterim, estudos hematológicos e bioquímicos

foram conduzidos, os quais revelaram alterações agudas em hepatócitos e transitórias na medula óssea. Para o estudo de atividade antitumoral, camundongos Swiss portadores de tumor sólido de Ehrlich receberam três doses de ^{159}Gd-SpHL e três doses de ^{159}Gd-FTSpHL (cada dose = 236mg/kg). O tratamento inibiu o aumento do volume dos tumores em comparação com os animais tratados com solução de NaCl (0,9% p/v). Houve ainda a inibição do ganho de massa corpórea e um aumento da sobrevida dos animais estudados. Considerando todos os resultados obtidos, os lipossomas pH-sensíveis contendo o complexo radioativo ^{159}Gd-DTPA-BMA podem ser considerados um potencial agente terapêutico do câncer.

Palavras-chave: Gd-DTPA-BMA, gadolínio-159, lipossomas pH-sensíveis, atividade antitumoral *in vitro*, atividade antitumoral *in vivo*

ABSTRACT

In Brazil, estimates of new cancer cases, valid for the years 2010 and 2011 show that the disease will be responsible for the deaths of about 500,000 people. As an alternative therapythe radiotherapy technique, widely used in treating various types of tumors, act indiscriminate tumoral and healthy cells. Seeking to minimize these effects, nanostructured carriers containing radioisotopes, such as liposomes, have been studied with the aim of improving the specificity of action of ionizing radiation, delivering and retaining adequate amounts of radioactive material in tumor cells, leading them to death. In this context, the present study, we prepared liposomes stealth pH-sensitive metal complex containing the radioactive ^{159}Gd-DTPA-BMA (^{159}Gd-SpHL) aiming to study in vitro and in vivo its effects in cancer treatment. The vesicles showed encapsulation rate of about 20%, average diameter of 100 nm and low release kinetics of radioactivity in biological media. The formulation was characterized through physic-chemical and morphological studies and the results revealed a low polydispersity index and negative Zeta potential. We studied in vitro and in vivo its action against the cells of Ehrlich tumor models and RT2 (rat glioma). The results of in vitro studies showed that the complex has significant radioactive cytotoxicity against the cells of two of the three models studied and that, being encapsulated in liposomes, the cytotoxicity was greatly enhanced. Additionally, we investigated the involvement of caspase-3 protein in Ehrlich and RT2 cell death. The results suggest that the main mechanism involved in the cytotoxic action of radioactive complex is related to apoptosis. The results of in vivo studies showed that liposomes containing ^{159}Gd-DTPA-BMA accumulated significantly in Ehrlich solid tumor in mice. Aiming to improve this uptake, we prepared pH-sensitive liposomes coated with folate containing the same radioactive complex (^{159}Gd-FTSpHL). The results revealed an increase of about three times the tumor uptake. However, we observed that the two formulations were also significantly accumulated in the liver and spleen. Therefore, studies were conducted biochemical and hematological, which revealed acute changes in hepatocytes and transient bone marrow. To study the antitumor activity, tumor-bearing Swiss mice solid Ehrlich received three doses of ^{159}Gd-SpHL and three levels of ^{159}Gd-FTSpHL (each dose = 236mg/kg). The treatment inhibited the increase in the

volume of tumors compared with animals treated with NaCl (0.9% w/v). There was also inhibition of body mass gain and an increased survival of animals studied. Considering all results, the pH-sensitive liposomes containing the radioactive complex ^{159}Gd-DTPA-BMA can be considered a potential therapeutic agent for cancer.

Keywords: Gd-DTPA-BMA, gadolinium-159, pH-sensitive liposomes, in vitro antitumor activity, antitumor activity *in vivo*

SUMÁRIO

LISTA DE ABREVIATURAS E SIGLAS

AFM	*Atomic Force Microscopy*
ALT	Alanina-amino-transferase
AMP	Adenosina Mono Fosfato
ANOVA	Análise de Variância
AUC	*Area under curve*
AST	Aspartato-amino-transferase
ASV	*Avian Sarcoma Virus*
CDK's	Quinases Ciclinas dependentes
CDTN	Centro de Desenvolvimento da Tecnologia Nuclear
CHEMS	Hemisuccinato de colesterila
CLAE	Cromatografia Líquida de Alta Eficiência
CE	Coxa Esquerda
CRT-3D	*Conformal Radiotherapy – 3 Dimensional*
DL_{50}	Dose letal que induz a morte de 50% dos animais
DMEM	*Dubelccos's Modified Eagle Medium*
DNA	Ácido desoxirribonucléico
DOPE	Dioleoilfosfatidiletanolamina
DPS	Decaimento por Segundo
DSPE-$MPEG_{2000}$	Diestearoilfosfatidiletanolamina associada a metoxipolietilenoglicol 2000
EC	Eficiência de encapsulação
EDTA	Ácido dietilenotriaminopentaacético
FA	Fosfatase Alcalina
FR	Receptor de Folato
FTIR	*Fourier Transformer Infrared Spectroscopy*
Gd-DTPA-BMA	Complexo Gadolínio-ácido dietilenotriaminopentaacético-bismetilamida
Gd-DTPA-BMA-L-Lisina	Complexo gadolínio-ácido dietilenotriaminopentaacético –bismetilamida-associada ao aminoácido L-lisina

Gd-FTSpHL	Lipossoma pH-sensível recoberto com folato contendo o complexo Gd-DTPA-BMA
^{159}Gd-FTSpHL	Lipossoma pH-sensível recoberto com folato contendo o complexo ^{159}Gd-DTPA-BMA
Gd-SpHL	Lipossoma pH-sensível contendo o complexo Gd-DTPA-BMA
^{159}Gd-SpHL	Lipossoma pH-sensível recoberto com folato contendo o complexo ^{159}Gd-DTPA-BMA radioativo
GGT	Gama-glutamil-transferase
Gy	Gray
HEGP	Colimador de aplicações gerais de elevada energia
HEPES	4- (2-hidroxietil) - ácido 1-piperazina-etano-sulfônico
HPLC	Cromatografia Líquida de Alta Eficiência
Hz	Hertz
IC_{50}	Concentração Inibitória 50% de células
ICP-AES	Espectroscopia de emissão atômica indutivamente acoplada a plasma
IMRT	Intensity Modulated Radiotherapy
INCA	Instituto Nacional do Câncer
IP	Intraperitoneal
IV	Intravenosa
keV	Quilo-elétron-volt
Kp	Coeficiente de Partição
LUV	Lipossoma unilamelar grande
M	Molar
MBq	Mega-Bequerel
MET	Microscopia Eletrônica de Transmitância
MeV	Milhoes-de-elétrons-volt
MLV	Lipossoma multilamelar
MTT	Sal de Metil-tetrazólio
P/V	Razão Peso Volume
PCS	Espectroscopia de Correlação de Fótons
pH	Potencial Hidrogeniônico
PL	Percentual de Liberação

PLL	Poli-L-Lisina
*p*NA	Para-nitro-anilina
Ri’s	Regiões de Interesse
SD	Desvio Padrão
SFB	Soro Fetal Bovino
SUV	Lipossoma unilamelar pequeno
TC	Tomografia Computadorizada
TF’s	Fatores de Transcrição de DNA
UV	Ultravioleta
Vis	Visível

INTRODUÇÃO GERAL

No Brasil, as estimativas de novos casos de cânceresválidas para o ano de 2010 e 2011 mostram que esta doença será responsável pela morte de cerca de 489.270 pessoas (INCA, 2010). Provavelmente, os tipos mais incidentes, conforme os últimos anos serão os cânceres de próstata e de pulmão, no sexo masculino, e os cânceres de mama e de colo do útero, no sexo feminino.

Durante as últimas décadas, as principais abordagens terapêuticas empregadas no tratamento de diversos tipos de câncer foram a cirurgia, a quimioterapia e a radioterapia. A cirurgia,antes do advento da quimioterapia e da radioterapia, era a única possibilidade de tratamento do câncer. Naquela época, acreditava-se que uma neoplasia maligna era um problema exclusivamente local e, portanto, a cura poderia ser alcançada a partir de procedimentos cirúrgicos que datam de 1600 anosantes de Cristo (Merck Index, 2009). Atualmente, a cirurgia, objetiva além da cura, o aumento da sobrevida e da qualidade de vida dos pacientes portadores de tumores sólidos.A quimioterapia, em suas várias formas, destina-se principalmente aatacar pequenos agregados de células tumorais remanescentes de uma intervenção cirúrgica ouque estão sendo disseminadas através do corpo por um processo conhecido como metástase, além de atuar também contra o tumor primário.A técnica de radioterapia, empregada clinicamente em grande escala em todo o mundo, desde a década de 70, tem como principais alvos os tumores sólidos não metastizados.Infelizmente, devido às suas próprias características, esta técnica atua de modo indiscriminado nos diferentes tecidos, destruindo tanto células tumorais quantocélulas sadias. Neste contexo, o emprego da radiação ionizante, em procedimentos terapêuticos, deve ser direcionado principalmente às células tumorais, minimizando as doses de radiação em tecidos sadios. Buscando estes objetivos, a radioterapia, através de diversas técnicas de colimação, blindagem e escalonamento de doses, busca concentrar elevadas doses de radiação exclusivamente nos tecidos tumorais. Entretanto, mesmo direcionando e/ou escalonando as doses e empregando-se modernas técnicas como a 3D-CRT (3-Dimensional Conformal Radiotherapy) e a IMRT (Intensity Modulated Radiation Therapy), observa-se em alguns casos uma modesta redução dos efeitos adversos sob tecidos sadios (Chen *et al*. 2004; Bos *et al.*, 2005).

Buscando-se minimizar os efeitos adversos da radioterapia, principalmente os efeitos imediatos, vários estudos vêm sendo conduzidos com o objetivo de desenvolver novas formas seletivas de aplicação da radiação ionizante às células tumorais, protegendo ao máximo as células sadias. Dentre estes avanços, observam-se nos últimos anos, intensas pesquisas focadas no desenvolvimento de formulações farmacêuticas nanoestruturadas, como lipossomas e nanopartículas capazes de encapsular radioisótopos emissores de radiação particulada visando o tratamento de diversos tipos de cânceres. Estes sistemas têm como objetivo principal a entrega e a retenção de radioisótopos com elevada atividade específica àscélulas tumorais, depositando nelas, elevadas doses de radiação, conduzindo-as à morte. Simultaneamente, estes sistemas objetivam a minimização dos efeitos adversos da radiação em tecidos sadios adjacentes (Oliveira *et al.,* 2006; Kwekkeboom *et al.,* 2003; Zelenetz *et al.,* 2003).

Alguns trabalhos publicados na literatura demonstram a potencialidade terapêutica de alguns radioisótopos contra o câncer, baseando-se exclusivamente em características físicas como o tipo de decaimento, a atividade específica e o tempo de meia-vida física. Nestes trabalhos, o radioisótopo Gadolínio-159 é citado como um promissor radioisótopo por apresentar um decaimento beta negativo com uma energia média de 1001 keV e elevada atividade específica (Soares *et al.,* 2010). Entretanto, nenhum estudo investigou a atividade antitumoral do radioisótopo e tampouco sua associação a algum sistema nanoestruturado com objetivos terapêuticos. Por estes motivos, o objetivo da presente tese é conduzir estudos *in vitro* e *in vivo* do radioisótopo ^{159}Gd encapsulado em lipossomas pH-sensíveis, visando avaliar seu potencial emprego no tratamento do câncer. A tese está dividida em duas partes. Na primeira parte, conduzimos uma revisão da literatura onde está apresentada uma fundamentação teórica do emprego da radiação ionizante e dos lipossomas no tratamento do câncer. Na segunda parte da tese, apresentada na forma de 5 capítulos, descrevemos o trabalho experimental.

O capítulo 1 descreve a preparação e quantificação da atividade específica do radioisótopo ^{159}Gd presente no complexo metálico Gd-DTPA-BMA comercializado como Omniscan®. Investigamos se a integridade estrutural do complexo foi mantida após o processo de irradiação e também avaliamos *in vitro* a atividade antitumoral do complexo radioativo frente ao modelo de células tumorais de Ehrlich.

O capítulo 2 descreve a preparação, caracterização físico-química, perfil de liberação em meios biológicos e atividade antitumoral *in vitro* de lipossomas pH-sensíveis contendo o complexo radiativo ^{159}Gd-DTPA-BMA contra os modelos de células tumorais RT2 (Glioma murino).

O capítulo 3 apresenta os estudos de biodistribuição e imagens cintilográficas do complexo radioativo ^{159}Gd-DTPA-BMA livre e encapsulado em lipossomas em camundongos Swiss contendo o tumor sólido de Ehrlich previamente implantado e desenvolvido.

O capítulo 4 mostra os estudos da atividade antitumoral e da toxicidade*in vivo* de lipossomas contendo ^{159}Gd-DTPA-BMA em camundongos Swiss contendo também o tumor sólido de Ehrlich previamente implantado e desenvolvido.

Por fim, no capítulo 5,investigamos*in vitro* o processo deapoptose mediadopela proteína caspase-3 e os efeitos anticancerígenos p53 dependente do complexo ^{159}Gd-DTPA-BMA sob células de glioblastoma murino ehumano (RT2 e T98, respectivamente).

PARTE 1 – REVISÃODA LITERATURA

1 – Câncer

O câncer não é apenas uma doença, mas um termo genérico, utilizado para descrever um grupo de mais de 200 enfermidades caracterizadas pelo comportamento de células que perdem a capacidade de limitar e controlar o seu próprio crescimento (Corner, 2002; Yarbro, 2000). O resultado desse processo desordenado de crescimento celular é a produção do que se conhece como tumor. Tumores podem ser classificados como benignos e malignos. Tumores benignos são aqueles em que as células crescem lentamente e são semelhantes ao tecido normal em estrutura e funções. Tumores malignos são caracterizados por células que crescem descontroladamente não respeitando a estrutura e funções do órgão em que estão crescendo. Além disso, são capazes de evadirem da ação de células do sistema imune, espalhando-se para diversas regiões do organismo, por um processo denominado metástase, responsável pela morte de cerca de 70% de seus portadores (Herzberg & Ferrari, 2005; Fynan & Reiss, 1993). Neste contexto, a detecção precoce da maioria dos cânceres promove um aumento considerável nas probabilidades de cura, sendo, portanto, objetivo dos profissionais que atuam em oncologia (Molina *et al.,* 2003).

Quando o diagnóstico do câncer é positivo, iniciam-se alguns procedimentos que permitem avaliar o grau de comprometimento do tecido ou do órgão acometido e, principalmente, da presença de metástases. Este conjunto de procedimentos, denominado “estadiamento”, consiste em exames anatomopatológicos, análise de imagens obtidas a partir de Radiografias, Ultrassonografias, Tomografia Computadorizada, Ressonância Magnética Nuclear, Cintilografia e recentemente a avançada técnica de Tomografia por Emissão de Pósitrons (Gary & Djulbegovic, 2005).

2 – Aspectosepidemiológicos do câncer no Brasil

Segundo o INCA (Instituto Nacional do Câncer), para o ano 2010, o número de novos casos de câncer chegará a 489.270, destes 236.240 serão para o sexo masculino e 253.030 para sexo feminino. A Tabela 1 mostra a estimativa, em números, dos principais novos casos de câncer parao ano de 2010, válido também para o ano de 2011.Como ocorrido nos anos anteriores, os cânceres de próstata e mama continuam sendo os líderes de novos casos para o sexo masculino e feminino, respectivamente.

Tabelas 1 – Estimativas para o ano 2010 de número de casos novos por câncer, em homens e mulheres, segundo localização primária (INCA, 2010).

Localização Primária da Neoplasia Maligna	Estimativa de Casos Novos		
	Masculino	**Feminino**	**Total**
Próstata	52.350	-	52.350
Mama Feminina	-	49.240	49.240
Traquéia, Brônquio e Pulmão	17.800	9.830	27.630
Cólon e Reto	13.310	14.800	28.110
Estômago	13.820	7.680	21.500
Colo do Útero	-	18.430	18.430
Cavidade Oral	10.330	3.790	14.120
Esôfago	7.890	2.740	10.630
Leucemias	5.240	4.340	9.580
Pele Melanoma	2.960	2.970	5.930
Outras Localizações	59.130	78.770	137.900
Subtotal	***182.830***	***192.590***	***375.420***
Pele não melanoma	53.410	60.440	113.850
Todas as Neoplasias	**236.240**	**253.030**	**489.270**

**Números arredondados para 10 ou múltiplos de 10.*

3 – Etiologiae principais fatores predisponentes do câncer

Os mecanismos etiológicos do câncer ainda não estão completamente elucidados e os estudos de suas características epidemiológicas têm permitido identificar vários fatores de risco que participam do seu desenvolvimento. Trabalhos demonstram que as causas do câncer são variadas, podendo ser de origem interna ou externa ao organismo, estando, de certa forma, inter-relacionadas (Thomas & Mack, 2004; Chisari, 2000; Parkin, 1998).As causas internas estão, na maior parte dos casos, ligadas à capacidade do organismo em se adaptar e reagir a agressões externas. As causas externas relacionam-se ao meio ambiente e aos hábitos individuais em relações sócio-culturais (Kuper *et al.*, 2002). Esses fatores podem se interagir, aumentando a probabilidade de transformações malignas nas células normais. Segundo várias pesquisas, os principais fatores causadores de mutações celulares são atribuídos à dieta, exposições às substâncias químicas carcinógenas, radiação ionizante, infecções de origem viral ou bacteriana, disfunções hormonais, disfunções do sistema imunológico, hereditariedade e outras causas ainda não bem elucidadas (Gillis, 1978; Cartmel & Reid, 2000; Willett, 1989).

4 – Aspectosfisiopatológicos do câncer

Existem vários padrões de crescimento celular, pelos quais os diferentes tecidos do corpo passam normalmente, durante o ciclo de vida de um dado indivíduo (Smeltzer & Bare, 2005). De acordo com Otto (2002), os quatro padrões não-neoplásicos mais comuns são:

Hipertrofia: aumento no tamanho da célula, devido a vários fatores dentre os quais se destaca o aumento de carga de trabalho, estimulação hormonal ou compensação relacionada com a perda ou redução funcional de outros tecidos.

Hiperplasia: aumento da quantidade de células de determinado tipo de tecido, resultando em aumento de massa. É considerado um processo proliferativo normal

comum, em períodos de desenvolvimento de um dado indivíduo, e durante a regeneração da pele e da medula óssea. Torna-se anormal quando o volume de células produzido ultrapassa a necessidade fisiológica.

Displasia: alteração reversível no tamanho ou formato de células normais, em decorrência de vários e diferentes fatores externos como agentes químicos, radiação, inflamação, irritação crônica.

Metaplasia: substituição de um tipo de célula adulta por outra, em geral, diferente daquela encontrada no tecido envolvido. Pode ser reversível ou evoluir para a displasia. A metaplasia também pode ser induzida por um processo inflamatório, deficiência de algum tipo de vitamina e exposições químicas.

Considerando alterações anormais, pode-se observar ainda, a existência de dois padrões de crescimentos:

Anaplasia: uma típica alteração celular reversível; em que se observam baixíssimos graus de diferenciação de células displásicas. As células têm formatos irregulares ou desorganizados no que se refere a crescimento e disposição.

Neoplasia: Discorre sobre uma massa tecidual anormal que se expande além dos limites do tecido e não consegue cumprir a função normal das células daquele tecido.

A classificação das neoplasias está baseada em seu comportamento biológico e suas características podem ser sumarizadas na Tabela 2.

Tabela 2 – Principais características de diferentes tipos de neoplasmas

Neoplasmas	
Malignos	**Benignos**
Não encapsulados	Encapsulados
Geralmente não invasivos	Invasivos
Poucos diferenciados	Altamente diferenciados
Crescimento acelerado	Crescimento lento
Anaplasias de vários graus	Baixa ou nenhuma anaplasia
Podem metastizar	Não podem metastizar

A maioria das diferenças entre neoplasmas benignos e malignos são relativas. A diferença crítica está relacionada à capacidade de metastização. Nenhum tumor benigno tem a capacidade de irradiar suas células, através de vasos linfáticos ou sanguíneos, de modo a produzir um clone, num tecido distante.

5 – Biologiacelular e molecular do câncer

Estudos realizados na área da biologia molecular e genética propiciaram o desenvolvimento do modelo de ciclo celular que permite uma melhor compreensão das etapas e mecanismos envolvidos no processo de divisão celular(Figura1).Durante as diferentes fases do ciclo celular, a maquinaria interna da célula desempenha diferentes funções, que permite à proliferação celular alcançar sucesso e assim, uma nova célula ser criada aos moldes da célula que a originou.

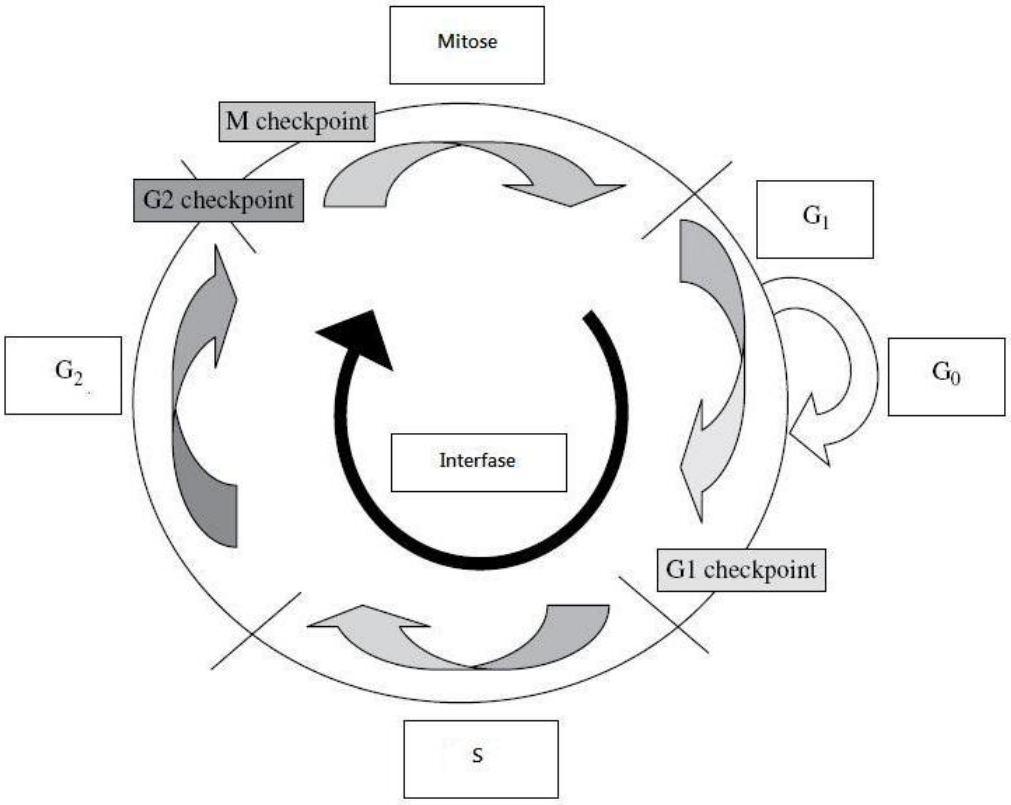

Figura 1 – Representação esquemática do ciclo celular e suas respectivas etapas. Adaptado de Gabriel, 2004.

O ciclo celular é dividido, basicamente, em cinco fases consecutivas denominadas G_0, G_1, S, G_2 e M (Nurse, 1998).Durante a fase G_0, a célula está quiescente e somente sairá desta condição quando for induzida a dividir-se a partir de estimulação externa, mediada pela interação de fatores de crescimento com receptores localizados na superfície celular. Externamente, fatores de crescimento iniciam a passagem de G_0 a G_1 e assim, uma cascata de sinais estimula a produção de RNA e proteínas que culminam na síntese de um novo DNA. Fatores de crescimento se ligam aos receptores de superfície celular específico e conduzem o sinal externo de crescimento para o interior das células.

A fase G_1 é caracterizada pelo início do processo de divisão celular. Nesta fase ocorre grande expressão de genes e produção de proteínas que participarão ativamente da síntese de DNA.Durante esta fase, complexos mecanismos bioquímicos regulam o processo de crescimento celular onde vários nutrientes como açúcares, sais,

aminoácidos essenciais e vitaminas são necessários (Baserga, 1985).Estudos *in vitro,* comparando padrões de crescimento de células cancerosas e de células normais, revelaram que a regulação da divisão celular é crucial para o desenvolvimento normal de organismos multicelulares e que esta regulação ocorre principalmente na fase G_1 do ciclo celular, antes do processo de síntese de DNA (Pardee, 1989).

Várias proteínas localizadas no citoplasma desempenham um importante papelneste processo, dentre as quais, podemos destacar as ciclinas e as quinases ciclinas dependentes (CDK's). Existem 15 subtipos de ciclinas (A-T) e treze tipos de quinases ciclinas dependentes (CDK1-CDK13). As CDK's de 1 a 8 estão envolvidas no controle do ciclo celular, enquanto que as CDK's 9 e 10 são importantes reguladores transcricionais. As funções das CDK's 11-13 ainda não foram bem elucidadas. A partir desses conhecimentos, algunsinibidores farmacológicos de CDK's foram desenvolvidos e são atualmente utilizados com fins terapêuticos, atuando sob as funções de transcrição, proliferação celular e apoptose em células tumorais (Ortega *et al.*, 2002).

A formação, a ativação e a separação dos complexos ciclinas-CDK's são eventos fundamentais na coordenação do ciclo celular. Se um problema com o ciclo celular é detectado a ativação dos complexos ciclinas-CDK's não ocorre. Caso contrário, se nenhum problema é identificado, a formação dos complexos ciclinas-CDK's será obtida com sucesso, permitindo assim, a ativação de fatores de transcrição (Murray, 2004). Os fatores de transcrição ou TF's são proteínas que se ligam ao DNA e que permitem que haja uma ligação entre a enzimaRNA-polimerase e o DNA e, contudo, uma perfeita transcrição. O envolvimento de diversas moléculas, bem como proteínas e fatores de crescimento sob o ciclo celular está sumarizado na Figura 2 (Murray & Hunt, 1993).

A fase S é caracterizada pela replicação de DNA e obtenção de duas fitas completas, as quais serão divididas entre as duas novas células filhas. Através da fase G_2, a célula novamente cresce e sintetiza proteínas, que serão distribuídas entre as duas futuras células filhas. Terminada esta fase, a célula finalmente entra na fase mitótica ou fase M. A fase mitótica é dividida em duas fases distintas, denominadas de cariocinese e

citocinese. A cariocinese está subdividida em quatro etapas distintas: prófase, metáfase, anáfase e telófase. Já a citocinese consiste no estrangulamento do citoplasma que ocorre ao final da mitose.Forma-se, no citoplasma, um anel de filamentos protéicos que se contraem, puxando a membrana plasmática para o lado interno da célula, de modo que as duas células se separam (Fay, 2005). Após, as duas novas células, podem novamente começar outro ciclo celular, entrando na fase G1 ou tornarem-se quiescentes, entrando em G_0 (Rashidian *et al.*, 2007; Pietenpol & Stewart, 2002).

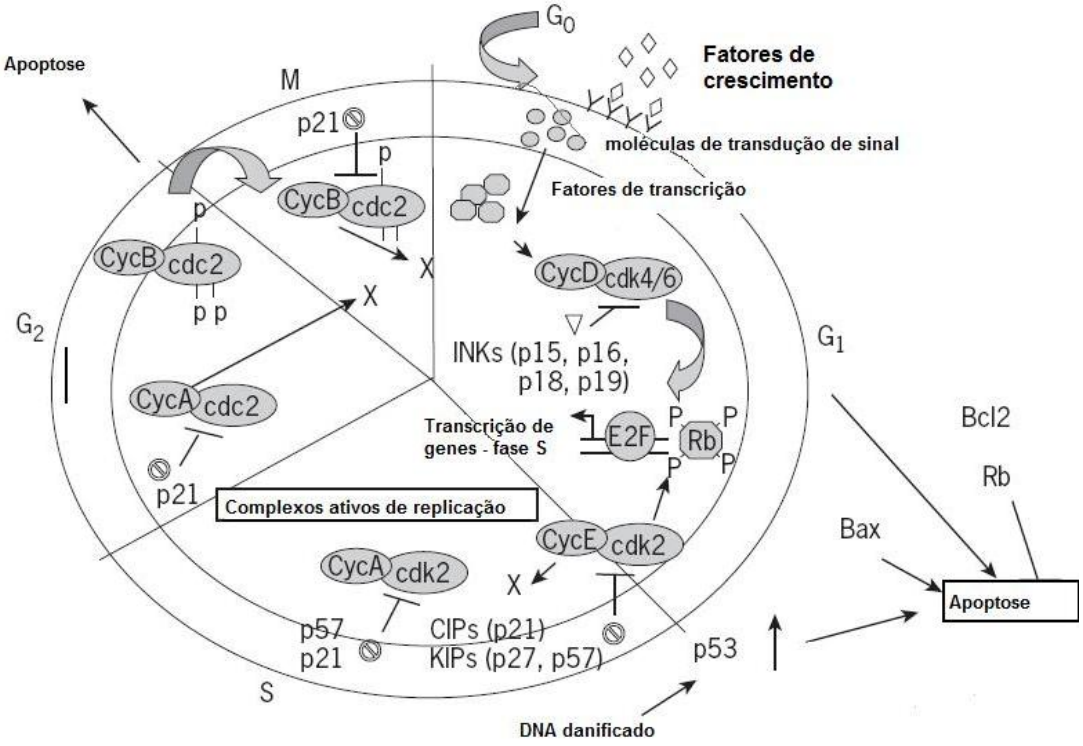

Figura 2 – Algumas de muitas moléculas que promovem sinais de regulação do crescimento celular. Adaptado de Pardee, 1989.

Um importante processo de auto verificação denominado “Checkpoint” está presente em todas as fases do ciclo celular. Os *checkpoints*, nome dado por Hartwell & Kastan em 1994, são pontos de verificação do ciclo celular que visam assegurar que uma fase do ciclo celular não se inicie antes do término da fase imediatamente anterior e, principalmente, conduz a célula a apoptose, caso algo esteja errado (Lukas *et al.*, 2004).

O primeiro *checkpoint*, geralmente denominado de ponto de restrição ou simplesmente ponto R, está localizado no final da fase G_1 do ciclo celular. Está localizado imediatamente antes do início da fase S, tendo como função chave decidir se a célula deve dividir-se, atrasar o processo de divisão ou entrar em estado quiescente na fase G_0. O segundo *checkpoint* está localizado no final da fase G_2, imediatamente antes do início da fase M. Durante este ponto de checagem, a célula verifica se o número de fatores promotores de mitoses é suficiente. O terceiro *checkpoint* está localizado na fase M.Neste ponto de controle, o alinhamento dos cromossomos metafásicos é devidamente processado e se todos os cromossomos estão ligados ao fuso mitótico, as células filhas serão idênticas (Lukas *et al.*, 2004).Os *checkpoints* constituem, portanto, etapas importantes do mecanismo de controle da progressão de células normais a células cancerosas. Numa dada célula cancerosa, a seqüência geral de organização e duração do ciclo celular é mantida, mas os mecanismos que atuam no processo de *checkpoints* são ineficientes (Pardee, 1989; Hartwell & Kastan, 1994). O principal defeito de regulação do crescimento celular pode ser observado no *checkpoint* G2/M. Em geral, este ponto de restrição é ignorado pelas células neoplásicas malignas e o resultado é um crescimento exagerado e descontrolado (Lukas *et al.*, 2004).

Células expostas a agentes danosos ao DNA (substâncias químicas ou radiação ionizante) respondem, com atraso no ciclo celular que busca promover tempo para o reparo do DNA. Uma falha no processo de reparação do DNA faz com que a célula siga dois caminhos distintos. Em um deles, a célula perde a capacidade de restringir seu crescimento, através de falhas nos mecanismos de *checkpoints*. Como resultado final observa-se a formação de células viáveis alteradas. No outro caminho, observa-se a

adequada ação dos mecanismos de restrição celular, e a célula é conduzida a apoptose (Pietenpol & Stewart, 2002).

Estudos indicam que uma proteína denominada p53 possui um papel central na regulação do checkpoint da fase G_1 em resposta ao dano no DNA (Millau *et al.*, 2008). Resultados experimentais sugerem que o checkpoint em G_1 mediado por p53 deve envolver a inativação de genes efetores.Assim, a proteína p53 desempenha um importante papel relacionado à preservação da integridade do código genético em cada célula, atuando de forma a manter coesa a mesma seqüência de nucleotídeos, ao longo de toda a molécula de DNA igualmente presente em cada célula do corpo (Finlay, *et al.*, 1989; Benjamin & Ananthaswamy, 2007). A proteína p53 faz uma verificação quanto à eventual ocorrência de mutação na seqüência do código genético em decorrência de um erro de replicação do DNA. Caso seja verificada a existência de uma mutação, é função da proteína p53, através de uma cascata de reações, impedir que esta célula entre na fase M do ciclo celular e complete o processo de divisão (Adimoolam & Ford, 2003). Para alcançar isto, a proteína p53 pode conduzir a maquinaria interna da célula por dois caminhos distintos que são a correção da mutação através da ativação de proteínas de reparo ou a indução da morte celular, através de mecanismos apoptóticos (Pluquet & Hainaut, 2005; Tokino & Nakamura, 2000).

Por exercer esta função, a proteína p53 é considerada a guardiã do genoma e é um importante elemento na prevenção do desenvolvimento de tumores, sendo seu gene codificador TP53, classificado como gene supressor tumoral. TP53 é um gene regulador-chave do ciclo celular, que ao sofrer mutações, leva ao desenvolvimento de neoplasias (Hollstein, 1991; Guimaraes & Hainaut, 2002; Hanahan&Weinberg, 2000).

A relação entre a proteína p53 e a carcinogênese têm sido amplamente comprovada através do elevado índice de mutações de seu gene, em tumores malignos de diferentes tecidos do organismo, conforme apresentado na Figura3 (Khan *et al.*, 2000; Baker *et al.*, 1990.

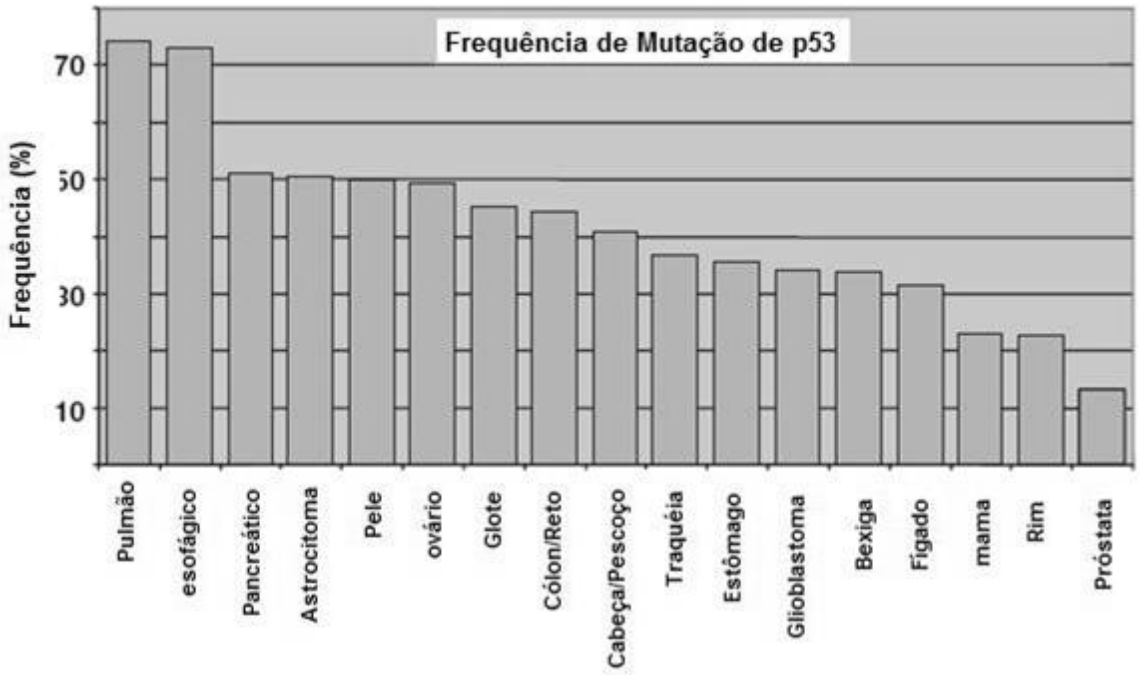

Figura 3 – Freqüência de mutação da proteína p53 em diferentes tipos de cânceres. Adptado de Khan *et al.*, 2000.

Também foram identificados genes homólogos ao TP53, denominados TP73 e TP63, provavelmente descendentes de um gene ancestral comum (De Laurenzi & Melino, 2000). O gene TP73 participa da ativação de vários genes responsivos ao TP53 e, ao serem expressos, inibem a ação de TP53. Sua ação sob tumorigênese humana ainda é motivo de inúmeras controvérsias, já que raramente encontra-se mutado em neoplasias (Kaghad, 1997). O gene TP63 está presente em células de vários órgãos epiteliais, como a pele e a próstata e desempenha importante papel na manutenção de uma população de células-tronco em diversos tecidos epiteliais (Mills *et al.*, 1999; Yang *et al.* 1999). Ele não é considerado supressor tumoral, entretanto, é um importante marcador de indiferenciação celular e seu papel no desenvolvimento de neoplasias em humanos ainda requer maior investigação (Ribeiro-Silva & Zucoloto, 2003).

Observou-se, em procedimentos experimentais, que células deficientes de TP53 não eram capazes de recuperar-se de danos celulares, causados por radiação ionizante e outros agentes tumoricidas e que houve uma multiplicação destas células mutadas, devido à perda da competência apoptótica (Timothy & Keyomarsi, 2004; Guimaraes & Hainaut, 2002).Em outros procedimentos experimentais, usando células tumorais não deficientes de TP53, os resultados mostraram que a radiação ionizante é capaz de promover a ativação de p53, elevando sua concentração no núcleo celular e conduzindo as células, a apoptose. Estas observações contribuíram enormemente para a elucidação do mecanismo de ação da radiação ionizante contra células tumorais (Zambetti, 2005; Choi *et al.*, 2004).

6 – Terapiasdo câncer utilizando radiação ionizante

6.1 – Efeitos da radiação ionizante sob células

Estudos da ação biológica da radiação ionizante em células e tecidos tem sido alvo de diversos estudos e são interessantes para aplicações terapêuticas na medicina e na proteção radiológica para trabalhadores (Cox, 1999).Quando está em discussão a ação da radiação ionizante sobre células vivas, um ponto importante a ser considerado é a compreensão dos mecanismos que conduzem, à morte, células tumorais. Dentre os principais mecanismos, podemos destacar a apoptose e a catástrofe mitótica (Chan *et al.*, 1999; Chu *et al.*, 2004; Brown & Attardi, 2005). Outra variável a ser considerada nesta discussão é a capacidade de recuperação das células quanto aos danos sofridos (Bernier *et al.*, 2004). Assim, em muitos casos, abre-se, então, a possibilidade de uma célula mutada tornar-se viável ou não. Em caso negativo, geralmente observa-se a ativação de enzimas (caspases, por exemplo) que ativam uma cascata de eventos em que a célula é conduzida à apoptose. Em caso positivo, a célula mutada viável pode multiplicar-se de forma descontrolada e formar uma massa tumoral.

Uma característica típica celularé sua capacidade de crescer e se duplicar em células filhas idênticas, através do processo de divisão celular que requer a exata duplicação

do DNA e a precisa distribuição de genes a cada uma das duas cópias. Resultados experimentais, utilizando diferentes vírus, bactérias e células de mamíferos têm demonstrado que a severidade do dano celular depende da localização da deposição da radiação ionizante. Observa-se que a deposição de radiação ionizante sobre o núcleo das células tem um efeito danoso de aproximadamente 100 vezes maior que os observados em células que foram irradiadas somente no citoplasma.Estes resultados sugerem que o DNA realiza um importante papel na resposta de uma célula a exposição à radiação ionizante, cuja ação sobre o núcleo das células conduz a eventos de ionização no DNA e, subseqüentemente, à quebra de ligações químicas e intermoleculares da fita simples ou dupla. Estes eventos são classificados como sendo “efeitos diretos”. Por outro lado, a radiação ionizante produz radicais livres altamente instáveis como grupamentos hidroxilas e peróxidos, através da radiólise das moléculas de água que estão ao redor do DNA.Estes radicais têm a capacidade de migrar a distâncias nanométricas e danificar o DNA. A estes efeitos denominam-se efeitos indiretos da radiação ionizante (Grubbé, 1933).A Figura 4 sumariza os principais tipos de danos ao DNA induzidos pela radiação ionizante.

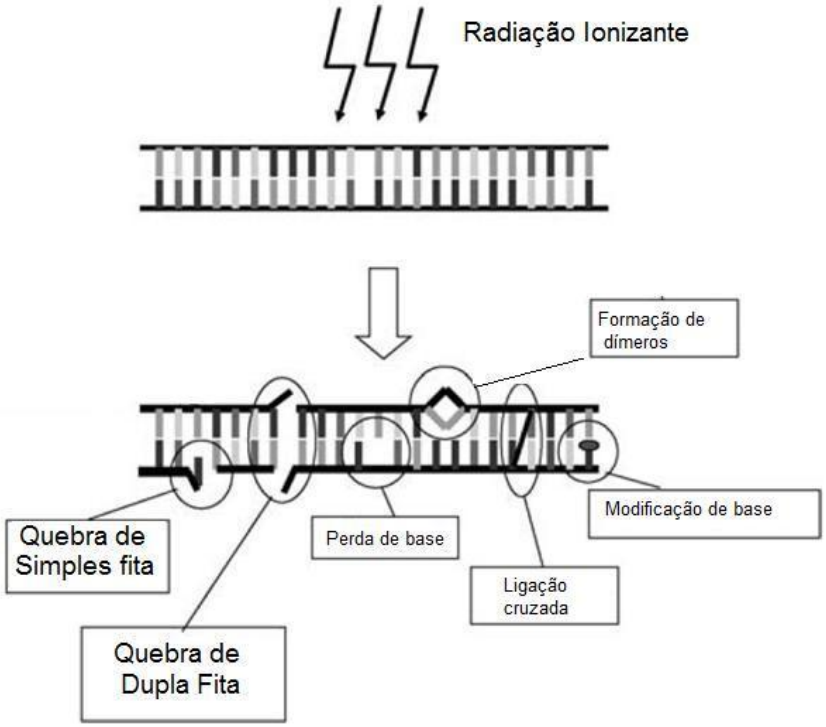

Figura 4- Principais tipos de danos ao DNA induzidos pela radiação ionizante. Adaptado de Grubbé, 1933.

Células de organismos desenvolvidos, como as dos mamíferos, são capazes de reconhecer e reparar determinadas alterações em seus DNA (Khanna & Jackson, 2001). As eficiências destes processos de reparo dependem da complexidade do dano induzido. A maquinaria interna da célula está inteiramente habilitada a corrigir tais danos em pequena escala. Por exemplo, uma fita simples de DNA quebrada pode ser facilmente reparada, uma vez que este evento ocorre naturalmente durante o ciclo normal de replicação. Porém, quando o dano é grande, como várias quebras de duplas fitas de DNA, este reparo pode não ser bem sucedido (Prise, 1994; Olive, 1998).

A caracterização dos mecanismos que participam da morte celular, causada pela exposição à radiação ionizante, é uma missão difícil e complexa.Os modelos atuais baseiam-se em organismos simples como bactérias e outros microrganismos. Já os

estudos com organismos mais desenvolvidos, como os dos mamíferos, devem ser conduzidos com um maior rigor para uma melhor compreensão dos danos causados. Sabe-se, no entanto, que diferentes tipos de células apresentam diferentes níveis de sensibilidade à radiação ionizante (Sparrow, 1965). Neste ínterim, em 1904, Bergonié, M. e Tribondéau, L. observaram os efeitos da radiação ionizante em ratos e formularam um importante conceito a respeito da radiossensibilidade celular que diz:

> *"A radiossensibilidade celular é diretamente proporcional ao seu grau de reprodutibilidade e inversamente proporcional ao seu grau de especialização."*

Assim, células da medula óssea, gônadas e intestino são mais radiossensíveis que as células do intestino, rins e cérebro por possuírem um grau de reprodutibilidade mais elevado. Alguns trabalhos publicados relacionam os níveis de AMP (Adenosina monofosfato) cíclico à radiossensibilidade celular. Células que se dividem rapidamente possuem baixos níveis de AMP cíclico. Assim, O AMP cíclico pode ser a justificativa da lei de Bergonié e Tribondéau, em termos moleculares. Goldfeder (1965) propôs que, em certos tipos de células, as mitocôndrias podem realizar um importante papel na determinação da radiossensibilidade celular. Foi demonstrado que células que possuem um elevado número de mitocôndrias são mais resistentes à radiação ionizante. Células tumorais *in vivo*, especialmente aquelas oriundas de recrescimento de tumores tratados por radioterapia e os linfócitos periféricos, por razões ainda desconhecidas, apresentam-se como uma das exceções a lei de Bergonié e Tribondéau (Prasad, 1995).

Estudos têm demonstrado que as células eucariontes apresentam relativa sensibilidade à radiação ionizante, dependendo da fase do ciclo celular em que se encontram. Na próxima Figura, curvas de sobrevivência celular são comparadas para células que se encontram em diferentes fases do seu ciclo. A fase mitótica se apresenta como a de maior radiossensibilidade enquanto a fase S tardia, a radiossensibilidade é menor. As fases G_1, S e G_2 apresentam radiossensibilidade intermediária (Figura 5) (Okayasu *et al.*, 1998).

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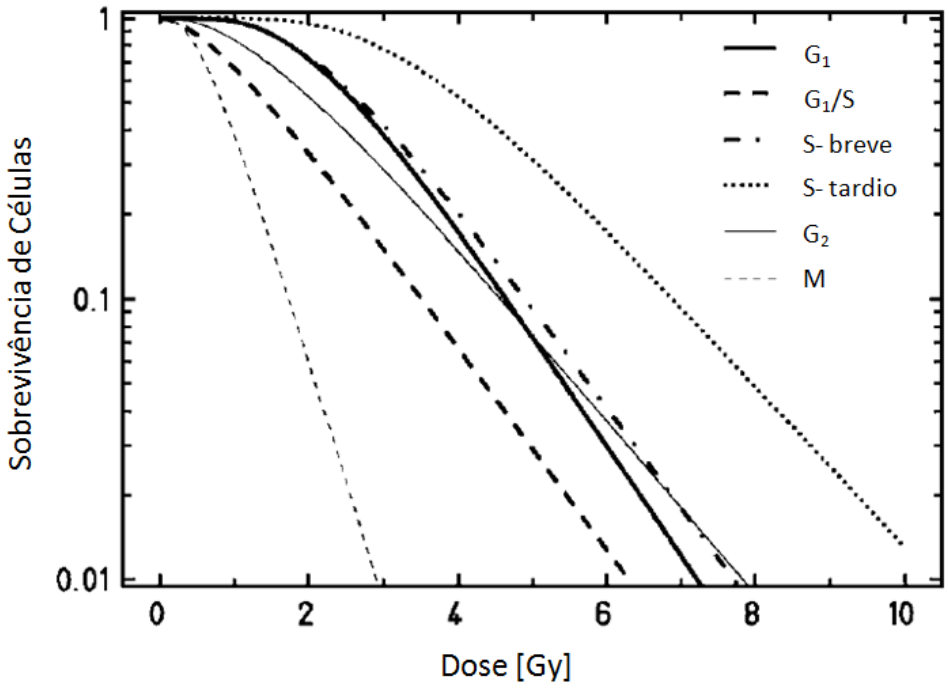

Figura 5 – Dependência da radiossensibilidade de uma célula em função da dose absorvida. Adaptado de Okayasu *et al.*, 1998.

A distribuição de doses de radiação é um importante parâmetro a ser considerado quando se estudam os efeitos biológicos sobre células (Kiefer, 1975). Dose elevadas e agudas, em geral, não permitem que reparos profundos sejam realizados e, assim, a morte celular é certa. Dose elevadas, porém fracionadas, permitem que reparos sejam feitos em células sadias. Este tipo de recuperação é muito explorado em procedimentos de radioterapia, em que a dose total é distribuída em trinta ou mais frações, durante várias semanas de tratamento. A vantagem dessa abordagem é a de permitir que haja a reparação de tecidos normais que estão próximos ao tumor em tratamento, já que a maioria dos tecidos tumorais mostra uma reduzida capacidade de reparação.

Alguns fatores podem potencializar ou minimizar os efeitos danosos da radiação ionizante sob células. Um dos mais gerais e bem conhecidos agentes modificadores da ação da radiação sobre células é a concentração de oxigênio molecular nestas (Kiefer, 1975). A habilidade em potencializar a ação da radiação ionizante é denominada “efeito

oxigênio". As bases físicas e biológicas envolvidas, bem como os mecanismos que medeiam a ação potencializadora do oxigênio ainda permanecem obscuras. Sabe-se, no entanto, que a presença do oxigênio molecular permite a formação de radicais livres do tipo hidroperóxidos (HO_2) e peróxidos (RO_2) que atuam de modo indireto, sobre o DNA, promovendo quebra de dupla e simples fita, dentre outras alterações (Figura6). Em experimentos *in vitro*, na presença de oxigênio em concentrações acima de 8,5 µM, observa-se respostas potencializadoras da ação da radiação ionizante.

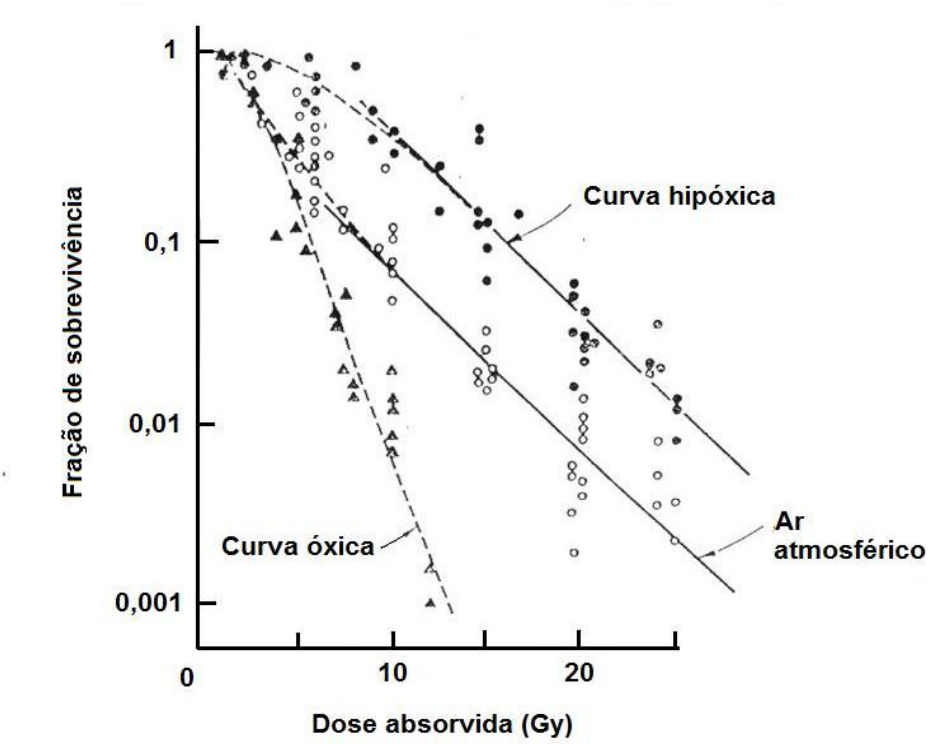

Figura 6 – Fração de sobrevivência de células renais em cultura. Adptado de Barendsen, *et al.* 1963.

6.2 – Terapia do câncer com radiofármacos

Radiofármacos são compostos empregados na medicina nuclear no diagnóstico e terapia de várias doenças, incluindo o câncer (European Pharmacopoeia, 2005). Os radiofármacos terapêuticos são formulações farmacêuticas constituídas necessariamente por um radioisótopo emissorde radiação ionizante particulada e um complexo carreador, geralmente formado por uma molécula, anticorpo ou um composto nanoestruturado que dá, ao radioisótopo, características de biodistribuição e eliminação adequada para uso em seres humanos (Saha, 1998; Volkert & Hoffman, 1999; Heeg &Jurisson, 1999; Vallabhajosula, 2001).

Um radiofármaco terapêutico ideal se liga ao tecido alvo com elevada afinidade, depositando neste tecido elevadas quantidades de energia ionizante e é diretamente eliminado sem que ocorra nenhum tipo de absorção em tecidos não alvo. Na prática, há, atualmente, algumas formulações que são amplamente absorvidas por tumores ou tecidos neoplásicos; mas efeitos adversos são observados em alguns casos (Volkert & Hoffman, 1999).

Os radiofármacos empregados em procedimentos terapêuticos devem apresentar algumas características: *(i)* ser emissor de radiação particulada (alfa, beta ou elétrons auger) com energias na faixa de 200 a 500 keV.As partículas beta ou elétrons auger têm um maior poder de penetração nos tecidos, se comparada às partículas alfa, porém com um LET (Linear Energy Transference) menor que os elétrons Auger. Partículas beta, com baixa energia (< 500 KeV), podem alcançar distâncias de poucos Angstroms. Quando possuem elevadas energias (> 1.000 KEV) como é o caso dos radioisótopos de P-32, Ho-166 e Gd-159, têm a capacidade de penetração que pode alçar vários milímetros o que é uma propriedade desejável quando estas partículas depositam suas energias em células neoplásicas de tumores sólidos.*(ii)* possuir meia vida física adequada. Radioisótopos com meia vida física muito curta não permitem aplicações *in vivo* pelo fato de alcançarem seus tecidos alvos com baixa atividade. Por outro lado, radioisótopos com meia vida física muito longa depositam elevadas doses de radiação

nos pacientes, caso não sejam adequadamente eliminados. *(iii)* apresentar elevada atividade específica (GBq/µg). Um radiofármaco de tratamento deve apresentar uma elevada atividade específica para que possa depositar o máximo de radiação ionizante em um tecido tumoral alvo, com um mínino de átomos radioativos (Saha, 1998; Lukas *et al.*, 2004).

6.2.1 – Radiofármacos emissores alfa

Os radiofármacos terapêuticos emissores de partículas alfa devem ser empregados no tratamento de tumores de fácil acesso, tais como células presentes no sangue ou medula óssea. As partículas alfa têm um baixo alcance no tecido, geralmente numa faixa menor que 100 µm e apresentam alta transferência linear de energia (LET) que promove uma grande capacidade de ionização do meio, podendo, portanto, depositar radiação letal ao tecido tumoral, em uma única dose. Como exemplo, podemos destacar os radioisótopos At-211 e Bi-212 com poder de penetração nos tecidos, respectivamente, de 65µm e 70µm em média. Existe mais de uma centena de radioisótopos emissores de partículas alfa e a maioria apresentam tempos de meia-vida extremamente longos que são incompatíveis com aplicações *in vivo*. Esta incompatibilidade dá-se, principalmente, em função da dificuldade de se produzir formulações farmacêuticas capazes de garantir uma completa eliminação do radioisótopo do corpo humano. Neste contexto, o desenvolvimento de formulações farmacêuticas que possam melhorar o perfil de biodistribuição desses radioisótopos permitiria um maior número de aplicações terapêuticas (Saha, 1998; Lukas *et al.*, 2004).

6.2.2 – Radiofármacosemissores beta

Radiofármacos emissores de radiação beta têm sido aplicados a diversos procedimentos clínicos e integram um importante grupo de radiofármacos aplicados na terapia de tumores. Radiação beta de baixa energia tem a capacidade de penetrar alguns centímetros no tecido biológico, o que permite o seu emprego desde o

tratamento de pequenos agregados celulares até tumores sólidos com alguns centímetros de diâmetro. Atualmente, o radioisótopo Ítrio-90 integra um dos mais populares radiofármacos terapêuticos emissores de radiação beta.Este radioisótopo é produzido a partir do bombardeamento de Ítrio-89 não-radioativo, com nêutrons térmicos em reator nuclear. Possui meia-vida física de 64 horas e suas partículas beta têm energia média de 0,936 MeV, alcançando uma penetração média de 2,5mm em tecidos (Garrean & Espat, 2005). Atualmente, são comercializados dois radiofármacos a base de Ítrio-90, associados a microesferas. Asformulações de microesferas de Ítrio-90 disponívies são “SIR-Spheres (Sirtex Medical, Sydney, Australia) e TheraSphere (MDS Nordion, Ottawa, Ontario, Canadá)”.

6.3 – Gadolínio-159 como potencial radioisótopo terapêutico

De acordo com Saha (1998), alguns radioisótopos apresentam simultaneamente, algumas das principais características físicas desejáveis para aplicações terapêuticas (Tabela 3), dentre os quais, podemos destacar o Iodo-131 como o radioisótopo com o maior número de aplicações em pacientes, o Samário-153 e Rênio-186 empregados como paliativo de dor óssea e o Ítrio-90 como um importante radioisótopo constituinte de preparações farmacêuticas, empregadas em procedimentos terapêuticos contra o câncer.O radioisótopo ^{159}Gdtem também sido citado em alguns trabalhos publicados na literaturacomo um potencial radioisótopo a ser empregado em procedimentos terapêuticos, na área de medicina nuclear, devido as suas características físicas, como tempo de meia-vida e emissão de partículas beta negativas, com energias médias de 1001 KeV (Tabela 3) (Bardies & Chatal, 1994; Goorley& Nikjoo, 2000).

Tabela 3 – Principais radioisótopos integrantes de radiofármacos terapêuticos de uso clinico ou em pesquisas (Adptado de Saha, 1998)

Radionuclídeo	Tempo de meia-vida (dias)	Modo de decaimento	Energia máxima β (MeV)	Energia raios γ (MeV)	Alcance máximo nos tecidos (mm)
^{131}I	8,0	β^-	0,81	0,364 (81%)	2,4
^{32}P	14,3	β^-	1,71	-	8,7
^{67}Cu	2,6	β^-	0,57	0,185 (48%)	-
^{177}Lu	6,7	β^-	0,50	0,208 (11%)	-
^{89}Sr	50,5	β^-	1,46	-	8.0
^{186}Re	3,8	β^-	1,07	0,137 (9%)	5.0
^{153}Sm	1,9	β^-	0,80	0,103 (29%)	3,0
^{90}Y	*2,7*	*β^-*	*2,27*	-	*12,0*
^{159}Gd	*0,75*	*β^-*	*1,01*	*0,363 (68%)*	-
^{188}Re	0,71	β^-	2,11	0,155 (15%)	10,8
^{117m}Sn	13,6	β^-	0,13	0,158 (87%)	0,3
^{213}Bi	0,76	A	-	0,440 (17%)	0,1
^{211}At	0,30	A	-	0,670(0,3%)	65
^{125}I	60,3	E.C.	-	0,025-0,035	-

Na Tabela 4 verifica-se que o radioisótopo Gadolínio-159 apresenta características adequadas para emprego terapêutico, segundo Saha, (1998).

Tabela 4 – Características físicas dos radioisótopos de aplicações terapêuticas (Saha, 1998)

Parâmetro	Característica desejada	Ítrio-90	Gadolínio-159
Meia vida física	Horas a 70 dias	2,27 dias	18,59 horas
Modo de decaimento	Alfa (5 - 8 MeV)	-	-
	Beta negativo (0,3 - 2 MeV)	Beta negativo (2,27 MeV)	Beta negativo (1,01 MeV)
			-
	Elétrons Auger	–	–
	Gama associado	–	Gama (363,4 KeV)
Atividade específica	Elevada		Elevada

Complexos de Gadolínio não-radioativo são utilizados na área médica como agentes de contraste aplicados à técnica de imagem de Ressonância Magnética Nuclear e, atualmente, a Gadodiamida (Omniscan®,GE Healthcare Company) é um dos complexos de gadolínio mais utilizados com este fim, devido a sua baixa osmolalidade e quimiotoxicidade.A Gadodiamida possui fórmula molecular $C_{16}H_{26}GdN_5O_8$ anidra ou $C_{16}H_{26}GdN_5O_9$ H_2O na forma hidratada e tem os dois grupos carboxilatos do DTPA substituídos por grupos amida, resultando num complexo não iônico de baixa dissociação em solução (Figura7) (CHANG *et al.,* 1992).Entretanto, em alguns pacientes submetidos exames de ressonância magnética nuclear com Gd-DTPA-BMA, observaram-se algumas reações adversas, especialmente em pacientes portadores de insuficiência renal crônica (Thomsen, 2006). Por esta razão o FDA, (Food and Drug Administration) agência americana de controle de alimentos e medicamentos, solicitou que um aviso fosse adicionado as bulas dos medicamentos alertando sobre o potencial risco de desenvolvimento de fibrose nefrogênica sistêmica em pacientes portatores de insuficiência renal crônica (US FDA, 2010).

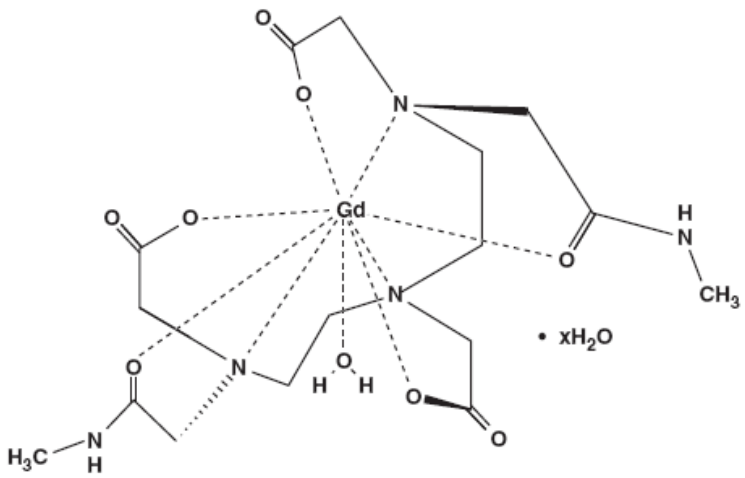

Figura 7 – Estrutura da Gadodiamida (Gd-DTPA-BMA).

O radioisótopo Gadolínio-159 pode ser obtido submetendo-se uma amostra natural de Gadolínio (por exemplo, a Gadodiamida) a um feixe de nêutrons térmicos de uma fonte apropriada (reator nuclear ou fontes de Cf-252). Observa-se a ocorrência de umareação nuclear do tipo (n, γ), em que o isótopo Gd-158, que representa 24,84% do total, captura um nêutron térmico e passa a configurar-se como Gd-159 radioativo que novamente decai, formando o elemento Térbio-159 estável (Figura 8). Contudo, o grande problema de se trabalhar com este radioisótopo é obtê-lo, já que ele não está disponível comercialmente.

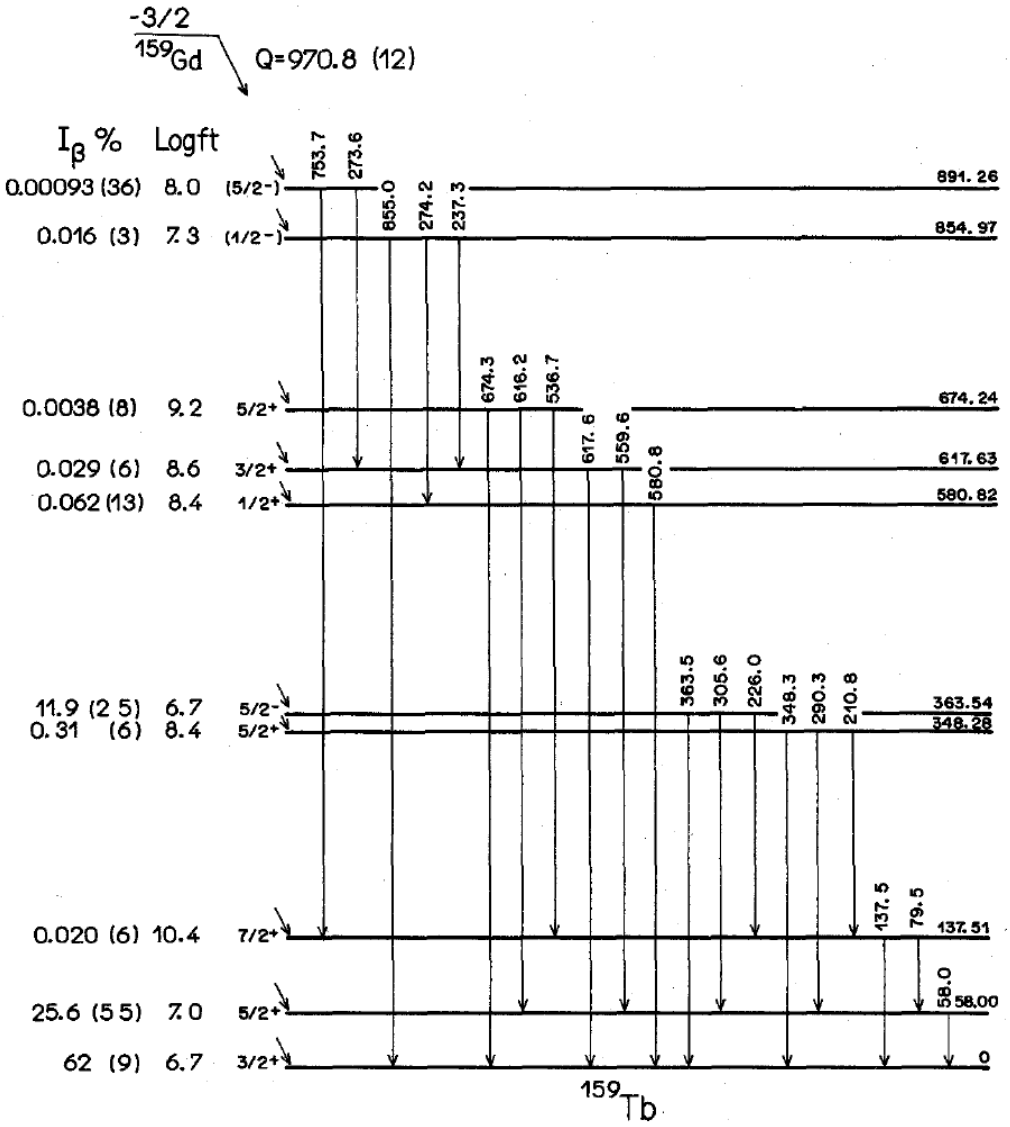

Figura 8 – Esquema de decaimento do Gd-159. Adptado de Moralles *et al*.,1995.

7 – Lipossomas como carreadores de radioisótopos

Lipossomas são sistemas nanoestruturados lipídicos constituídos geralmente por fosfolípides, os quais em meio aquoso se organizam espontaneamente em bicamadas formando vesículas esféricas (Figura9). Essas bicamadas circundam uma cavidade aquosa interna e se encontram envolvidas por um meio aquoso. Considerando que os

lipossomas são constituídos por moléculas anfifílicas, fármacos polares e apolares e bem como radioisótopos podem ser encapsulados, permitindo assim que o perfil de biodistribuição *in vivo* dos compostos encapsulados seja moduldado de forma que um tecido alvo seja alcançado em maior magnitude minimizando os efeitos colaterais (Vemuri & Rhodes, 1995; Tokumitsu *et al.*, 2000; Watanabe *et al.*, 2002; Le & Cui, 2006ab).

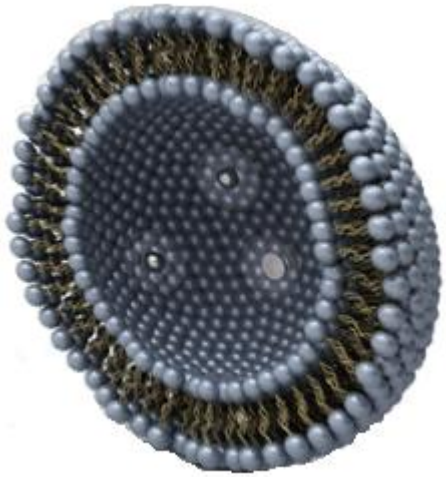

Figura 9 – Representação esquemática da estrutura básica de um lipossoma. Adptado de Stuart, 2010.

Esses sistemas lipídicos foram descritos na década de 60 por Bangham e colaboradores (1965) como modelos de membranas biológicas. A utilização dos lipossomas como sistema de liberação de fármacos foi proposta na década de 70. Entretanto, as primeiras formulações de lipossomas estudadas não produziram os resultados esperados devido à instabilidade das vesículas, baixa taxa de encapsulação dos fármacos e à escolha errônea da via de administração (Lasic, 1998).Os lipossomas podem ser classificados em relação ao mecanismo de liberação intracelular, diâmetro, número de bicamadas e composição. Lipossomas que apresentam apenas um compartimento aquoso circundado por uma bicamada lipídica são denominados unilamelares de tamanho pequeno (SUV) com diâmetro médio de 25 a 100 nm ou

unilamelares de grande tamanho (LUV) com diâmetro médio superior a100 nm. Aqueles que apresentam vários compartimentos aquosos circundados por várias bicamadas lipídicas são denominados lipossomas multilamelares (MLV) e possuem diâmetro médio superior a 300 nm (Lasic, 1998). Em relação à composição e ao mecanismo de liberação intracelular, as vesículas ainda podem ser classificadas como convencionais, de circulação prolongada (Steath) e polimórficos (catiônicos, pH-sensíveis e termosensíves) (Immordino *et al*., 2006; Batista *et al*., 2007).

Buscando melhorar a interação dos lipossomas com os tecidos alvo e bem como aumentar a quantidade do fármaco liberado nestas células, lipossomas pH-sensíveis, foram propostos. Este sistema foi sugerido a partir da observação de que tecidos patológicos comotumores, tecidos inflamados e infecciosos apresentam um pH extracelular menor doque os tecidos normais (Gulino *et al.*, 1967). Neste contexto, o emprego de formulações pH sensíveis permitiram que o conteudo encapsulado fosse liberado na presença de um ambiente com menor pH através da perda da estabilidade das vesículas. Os lipossomas pH-sensíveisconsistem na combinação de lípides insaturados, como a fosfatidiletanolamina (PE) e seu derivado a dioleoilfosfatidiletanolamina (DOPE), com compostoscarboxilados como o hemisuccinato de colesterila (CHEMS). Diferente da maioria dos fosfolípides, a PE possui uma cabeça polar pequena e com pouca hidratação, permitindo um volume menor que a sua respectiva cadeia principal, exibindo, portanto, uma organizaçãomolecular do tipo cônica. Estadistribuição espacial permite o estabelecimento deforças intermoleculares entre os grupamentos amina e fosfato da cabeça polar, o que pode explicar atendência dessas moléculas em adquirir a fase hexagonal invertida (HII) e a conseqüente incapacidade de formar vesículas. A preparação de lipossomas com estes fosfolípides exige a adição de compostos estabilizantes carboxilados, como o CHEMS, os quais se encontramsob a forma ionizada em pH fisiológico. O CHEMS se distribui entre as moléculas de PE homogeneamente e o aparecimento de forças eletrostáticas repulsivas entre osgrupos carboxílicos presentes no CHEMS e os grupos fosfatos presentes nosfosfolípides

favorece a organização lamelar e a consequenteobtenção das vesículas como pode se observado na Figura 10.

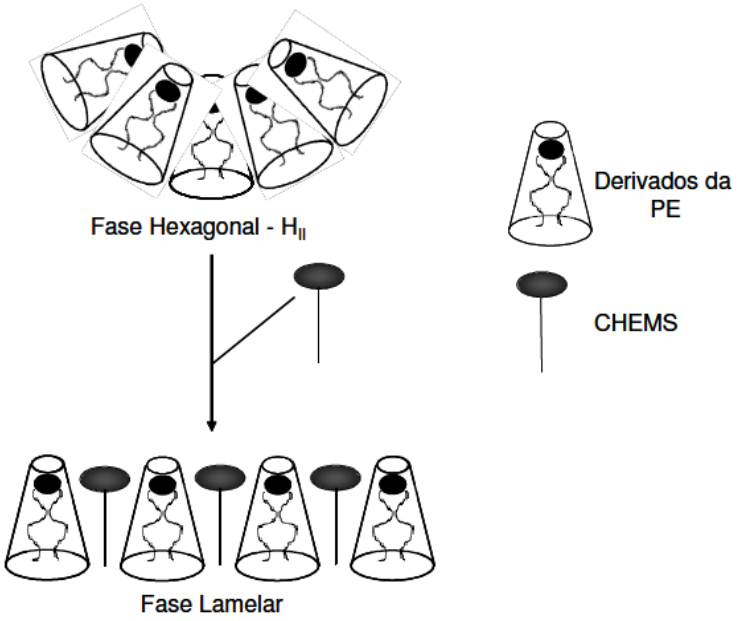

Figura 10 - Organização estrutural esquemática de PE e derivados sob a ausência e presença de CHEMS. (Extraído de Leite, 2010).

Na presença de um pH fisiológico, esses lipossomas são estáveis. Aexposição dessas vesículas a um meio adequadamente ácido permite que os grupos carboxilas presente nos agentes estabilizantes sejam protonados, contribuindo assim, significativamente para a desestabilização das vesículas e consequente liberação do material encapsulado (De Oliveira *et al.*, 1998; Simões *et al.*, 2004).

Lipossomas quando administrados por via endovenosa podem sofrem o fenômeno de opsonização(adsorção de proteínas séricas), ocasionando sua rápida identificação pelo sistema imune e posterior captura pelas células do sistema mononuclear fagocitário(SMF) (Delattre *et al*., 1993). Assim, buscando evitar este fenômeno, lipossomas de longa circulação foram propostos baseando-se na imobilização de

moléculas hidrofílicas em suas superfícies (Figura 11). Estas moléculas, como exemplo o polietilenoglicol (PEG) associado à fosfolípides, evitam a adsorção de proteínas séricas na superfície dos lipossomas, reduzindo a sua captação pelo SFM, e consequentemente, prolongando seu tempo de circulação na corrente sanguínea.

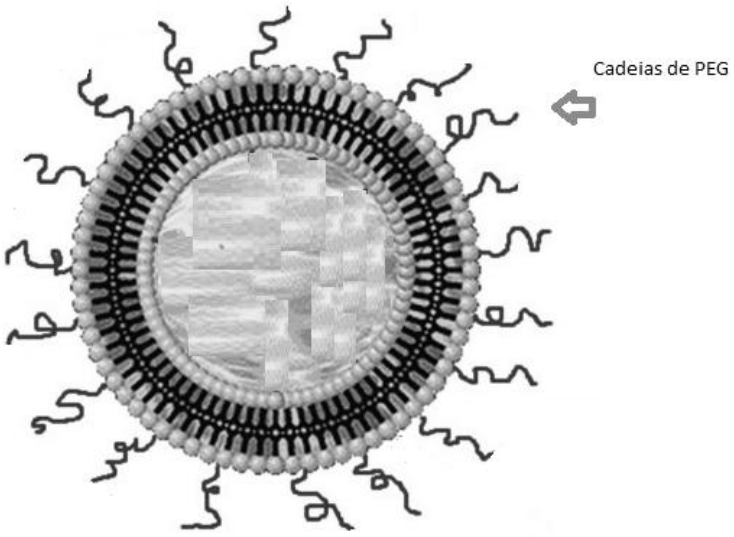

Figura 11 – Representação esquemática de lipossomas contendo o polímero PEG imobilizado em sua superfície.

O desenvolvimento eo uso de lipossomas como carreadores de radioisótopos terapêuticos têm focado principalmente na melhoria da absorção e retenção dos radioisótopos nos tumores. Embora um grande número de trabalhos publicados na literatura mostra a utilização de lipossomas radiomarcados em pesquisas na área de medicina nuclear diagnóstica, objetivando a aquisição de imagens de tumores, poucos trabalhos abordaram o usodesses lipossomas objetivando a terapia tumoral. Um dos primeiros trabalhos publicados com este objetivo foi conduzido por Hafeli e colaboradores (1991). Neste estudo os autores prepararam lipossomas com um diâmetro médio de 70 nmencapsulando os radioisótopos ^{186}Re e ^{188}Re. Outro estudo semelhante foi conduzido por Utkhede e colaboradores (1994). Neste trabalho, os autores descrevem a encapsulação bem sucedida de Ítrio-90 em lipossomas peguilados do tipo SUV. Um estudo mais aprofundado conduzido por Emfietzoglou e colaboradores

(2005) empregou elevadas doses de radiação às células de tumores avasculares esferoidais por meio de lipossomas contendo radioisótopos. Os resultados mostraram que uma cuidadosa seleção de radioisótopos pode aumentar de 2 a 10 vezes a dose de radiação absorvida na região central do tumor. Nos estudos conduzidos por Syme e colaboradores (2003) as características superficiais dos lipossomas foram modificadas de modo a melhorar as doses de radiação absorvida por células de câncer de ovário.A incorporação de radioisótopos emissores de partículas alfa encapsulados emlipossomas foram descritos porHenriksen e colaboradores (2004). Os experimentos consistiram na preparação e caracterização de lipossomas estericamente estabilizados e imobilizados $F(ab')_2$ contendo ^{223}Ra, ^{224}Ra ^{225}Ac. Os resultados mostraram uma boa estabilidade*in vitro* da formulação.

Buscando dar especificidade de ação aos lipossomas, estudos têm sido conduzidos com o intuito de adicionar a superfície dos lipossomas, além de moléculas hidrofílicas como o PEG, moléculas ou anticorpos específicos para receptores localizados na superfície de células tumorais. Diversas linhagens de carcinomas como Ovcar-3, IGROV1, SKOV3, câncer de ovário; SW626 e adenocarcinoma humano expressam na superfície de suas células receptores para o ácido fólico. Nestes trabalhos, os lipossomas contendo folato apresentaram uma maior especificidade de interação por estas células em relação à mesma formulação desprovida de ácido folico (Sen *et al*., 2006; Parker *et al*., 2005; Gosselin & Lee, 2002).

8 – Conclusão

Como conclusão dessa revisão bibliográfica verificamos que os dados disponíveis na literatura mostramque o radioisótopo ^{159}Gd apresenta características físicas úteis no tratamento do câncer. Entretanto, até o presente, nenhum estudo *in vitro* e *in vivo* mostrou esta aplicação. Além disso, não se conhecem também os efeitos que serão obtidos associando este radioisótopo a um carreador nanoestruturado, como os lipossomas.

PARTE 2 – TRABALHO EXPERIMENTAL

CAPÍTULO 1

Gadolinium-159: preparation and preliminary evaluation as a potential antitumoral radionuclide.***Journal of Radioanalytical and Nuclear Chemistry* 284, 315-320, 2010.**

Gadolinium-159: preparation and preliminary evaluation as a potential antitumoral radionuclide

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Abstract

Beta emitting radionuclides, such as ^{90}Y, ^{153}Sm, and ^{131}I, with short half lives and that deliver a higher radiation dose rate, have been used effectively in alleviating bone pain due to metastases, synovectomy, and tumor therapy. The ^{159}Gd is a radionuclide that presents these characteristics; however, its antitumoral potential has yet to be investigated. The present study aims to identify the ^{159}Gd production, structural characterization, and antitumoral activity. To achieve these objectives, Gadodiamide®, the most non-ionic dye used in nuclear magnetic resonance imaging, was neutron irradiated for 8 hours to obtain ^{159}Gd-Gadodiamide. A final product with high specific activity was obtained, and its structural integrity was determined by Fourier Transformer Infrared Spectroscopy and High Performance Liquid Chromatography methods. Finally, the irradiated product presented good stability and increased to approximately 95 times that of the *in vitro* cytotoxicity of the gadodiamide against Ehrlich tumor cells. These results suggest that there is a high potential for the use of ^{159}Gd against tumoral cells.

Keywords: Neutron irradiation, Gadolinium-159 radionuclide, antitumoral radionuclide

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1 – Introduction

Beta emitting radionuclides, such as ^{90}Y, ^{153}Sm, and ^{131}I, with short half lives and that deliver a higher radiation dose rate, have been used effectively in alleviating bone pain due to metastases, synovectomy, and tumors[1-3]. Beta particles with low energy (<500 keV) can penetrate a few Angstroms in human tissues, but high energy beta particles (>1000 keV), as is the case of ^{32}P, ^{188}Re, ^{89}Sr, and ^{90}Y, have the ability to penetrate many millimeters, depositing high amounts of ionizing energy in neoplastic cells[4] (Table 1).

Table 1 – Main radionuclides employees currently in therapeutic nuclear medicine

Radionuclide	Half life (days)	Decay mode	Max. β energy (MeV)	γ Energy (MeV)	Maximum range in tissues (mm)
^{131}I	8.0	β^-	0.81	0.364 (81%)	2.4
^{32}P	14.3	β^-	1.71	–	8.7
^{67}Cu	2.6	β^-	0.57	0.185 (48%)	–
^{177}Lu	6.7	β^-	0.50	0.208 (11%)	–
^{89}Sr	50.5	β^-	1.46	–	8.0
^{186}Re	3.8	β^-	1.07	0.137 (9%)	5.0
^{153}Sm	1.9	β^-	0.80	0.103 (29%)	3.0
^{90}Y	2.7	β^-	2.27	–	12.0
^{188}Re	0.71	β^-	2.11	0.155 (15%)	10.8
^{117m}Sn	13.6	β^-	0.13	0.158 (87%)	0.3
^{213}Bi	0.76	α	–	0.440 (17%)	0.1
^{211}At	0.30	α	–	0.670 (0.3%)	65
^{125}I	60.3	E.C.	–	0.025–0.035	–

The ^{159}Gd radionuclide is a beta (1001 keV) and gamma (main energy: 363.54 keV) emitter with a half life of 18.59 hours[5]. The physical characteristics of the ^{159}Gd isotope suggest that it has the potential to be used in nuclear medicine research[6,7]. Currently, studies showing the *in vitro* cytotoxic activity of ^{159}Gd are limited. Gadodiamide® (Omniscan - General Electric Healthcare Company), is a frequently non-ionic Gd complex (Figure 1) dye used in MRI's[8,9]. In this study, Gadodiamide was used to investigate the antitumoral potential.

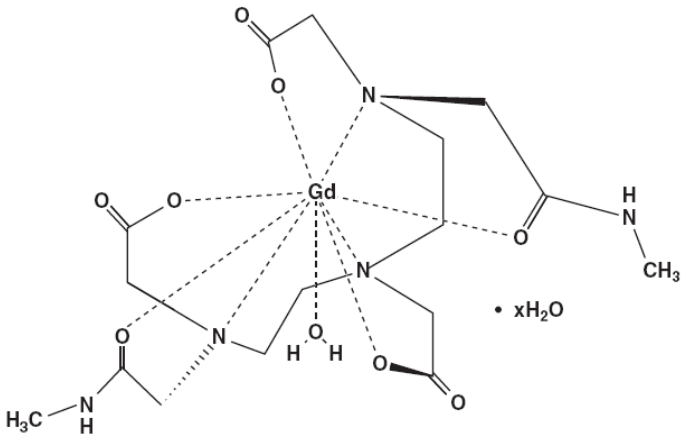

Figure 1: Gd-DTPA-BMA (Gadodiamide®)structure.

The gadolinium present in Gadodiamide is a mixture of several isotopes: ^{152}Gd (0.20%), ^{154}Gd (2.18%), ^{155}Gd (14.80%), ^{156}Gd (20.47%), ^{157}Gd (15.65%), ^{158}Gd (24.84%), and ^{160}Gd (21.86%)[10]. Through neutron irradiation, the isotope ^{158}Gd captures a neutron and becomes ^{159}Gd [^{158}Gd(n, y)^{159}Gd]. The other formed radionuclides (except ^{153}Gd) either remain stable or have a shorter half life, and they are not detected in conventional procedures.

During the neutron irradiation process, some changes in the molecular structure may appear, which are caused by the recoiling energy of the activated nucleus during beta or gamma ray emission as well as the collision of neutron flux in the nuclear reactor

core[11]. Thus, after every irradiation process, the integrity of the molecular structure target was evaluated through analytical techniques, such as FTIR and HPLC.
The aim of this work was to prepare and quantify the specific activity of a ^{159}Gd radionuclide to determine if the structural integrity of the gadodiamide complex was in fact maintained throughthe neutron irradiation process and to investigate the cytotoxic activity of radioactive and non-radioactive gadodiamide on Ehrlich tumor cells*in vitro*.

2 – Experimental

All procedures were performed at different laboratories of the Centro de Desenvolvimento da Tecnologia Nuclear - Comissão Nacional de Energia Nuclear (CDTN-CNEN), Brazil.

2.1 – Materials

Gadodiamide® was purchased from Farmasa (Laboratório Americano de Farmacoterapia S.A, São Paulo, Brazil). The Gadolinium Standard was purchased from Sigma-Aldrich (Brazil). The MTT [bromide 3 - (4, 5-dimethyl-2-thiazolyl-2,5-diphenyl)-2H-tetrazolium] salt was purchased from Sigma (St. Louis, MO, USA), and the DMEM culture medium was purchased from Gibco BRL (Grand Island, NY, USA). The HPLC columns were purchased from Sigma-Aldrich-Supelco (Belo Horizonte, Brazil). All the solvents used were of analytical grade, and other chemicals were commercially available, of reagent grade, and used without further purification. MilliQ® water (Simplicity 185, Millipore, Bedford, USA) was used throughout.

2.2 – Methods

2.2.1 – Irradiation of Gadodiamide

The irradiation of Gadodiamide into polyethylene vials was carried out in a TRIGA MARK I IPR-R1 nuclear reactor. Two samples, in triplicate, were irradiated at 100 kW,

under an average thermal neutron flux of 6.6×10^{11} $n.cm^2.s^{-1}$ in two time periods – 10 minutes and 8 hours – to evaluate its chemical integrity during irradiation. After 8 hours of cooling-time, gamma spectrometry was carried out for 300 seconds, which was enough time to collect a statistically suitable number of counts. The gamma counting system used was a CANBERRA hyper pure germanium detector (HPGe) with a full-width at half-maximum resolution (FWHM) of 1.75 keV at 1332 keV and 15% relative efficiency. All measurements were performed with the same geometry to maintain the dead-time below 5%. The Genie-2000 software (CANBERRA) was used for spectral data acquisition and peak area.

2.2.2 – Specific activity determination

The specific activity of gadodiamide was determined by two different methods. For the first method, samples containing 10 mg of gadodiamide were irradiated following the previously described procedure, using K_0-instrumental neutron activation analysis[12]. In the second method, a Gadolinium chloride standard (Sigma-Aldrich) was used jointly with gadodiamide samples. The Genie software was used in both methods for specific activity evaluation. The results were compared, and no statistically significant differences were observed.

2.2.3 – Determination of gadodiamide stability by Fourier Transformer Infrared spectroscopy (FTIR)

FTIR spectroscopy was used to evaluate if the structural integrity of gadodiamide was maintained after neutron irradiation. Triplicate samples were irradiated for 10 minutes and 8 hours. After adequate cooling, the samples were analyzed using the Ca_2F window of a BB Bomen Spectrometer (Model MB 102). The FTIR spectra were acquired using a 400–4000 cm^{-1} spectral range, with 128 scans (20 per minute) and 1 cm^{-1} ofresolution.

2.2.4 – Determination of gadodiamide stability by HPLC Chromatography

The procedure for determining gadodiamide stability by HPLC was based on that previously described by Hvattum et al[13]. Briefly, the calibration standards contained 2.00–800 mM of gadodiamide, and the samples contained approximately 5.00, 100, and 400 mM of gadodiamide. HPLC analysis was performed using a Supelcosil TM LC-18-DB 250 x 4.6 mm 5 µm reversed-phase column with a corresponding guard column, a Supelguard TM LC-18-DB 20 x 2.1 mm 5 µm by Supelco (Bellefonte, USA), a Shimadzu pump LC-10AD detector UV/Vis SPD 10AD, and an auto-injector supplied by Shimadzu (Osaka, Japan). The elluent consisted of triethylammonium acetate (10 mM) and EDTA (2 mM). The pH was adjusted with acetic acid (1 M) or sodium hydroxide (1 M) to 6.5–7.0. The flow rate of the mobile phase reagent was 0.3 $ml.min^{-1}$. These were mixed in a peek tee before being transferred to the detector. The separation was performed at 30°C. The samples were kept at 4°C, and 10 µl was injected for each analysis.

2.2.5 – *In vitro* cytotoxic evaluation

Ehrlich tumor cells were obtained from ascites tumors which had been previously induced in Swiss mice according to the protocol number 019/09, which was approved by the Animal Experimentation Ethical Committee from the Federal University of Minas Gerais (CETEA-UFMG). After the appropriate time period for tumor development in animal donors, the Ehrlich cells were extracted and placed on a culture plate of 96 wells. This was performed to evaluate the metabolic activity after a minimal confluence of 90% using a DMEM (Dubelcco's modified Eagle Medium) culture medium. The cells were subjected to treatment for 48 hours, under increasing concentrations of non-radioactive gadodiamide. Eight replicates were investigated for statistical evaluation. The metabolic viability tests were performed using the MTT assay to evaluate the effectiveness of the treatment. The determination of the absorbance at 570 nm of the formed product is a measure of metabolic cell viability, and the two quantities are directly proportional[14].

2.2.6 – Statistics

All experiments were performed in triplicate and expressed as the mean ± standard deviation, unless otherwise stated. The specific activity data were compared by means of the Student's t-test, using the prism 4.0 software, considering a 95% confidence interval.

3 – Results and discussion

3.1 – Irradiation of Gadodiamide and determination of specific activity

After irradiation, the gamma spectrum of gadodiamide was evaluated using the Genie 2000 software. The characteristic photopic radionuclide ^{159}Gd could be observed at 348.28 keV, 363.50 keV, and 560.80 keV (Figure 2). The energies and their intensities were compared and were found to be in accordance with Moralles et al.[5] and Blaauw[15]. The specific activity was determined by the K_0-standardization method to be 240.8 ± 42 MBq.mg^{-1} and by the gadolinium chloride standard method to be 238.0 ± 36 MBq.mg^{-1} after 8 hours of irradiation. These results are not statistically different (p=0.301) and are considered sufficient for therapeutic applications based on current, commercially available radiopharmaceuticals[16].

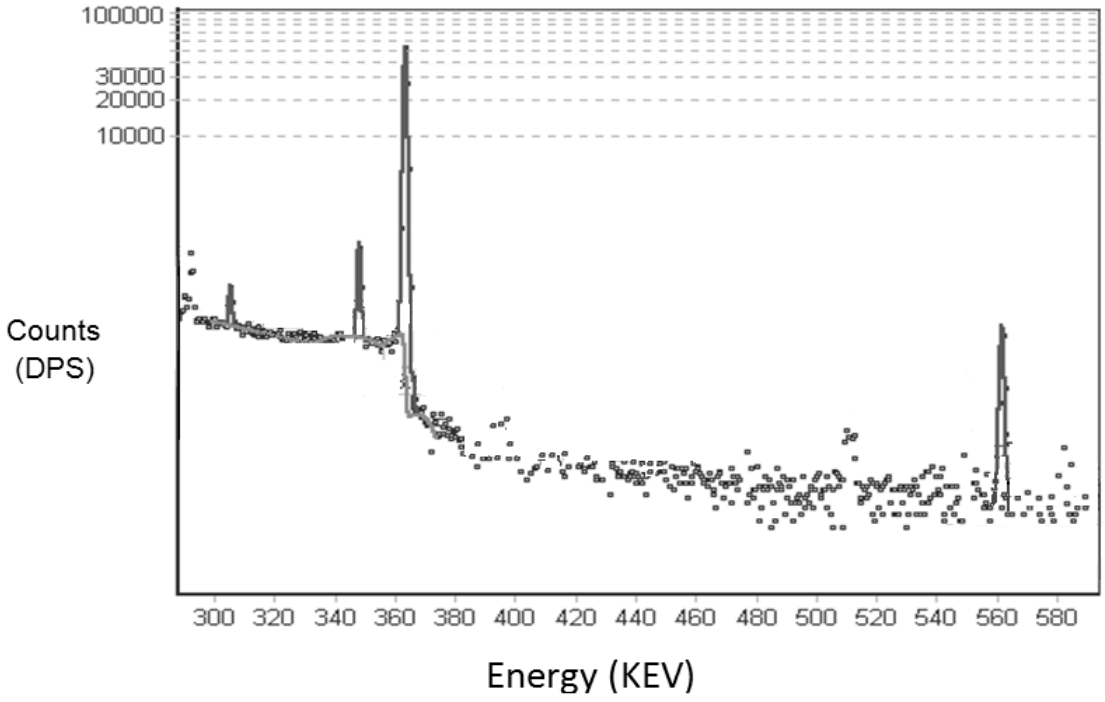

Figure 2 – Gamma spectrum of ^{159}Gd in Gadodiamide sample. The characteristic photopic could be observed at 348.28 keV, 363.50 keV, and 560.80 keV.

3.2 – Determinationof gadodiamide stability

From the FTIR spectrum, it is possible to observe that the same peaks of the non-irradiated samples are present in the samples irradiated for 10 minutes and 8 hours (Figure 3). Furthermore, no new absorption peaks were found in the samples that underwent the irradiation process, suggesting that there were no broken or newly formed chemical bonds. The HPLC procedures confirm the results found via infrared spectroscopy. No new species was determined in the different chromatograms (Figure 4). From these results, it can be said that, even after 8 hours of irradiation in a nuclear reactor, the structural integrity of the Gd-DTPA-BMA complex was maintained.

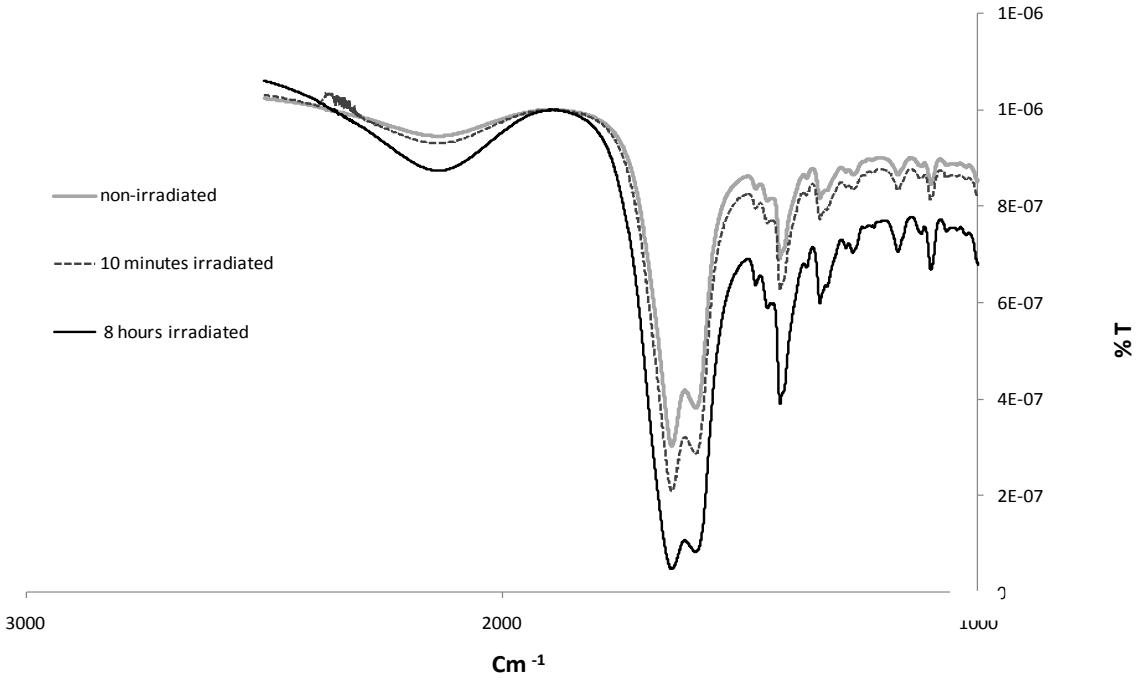

Figure 3 – FTIR spectra of different samples of Gadodiamide after irradiation.

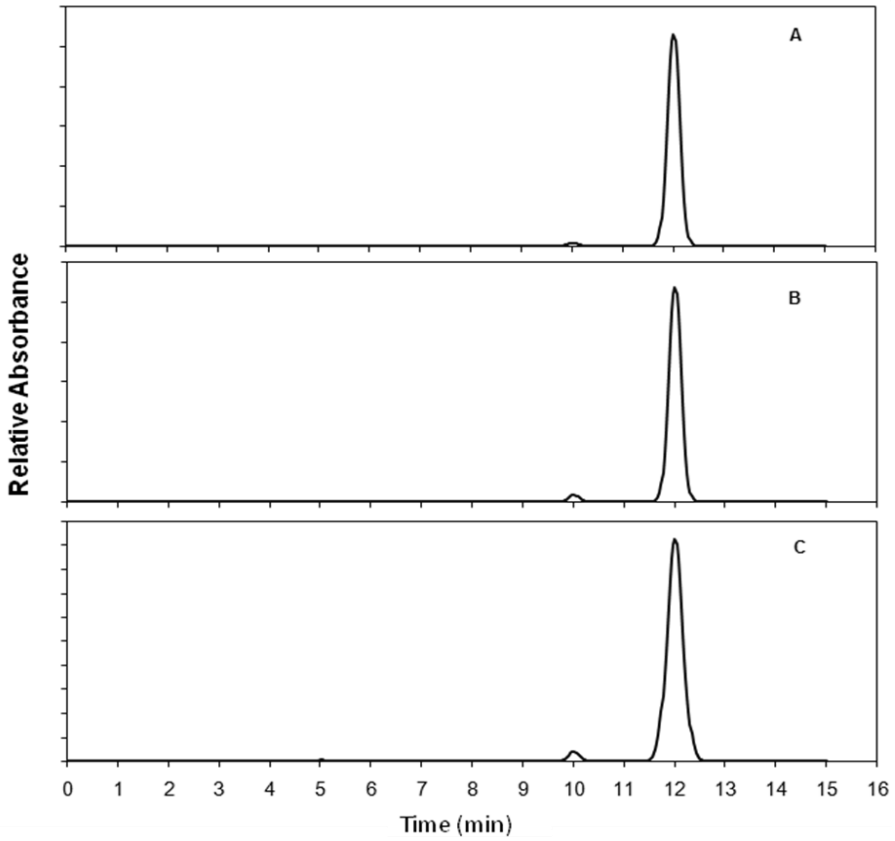

Figure 4 - Representative HPLC chromatograms of gadodiamide in different samples. (A) Non-irradiated, (B) 10 minute irradiation, (C) 8 hour irradiation

3.3 – In vitro cytotoxic evaluation

Radiotherapy is a therapeutic modality employed in very different treatments of malignant tumors. However, this technique has encountered serious difficulties in discriminating tumor cells from healthy cells. In this context, several radionuclides which emit particulate radiation have been studied as an alternative to conventional radiation therapy, where the major objective is to minimize the effects of ionizing radiation on healthy tissue[17-19]. In work published by Neves et al.[6] the radioisotope ^{159}Gd, among other radionuclides, was presented as a potentially new radioisotope to be used in therapeutic procedures.

In the present study, this radionuclide, in complex form (Gd-DTPA-BMA), had its therapeutic potential evaluated through antitumor activity against Ehrlich tumor cells (*in vitro)*. After an appropriate time period for tumor development, Ehrlich cells were placed on 96-well plates, where increasing concentrations of radioactive or non-radioactive Gd-DTPA-BMA were added. Next, a curve that compares the percentage of cell survival to the log molar concentration of Gd-DTPA-BMA was obtained by the Prism® 5.0 software (Figure 5). The subsequent results showed an IC_{50} of 134 mM for the non-radioactive compound (Figure 5A) and 1.4 mM for the radioactive compound (Figure 5B). Thus, we can say that the radioactive compound presents a 96-times greater cytotoxic action.

The dose rate in Ehrlich cells was estimated for the IC_{50} concentration[20] using Martin's mathematic model for beta dosimetry on contaminated skin surfaces. The results showed that the dose rate value is approximately 150 Gy/h in the Ehrlich cell culture.

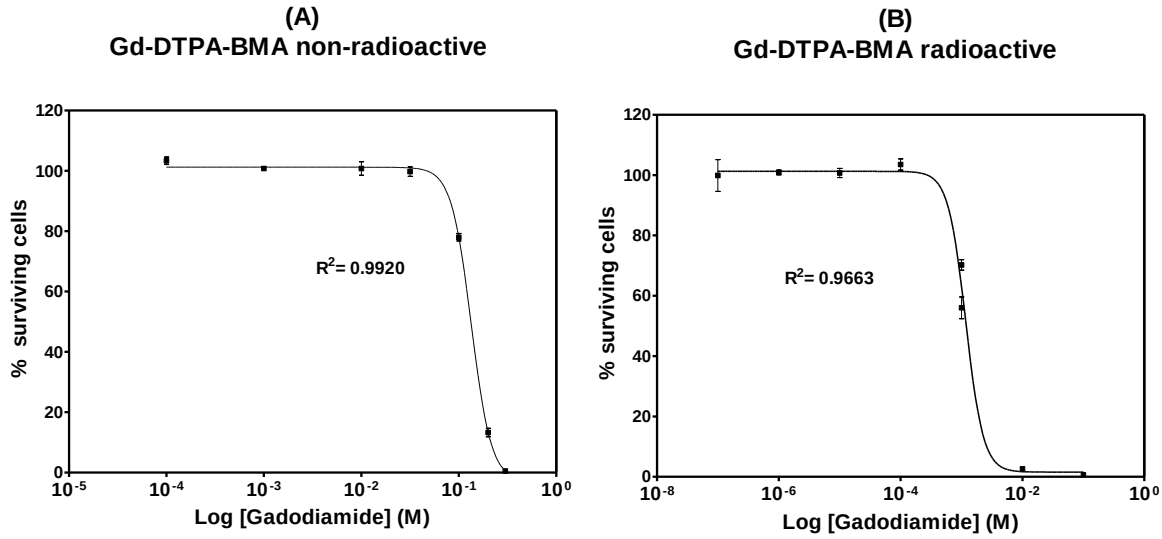

Figure 5 – Representative curves of surviving Ehrlich cells vs. the Log of Gd-DTPA-BMA molar concentration:(A) Non-radioactive (b) Radioactive. The IC_{50} value was determined through non-linear regression (n = 8).

Although malignant tumor cells exhibit varied characteristics, evidence shows that resistance to apoptosis is a prominent feature of most malignant tumors and is an important objective of therapeutic approaches in medicine[21]. Studies indicate that the p53 protein plays a central role in regulating the G1 phase checkpoint, in response to damage in DNA[22]. The relationship between the p53 protein and carcinogenesis has been proven by the high rate of mutations of its gene in malignant tumors from different tissues[23,24]. Experimental procedures have shown that ionizing radiation can promote the activation of p53, increasing its concentration in the cell nucleus, and lead these cells to death by apoptosis. These observations have greatly contributed to a better understanding of the action mechanism of ionizing radiation in tumor cells[25-27]. We believe that this mechanism may explain the cytotoxic activity of radioactive Gd-DTPA-BMA used against Ehrlich cells. However, more studies are necessary to elucidate the mechanism of Ehrlich cell death by ^{159}Gd radionuclide.

4 – Conclusion

The highly specific activity of Gd-DTPA-BMA was obtained after 8 hours of neutron irradiation and may be considered an appropriate, future therapeutic research procedure. Through FTIR spectroscopy, it could be verified that the irradiation process does not disrupt the molecular structure of the metal complex. These results showed that it is possible to obtain a radiolabeled gadolinium complex directly from a TRIGA reactor following irradiation and that no subsequent modifications were observed in the gadolinium complex structure.

Non-radioactive Gd-DTPA-BMA did in fact present cytotoxicity in Ehrlich tumor cells due to the fact that it’s highly specific activity and the physical characteristics of the cytotoxic activity of radioactive Gd-DTPA-BMA was greatly increased. This clearly demonstrates that ionizing radiation has played an important role in Ehrlich cell death. In this light, the ^{159}Gd radionuclide can be considered a potential antitumor radionuclide.

These findings, considering the local technology, are relevant and may represent a useful tool for future *in vivo* studies, such as the development of new formulations using gadolinium as a radiopharmaceutical treatment.

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CAPÍTULO 2

Liposomes Radiolabeled with ^{159}Gd-DTPA-BMA: Preparation, Physicochemical Characterization, Release Profile and *in vitro* Cytotoxic Evaluation.***European Journal of Pharmaceutical Sciences Vol*. 42, 462-469, 2011.**

Liposomes Radiolabeled with ^{159}Gd-DTPA-BMA: Preparation, Physicochemical Characterization, Release Profile and *in vitro* Cytotoxic Evaluation

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Abstract

The present work describes the preparation, labeling, physicochemical characterization, and *in vitro* cytotoxic evaluation of long circulating pH-sensitive liposomes containing ^{159}Gd-DTPA-BMA. These liposomes were successfully obtained and submitted to neutron irradiation for gadolinium labeling. Their size, distribution, and homogeneity were determined by photon correlation spectroscopy, while their zeta potential was determined by laser Doppler anemometry. The morphology and structural organization were evaluated by atomic force microscopy. The stability and release profiles of Gd-DTPA-BMA in the liposomes were determined *in vitro* in Dubelco's Modified Eagle's Medium and rat serum at 70%. The results showed that liposomes remained physically stable after 8 hours of irradiation and presented a low release profile of its content in two

different biological mediums. The formulation of liposomes containing ^{159}Gd and its respective controls were evaluated *in vitro* cytotoxicity against tumor cells RT2. The results showed increased cytotoxic activity of approximately 1170 fold in relation to free Gd-DTPA-BMA.

Keywords: Gd-DTPA-BMA, Liposome characterization, Release profile, *in vitro* cytotoxic evaluation

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1 – Introduction

In nuclear medicine, many radioisotopes have been used in diagnostic or therapeutic procedures to treat a wide range of diseases, including cancer. When used exclusively in therapeutic procedures, the radioisotopes should emit particulate radiation (auger, beta, or alpha) with energy levels between 50 and 2,500 keV (Saha, 1998; Scholz, 2006). Some radioisotopes, such as Yttrium-90, Renium-186, and Samarium-153, present these characteristics, which have been applied in many clinical procedures, including the treatment of some types of cancer and chronic inflammatory processes (Bauman *et al.,* 2005; Finlay *et al.,* 2005; Garrean & Espat, 2005).

Gd-DTPA-BMA, a non-ionic complex containing one molecule of H_2O coordination, presents a low disassociation in aqueous solutions (Chang *et al.,* 1992), has proven to be of great use as a dye in MRI procedures, due to its low osmolality and chemotoxicity (Figure 1) (Meyer *et al.,* 1990). However, Gd-DTPA-BMA has shown some adverse reactions, especially in patients suffering from chronic renal insufficiency (Thomsen, 2006). For this reason, the FDA requested that a warning be added to the product descriptions regarding the potential risk of nephrogenic systemic fibrosis (NFS) in patients with chronic renal failure (US FDA, 2010).

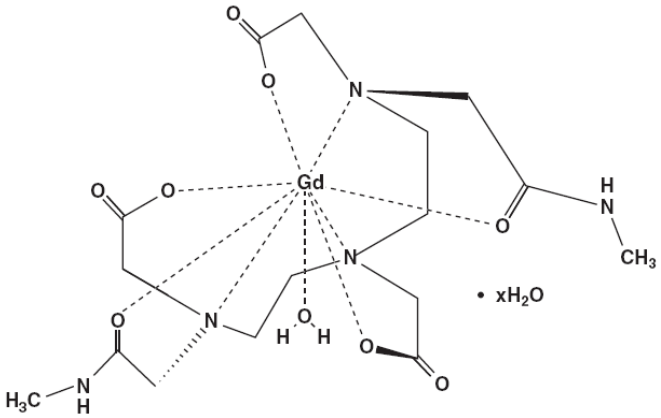


Figure 1 – Gd-DTPA-BMA (gadolinium diethylenetriaminepentaacetic acid bis(methylamide)) complex.

The gadolinium metal contains a mixture of the following isotopes: ^{152}Gd (0.20%), ^{154}Gd (2.18%), ^{155}Gd (14.80%), ^{156}Gd (20.47%), ^{157}Gd (15.65%), ^{158}Gd (24.84%), and ^{160}Gd (21.86%) (Browne *et al.,* 1986). By submitting a natural gadolinium sample to an appropriate nêutron irradiation process, the radioisotope ^{159}Gd is obtained (Alfassi, 1985). The ^{159}Gd radioisotope has a physical half-life of 18.59 hours and emits negative beta particles with a maximum energy of 1.01 MeV and a characteristic gamma emission of 363.54 keV (Moralles *et al.,* 1995). Based on these characteristics, some works demonstrate the potential use of ^{159}Gd radioisotopes in cancer treatment with some advantages over other potentially applicable radioisotopes (Bardies & Chatal, 1994; Goorley & Nikjoo, 2000). For example, in relation to ^{77}Lu, ^{159}Gd presents a longer half-life, and a negative beta emission with higher energy is particularly interesting in the treatment of solid tumors with significant mass.

Recently, our research group also demonstrated this capability in a preliminary *in vitro* study using the Ehrlich tumor model (Soares *et al.,* 2010). However, the use of this type of radioisotope requires its direct application in the target organ to prevent the healthy tissue from receiving excessively high radiation doses. In this context, the use of

nanostructured systems, such as liposomes or nanoparticles, allows many substances, including radioisotopes, to be delivered to target tissues in greater quantities, in turn minimizing the radiation dose within normal surrounding tissues (Vemuri & Rhodes, 1995; Tokumitsu *et al.,* 2000). These nanostructured systems have the ability to permeate the interstitial space by passing through leaky capillaries, thus allowing for radiotracer delivery at different locations outside the Mononuclear Phagocyte System (MPS) (Oyen *et al.,* 1996). Some authors have demonstrated that the mean diameter of nanoparticles has influenced biodistribution studies. Nanoparticles of larger than 300 nm and smaller than 70 nm are rapidly cleared from circulation by MPS cells (Gref *et al.,* 1994). However, the use of a PEG (polyethyleneglycol) chain, through the addition of PEG-DSPE on the surface of the liposome, results in a decreased recognition by MPS cells, thereby increasing the half-life of their circulation in the blood (Jones, 1995; Pereira *et al.,* 2008; Woodle, 1993). Another important factor to be considered is the size and capacity needed to reach inflammatory foci through loose junctions of the endothelium.

Prior studies have reported that the inclusion of amphipathic poly(ethyleneglycol) lipids, such as diestearoylphosphatidylethanolamine-polyethyleneglycol 2000 (DSPE-PEG 2000), as can be seen in liposome compositions, can significantly reduce the uptake by the MPS cells, thus resulting in the liposomes prolonged circulation (Allen *et al.,* 1989; Garbuzenko *et al.,* 2005; Klibanov *et al.,* 1990; Torchilin & Papisov, 1994;). The pH-sensitive liposomes are internalized in a low pH compartment within endosomal vesicles, and the encapsulated drug are released into the cytoplasm, in turn inducing cellular death. The construction of pH-sensitive liposomes takes advantage of the polymorphic phase behavior of unsaturated phosphatidylethanolamine, which generates inverted hexagonal phase two (HII) forms rather than bilayers. Liposome stabilization within bilayers can be achieved by using a titratable acid lipid, such as CHEMS (cholesteryl hemisuccinate), which is negatively charged at neutral pH (Massey, 1998). This lipid, homogenously distributed among dioleoylphosphatidylethanolamine (DOPE) molecules, provides electrostatic repulsions which decrease DOPE intermolecular interactions, thus preventing HII phase formation under physiological conditions. The

protonation of CHEMS molecules in an acidic medium (e.g., endosomal vesicles), neutralizes their negative charges. In the process, the liposomes undergo destabilization and release their aqueous contents (Carvalho Junior *et al.,* 2007). Thus, the encapsulation of Gd-DTPA-BMA in this liposome formulation and its irradiation can lead to a higher retention of the ^{159}Gd radioisotope within liposomes and can subsequently be released in acidic mediums, such as endosomal vesicles in tumor cells. This behavior can reduce its systemic toxicity and improve the antitumor effect, considering that a higher dose of ^{159}Gd will be accumulated in the tumor.

The present work aimed to prepare and radiolabel long-circulating pH-sensitive liposomes with ^{159}Gd from Gd-DTPA-BMA. To study its physicochemical characteristics (mean size, zeta potential, and polydispersity index), morphologic stability of the system after neutron irradiation in a nuclear reactor using the atomic force microscopy (AFM) technique was applied. To investigate the release profiles of encapsulated ^{159}Gd in different physiological mediums: DMEM (Dubelco's Modified Eagle's Medium) and rat serum at 70% were administered and the cytotoxic activity of liposomes containing ^{159}Gd-DTPA-BMA to combat RT2 (Murine Glioblastoma cells) tumors cells, as wells as its controls, was evaluated.

2 – Materialsand Methods

2.1 – Materials

DOPE, DSPE-PEG, and CHEMS lipids were purchased from Lipoid GmbH (Ludwigshafen, Germany) and Sigma (St. Louis, USA), respectively. Gd-DTPA-BMA (Gadodiamide - Omniscan® - General Electric Healthcare Company) was kindly provided by FARMASA (São Paulo, Brazil). The MTT [bromide 3-(4,5-dimethyl-2-thiazolyl-2,5-diphenyl)-2H-tetrazolium] salt was purchased from Sigma (St. Louis, MO USA), and the DMEM culture medium was purchased from Gibco BRL (Grand Island, NY USA). All solvents used in this study were of analytical grade. All other chemicals used in this study were available commercially at a reagent grade and were used without further

purification. MilliQ® water (simplicity 185, Millipore, Bedford, USA) was used throughout the study. Parafilm® was purchased from Pechiney Plastic Packaging, Inc., Chicago, USA.

2.2 – Methods

2.2.1 – Liposome preparation

A volume of 10 mL of liposomes was prepared according to the reversed phase evaporation method described by Szoka & Papahadpoulos (1978). Chloroformic aliquots of DOPE, CHEMS, and DSPE-PEG, with molar ratios of 5.7 / 3.8 / 0.5, in a total lipid concentration of 40 mM, were transferred to a bottom flask where the solvent was then removed under vacuum until a lipid film on the walls of the flask had formed. An aliquot of Gd-DTPA-BMA and diethyl ether was added to the lipid solution (aqueous phase / organic phase ratio of 1/3). The film was then dissolved and shaken vigorously in a vortex. The mixture obtained was submitted to a vortex apparatus for 3 minutes, producing an A / O emulsion-type (water in oil).

The emulsion was submitted to evaporation under vacuum to remove the organic solvent, allowing the formation of lipid vesicles. The non-encapsulated Gd-DTPA-BMA was separated from the encapsulated Gd-DTPA-BMA in liposomes, using ultracentrifugation at 150,000 x g, for 1 hour and a temperature of 4°C. The precipitate was re-suspended in an attempt to obtain the same volume it had before the process of ultracentrifugation. The obtained liposomes were calibrated by passing through polycarbonate membranes of 0.4 μm, 0.2 μm, and 0.1 μm, respectively. This procedure was repeated ten times in each membrane.

2.2.2 – Gd-DTPA-BMA labeling procedure

The Gd-DTPA-BMA labeling procedure which was encapsulated into pH-sensitive liposomes was conducted within polystyrene containers and performed using a TRIGA MARK-I IPR-R1 nuclear reactor at the Center for the Development of Nuclear Technology – National Commission on Nuclear Energy (CDTN-CNEN, Brazil). Liposomes samples (1 mL) containing Gd-DTPA-BMA were irradiated at 100 kW, under a thermal neutron flux of 6.6×10^{11} $n.cm^{-2}.s^{-1}$ for 8 hours, reaching an activity of 13.8 ± 1.4 GBq.

To verify whether or not the ^{159}Gd radioisotope had been formed, the gamma spectroscopy technique was conducted in a hyper-pure germanium detector apparatus (Canberra, France) with a resolution (FWHM) of 1.75 keV at 1332 keV, with a 15% relative efficiency. All counts were taken at a constant geometry in a position where the analyzer dead-time indicator registered less than 5%. Decay correction was conducted during the sample count. The software Genie-2000 was used for spectra processing and peak area determinations.

2.2.3 – Liposome characterization

2.2.3.1 – Photon correlation spectroscopy analysis

This analytical procedure allows one to determine the mean diameter of the liposomes and the polydispersity index (P.I.), which is a dimensionless measure of the broadness of the particle size distribution. The analytical procedure was conducted in a Zetasizer HS 3000 (Malvern Instruments, Malvern, UK) apparatus after its adequate dilution in ultra-pure MilliQ® water. The results are expressed as mean ± standard deviation for at least three different batches of each liposome formulation.

2.2.3.2 – Zeta potential analysis

The zeta potential was determined by Laser Doppler Anemometry (LDA) in a Zetasizer HS3000 (Malvern Instruments, Malvern, UK). The samples of liposomes were analyzed following a dilution of 1 / 1000 in 1 mM of NaCl at a conductivity of approximately 120±20 S/cm^2. The results are reported as the mean ± standard deviation of at least three different batches of each liposome formulation.

2.2.3.3 – Atomic force microscopy

The atomic force microscopy technique (AFM) has been widely applied to investigate biological processes, cells, drug carriers, and other soft samples (Kasas *et al.,* 1998). This technique allows one to visualize nanoparticles and liposomes in aqueous systems and in air, thus eliminating sample preparation (Pereira *et al.,* 2008).

The procedure was performed in air at room temperature, using a Dimension 3100, monitored by a NanoScope IIIa controller from Digital Instruments (Santa Barbara, CA, USA). Samples of irradiated liposomes (5 µL) and control (non-irradiated) were deposited on a freshly cleaved mica surface, spread, and partially dried with an argon stream. The images were obtained in a tapping mode using commercial silicon probes, by means of Nanosensors^{TM}, together with cantilevers at a length of 228 µm, with resonance frequencies of 75-98 KHz, spring constants of 3-7 N/m, and a nominal tip curvature radius of 5-10 nm. The scan rate used was 1 Hz. Dimensional analyses were performed using the “section analysis” program of the system. A minimum of ten images from each sample was analyzed to assure reproducible results. The values represent an average ± standard deviation of approximately 40 particle measurements.

2.2.4 – Gd-DTPA-BMA encapsulation efficiency and release profile

Recently, Le & Cui (2006ab) studied the potential use of liposomes that encapsulate gadolinium (Gd-DTPA) by means of the neutron capture therapy technique. The results of these studies showed that the addition of chloride poly-L-lysine (PLL) to the complex increased the encapsulation efficiency of Gd-DTPA while significantly limiting the release of the liposome compound. The present study investigated the encapsulation efficiency of Gd-DTPA-BMA with and without PLL in the liposomes (ratio 2/1 w/w), following the same aforementioned methodology. The separation of non-encapsulated and encapsulated Gd-DTPA-BMA in the liposomes was obtained by ultra-centrifugation at 150,000 x g for 1 hour at 4ºC. The precipitate generated after ultracentrifugation was dispersed within a volume equal to that which had been used before the purification process together with a pH 7.4 HEPES buffer. The liposomes obtained were calibrated by being passed ten times through polycarbonate membranes of 0.4, 0.2, and 0.1 micrometers, respectively. Next, the liposomes containing Gd-DTPA-BMA were irradiated as mentioned above. Triplicate samples containing 5 mL of liposomes were sent for elemental analysis through the ICP-AES technique. The encapsulation efficiency was calculated by dividing the number of moles of encapsulated Gd-DTPA-BMA in liposomes and the number of total moles used in preparing the formulation.

In an attempt to simulate the physiological conditions, the study of Gd-DTPA-BMA release from liposomes was performed either in DMEM (Dulbecco's Eagle modified Medium) or in rat serum at 70%, under sink conditions, at different times. This study followed a protocol based on Assis *et al.,* (2008), which is briefly described below. Samples in triplicate, containing 100 µL of Gd-DTPA-BMA liposomes, with or without PLL, previously purified, and calibrated at 100 nm, were incubated in eppendorf tubes (500 µL) within 400 µL of DMEM or rat serum at 70% for periods of 30 minutes, 1, 2, 3, 8, and 24 hours. After each time period, the samples were ultra-filtered through polycarbonate membranes with a pore size of 30 nm (Millipore®, Brazil). After separation, the concentration of Gd-DTPA-BMA present in the supernatant was analyzed by the ICP-AES technique. The study conducted with rat serum at 70%

attempted to verify the release profile of a Gd-DTPA-BMA compound of liposomes within a real biological medium. The evaluation of the stability of liposomes within a DMEM medium had the purpose of checking the stability of liposomes in common mediums used for *in vitro* studies of antitumor activity.

2.2.5 – in vitro cytotoxic evaluation

The *in vitro* cytotoxic activity of samples containing the non-radioactive complex Gd-DTPA-BMA, the radioactive complex ^{159}Gd-DTPA-BMA, blank liposomes (encapsulating water only), Gd-DTPA-BMA encapsulated into liposomes (Gd-SpHL) and liposomes encapsulated with ^{159}Gd-DTPA-BMA (^{159}Gd-SpHL) were evaluated against RT2 tumor cells (Murine Glioblastoma cells) obtained from American Type Cell Collection (ATCC). The cells were thawed and replicated, and after having reached an adequate confluence, were placed on three 96-well plates (500/well), totaling 120,000 cells. Through serial dilution, with a maximum radioactivity concentration of 650 MBq. mL^{-1}. $well^{-1}$ in a volume of 100 µL, the cells were treated for 48 hours within a CO_2 incubator (5% CO_2 - Pol-Eko Aparatura – ST line) in a humid atmosphere at 37°C and in a culture medium of Dulbecco's modified eagle medium (DMEM) supplemented with sodium bicarbonate (3.7 g / L), penicillin (5,000 units / mL) and 10% w/w sterile fetal calf serum.
A total of eight replicates were used for appropriate statistical evaluation. By means of MTT assay, metabolic feasibility tests were performed to evaluate the efficacy of the treatment employed. The curve comparing the percentage of cell survival to the molar concentration of samples was obtained using the Prism 4.0 software. A non-linear regression model (sigmoidal dose-response option) was used to determine the IC_{50} values.

2.2.6 – Statistics

All experiments were performed in triplicate and expressed as mean ± standard deviation, unless otherwise stated. Mean size and zeta potential data within each time

period were compared by means of the ANOVA test, using the prism 4.0 software and considering a probability of 5% as significant.

3 – Results and discussion

3.1 – Liposome labeling

The isotope ^{159}Gd is not available on the market. Therefore, the only way to produce this isotope is by irradiating a natural gadolinium sample with neutron flux in a suitable nuclear reactor or from a source of ^{252}Cf (Alfassi, 1985). In a previous study, our research group irradiated the Gd-DTPA-BMA complex (Omniscan®) with neutrons to obtain the isotope ^{159}Gd-DTPA-BMA, reaching the conclusion that this complex was stable and that no significant change could be observed after 8 hours of irradiation (Soares *et al.*, 2010).The preparation of liposomes containing radioisotopes involves complex operations, which require licensing from government agencies as well as the handling of radioactive material in liquid form, which is typically performed by highly trained personnel. Faced with these difficulties, our research team formulated liposomes containing the complex Gd-DTPA-BMA by means of neutron activation, in turn obtaining a radioactive ^{159}Gd-DTPA-BMA complex.

Through the previously described procedures, the and Gd-DTPA-BMA encapsulated in liposomes was prepared and irradiated with neutrons for 8 hours. According to Soares *et al*. (2010), when using the same irradiation parameters, the radioactive complex presents a specific activity of 240.8 ± 42 MBq. mg^{-1}. The present study identified a specific activity of 12.6 ± 2.1 MBq. mg^{-1} (value calculated and confirmed by gamma spectroscopy) for the formulation containing the radioactive complex encapsulated in liposomes. The ^{159}Gd radioisotope's characteristic peaks were determined at 58.7, 79.5, 137.5, 305.5, 348.2, and 363.5 keV (Figure 2). The energies and their intensities are consistent with the values published by Moralles *et al.* (1995) and Soares *et al.* (2010).

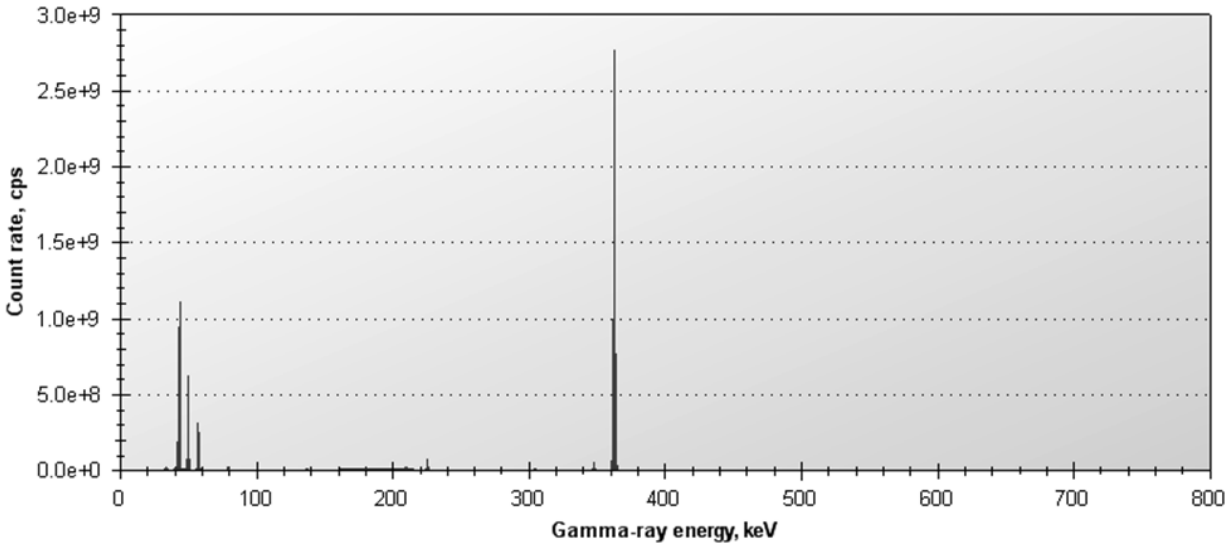


Figure 2 – Gamma spectrum of ^{159}Gd in liposome sample.

3.2 – Liposome characterization

3.2.1 – Photon correlation spectroscopy, polydispersity index, and zeta potential analysis

The photon correlation spectroscopy (PCS) was conducted on 5 samples at different times. The samples were irradiated for 1, 2, 3, 5, and 8 hours, respectively. This procedure evaluated the exposure time in which the liposomes were able to maintain their integrity. The results were calculated from three independent samples by software provided by the manufacturer, which revealed that even after 8 hours of irradiation with neutrons (epithermal, thermal, and fast), the average size of the liposomes were maintained nearly constant at 100 nm (Table 1). These results show that the neutron activation process brought about no changes in the size distribution of the liposomes, thus indicating the maintenance of their physical integrity (Mumper & Jay, 1992; Ostrowsky, 1993).

The polydispersity index (P.I.) found in this study is presented in Table 1. All samples presented a P.I. of above 0.3, including the control. These results indicate that the process used in liposome preparation allows one to obtain a monodispersed system (<0.3) (de Assis *et al.,* 2008; Lamprecht *et al*., 1999; Tobío *et al.*, 1998).

The zeta potential of liposome preparation can aid in predicting the fate of *in vivo* liposomes, given that the presence of electrical charges minimizes agglomeration and fusion phenomenon (Casals *et al.,* 2003). The Zeta potential from the formulation exhibited a negative charge, with values of -53 ± 4.9 mV (Table 1), during the 3 hours of neutron irradiation.

Table 1- Physicochemical characteristics of the liposomes under different times of neutron irradiation

Irradiation time (Hour)	**Mean Size ± S.D. (nm) (PCS)[a]**	**Polydispersity Index[b]**	**ζ potential ± S.D. (mV)[c]**
Control	100 ± 11	0.25 ± 0.01	- 46 ± 8.3
1	99 ± 10	0.18 ± 0.02	- 49 ± 5.5
2	102 ± 12	0.24 ± 0.05	- 47 ± 6.1
3	99 ± 8	0.21 ± 0.01	- 53 ± 4.9
5	100 ± 13	0.24 ± 0.04	- 51 ± 7.3
8	100 ± 22	0.20 ± 0.02	-52 ± 8.1

[a] Standard deviation (n = 3) of the population that was reported by the instrument.

[b] Monodispersed samples (≤0.3).

[c] Measurement after 1:1000 dilution in 1mM NaCl (conductivity, 120±20_S/cm).

The negative zeta potential conveyed by CHEMS was not masked by the presence of PEG chains. In cell biology, the concept of the zeta potential has commonly been used to study many cellular processes, such as activation, agglutination, and adhesion. Cook & Jacobson (1968) pioneered this research by finding significant differences in surface electrokinetic properties between normal and cancer cells. Recently, Zhang *et al.* (2008) reported a significant reduction in the zeta potential values between normal breast epithelial cells (MCF10A) and cancer breast epithelial cells (MCF7), showing an important relationship between the zeta potential and tumor malignancy. Gabizon & Papahadjopoulos (1992) demonstrated that, *in vivo,* negative charges heavily increase the clearance of liposomes due to the interaction of charged phospholipid head-groups with certain opsonizing proteins present in blood circulation. In addition, PEG-liposomes prevent clearance by preventing the opsonization of various serum proteins in the blood (Needham *et al.,* 1992; Shimada *et al.,* 1995).

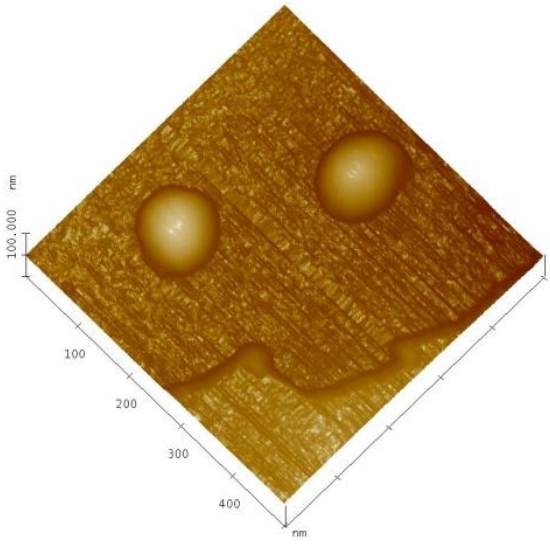
3.2.2 – Atomic Force Microscopy

The environment of a nuclear reactor tends to produce harmful lipid formulations, mainly due to the presence of ionizing radiation capable of promoting lipid oxidation, thereby destabilizing the formulations. The AFM technique was used in this study to compare the morphology of liposomes subjected to the irradiation with neutrons to the groups that were not irradiated (control) so as to verify the physical instability of liposomes after irradiation. The irradiated and control groups underwent the AFM technique as a substrate using a paraffin wax film (Parafilm®). The results showed that the liposomes were not irradiated, isolated spherical structures with an average size of 92 ± 4 nM (Figure 3). The size distribution of this preparation was in fact homogeneous, showing no tendency to disseminate along the substrate surface, thus proving to be a stable formulation.

The liposomes submitted to the irradiation process studied by the AFM technique revealed the same isolated spherical structures with an average diameter of 81 ± 3 nm (Figure 4), indicating that the size of vesicles, as well as their structural integrity, had not

changed during the process of neutron activation. These results aid in evaluating neutron irradiation as a new method for obtaining liposomes which have been radiolabelled directly in a nuclear reactor. Additionally, this methodology allows for a significant reduction in researcher exposure to ionizing radiation, given that there is no laboratory procedure to be conducted after the neutron irradiation process. In this study, the AFM images were obtained after placement and drying of liposomes on a substrate surface. Liposomes, when removed from water after drying on the substrate, may undergo changes, such as losing their internal aqueous content. Figure 4 shows that liposomes have a height of 10nm. This may well be attributed to the height of two lipid bilayers resulting from the loss of internal content.

(A)



(B)

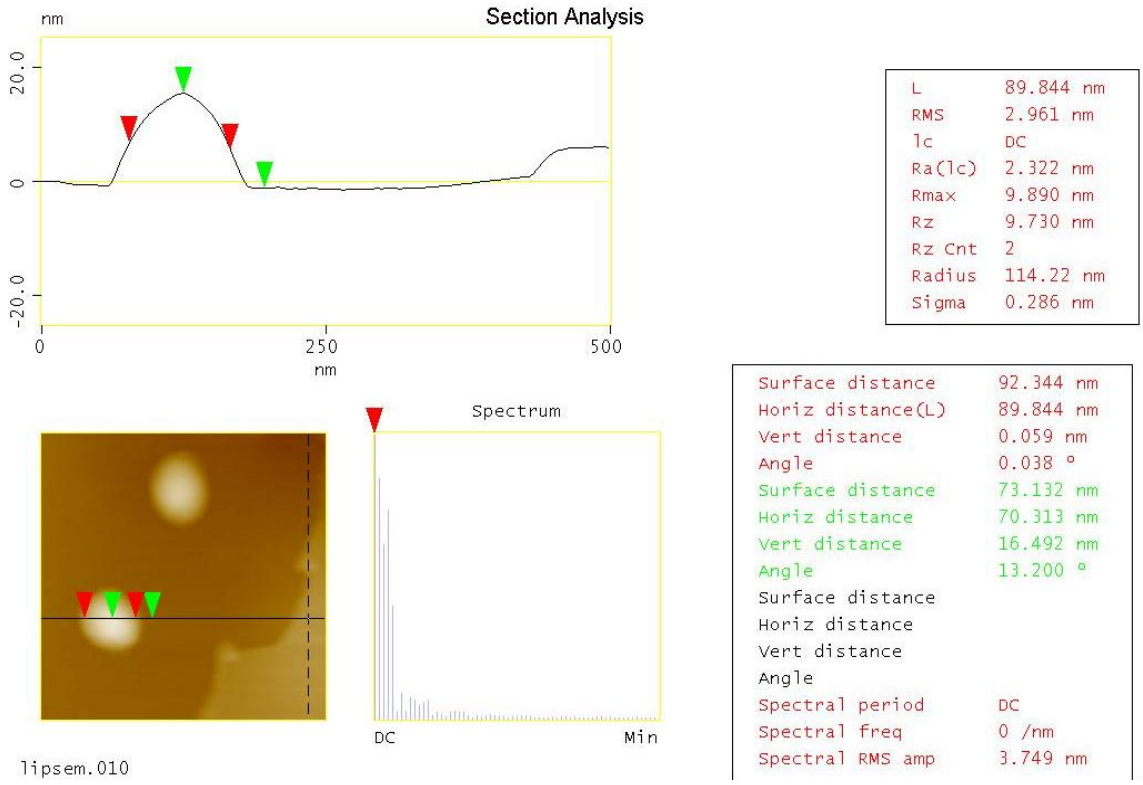
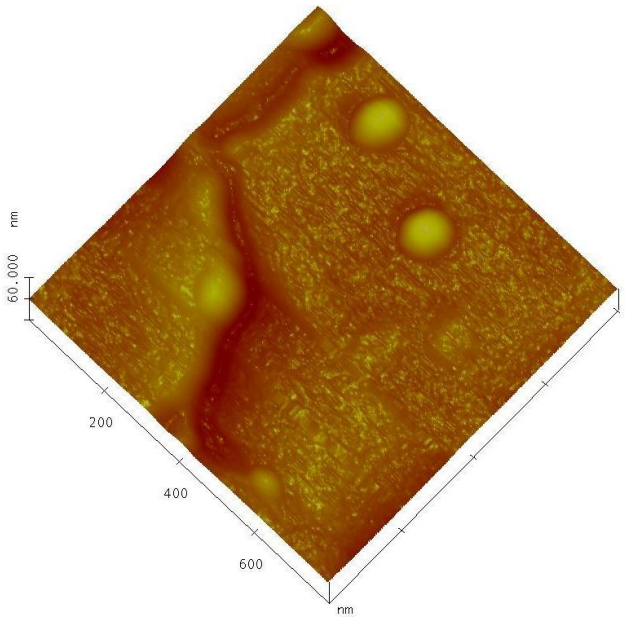


Figure 3 – Atomic Force Microscopy of Liposomes containing Gd-DTPA-BMA (control group) (A) 3D plan and (B) Top view. Using the software option "section analysis", the figure shows liposome height and diameter with gray and black arrows, respectively. The measured sizes are indicated in tables with the same colors.

(A)



(B)

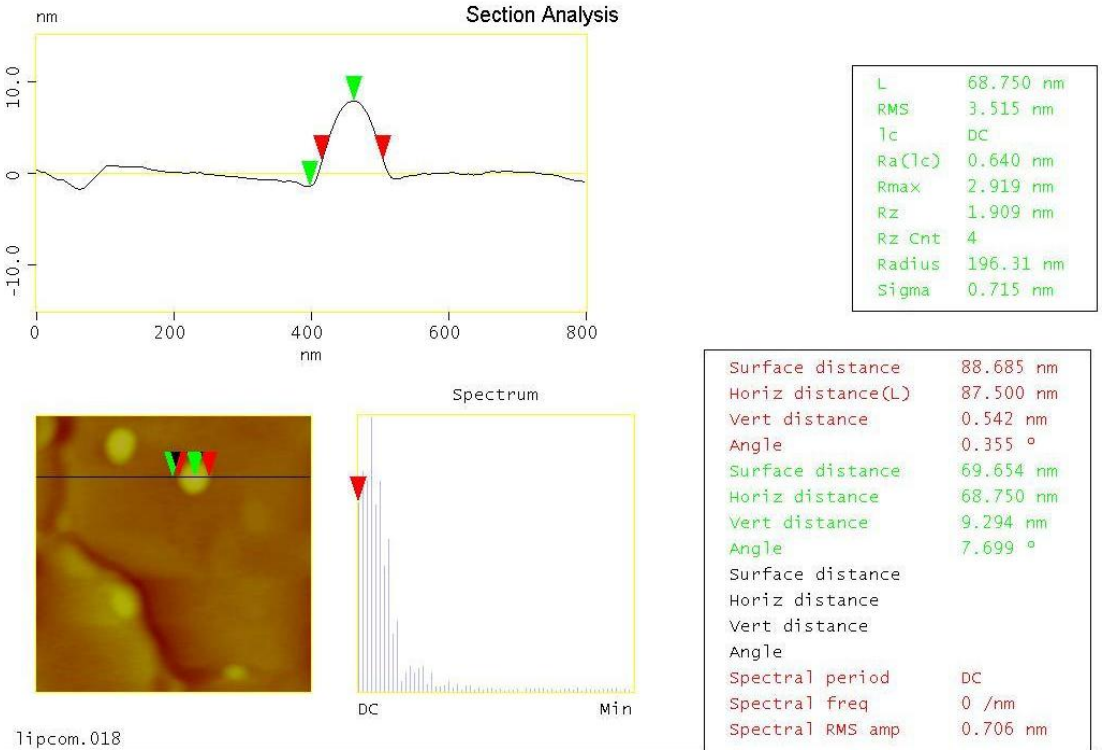


Figure 4 – Atomic Force Microscopy of Liposomes containing ^{159}Gd-DTPA-BMA (irradiated samples) (A) 3D plan and (B) Top view. Using the software option "section analysis", the figure shows liposome height and diameter with gray and black arrows, respectively. The measured sizes are indicated in tables with the same colors.

3.2.3 – Gd-DTPA-BMA encapsulation efficiency and release profile

The encapsulation rates of different substances in the internal compartment, or in the membrane of liposomes, are important parameters that fundamentally depend on the methods of preparation and concentration of the lipid (Frézard *et al.,* 2005). Le (2008) and Le & Cui (2006ab) improved the encapsulation efficiency of the Gd-DTPA complex in different liposomal formulations made up of hydrogenated phosphatidylcholine (HPC), distearoylphosphocholine (DSPC), and distearoylphosphoethanolamine-methoxy-polyethyleneglycol 2000 (MPEG-DSPE) in a respective molar ratio of 50 / 35 / 5, adding the poly-L-lysine (PLL) amino acid to the liposome preparation. With the same purpose, the present study also added the the Poly-L-lyisine amino acid to the Gd-DTPA-BMA complex (1 / 0.25, w/w) during liposome production. However, the results obtained show no significant difference in the encapsulation efficiency between the two formulations (p= 0.7) (Table 2).

Table 2 – Gd-DTPA-BMA encapsulation efficiency in liposomes (n=3)

Statistic Parameter	Liposome	Poly-L-lysine Liposome
Encapsulation Average (%)	19,01	21,72
Standard Deviation	0,92	1,87
Percentual Error	4,77	8,61

The release kinetics of Gd-DTPA-BMA are shown in Figure 5 for the DMEM (Dubelco's Modified Eagle's Medium) culture medium and rat serum at 70%. It can be observed that, after 24 hours, only the release of approximately 2.7% of the formulation without PLL actually occurs. For liposomes containing the PLL formulation, a release of approximately 2.2% occurred. Within 8 hours of incubation, a greater tendency toward stabilizing both thermodynamic formulations occurred. Therefore, it could be observed

that the addition of PLL to the Gd-DTPA-BMA liposome compound did not contribute significantly to reducing the release rate of Gd-DTPA-BMA from these liposomes ($p>0.05$).

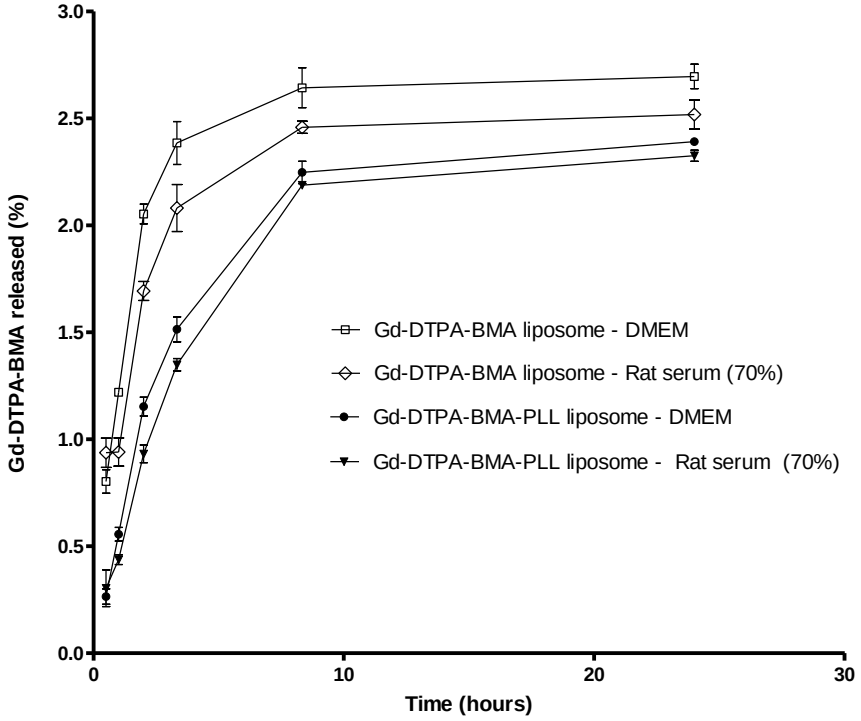


Figure 5 - Kinetics of release of Gd-DTPA-BMA liposomes (100 nM) in DMEM and rat serum at 70% (n=3). The one-way ANOVA analysis followed "Bonferroni's Multiple Comparison Test" show $p>0.05$.

The PLL action mechanism regarding the release of the liposome content is not well explained in the literature. According to Le (2008), the effect of PLL on the release of the gadolinium compound is due to a relative increase in the size of the Gd-DTPA-PLL compound. This finding is supported by Frézard *et al.* (2005), who stated that the larger the size of the substances encapsulated in liposomes, the slower its release. However, it is also emphasized that the Gd-DTPA-BMA compound is sufficiently large, which minimizes the action of poly-L-lysine.

3.2.4 – In vitro cytotoxic evaluation

The cytotoxic activity of the blank liposomes, Gd-DTPA-BMA, Gd-SpHL, and ^{159}Gd-DTPA-BMA were evaluated as a control of the cytotoxic activity of the ^{159}Gd-SpHL formulation (Table 3). The results revealed an IC_{50} of 13.8 mM for blank liposomes. Moreover, lipid concentrations of below 9 mM present a cell feasibility of greater than 90%. From these results, studies aimed at evaluating the cytotoxic activity of the complex within radioactive liposomes were conducted with a lipid concentration below 9 mM. Thus, the cytotoxic activity was removed from the lipids in the formulation.

Table 3- IC_{50} of control samples within RT2 cells

IC_{50} values within RT2 cells (mM) n=8				
Blank Liposomes	**Gd-DTPA-BMA**	**Gd-SpHL**	**^{159}Gd-DTPA-BMA**	**^{159}Gd-SpHL**
13.8 ± 0.4	19.9 ± 0.9	0.066 ± 0.015	0.12 ± 0.01	0.017 ± 0.002

The Gd-DTPA-BMA complex has a cytotoxic activity that acts against RT2 tumor cells. However, within the liposomes, the cytotoxic activity is increased by approximately 300 fold (19.9 mM to 0.066 mM). As the Gd-DTPA-BMA complex is quite hydrophilic, its absorption in the intracellular environment has proven to be relatively insignificant, which may well explain the low cytotoxicity observed in RT2 cells. However, when encapsulated in liposomes, an increase in the cytotoxic activity of the complex could be observed. This behavior can be explained by the fact that the liposomes are structures that allow the intracelular internalization of their contents, in turn allotting greater potential to the cytotoxic effect (Miller *et al.,* 1998; Lukyanov *et al.,* 2004).

By means of ionizing radiation, the cytotoxic activity of the free complex is incremented approximately 166 fold. For ^{159}Gd-SpHL, an IC_{50} value of 0.017 mM (Figure 6) could be observed, which is considered to be of the same magnitude as some cytotoxic agents used in cancer treatment. Comparing this figure to the IC_{50} of free and non-radioactive complexes it can be observed that the sum of factors – presentation form of tumor cells and radiation presence – allows for an increase of approximately 1,170 times the cytotoxic activity of metal complex.

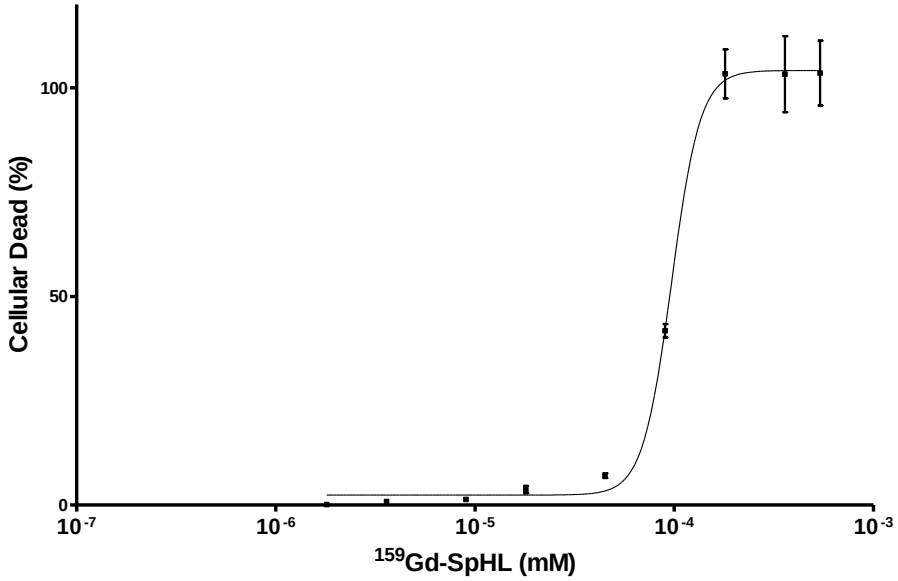


Figure 6 - Representative curves of surviving RT2 cells vs. the log of ^{159}Gd-SpHL molar concentration. The IC_{50} value was determined through non-linear regression (n=8).

The absorbed dose rate in RT2 cells was estimated for the IC_{50} concentration using Martin's mathematic model for beta dosimetry on contaminated skin surfaces (Martin, 2006). The results showed that the absorbed dose rate value is approximately 8.5 $Gy.h^{-1}$ in RT2 cell cultures. It can therefore be concluded that the p53 activation mechanism may be used to explain the cytotoxic activity of radioactive Gd-DTPA-BMA used against RT2 cells.

Prior studies have pointed out the development of nephrogenic systemic fibrosis (NSF) in groups of patients with severe chronic kidney disease, through the use of free Gd-DTPA-BMA (Omniscan®). However, no study has demonstrated the relationship between the use of Gd-DTPA-BMA encapsulated in liposomes as regards the development of NSF. Carvalho Junior *et al.* (2007) demonstrated that the encapsulation of cisplatin (CDDP) in liposomes (SpHL) tends to lead to a reduction in CDDP retention via renal tissues, indicating that the SpHL-CDDP may indeed be useful in alleviating kidney damage induced by CDDP. For this reason, in patients with severe chronic kidney disease, the possible use of the ^{159}Gd-SpHL formulation, geared toward treating cancer, still warrants further investigation.

4 – Conclusion

The findings in this study revealed that during and after the process of neutron irradiation (labeling process), the liposomes maintained similar physicochemical characteristics in terms of size and zeta potential. After the labeling procedure, the vesicles were also considered to be stable from a morphological point of view and showed low release kinetics in their contents' biological environment. This finding is quite suitable for systems that selectively accumulate radioisotopes within target tissues (e.g. tumor cells), in detriment of healthy tissues, which leads to the need for the safe use of this formulation in *in vivo* studies. The present results, together with further studies, may reveal a potential application of pH-sensitive liposomes containing a therapeutic radioisotope for cancer treatment.

5 – Acknowlegments

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CAPÍTULO 3

Liposomes Radiolabeled with ^{159}Gd: *In Vitro* Antitumoral Activity, Biodistribution Study and Scintigraphic Image in Ehrlich Tumor Bearing Mice.***European Journal of Pharmaceutical Sciences,vol. 43, 290-296, 2011.***

Liposomes Radiolabeled with ^{159}Gd: *in vitro* Antitumoral Activity, Biodistribution Study and Scintigraphic Image in Ehrlich Tumor Bearing Mice

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Abstract

PEG-coated pH-sensitive and PEG-folate-coated pH-sensitive liposomes containing the Gd-DTPA-BMA complex were prepared and radiolabeled by neutron activation. The radiolabeled liposomes presented significant *in vitro* cytotoxic activity against Ehrlich tumor cells when compared to controls. The biodistribution profile of these liposomes and free ^{159}Gd-DTPA-BMA were studied in mice bearing a previously-developed solid Ehrlich tumor. The results demonstrated an important uptake of the formulations by the tumor tissue, with a tissue/blood partition coefficient (Kp) 3.88 and 14.16 times higher than that of the free complex for pH-sensitive PEG-coated and PEG-folate-coated liposomes containing the ^{159}Gd-DTPA-BMA complex, respectively. Both formulations accumulated in the liver and spleen, thereby revealing some difficulty in escaping the action of the MPS cells. The formulation without folate presented a lower renal uptake, which is desirable in patients with chronic renal failure because of the potential risk of nephrogenic systemic fibrosis (NFS). The scintigraphic study revealed that the target/non-target ratio is always greater than three for pH-sensitive PEG-coated liposome formulations and above nine for pH-sensitive PEG-folate-coated liposome formulations. The results obtained in this study demonstrated that the formulations employed can be considered to be a potential alternative for the treatment of cancer, including patients with chronic renal failure.

Keywords: Gadolinium-159 liposomes, in vitro antitumoral activity, Biodistribution study, Scintigraphic Image

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1 – Introduction

Different therapeutic radiopharmaceuticals containing the ^{32}P, ^{188}Re, ^{89}Sr, and ^{90}Y radioisotopes have been used effectively in alleviating bone pain resulting from metastases, synovectomy, and tumors (Vallabhajosula, 2001; Ferro-Flores & Murphy, 2008). These radioisotopes all present the ability to emit high doses of beta radiation, leading to death of tumor cells. Similarly, the ^{159}Gd isotope emits a negative beta (1001 keV) and gamma (main energy: 363.54 keV) radiation suitable for therapeutic applications in nuclear medicine according to Saha (1998). *In vitro* studies conducted by Soares *et al.* (2010) showed that free ^{159}Gd-DTPA-BMA has a high *in vitro* cytotoxicity against Ehrlich tumor cells. However, when administered internally, alone,this radioisotope does not accumulate in target tissues. Thus, to meet these requirements, different pharmaceutical formulations constituted of nanostructured carriers such as liposomes have been proposed to reduce the toxicity to non-target organs, especially in patients with chronic renal insufficiency (Thomsen, 2006), while increasing the effective concentration and contact time in target tissues (Vemuri and Rhodes, 1995; Tokumitsu *et al*., 2000; Watanabe *et al.,* 2002; Le & Cui, 2006ab).

The *in vivo* behavior of "classical or conventional" liposomes showed that they accumulate rapidly in cells of the monocytic phagocyte system (MPS), especially in the liver, spleen and bone marrow (tissues rich in macrophages) and are quickly eliminated. This elimination limits their use in other tissues. Seeking to overcome these problems, advances in the biophysics of membranes have permitted the development of liposomes with a modified surface so that the vesicles undergo opsonization of serum proteins to a

lesser extent, leading to a low degree of recognition by cells of the MPS. These liposomes are referred to as "stealth", "long circulating" or "sterically stabilized" and represent an important step forward for *in vivo* applications because of the ability to evade the action cells of the MPS, and they have a longer circulation time in the bloodstream that facilitates the selection of the tumor regions that are targeted Since the pH of oncogenic tissues is lower than that observed in normal tissues, the use of pH-sensitive liposomes has been suggested as a new therapeutic strategy (Carvalho Junior *et al.,* 2007). This system is capable of releasing, for example, an antineoplasic drug directly into the tumor, thereby decreasing the absorption by non-target tissues. In studies conducted by our group, a stable radiolabeled gadolinium (^{159}Gd-DTPA-BMA) complex suitable for *in vitro* studies in stealth PEG-coated pH-sensitive liposomes (^{159}Gd-SpHL) was produced using a neutron-activation technique in a nuclear reactor and was successfully characterized. In addition, the ^{159}Gd-SpHL presented an *in vitro* cytotoxic activity 1170 times greater than that of the free complex in RT2 tumor cells (mouse glioma) (Soares *et al.,* 2011*).*

Several tumor cell lines (OVCAR-3, IGROV1, SKOV3, ovarian cancer; SW626, human adenocarcinoma and Ehrlich) express receptors for folic acid on their cell surfaces. Thus, seeking better targeting results for the application of drugs and radioisotopes in tumor tissues, studies have been conducted that involved the immobilization of molecules of folic acid on the surface of liposomes and other nanostructures to increase the specificity of liposome action in interacting with receptors located on the surface of tumor cells (Sikora & Grzelakowska-Sztabert, 1984; Parker *et al*., 2005; Gosselin & Lee, 2002; Sen *et al.*, 1996). In the present work, the preparation of pH-sensitive PEG-coated and PEG-folate-coated liposomes containing the ^{159}Gd-DTPA-BMA complex, the characterization of the *in vitro* cytotoxic activity of these formulations against Ehrlichtumor cells, and a biodistribution study and scintigraphic image in mice bearing a solid Ehrlich tumor were performed.

2 – Experimental

2.1 – Materials

The Gd-DTPA-BMA complex, commercially known as Gadodiamide (Omniscan® - General Electric Healthcare Company), was purchased from FARMASA (São Paulo, Brazil). The lipids dioleylphosphatidylethanolamine (DOPE), distearoylphosphatidylethanolamine-polyethyleneglycol 2000 (DSPE-PEG_{2000}) and cholesteryl hemisuccinate (CHEMS) were purchased from LipoidGmbH (Ludwigshafen, Germany) and Sigma (St. Louis, USA), respectively. The DSPE-PEG-Folate lipid was purchased from Avanti Polar Lipids (Alabaster, USA). The MTT [3-(4,5-dimethyl-2-thiazolyl-2,5-diphenyl)-2H-tetrazolium bromide] salt was purchased from Sigma (St. Louis, MO USA), and the DMEM culture medium was purchased from Gibco BRL (Grand Island, NY USA). All the solvents used were analytical grade, and the other chemicals were commercially available reagent grade and were used without further purification. MilliQ® water (simplicity 185, Millipore, Bedford, USA) was used throughout. For quantification of caspase-3, the *CleavaLite®Caspase 3Activity Assay Kit* was purchased from Millipore (Billerica, USA).

2.2 – Methods

2.2.1 – Liposome Preparation

The pH-sensitive PEG-coated and PEG-folate-coated liposomes containing the Gd-DTPA-BMA complex (Gd-SpH and Gd-FTSpHL, respectively) were prepared according to the procedure of Szoka and Papahadjopoulos, (1978). For the Gd-SpHL formulation, the DOPE, CHEMS, and DSPE-PEG2000 lipids were dissolved in chloroform (lipid concentrations of 20, 30, and 40 mM and molar ratio of 5.7:3.8:0.5) and transferred to a round-bottom flask where the solvent was removed under vacuum until a lipid film formed on the walls of the flask. An aliquot of Gd-DTPA-BMA in diethyl ether was added to the lipid solution (ratio of aqueous phase/organic phase = 1/3). The film was dissolved

and stirred vigorously on a vortex stirrer. The mixture obtained was stirred on a vortex stirrer for 3 minutes, producing an W/O type emulsion (water in oil). The emulsion was submitted to evaporation under vacuum to remove the organic solvent, resulting in the formation of lipid vesicles. The non-encapsulated Gd-DTPA-BMA was separated from the encapsulated Gd-DTPA-BMA by ultracentrifugation on a Sorvall Ultra 80 ultracentrifuge (Albertville, USA) at 150,000 x g for 1 hour at 4 °C. The precipitate was re-suspended in the same volume it had before the ultracentrifugation. The obtained liposomes were calibrated by passing through polycarbonate membranes having pore diameters of 0.4 μm, 0.2 μm, and 0.1 μm. This procedure was repeated ten times with each membrane.

Following this same method, the formulation Gd-FTSpHL, were prepared by chloroform-aliquots of DOPE, CHEMS, DSPE-PEG_{2000} and DSPE-PEG_{2000}-folate, molar ratio of 57 / 38 / 4.5 / 0 5 in a total lipid concentration of 40 mM. The ratio between DSPE-PEG_{2000} and DSPE-PEG_{2000}-folate was 1/0.1 and adopted according to studies conducted by Gabizon *et al.,* (1999).

2.2.2 – Gd-DTPA-BMA labeling procedure

The procedure for labeling the Gd-DTPA-BMA complex encapsulated in liposomes was performed in polystyrene containers in a TRIGA MARK-I IPR-R1 nuclear reactor at the Division for Radiation Technology, Nuclear Technology Development Centre/National Commission for Nuclear Energy (CDTN-CNEN, Brazil). Liposome samples (1 mL) containing Gd-DTPA-BMA were irradiated at 100 kW with a thermal neutron flux of 6.6 x 10^{11} $n.cm^{-2}.s^{-1}$ for 8 hours, reaching an activity of 13.8 ± 1.4 GBq.

To verify whether the ^{159}Gd radioisotope had been formed, the gamma spectroscopy procedure was conducted in a hyper-pure germanium detector (Canberra, France) with a resolution (FWHM) of 1.75 keV at 1332 keV and a 15% relative efficiency. All counts were obtained at a constant geometry in a position where the analyzer dead-time indicator registered less than 5% decay correction. The Genie-2000 software was used for processing of spectra and determination of peak area.

2.2.3 – In vitro cytotoxic evaluation

In previous studies conducted by Soares *et al*. (2010), the IC_{50} values of free 134 mM PA-BMA and 1.4 mM ^{159}Gd-DTPA-BMA were determined against the Ehrlich tumor model. In the present study, the *in vitro* cytotoxic activity of samples containing blank liposomes (encapsulating HEPES buffer), PEG-coated (^{159}Gd-SpHL) and PEG-folate-coated (^{159}Gd-FTSpHL) pH-sensitive liposomes were determined using the same tumor model. The Ehrlich tumor cells were obtained from ascites tumors previously induced in Swiss mice. Thecells were extracted at the appropriate time for tumor development in animal donors and placed in a 96-well culture plate (500/well), totaling 120,000 cells. After serial dilution to obtain a maximum radioactivity concentration of 650 $MBq.mL^{-1}.well^{-1}$ in a volume of 100 µL, the cells were treated for 48 hours (5% CO_2 - Pol-Eko Aparatura – ST line) in a humid atmosphere at 37 °C in a CO_2 incubator. A culture medium of Dulbecco's modified eagle medium (DMEM) supplemented with sodium bicarbonate (3.7 g/L), penicillin (5,000 units/mL) and 10% w/w sterile fetal calf serum was employed. A total of eight replicates were used for appropriate statistical evaluation. By means of an MTT assay, metabolic feasibility tests were performed to evaluate the efficacy of the treatment. The IC_{50} curve comparing the percentage of cell survival to the molar concentration of samples was obtained by employing a non-linear regression model (sigmoid dose-response option) using the GraphPad Prism, version 5.0 software. Data were analyzed by a two-way ANOVA,. and the means were compared by applying the Bonferroni test (Post-hoc), where $P \leq 0.05$ was considered to be statistically significant.

2.2.4 – Ehrlichsolid tumor development

One milliliter of the ascitic fluid containing viable Ehrlich tumor cells was transfered to a 50 mL Falcon tube. The Ehrlich cells were centrifuged at 3000 x g for 5 minutes. The supernatant was removed and sediment was dissolved in sufficient saline solution (0.9 %) to obtain the original volume. This process was repeated, and the cells were stained

with a solution of Trypan blue (0.4 %) and counted using a Newbauer chamber with a microscope with a magnification of 400 X.

A suspension of viable cells with an average density of 5.0 x106 cells/mL was prepared for the development of the Ehrlich solid tumor and a 100-µL volume of this suspension was implanted in the right flank of female Swiss mice having approximately 25 g body mass (n = 36). The mice were kept in an area with controlled lighting and had free access to food and water. Twenty days after implantation, the solid Ehrlich tumor was visible and palpable.

All the protocols involving animal experiments in this study were approved by the Ethics Committee on Animal Experimentation of the Federal University of Minas Gerais (CETEA) with the code number 019/09 and comply with the requirements of the Guide for Care and Use of Laboratory Animals recommended by the Institute of Laboratory Animal Resources (USA).

2.2.5 – Determination of Apoptosis in Ehrlich tumor Cells

The test was performed by the method of the CleavaLite® Caspase 3 Activity Assay Kit (Millipore). For this study, Ehrlich cells were placed in 96-well culture plates and incubated for 24 hours for adhesion. Then, 3.0 x 10^6 cells were divided into three different groups, each containing 1.0 x 10^6 cells. The cells of Group 1 (the control group) were treated with saline. The cells of Group 2 and 3 were treated with Gd-DTPA-BMA and 159Gd-DTPA-BMA at 1.0 x10-1 mM and 1.0 x10^{-3} mM, respectively. After the treatment period, the cells of group 2 and group 3 were lysed with 500 µL of lysis buffer provided by the manufacturer of the kit and incubated in an ice bath for a period of 10 minutes.

The cells were centrifuged at 10,000 rpm (9,600 x g) for 5 minutes at 4 °C and the supernatant (cytosolic extract) was transfered to an Eppendorf tube. This cytosolic extract was used to test the activity of caspases 3. A 20-µL volume of extract was

incubated with 100 µL of assay buffer (supplied in the kit) in the presence or absence of caspase 3 inhibitor. The samples were then transfered and incubated at 37 °C in a 96-well microplate for 1 hour. The concentration of pNA (p-nitroaniline) formed was monitored in a 96-well card reader at 405 nm, and compared with the control group using a previously-standardized reference curve of optical density versus concentration of pNA.

2.2.6 – BiodistributionStudies

Biodistribution studies were conducted using two different protocols: (1) sacrifice and subsequent analysis of organs; (2) scintigraphic study and subsequent sacrifice. The studies were conducted based on the presence of radioactive gadolinium in the formulations, and the animals received implants of Ehrlich solid tumors with an average development time of 20 days prior to the study.

2.2.6.1 – Sacrifice and organs collection

Samples of ^{159}Gd-DTPA-BMA (100 µL; 0.5 mmol/mL), ^{159}Gd-SpHL and ^{159}Gd-FTSpHL (236 mg/kg) were injected intravenously into the caudal artery of Swiss mice. After periods of 10 minutes and 1, 8 and 24 hours, each animal (three per group) was anesthetized intraperitoneally with a mixture of ketamine and xylazine at a dose of 100 mg/kg and 8 mg/kg, respectively. The blood was collected immediately by cardiac puncture, and the mice were sacrificed by cervical dislocation. The lungs, stomach, spleen, liver, kidneys, and solid Ehrlich tumor were collected, washed with distilled water, dried on filter paper, and weighed. The determination of radioactivity present in the organs was achieved by automatic scintillation (ANSR-Abott, USA). The readings were conducted at the 350-380 keV energy window for 4 minutes. The radioactivity contained in each organ was divided by its mass to furnish the radioactivity in counts per minute per gram of tissue (cpm/g). To fix the radioactive decay of 159Gd, a separate tube with the standard dose containing the same activity and volume of the dose injected into animals was defined as 100% of radioactivity. The ratio %ID/g of tissue

was obtained from the relationship between the radioactivity measured in tissue and radioactivity measured in the standard. The data were statistically analyzed by analysis of variance (ANOVA) using PRISM 5.0 software. The tissue-blood partition coefficient (Kp) was determined by dividing the area under the tissue concentration vs time curve by the area under the blood concentration vs time curve.

2.2.6.2 – Scintigraphicbiodistribution study

For the scintigraphic study, ^{159}Gd-SpHL and ^{159}Gd-FTSpHL with 125 MBq activity were produced to obtain a minimum activity sufficient to acquire the images. The animals were anesthetized intraperitoneally with a solution of ketamine and xylazine at a dose of 40 mg/kg and 5 mg/kg, respectively, and then placed on the table in a supine position so that the detector was positioned on the anterior region of the animal. The images were obtained with a gamma camera with a collimator-type High Energy General Purpose (HEGP) 128 x 128 x 8 array and an acquisition time of 900 seconds. Nine animals were used, with the Ehrlich tumor implanted in the back of the left thigh. Images were obtained from the same animals eight hours after administration of 11 MBq of radiolabeled sample.

3 – Statistical analysis

The results were calculated and presented as the mean for each group ± standard error of the mean (mean ± S.D.). Statistical evaluation of the data was performed using analysis of variance (ANOVA), followed by the Bonferroni's test (Post-hoc), where $P \leq 0.05$ was considered to be statistically significant.

4 – Results and discussion

4.1 – Liposome labeling

The ^{159}Gd isotope is not available on the market. Therefore, the only way to produce this isotope is by irradiating a natural gadolinium sample with a neutron flux in a suitable

nuclear reactor or by obtaining it from a ^{252}Cf source (Alfassi, 1985). In a previous study, the Gd-DTPA-BMA complex was irradiated with neutrons to obtain the ^{159}Gd-DTPA-BMA isotope. It was concluded that this complex was stable and that no significant change in the structure of the complex could be observed after eight hours of irradiation (Soares *et al.*, 2010a). The preparation of liposomes containing radioisotopes involves complex operations that require licensing from government agencies as well as the handling of radioactive material in liquid form, which is typically performed by highly trained personnel. Faced with these difficulties, Gd-SpHL was prepared in a previous study and irradiated with neutrons during eight hours to produce a radioactive liposome (^{159}Gd-SpHL) (Soares *et al.* 2011). The results showed that the neutron activation process resulted in no changes in the size distribution, Zeta potential, and morphology of the liposomes, thus indicating that their physical integrity was maintained (Mumper & Jay, 1992; Ostrowsky, 1993; Soares *et al.* 2011).

In the present study, Gd-SpHL and Gd-FTSpHL were irradiated for eight hours using the same procedures described above. The characteristic peaks of the ^{159}Gd radioisotope were determined at 58.7, 79.5, 137.5, 305.5, 348.2, and 363.5 keV, and the specific activity of 12.6 ± 2.1 MBq.mg^{-1} was calculated and confirmed through gamma spectroscopy. The energies and their intensities were consistent with the values published by Soares *et al.* (2011).

4.2 –*In vitro* cytotoxic study

The cytotoxic activity of the blank liposomes and the Gd-SpHL were evaluated as a control of the cytotoxic activity of the ^{159}Gd-SpHL formulation. The results revealed an IC_{50} of 13.8 ± 0.4 mM for blank liposomes (Figure 1). Research published since the 1970s demonstrate the cytotoxic activity of liposomes (Panzner & Jansons, 1979; Cortesi *et al.*, 1996).

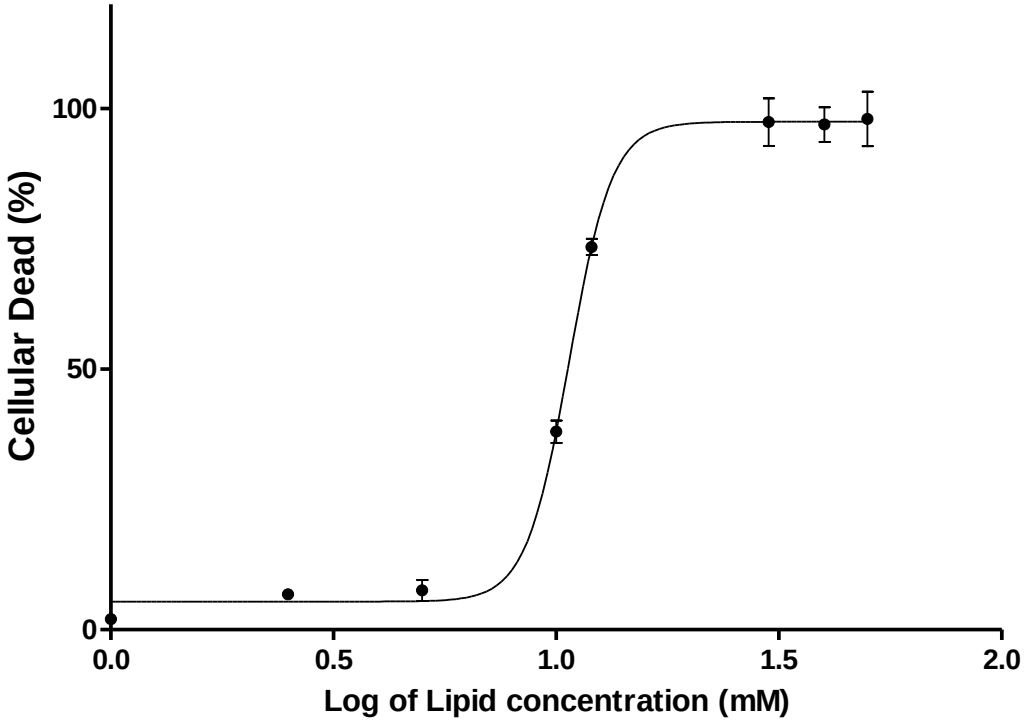

Figure 1 – Cytotoxity of blank liposomes in Ehrlich cells (n = 8).The curve fit presents R^2= 0.981 and degrees of freedom = 30.

Because of the similarity of chemical structures in the cell membranes and liposomes, fluidization phenomena and destabilization of the cytoplasmic membrane can be observed that lead to apoptosis, especially in tumor cells. Furthermore, the results also showed that the use of blank liposomes containing a total lipid concentration lower than 9 mM lead to a viability of the Ehrlich cells of over 90% (data not presented). Therefore, lipid concentrations lower than 9 mM were employed for all the experiments, thereby avoiding the contribution of the phospholipid constituents of the preparations to the cytotoxic activity of formulations containing the Gd-DTPA-BMA.

IC_{50} values of 0.39 ± 0.4 mM and 0.024 ± 0.004 mM, respectively, were observed for Gd-SpHL and ^{159}Gd-SpHL samples,. The radioactivity caused a 16-fold enhancement of

the cytotoxic activity of the formulation against Ehrlich tumor cells. By comparing these results with data published by Soares *et al.* (2010), where Gd-DTPA-BMA and ^{159}Gd-DTPA-BMA presented IC_{50} values of 134 mM and 1.4 mM, respectively, it can be verified that, regardless of the form used (radioactive or not), the encapsulation of Gd-DTPA-BMA in liposomes enhanced the *in vitro* cytotoxic effect against the Ehrlich tumor model. Furthermore, when comparing the results for the IC_{50} values of Gd-DTPA-BMA and ^{159}Gd-SpHL, the radioactivity was observed to supplement the action of the liposomes as carriers and lead to a 5,580-fold increase in the cytotoxic activity.

4.3 – A study of apoptosis in Ehrlich cells

The determination of caspase-3 activity in Ehrlich cells was employed in a study of the apoptosis process in Ehrlich cells. The results for the activityofcaspase-3 inthreegroups of samplesis presented in Figure2. These resultsrevealedthat thepresence of a non-radioactivemetal complexdid notalterthe activityof caspase-3. However, there wasasignificant increase in the caspase-3 group, when comparedtocontrol cells, when they were under the influence of^{159}Gd-DTPA-BMA, indicating that apoptosis is probably the principal mechanism of death suffered by the cells.

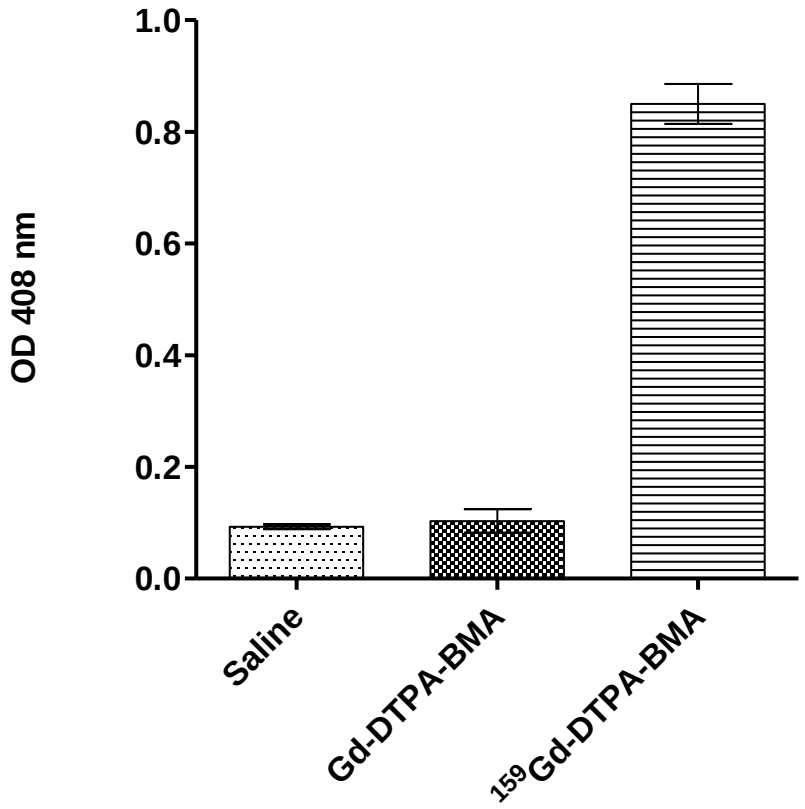

Figure 2 - Caspase-3 activity in Ehrlich cells treated with saline, Gd-DTPA-BMA and ^{159}Gd-DTPA-BMA. Values Expressed in Optic Density ± Standard Deviation.

Although malignant tumor cells exhibit various characteristics, evidence shows that resistance to apoptosis is a distinguishing characteristic of most malignant tumors and is an important goal of therapeutic approaches (Okada & Mak, 2004). Studies indicate that the p53 protein has a central role in regulating the G1 phase checkpoint in response to DNA damage (Millau *et al.*, 2008). The relationship between p53 protein and carcinogenesis has been widely confirmed by the high rate of mutation of the gene in malignant tumors from different tissues (Khan *et al.*, 2000, Baker *et al.*, 1990). Experimental procedures revealed that ionizing radiation can promote the activation of p53 protein, thereby increasing its concentration in the cell nucleus and leading to apoptosis. These observations have greatly contributed to the elucidation of the

mechanism of action of ionizing radiation against tumor cells (Burger *et al.*, 1998; Choi *et al.*, 2004; Zambetti, 2005). The migration of pro-apoptotic proteins (BAX and BID) from the cytoplasm to the mitochondria can occur in the presence of intracellular stress, such as exposure to ionizing radiation from ^{159}Gd, thereby altering the permeability and allowing the release of other pro-apoptotic proteins as well as activation of caspase-9. This activation activates the caspase-3 effector identified in this study. These results suggest that the cytotoxic effect triggered by ^{159}Gd-DTPA-BMA is associated with activation of apoptotic pathways that lead cells to Ehrlich death.

4.4 – Biodistribution Studies

4.4.1 – Sacrifice Biodistribution Study

The results of sacrifice biodistribution studies are shown in Figure 3. The values encountered in the lungs and stomach were suppressed because these values were lower than 0.01% ID/g and were considered negligible. For the other organs investigated (blood, liver, spleen and kidneys), a rapid blood clearance within one hour after the administration was observed for ^{159}Gd-DTPA-BMA. This result is in agreement with those presented in the monograph of the drug (GE Healthcare, 2006). The values observed for the formulations after one hour were significantly higher than those found for the free drug. The values for the area under the curve (AUC) were 6.55 and 5.81 times higher for the ^{159}Gd-SpHL and ^{159}Gd-FTSpHL formulations, respectively. Thus, the circulation time of the formulations in the bloodstream (Tables 1 and 2) is greater. The biodistribution studies of the liver and spleen showed that the concentration of free drug was lower than those of the formulations. According to Zong *et al*. (2008), the Gd-DTPA-BMA complex does not undergo hepatic metabolism and is eliminated mainly in the form of the original complex. This fact could explain the low observed hepatic uptake. Moreover, it is known that liposomes are taken up by cells of the MPS, which are widely present in the liver and spleen, although the formulations have PEG or PEG-folate chains.

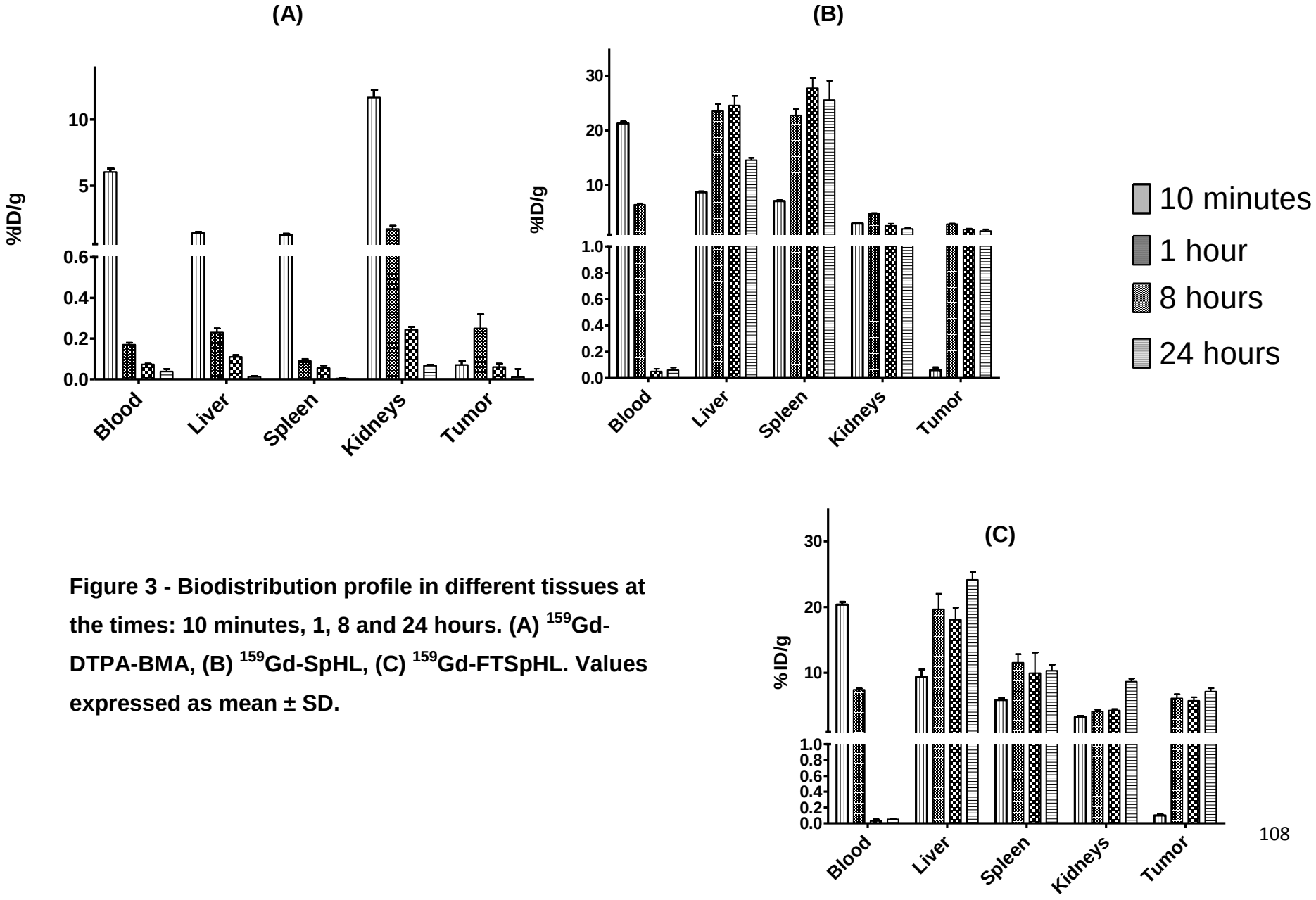

Figure 3 - Biodistribution profile in different tissues at the times: 10 minutes, 1, 8 and 24 hours. (A) ^{159}Gd-DTPA-BMA, (B) ^{159}Gd-SpHL, (C) ^{159}Gd-FTSpHL. Values expressed as mean ± SD.

Table1 – Partition coefficient tissue/blood (Kp) in mouse different tissues after I.V. administration of ^{159}Gd-DTPA-BMA and ^{159}Gd-SpHL

Tissue	AUC ^{159}Gd-DTPA-BMA (A)	AUC ^{159}Gd-SpHL (B)	Kp (A)	Kp (B)	Kp(B)/Kp(A)
Blood	4,27	27,98			
Liver	2,86	495,00	0,67	17,69	26,44
Spleen	1,55	615,20	0,36	21,99	60,45
Kidneys	14,95	57,45	3,50	2,31	0,66
Tumor	1,79	45,52	0,42	1,63	3,88

Kp calculed from ratio $AUC_{0\text{-}24h}$/tissue and $AUC_{0\text{-}24h}$ blood.

Table 2 – Tissue/blood partition coefficient (Kp) in different tissues of the mouse after I.V. administration of ^{159}Gd-DTPA-BMA (A) and ^{159}Gd-FTSpHL (B)

Tissue	AUC ^{159}Gd-DTPA-BMA (A)	AUC Gd-FTSpHL (B)	Kp (A)	Kp (B)	Kp(B)/Kp(A)
Blood	4,27	24,81			
Liver	2,86	481,60	0,67	19,41	29,01
Spleen	1,55	244,50	0,36	9,85	27,10
Kidneys	14,95	139,20	3,50	4,97	1,42
Tumor	1,79	147,30	0,42	5,93	14,16

Kp calculed from ratio $AUC_{0\text{-}24h}$ /tissue and $AUC_{0\text{-}24h}$ blood.

There was a significantly higher uptake of the ^{159}Gd-FTSpHL formulation by the liver than the formulation without folate after 24 hours. This observation can be explained by the fact that the liver has cells that express receptors for folic acid (FR) (Parker *et al*., 2005). The formulation containing folate had a lower uptake by the spleen than that of

the ^{159}Gd-SpHL formulation. It is known that the addition of hydrophilic polymers to the surface of liposomes results in a lower serum protein opsonization of the vesicles, thus reducing the uptake by cells of the MPS. It is believed that the addition of folic acid, a very hydrophilic molecule, to the surface of liposomes contributed to further reduce the process of opsonization and lead to a lower uptake by cells of the MPS. This fact could explain the results for uptake by the spleen (Torchilin, 2006). All the results obtained for the biodistribution of the formulations in the liver and spleen are in agreement with the findings of Carvalho Junior *et al*. (2007). The biodistribution values for free ^{159}Gd-DTPA-BMA are also consistent with the results published by Tweedle *et al.* (1995).

Similar to the results obtained for the hepatic uptake, a significant increase in renal uptake of ^{159}Gd-SpHL and ^{159}Gd-FTSpHL during a 24-h period was observed. These results revealed that this is an important elimination route for the two formulations. However, the increased uptake observed for the ^{159}Gd-FTSpHL formulation concerning ^{159}GdSpHL can be explained by the presence of folate receptors in this organ (Parker *et al.*, 2005). Adverse effects, especially in patients suffering from chronic renal insufficiency, were observed afteradministration of the Gd-DTPA-BMA complex in MRI procedures (Thomsen, 2006). For this reason, the FDA requested that a warning be added to the descriptions of the product (Omniscan®) regarding the potential risk of NFS in patients with chronic renal failure (US FDA, 2010).

The ^{159}Gd-SpHL formulation has a Kp of 2.31, versus 3.50 for the free drug (Table 1). A lower renal uptake may result in fewer possibilities for development of NFS in patients with renal failure. However, a Kp of 4.97 was observed for the ^{159}Gd-FTSpHL formulation versus 3.50 for the free drug, implies signifies that the former presented a higher renal accumulation (Table 2). Therefore, the possible use of the ^{159}Gd-FTSpHL formulation for the treatment of cancer by patients with severe chronic kidney disease still warrants further investigation.

The biodistribution studies in solid Ehrlich tumors revealed a low uptake of the free drug, which presented a maximum value of 0.25 ± 0.7% ID/g after one hour (Figure 3). The formulations presented a higher uptake by tumor tissue during the same period. The uptake of the ^{159}Gd-SpHL formulation (2.92 ± 0.1% ID/g) was 11.7 times higher, and that of the ^{159}Gd-FTSpHL formulation (6.12 ± 0.3% ID/g) was 24.5 times higher. For other times, it was verified that the other is manintenance of levels of tumor uptake for both formulations. Several studies have been published showing that folic acid receptors are expressed in a broad spectrum of tumors (Sudimack *et al.*, 2000; Gabizon, *et al*., 2003). In the present study, the formulation containing folic acid presented an uptake by solid Ehrlich tumors significantly higher than that of the formulation without of folic acid. This result is consistent with the fact that tumor cells of Ehrlich's, second Sztabert Grzelakowska & Sikora (1984), express receptors for folic acid on their surface. More detailed studies regarding the quantity and avidity of folic acid receptors in these cells can serve as guidelines for the development of more effective formulations of therapeutic drugs for targeting these cells.

By utilizing the AUC/tissue and AUC/blood ratios for the studied period, the partition coefficient was calculated and revealed that the formulations have a more expressive perfusion in the liver, spleen, kidneys and tumor with Kp values of 17.69, 21.99, 2.31 and 1.63, respectively, for ^{159}Gd-SpHL (Table 1) and Kp values of 19.41, 9.85, 4.97 and 5.93, respectively, for ^{159}Gd-FTSpHL (Table 2). These results showed that the formulation containing folate induced a greater perfusion in all the tissues studied, except for the spleen, where a 2.2-fold reduction in splenic accumulation was observed, with Kp values varying from 21.99 to 9.85 (Table 2). With regard to the tumor, the free complex had a low degree of perfusion. However, a Kp value of 1.63 was obtained for the ^{159}Gd-SpHL formulation. This value represents an increase of approximately 3.88-fold over that of the free complex (Table 1). The ^{159}GdFTSpHL presented a Kp value of 5.93, which represents an increase of about 14.41-fold that of the free complex (Table 2). When comparing the Kp of the both formulations, the addition of folate led to a 3.63-fold increase in tumor tissue permeation and an improvement in the delivery of the radioactive complex to these cells.

4.4.2 – Scintigraphy biodistribution study

Scintigraphic images of mice that received ^{159}Gd-SpHL or ^{159}Gd-FTSpHL are shown in Figure 4. Since the amounts of radiation were insufficient, it was not possible to acquire images of animals that received ^{159}Gd-DTPA-BMA.

The quantitative analysis of the images revealed that the region of interest (ROI's) showed that the targeted/non-target ratio was always greater than three for the ^{159}Gd-SpHL formulation. This fact means that the tumor region presented at least 300% more radioactivity than that of the control (muscle) (Table 3). These results support data obtained from the biodistribution study, confirming that the Gd-SpHL formulation has a greater affinity for the tumor region than for muscle tissue. The ROI's determined for the ^{159}Gd-FTSpHL formulation showed that the target/non-target ratios were always greater than nine (Table 3). This fact means that the tumor region presented at least 900% more radioactivity than the control (muscle) and that the formulation presented a high affinity for tumor tissue. By comparing the results of the target/non-target ratio for ^{159}Gd-SpHL and ^{159}Gd-FTSpHL formulations, an increase of about 3-fold in the tumor uptake for the formulation containing folate was calculated. This result is in agreement with the findings obtained in the sacrifice biodistribution study.

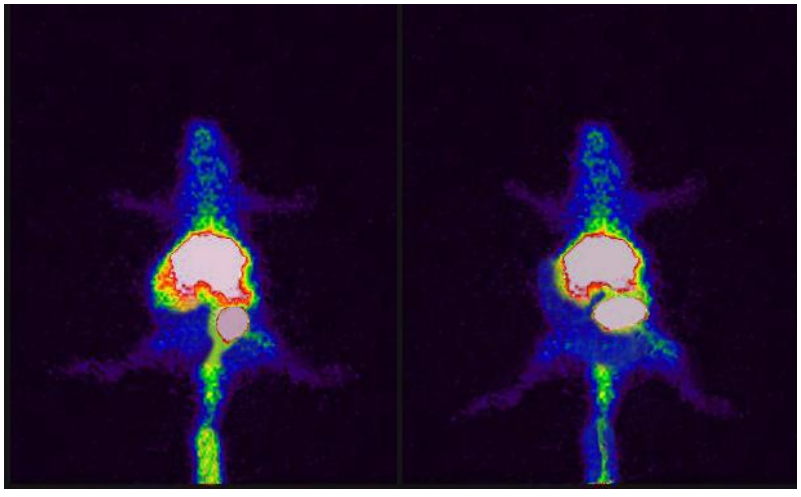

Figure 4 – Scintigraphic image obtained after I.V. administration of ^{159}Gd-SpHL (left image) and ^{159}Gd-FTSpHL (right image).

Table 3 – Target/non target ratio obtained in gamma camera (%dose/cm^2).

Sample	1h	4hs	6hs	8hs	18hs	24hs
^{159}Gd-SpHL	3,19±0,04	3,03±0,08	3,21±0,03	3,18±0,02	3,16±0,09	3,14±0,02
^{159}Gd-FTSpHL	9,14±0,06	9,00±0,03	9,45±0,12	9,58±0,11	9,67±0,08	9,75±0,17

Results expressed as mean ± standard deviation. The values aren't statistically different ($p>0.05$).

5 – Conclusion

Stealth pH-sensitive PEG-coated and PEG-folate-coated liposomes containing the ^{159}Gd-DTPA-BMA complex were successfully prepared and radiolabeled. These formulations presented significant cytotoxic activities against Ehrlich tumor cells, and the apoptosis was determined to be the most probable mechanism that mediated the death of the cells. The biodistribution study revealed that free ^{159}Gd-DTPA-BMA has a low affinity for the studied tissues, including the solid Ehrlich tumor, being eliminated mainly by renal excretion. However, the formulations exhibited different biodistribution profiles. The accumulation in tumor tissue was significantly greater than that observed in the, liver, spleen and kidney tissues. The immobilization of folate on the liposome surface resulted in a 3-fold increase in the uptake of the radioactive complex by the tumor, in addition to a significant reduction in uptake by the spleen. This increase resulted in the delivery of higher radiation doses to the tumor region and improved the chances of the successful treatment of cancer with this formulation.

Because of the presence of folate receptors in liver and kidney cells, the increase in the uptake of the formulation by these tissues resulted in an increase in the radiation dose in these organs. More detailed studies to evaluate whether the radiation doses received by these organs may impair their functions should be conducted. Finally, the investigation of the scintigraphic image showed that the ^{159}Gd-SpHL formulation presented a target/non target ratio above three, while ^{159}Gd-FTSpHL presented a target/non-target ratio above nine, thereby demonstrating that they are promising therapeutic cancer formulations.

6 – Acknowledgements

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CAPÍTULO 4

Antitumoral activity and toxicity of PEG-coated and PEG-folate-coated pH-sensitive liposomes containing ^{159}Gd-DTPA-BMA in Ehrlich tumor bearing mice. ***European Journal of Pharmaceutical Science (in press, 2011)*.**

Antitumoral activity and toxicity of PEG-coated and PEG-folate-coated pH-sensitive liposomes containing ^{159}Gd-DTPA-BMAin Ehrlich tumor bearing mice

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Abstract

In the present study, PEG-coated pH-sensitive and PEG-folate-coated pH-sensitive liposomes containing the ^{159}Gd-DTPA-BMA were prepared and radiolabeled through neutron activation technique, aiming to study the in vivo antitumoral activity and toxicity on mice bearing a previously-developed solid Ehrlich tumor. The treatment efficacy was verified through tumoral volume increasement and histomorphometry studies. The toxicity of formulations was investigated through animal weight variations, as well ashematological and biochemical tests. The results showed that after 31 days of treatment, animals treated with radioactive formulations had a lower increase in tumor volume and a significantly higher percentage of necrosis compared with controls revealed by histomorphometry studies. Furthermore, mice treated with radioactive formulations exhibited lower weight gain without significant hematological or biochemical changes, except for toxicity to hepatocytes which requires more detailed studies. From the results obtained to date, we believe that the radioactive formulations can be considered potential therapeutic agents for cancer.

Keywords: Gd-159 Liposomes, in vivo antitumoral activity, Toxicity study
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1 – Introduction

Although radiotherapy has various and modern methods such as 3D-CRT (3-Dimensional Conformal Radiotherapy) and IMRT (Intensity Modulated Radiation Therapy) for cancer treatment, it is observed in some cases, that only a small reduction of adverse effects are seen in adjacent normal tissues, thus exposing these tissues to high doses of radiation (Chen et al. 2004; Bos et al., 2005). Seeking to minimize these adverse effects, several studies have been conducted with the aim of developing new forms of selective application of ionizing radiation in tumors, protecting the healthy cells. Among these improvements, we highlight the development of nanostructured pharmaceutical formulations, capable of encapsulating particulate emitting radioisotopes. These systems aim to deliver and retain particulate radiation with high specific activity in tumor cells, depositing in then, high doses of radiation, leading them to death (Kwekkeboom et al. 2003; Zelenetz et al., 2003).

In previous studies, our research group tested in vitro antitumor activity of the isotope ^{159}Gd-DTPA-BMA encapsulated in stealth pH-sensitive liposomes against RT2 tumoral cells (murine glioma). The results revealed that the presentation of a radioisotope to tumor cells, coupled with the effect of ionizing radiation, potentiated the cytotoxic effect by a factor of 1,170 (Soares *et al*., 2011a). Additionally, we investigated the biodistribution profile of liposomes encapsulating the complex radioactive ^{159}Gd-DTPA-BMA in mice containing the previously inoculated and developed solid Ehrlich tumor. The results revealed a significant accumulation of the formulations in tumor tissue, showing that the formulation has potential for use in therapeutic procedures for cancer treatment (Soares *et al.,* 2011b). Considering these results, this paper aims to study the in vivo antitumor activity of PEG-coated pH-sensitive (SpHL) and PEG-folate-coated pH-

sensitive liposomes (FTSpHL) containing the ^{159}Gd-DTPA-BMA in Ehrlich tumor-bearing mice. Furthermore, we investigated the toxicity of radioactive compositions in the liver, spleen and kidneys of treated animals.

2 – Experimental

2.1 – Materials

The Gd-DTPA-BMA complex, commercially known as Gadodiamide (Omniscan® - General Electric Healthcare Company), was purchased from FARMASA (São Paulo, Brazil). The drugs used in animal analgesia (ketamine and xylazine) were purchased from the laboratory Ceva Santé Animale (São Paulo, Brazil). The sterile injectable saline solution was acquired at the Pharmaceutical Industry Halex Istar (Goiânia, Brazil). All solvents used were HPLC analytical grade. All animals were acquired at the bioterium of Faculty of Pharmacy - Federal University of Minas Gerais. The experimental protocols were approved by the Ethics Committee for Animal Experiments (CETEA), of Federal University of Minas Gerais, under the code 019/09 and comply with the requirements of the guide for care and use of laboratory animals recommended by the Institute of Laboratory Animal Resources.

2.2 – Methods

The samples used in the present work may be identified through the acronyms listed in Table 1.

Table 1 – Identification acronyms of employed formulations

Acronyms	Formulation
SpHL	Blank PEG-coated pH-sensitive liposomes
FTSpHL	Blank PEG-folate-coated pH-sensitive liposomes
Gd-SpHL	PEG-coated pH-sensitive liposomes containing gadolinium
Gd-FTSpHL	PEG-folate-coated pH-sensitive liposomes containing gadolinium
^{159}Gd-SpHL	PEG-coated pH-sensitive liposomes containing radioactive gadolinium
^{159}Gd-FTSpHL	PEG-folate-coated pH-sensitive liposomes containing radioactive gadolinium

2.2.1 – Liposome preparation

The pH-sensitive PEG-coated and PEG-folate-coated liposomes were prepared according to the procedure described by Soares *et al*. (2011b). For SpHL, Gd-SpHL and ^{159}Gd-SpHL formulations, the DOPE, CHEMS, and DSPE-PEG_{2000} lipids, in a molar ratio of 5.7 / 3.8 / 0.5 were employed. For the FTSpHL, Gd-FTSpHL and Gd-^{159}FTSpHL formulations, the lipids of DOPE, CHEMS, DSPE-PEG_{2000}, and DSPE-PEG_{2000}-folate presented a molar ratio of 5.7 / 3.8 / 0.45 / 0.05 respectively in a total lipid concentration of 40 mM, adopted from studies conducted by Gabizon *et al.* (1999). The procedure for radiolabeling the Dg-DTPA-BMA complex, encapsulated in liposomes, was performed based on studies conducted by Soares et al., 2011a. According to this study, the neutron activation process brought about no significant changes to the liposomes structure, even after 8 hours of irradiation, showing that liposomes presented same morphological and physic-chemical characteristic before and after neutron irradiation process.

2.2.2 – Implantation of Ehrlich solid tumor in Swiss mice

The implantation of Ehrlich solid tumor in Swiss mice was based on the experimental protocol conducted in previous studies (Soares *et al.,* 2011b). In order, to implant the solid tumor, a suspension of ascetic fluid, containing viable cells of the tumor was prepared. The volume of 100 µL, containing 1.0 x 10^6 cells, was injected in the right flank of female Swiss mice with approximately 25 g body mass. The mice were kept in an area with controlled lighting and had free access to food and water. After about 20 days of implantation, a solid Ehrlich tumor was made visible and palpable. The tumor growth was assessed with the use of a micrometer (Mitutoyo, measurement 0.01mm, series number 7301) performed before tumor cell inoculation and every day following.

2.2.3 – Antitumoral activity protocol study

The protocol of antitumoral activity employed in this study was based on the experimental protocol conducted by Carvalho Junior *et al*. (2007). The number and the range of doses of radioactive formulations and their respective controls followed the availability of the nuclear reactor at the CDTN/CNEN (Belo Horizonte, Brazil). Experiments were organized so that there was a synchrony between animal tumor development and the achievement of the radioisotope. Obtained from three irradiations CDTN/CNEN, the doses were divided into two sections, followed by an interval of seven days between each dose and a third section, 10 days after the second dose (Figure 1).
The animals (n = 10) received through tail vein, three doses of 100 µL of ^{159}Gd-SpHL or ^{159}Gd-FTSpHL (236 mg/kg/dose) distributed in 15, 22 and 32 days after implantation of solid Ehrlich tumors.

Due to the results of biodistribution which have pointed to a low tumor uptake of radioactive samples containing the free complex (Soares *et al.* 2011b), we chose not to use it in this study. Following the same procedure, non-radioactive control samples consisted of: (*i*) saline, (*ii*) SpHL (blank liposomes), (*iii*) FTSpHL (blank liposomes), (*iv*)

Gd-SpHL and (*v*) Gd-FTSpHL. The animals were sacrificed 45 days after tumor cell inoculation.

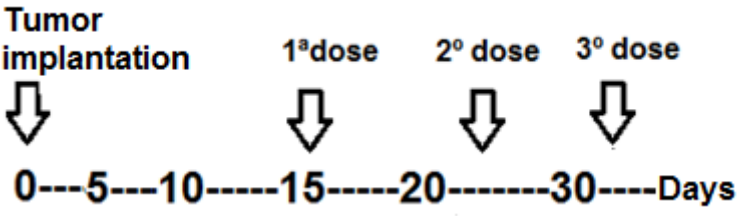

Figure 1 – Treatment doses distribution administrated in animals (n=8). The first dose was administered after 15 days tumor implantation. The second dose, 22 days after tumor implantation and third dose, 32 days after tumor implantation.

2.2.4 – Antitumoral activity evaluation

The treatment efficacy was investigated in terms of histomorphometry study and tumor growth restriction of animals (n=8). The histomorphometric analyses were performed in the Histopathology Laboratory of the School of Veterinary Medicine and Pathology Laboratory, Institute of Biological Sciences, both from the Federal University of Minas Gerais. In histological sections of the Ehrlich solid tumor, using a 40x objective, was used to determine the areas of neoplasia, necrosis and inflammation percentage. To determine these variables, the images were acquired by digital camera Spot Color Insight adapted to an Olympus BX-40 microscope and SPOT software version 3.4.5. Analysis of these Images was conducted with the Corel Draw® software and the count was determined in a total of 15 fields, covering the entire area of histological section. Twenty-five equidistant points were determined in the image field, a total of 75 points per slide examined.

The tumor growth restriction was evaluated by two orthogonal measures with a caliper: Mitutoyo, MIP/E-103. The tumoral volume (V) in centimeters was calculated following Eq. (1), where (a) is the largest diameter and (b) is the smallest diameter (Hruby *et al.*, 2011):

$$V = \frac{3.1416}{6 \cdot a \cdot b^2} \qquad (1)$$

2.2.5 – Toxicity evaluation

Previous studies conducted by Soares et al. (2011b) showed that the radioactive formulations (^{159}Gd-SpHL or ^{159}Gd-FTSpHL) have a biodistribution profile quite distinct from the free complex. The animals treated with these formulations, accumulated relevant quantities of radioactive complex in their liver, spleen and kidneys. For this reason, the evaluation of liver, spleen and kidney functions may reveal metabolic abnormalities in these organs. Thus, biochemical and hematological tests were performed all surviving mice after 39 days of the tumor implantation (7 days after the 3rd treatment dose). The mice were anesthetized with a mixture of Ketamine (40 mg/kg) and Xylazine (5 mg/kg). The blood was collected by cardiac puncture and after, the mice were sacrificed by cervical dislocation.

The hematologic parameters evaluated included erythrocyte count, hemoglobin, hematocrit, WBC (White Blood Cells - total and differential) and platelet count. The liver and kidney functions were assessed using the technique of clinical chemistry. Liver function was determined by measurement of ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALP (alkaline phosphatase), GGT (gamma-glutamyl transferase), total protein, globulin and albumin. The renal function was assessed by measurement of blood urea and creatinine and their ratio. All tests were performed in an outsourced laboratory by the company TECSA Laboratorios (Belo Horizonte, Brazil). The toxicity was also investigated through animals relative weight gain which were conducted 14, 21, 31 and 41 days after tumor implantation. The animals were sacrificed

4 days after the last dose of the treatment and during necropsies, the tumors were removed for histopathological analysis.

3 – Statistical Analysis

The relative weights gains by animals were statistically analyzed using the Lilliefors and Bartlett test to check the normality and homocedasticity of the variance, respectively. The data were analyzed in installments, with differents groups in the plots and time as subplots. All data on tumor volume and weight of the animals was processed by the equation: y = log (variable) + ½. The Scott-Knott test was used to compare measurements. The estimated regression models were used in the time interval for research of the evolution of the tumor and body weight change. Analyses of survival were performed by the Kaplan-Meier test (different groups were compared by log rank test). Statistical significance was considered as $p < 0.05$.

4 – Results and discussion

4.1 – Antitumoral activity evaluation

Thetumor volumeversus time curvefor control samplesandradioactiveformulations(^{159}Gd-SpHL and^{159}Gd-FTSpHL) are showninFigure2. The results displayedthatallsampleswithintheprocessing time ofthe first 20 dayswere not significantly different($P>0.05$). However, from the twentieth day, thetest showedthatwhencomparingtheformulations of radioactiveandcontrolsaline w / v, the valuesaresignificantly different ($p < 0.001$).

Given the average values obtained in the study period, we observed that there was a slower rate of tumor growth in animals treated with radioactive formulations. Thus, we believe that ionizing radiation has played an important role in reducing the rate of tumor development, due to its cytotoxic activity (Weichselbaum et al. 1994; Barcellos-Hoff, 2005).

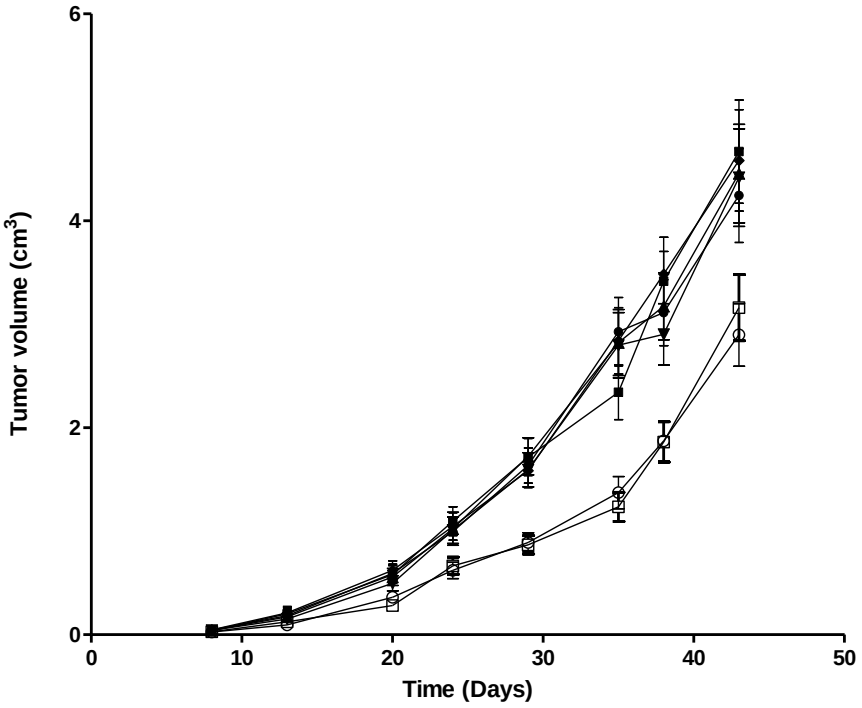

Figure 2 - Growth profile of solid Ehrlich tumor in Swiss female mice subjected to treatment with: (•) Saline; (■) control Gd-SpHL; (▲) control Gd-FTSpHL; (▼) Control SpHL blank; (◊) Control FTSpHL blank; (○) ^{159}Gd-SpHL e (□) ^{159}Gd-FTSpHL (n= 8). The radioactive formulations show statistic differences to controls at p<0,001.

The specific radioactivity and total radioactivity of all formulations were determined in 11.8 ± 0.9 GBq. mg^{-1} and 1.41 ± 0.4 GBq, respectively (Soares et al., 2010). The dose rate (whole body) in animals, using Martin's mathematics model for beta dosimetry was estimated in approximately 26 Gy/h/dose (Martin, 2006). These doses are considered sufficiently great to treat different types of cancer in humans, including breast cancer. However, this level of dose in non-target organs and tissues can conduct to highly toxic effects. Thus, the carrier system plays a decisive role to deliver the radioactive complex selectively to the tumor tissue. (Farhanghi et al., 1992; Maxon & Smith, 1990; Meléndez-Alafort et al., 2009).

In the histomorphometric evaluation of Ehrlich solid tumor, no differences were detected in the quantity of neoplastic or inflammatory areas. However, for necrotic areas, we observed a significant difference between radioactive treated groups and controls. The animals treated with saline solution, showed mean 8.2 % necrotic area (Figure 3 and 4A), while the animals treated with control formulations Gd-SpHL and Gd-FTSpHL showed necrotic mean area reaching respectively 14.9 % and 17.2 %, respectively (Figures 3, 4B and 4C).These results do not show a statistically significant difference regarding the animals treated with saline ($p > 0.05$). However, the animals treated with the radioactive formulations ^{159}Gd-SpHL and ^{159}Gd-FTSpHL showed necrotic mean area reaching 49.9 % and 65.4 % respectively (Figures 3, 4D and 4E). Thus, Between these two groups, no statistically significant differences was found ($p > 0.05$). However, when comparing the results of animals treated with the controls formulations and radioactive preparations, we observed a significant difference in the percentage of necrosis ($p < 0.01$). This fact can be attributed to the cytotoxic action of ionizing radiation, leading to the death of a significant portion of cells investigated.

Previous studies conducted by Soares et al. (2011b) showed high cytotoxic activity of complex liposomes containing radioactive ^{159}Gd-DTPA-BMA against Ehrlich tumor cells in vitro. In this study the authors verified that ionizing radiation can lead to the death of a significant amount of tumor cells by apoptosis mediated by Caspase-3. The results presented here, show that tumors undergoing treatment with ionizing radiation had a significant increase in necrotic areas compared with control groups treated without radiation. Therefore, ionizing radiation has played an important role in cytotoxic against Ehrlich tumor cells, booth in laboratory conditions in vitro and in living animals.

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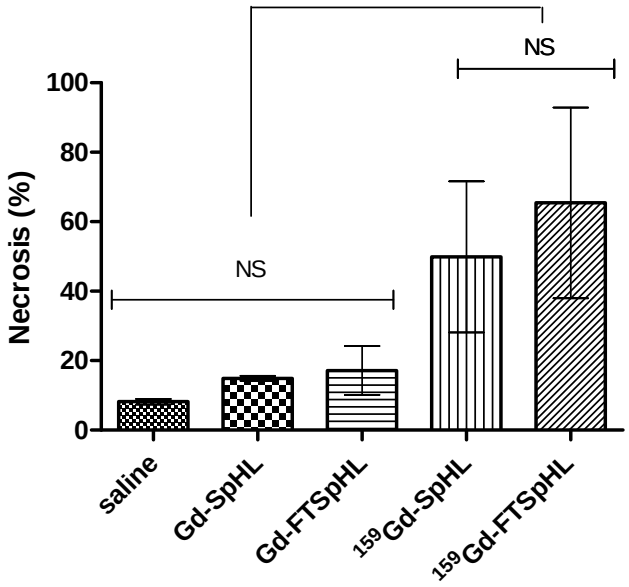

Figure 3 - Average percentage of necrosis in solid Ehrlich tumor cells treated with: Saline, control Gd-SpHL, control Gd-FTSpHL, ^{159}Gd-SpHL e ^{159}Gd-FTSpHL (n=8). Statistical difference is significant at the p< 0.01 level.

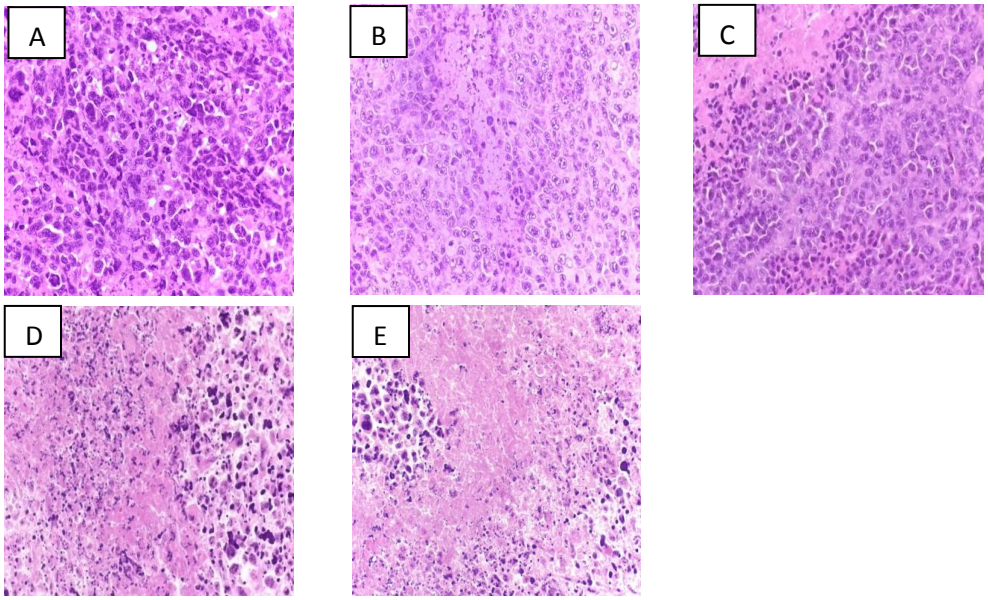

Figure 4 - Photomicrographs of Ehrlich solid tumor subjected to different treatments with: (A) saline, (B) control Gd-SpHL, (C) control Gd-FTSpHL, (D) ^{159}Gd-SpHL e (E) ^{159}Gd-FTSpHL. Hematoxylin-eosin, magnification 100 x.

4.2 – Toxicity evaluation

Toxicity studies of Gd-DTPA-BMA complex were conducted by its manufacturer, GE Healthcare Company. The LD_{50} was determined to be 19.7 mg/kg in healthy mice (GE Healthcare, 2006). In the present study, the doses of Omniscan® (236 mg/kg/dose) are about 84 times lower than the LD_{50} for mice, thus indicating a low probability of occurrence of acute toxic effects such as necrosis and renal tubular vacuolization.

Biodistribution studies of the formulations used in this study were conducted by Soares et al. (2011b). The results showed that vesicles were accumulated in the liver, spleen, kidney and Ehrlich solid tumor. Thus, studies indicative of the liver, spleen and kidney may reveal possible changes caused by liposome accumulation as well as the ionizing radiation deposited. Table 2 summarizes the indicative parameters of the liver function in female mice bearing Ehrlich solid tumor undergoing treatment with the formulations, radioactive ^{159}Gd-SpHL, ^{159}Gd-FTSpHL and respective controls (mean ± standard deviation) (n=8). The parameters ALT and AST may be indicative of the integrity of hepatocytes. The ALT is found almost exclusively in the cytoplasm of hepatocytes. The AST enzyme can be found in the cytosol and inside mitochondria of hepatocytes. For these reasons, these parameters can be used as markers of acute injury (ALT) or chronic (AST) from hepatocytes, respectively (Devi, 2010; Plaa, 2010). We can see in Table 2, the values of International Units (UI) of both enzymes, are increased in animals treated with radioactive and non-radioactive formulations, indicating their cytotoxicity to the hepatocytes. Additionally, we observed that the parameters relating to radioactive formulations are still higher than controls (Gd-SpHL, FTSpHL), thus indicating a higher cytotoxicity of these formulations on hepatocytes. This increased cytotoxicity can be attributed to radiation doses deposited in these cells leading them to destruction by radiotoxic mechanisms (Harrison *et al*., 2007; Pierotti, 2008).

Table 2 - Biochemical parameters indicative of liver function in female mice with Ehrlich solid tumor under treatment with ^{159}Gd-SpHL, ^{159}Gd-FTSpHL and controls (mean ± SD) (n=8) analyzed 39 days after tumor implantation.Means followed by different letters differ at the P<0.05.

Parameters	Controls			^{159}Gd-SpHL	^{159}Gd-FTSpHL
	NaCl (0,9% w/v)	Gd-SpHL	Gd-FTSpHL		
ALT (IU/L)	57 ± 6[a]	78 ± 3[a]	76 ± 5[a]	98 ± 8[b]	105 ± 8[b]
AST (IU/L)	103 ± 4 [a]	145 ± 8[a]	154 ± 7[a]	160 ± 7[b, c]	194 ± 9[b, c]
ALP (IU/L)	108 ± 8	112 ± 7	105 ± 8	115 ± 7	104 ± 7[b]
GGT (g/dL)	2.1 ± 0.4	2.0 ± 0.3	1.8 ± 0.1	2.2 ± 0.2	2.0 ± 0.3
Total protein (g/dL)	5.9 ± 0.2	5.7 ± 0.3	5.6 ± 0.2	5.5 ± 0.2	5.4 ± 0.3
Albumin (g/dL)	3.0 ± 0.2	3.1 ± 0.2	3.0 ± 0.1	2.8 ± 0.4	2.6 ± 0.3
Globulin (g/dL)	3.1 ± 0.2	3.0 ± 0.1	2.8 ± 0.2	2.9 ± 0.3	2.7 ± 0.2

ALT (alanine aminotransferase); AST (Aspartate aminotransferase); ALP (Alkaline Phosphatase); GGT (gamaglutamyl -transferase)

The parameters ALP (alkaline phosphatase) and GGT (gamaglutamyltrasferase) are serum markers of cholestasis processes and are important in the diagnosis of hepatic excretory function of bile and bile salts. We found that among all groups investigated the values found do not show a statistically significant difference compared with the control group treated with saline (Table 2). Thus, we deduce that the radioactive formulations did not significantly alter the metabolic enzyme-related cholestasis (Zimniak et al., 1990; Gray et al., 1990). The hepatic metabolism of proteins was assessed by serum concentrations of albumin, globulin and total protein. The values found are also detailed in Table 2 and reveal no statistically significant difference between the groups investigated and the control group treated with saline. Thus, we deduce that the formulations did not significantly alter the metabolism of proteins in hepatocytes (Jirtle et al., 1985; Srinivasan et al., 2008).

The parameters indicative of renal function in mice treated with different formulations are listed in Table 3. The serum dosage of urea can be used as an indicator of renal tubular function. The serum creatinine dosage can be used to verify the glomerular function. The results showed that serum levels of creatinine and urea compared with the control group treated with saline did not show statistical differences and thus we deduce that the glomerular and tubular functions of the treated animals were not altered by the formulations (Clifton Stephens et al., 1995; Robbins et al., 1995).

Table 3 - Biochemical parametersindicativeofkidney functioninfemale micewithEhrlich solid tumorundertreatment with ^{159}Gd-SpHL, ^{159}Gd-FTSpHL and controls (mean ± SD) (n=8) analyzed 39 days after tumor implantation. The results showed no statistically significant difference (p <0.05).

Parameters	Controls			^{159}Gd-SpHL	^{159}Gd-FTSpHL
	NaCl (0,9% w/v)	Gd-SpHL	Gd-FTSpHL		
Urea (mg/dL)	53 ± 2	51 ± 5	50 ± 1	55 ± 3	54 ± 3
Creatinine (mg/dL)	0.67 ± 0.04	0.68 ± 0.05	0.69 ± 0.04	0.69 ± 0.03	0.68 ± 0.03
Rate urea/creatinine	78 ± 5	74 ± 7	71 ± 6	78 ± 5	79 ± 4

In the beginning of renal impairment, a compensatory mechanism can be triggered where there is a reduction of creatinine release, maintaining their plasma levels within normal limits. Thus, the assessment ratio urea/creatinine can be used to identify the occurrence of renal toxicity in early stages (Leite *et al*., 2009; Domokos Máthé *et al.*, 2010). The results of this ratio also did not reveal statistically significant difference between the groups treated with the formulations and the control group treated with saline.

The main hematological parameters of mice subjected to treatment with formulations of ^{159}Gd-SpHL, ^{159}Gd-FTSpHL and respective controls are summarized in Table 4. The determined values of all variables showed no statistically significant difference. No hematopoietic suppression was observed. In addition, the liver and spleen were kept on blood homeostasis.

Table 4 – Hematological parameters micewithEhrlich solid tumorundertreatment with ^{159}Gd-SpHL, ^{159}Gd-FTSpHL and controls (mean ± SD) (n=8) analyzed 39 days after tumor implantation. The results showed no statistically significant difference (p <0.05).

Parameters	Controls			^{159}Gd-SpHL	^{159}Gd-FTSpHL
	NaCl (0,9% w/v)	Gd-SpHL	Gd-FTSpHL		
Erythrocytes (10^6/mm^3)	8.6± 0.2	8.7 ± 0.2	8.8 ± 0.2	9.0 ± 0.3	8.9 ± 0.2
Hematocrit (%)	43.3 ± 1.3	43.3 ± 1.0	42.9 ± 1.3	42.3 ± 0.7	43.8 ± 1.2
Leukocytes (10^3/mm^3)	5.6 ± 0.3	6.4 ± 0.4	5.7 ± 0.5	5.8 ± 0.6	5.4 ± 0.2
Monocytes (10^3/mm^3)	0.11 ± 0.01	0.09 ± 0.01	0.10 ± 0.02	0.11 ± 0.01	0.12 ± 0.02
Neutrophils (10^3/mm^3)	2.1 ± 0.3	2.3 ± 0.6	2.4 ± 0.1	2.5 ± 0.4	2.4 ± 0.7
Lymphocytes (10^3/mm^3)	3.8 ± 0.2	3.6 ± 0.4	3.7 ± 0.4	3.9 ± 0.03	3.4 ± 0.03
Platelets (10^3/mm^3)	508 ± 12	524 ± 7	504 ± 6	489 ± 7	404 ± 2
Hemoglobin (g/dL)	13.0 ± 0.4	13.5 ± 0.5	12.9 ± 0.1	12.7 ± 0.3	13.1 ± 0.2

The assessment of the results of biodistribution and hematological analysis revealed that although there was considerable accumulation of the formulations in the liver, spleen and kidney, no significant change was observed in metabolic functions, except for cytotoxicity on hepatocytes. More detailed investigations by histopathologic and histomorphometric measurements in liver tissue may elucidate with greater clarity the nature and extent of these lesions.

The relative weights gain of the Swiss mice containing the Ehrlich solid tumor undergoing treatment with ^{159}Gd-SpHL and ^{159}Gd-FTSpHL and their non-radioactive controls within 45 days after tumor implantation are presented in Figure 5. Fourteen days after inoculation of the solid tumor, it was found that the weight percentage change of the animals are fairly uniform, suggesting that the animals suffered no clinically significant changes resulting from tumor implants (data not shown). After 21, 31 and 41 days elapsed, following implantation of solid tumor, we also observed that there was no statistical difference among the control group members themselves. Furthermore, no statistical difference among radioactive formulations was observed (data not shown). However, when evaluating the entire period after implantation of solid tumor, we observed that in the groups treated with radiation, there was less weight gain compared to control groups (Figure 5). When statistically compared, the control group (saline) with the formulations using the radioactive test: "Bartlett's test for equal variances" found a value of $p < 0.001$, indicating a significant difference between the groups.

Among cancer patients treated with radiotherapy, the vast majority report an altered taste sense during and after treatments. Taste impairment has effects on quality of life because is associated with weight loss through reduced appetite and altered patterns of food intake (Ruo Redda and Allis, 2006). The lower weight gain observed in animals treated with radiation may be related to increased toxicity of the radioactive formulations that could have led to the animals lower food intake. However, more detailed studies need to be conducted in order to elucidate more clearly the causes of lower weight gain observed.

5 – Conclusion

Through the results, we found that animals under treatment with radioactive formulations gained less tumor volume compared to control group animals, demonstrating that ionizing radiation has played an important cytotoxic activity against solid tumor cells. However, from the experimental protocol adopted, it was not possible to observe a statistically significant difference between the results of radioactive formulations, indicating that immobilization of folate on the surface of the liposomes had low relevance for the treatment employed. The study of the toxicity of the formulations revealed no significant permanent hematological or biochemical changes, except for toxicity to hepatocytes which requires more detailed studies. Thus, we believe that the radioactive formulations can be considered potential therapeutic agents for cancer.

6- Acknowledgements

The authors thank FAPEMIG (Fundação de Amparo a Pesquisa do Estado de Minas Gerais), CNPQ (Conselho Nacional de Desenvolvimento Científico e Tecnológico), and CNEN (Comissão Nacional de Energia Nuclear) for their financial support.

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CAPÍTULO 5

Apoptosis mediated by caspase-3 and p53-dependent anticancer effects of ^{159}Gd-DTPA-BMA complex on RT2 glioblastoma cells.***Bioorganic and Medicinal ChemistryLetters (submitted, 2011)*****.**

Apoptosis mediated by caspase-3 and p53-dependent anticancer effects of ^{159}Gd-DTPA-BMA complex on RT2 glioblastoma cells

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ABSTRACT

New antitumor drugs based on metal complexes constitute an important class of new and potent therapeutic agents against cancer. In this work the apoptosis mediated by caspase-3 and p53-dependent anticancer effects of ^{159}Gd-DTPA-BMA metal complex were evaluated against RT2 glioblastoma cells. The results showed that the mechanism of cell death involves apoptosis by caspase-3 activation. Furthermore, we confirm preliminary the involvement of p53 protein triggered by ^{159}Gd-DTPA-BMA in these cells. These results suggest this radioactive complex as alternative therapeutic agent against cancer.

The research and development of new antitumor drugs based on metal complexes has been received attention from several research groups around the world and constitute an important class of new and potent therapeutic agents against cancer. The use of metal complex against tumors began with the discovery of cisplatin by Rosenberg and Van Camp in the late '60s that served as a stimulus for the research of new metal complexes containing gold, gallium, titanium and palladium[1-3]. Complexes containing the metal ions Ru^{+2} and Ru^{+3} are reported in several papers published recently as important

antitumor agents against different tumors lineage presenting, in some cases, equal or superior cytotoxicity to cisplatin[4, 5].

The vulnerability of some tumor cells to oxidative signals is a therapeutic objective for development of new anticancer agents[6]. Several cytotoxic anticancer entities can promote oxidative stress by changings levels of reactive oxygen species (ROS), thus leading tumor cell to apoptosis[7]. Based on this approach, in the past decade, works have been published where a new drug, consisting of a complex of gadolinium called Motexafin-Gadolinium (MGd), showed significant *in vitro* and *in vivo* antitumor activity against different cell linages[8, 9]. This drug belongs to porfirins molecule classes, called texafirins. Inside the cells, MGd disrupts redox-dependent activating apoptosis pathways. It accepts electrons, in the presence of oxygen, from reducing metabolites and forms reactive oxygen species (ROS) by redox cycling[10-12].

Cytotoxic stress, including chemotherapy, radiation and ROS, frequently activates an apoptotic cell death program through a cytosol or mitochondrial pathways leading to caspases activation[13]. Since MGd can catalyze the oxidation of reducing molecules and generate ROS, Chen et al., (2005)[14] verified that it might activate apoptotic pathways via caspase-3 can then cleave other caspases including caspase-2, -9, and -6 which in turn can cleave caspase-8 and capase-10. In other study, Singh et al. (2010)[15] investigated and correlated the effect of MGd on p53 expression, ROS, and apoptosis in lymphoma cells. The results showed that MGd was capable to induce apoptosis in lymphoma cell lines, that this is strongly related to p53 protein activation and formation of ROS.

In previous studies, performed by our research group, the gadolinium complex Gd-DTPA-BMA (Gadolinium-acid-diethylene-tri-amine-penta-acetic-bis-methyl-amide) showed antitumor activity against murine glioblastoma cancer cells (RT2)[16]. Additionally, we test the radioactive analog of the same complex (^{159}Gd-DTPA-BMA). The results showed an enhanced in cytotoxic activity in approximately 166 times, demonstrating that the radiation alone was responsible for the increase of activity. Any further investigation was conducted to evaluate the mechanisms involved.

Considering these results and published works about MGd and its correlating on p53 expression, ROS, and apoptosis mediated by caspase-3 in the present work, we conducted morphological *in vitro* study of cytotoxicity ^{159}Gd-DTPA-BMA in RT2 cells seeking to understand the involvement of proteins caspase-3 and p53 in cell death as verified in MGd complex.

The ^{159}Gd-DTPA-BMA was successfully prepared through neutron irradiation in nuclear reactor at CDTN. The characteristic photopeak of the radioisotope ^{159}Gd were confirmed at 348.28 keV, 363.50 keV and 560.80 keV by gamma spectroscopy. The energies and their intensities are found according to Moralles et al., (1995)[17]. The morphological cytotoxic *in vitro* study in RT2 cells was conducted according Soares et al., (2011)[16]. Briefly, the cells were thawed and cultivated until they reached 80% confluence in DEMEM culture medium. The pH was adjusted to 7.4 with hydrochloric acid or sodium hydroxide. In cells, were added 100 µL ^{159}Gd-DTPA-BMA at concentrations of 0.12 and 0.017 mM. After 48 hours of treatment, cells were viewed by light microscope and photographed (Nikon).

The cells in the control group (untreated) showed good adhesion with the matrix and typically morphology in star (Figure 1). Cells treated with ^{159}Gd-DTPA-BMA showed significant cytotoxicity at concentrations of 0.12 mM and 0.017mM (Figure 1). It is observed, for both concentrations, retraction of cells, loss of adhesion to extracellular matrix and neighboring cells, chromatin condensation, nucleosome fragmentation and formation of blebs characteristic of death by apoptosis.

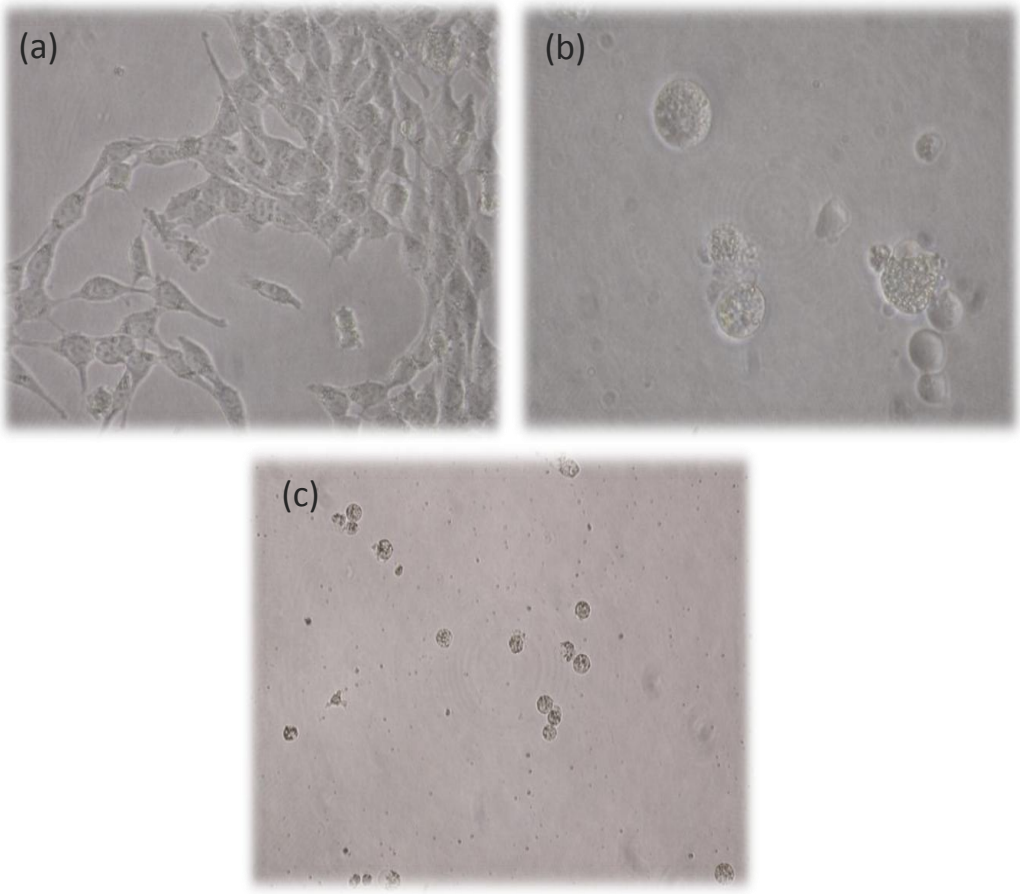

Figure 1. (a) Untreated control group of RT2 cells (amplification 250 x). (b) and (c) RT2 cells treated with ^{159}Gd-DTPA-BMA in 0.12 mM and 0.017mM respectively [amplification (a) 500 x (b) 250 x].

Apoptosis in RT2 Cells was performed by applying the method of the CleavaLite® Caspase-3 Activity Assay Kit (Millipore). The results revealed that the presence of the Gd-DTPA-BMA metal complex does not alter significantly the activity of the protein caspase-3, demonstrating that the cytotoxic activity of the complex on these cells does not involve the participation of this protein (Figure 2). However, when the radioactive analog (^{159}Gd-DTPA-BMA) was used, there was a significant increase in the activity of the caspase-3 in the RT2 tumor cells compared to the observed in control group at 4 h post-treatment, indicating that apoptosis is probably the main mechanism in cell death.

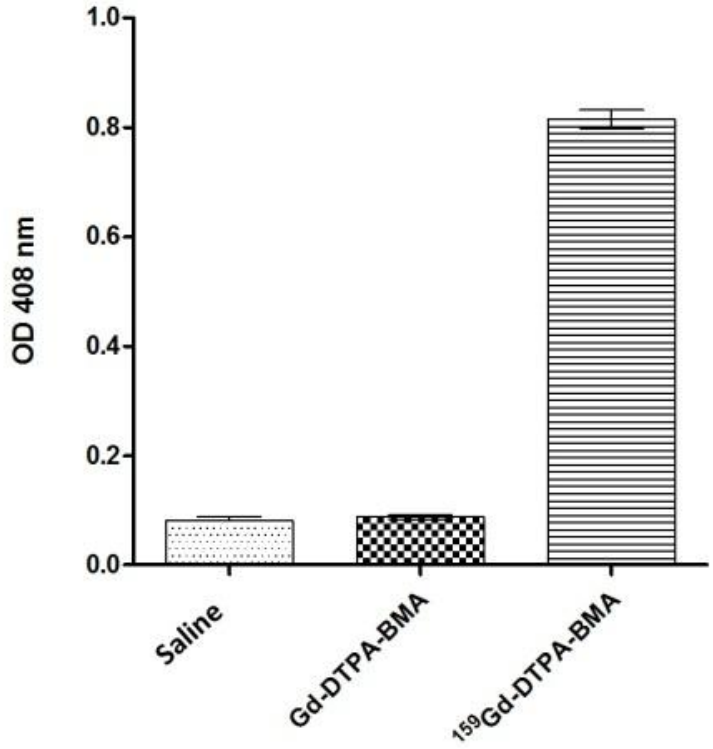

Figure 2. Caspase-3 activity in RT2 cells treated with saline (control), Gd-DTPA-BMA and ^{159}Gd-DTPA-BMA. Values expressed in Optic Density (OD) ± standard deviation. Values between Gd-DTPA-BMA and saline are not statistically significant ($p < 0.05$). Between ^{159}Gd-DTPA-BMA and others samples are extremely significant ($p<0.001$).

T98 cells (human glioblastoma) were chosen to evaluate the importance of p53 protein in the cytotoxic activity of the complex ^{159}Gd-DTPA-BMA due to the fact that T98 cells have a mutant inactivated p53[18]. Thus, differences in cytotoxic activity between cells treated with Gd-DTPA-BMA (non-radioactive) and ^{159}Gd-DTPA-BMA may reveal the involvement of this protein in cytotoxic mechanism trigger by ^{159}Gd-DTPA-BMA. The protocol used in this study was based on previous works conducted by Soares et al., (2011)[16].

The Figure 3a and 3b show the IC_{50} curves for samples Gd-DTPA-BMA and ^{159}Gd-DTPA-BMA, respectively. The IC_{50} of the sample Gd-DTPA-BMA was determined in 103 ± 2,5 mM and the sample ^{159}Gd-DTPA-BMA in 97 ± 1,1 mM. These results show that in the absence of active p53, no significant differences were observed in cytotoxic activity between samples tested. On the other hand, when RT2 tumor cells (p53 active) was evaluated the cytotoxic action of the radioactive complex was significantly enhanced as demonstrated in studies conducted by Soares et al., (2011)[16]. Thus, albeit preliminary, we found that the cytotoxic mechanism mediated by radioactive complex involved the participation of p53. However, more detailed studies should be conducted in order clarity the involved mechanisms.

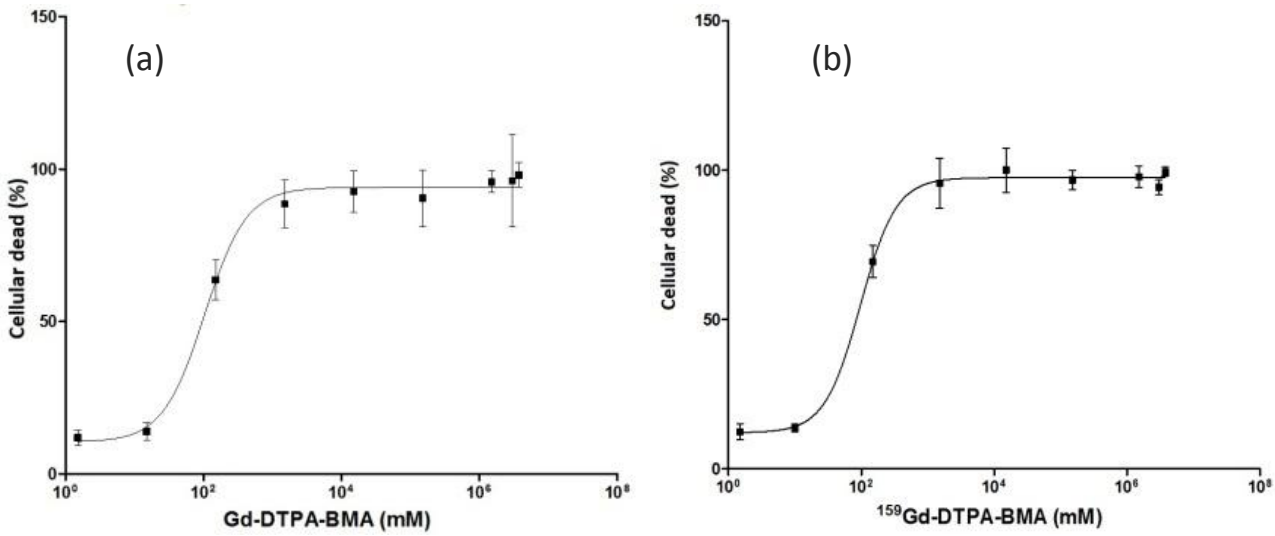

Figure 3. IC_{50} curves of (a) Gd-DTPA-BMA and (b) ^{159}Gd-DTPA-BMA in T98 cells (n = 8). The curve fit allowed for: (a) R^2= 0,983 and (b) R^2= 0,954. No statistical difference between the 2 samples were verified (ANOVA followed "Bonferroni's Multiple Comparison Test"p<0.05).

In summary, ^{159}Gd-DTPA-BMA was capable to induce a cytotoxic stress in cancer cells, as observed for MGd. In addition, the results presented by this paper suggest that the apoptosis mediated by caspase-3 and p53-dependent anticancer effects is an important pathway used for radioactive complex on RT2 cells. Further investigations will be conducted in order to reveal the potential application of the radioactive complex ^{159}Gd-DTPA-BMA as alternative therapeutic agent against cancer, and will be reported in due course.

Acknowledgement

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DISCUSSÃO GERAL

Diversos trabalhos publicados na literatura sugerem que alguns radioisótopos podem ter uma potencialidade terapêutica no tratamento do câncer, entretanto, em grande parte desses estudos esta sugestão é baseada apenas nas características físicas desses radioisótopos como o tipo de decaimento, a atividade específica e o tempo de meia-vida física. Dentre os radioisótopos sugeridos baseados apenas nestas características, o gadolínio-159 é citado como um promissor radioisótopo por apresentar um decaimento beta negativo com uma energia média de 1001 keV e elevada atividade específica (Bardies & Chatal, 1994; Goorley & Nikjoo, 2000; Saha, 1998). Ao nosso conhecimento, até o presente, nenhum estudo investigou a atividade antitumoral deste radioisótopo e tampouco sua associação a um sistema nanoestruturado, como os lipossomas, visando minimizar os efeitos da radiação em tecidos sadios adjacentes. O planejamento desta tese baseou-se em tentar responder as seguintes perguntas.

Existe uma correlação entre as propriedades físicas do ^{159}Gd e a sua real capacidade em tratar o câncer?

É possível direcionar o radioisótopo às células tumorais diminuindo os efeitos colaterais da radiação em outros tecidos?

Para responder as estas perguntas, o objetivo desta tese consistiu em estudar *in vitro* e *in vivo* o emprego do radioisótopo ^{159}Gd encapsulado em lipossomas visando avaliar seu potencial emprego no tratamento do câncer. Do ponto de vista físico-químico, inicialmente precisávamos obter o ^{159}Gd a partir do complexo Gd-DTPA-BMA, já que este radioisótopo não é disponibilizado comercialmente. Na sequência, quantificamos sua atividade específica e avaliamos sua integridade estrutural após o processo de irradiação com nêutrons em reator nuclear. Adicionalmente, preparamos, caracterizamos, verificamos o perfil de liberação em meios biológicos e a estabilidade dos lipossomas contendo ^{159}Gd-DTPA-BMA após o processo de irradiação. Do ponto de vista biológico, nosso interesse foi verificar a atividade antitumoral *in vitro* do complexo radioativo encapsulado em lipossomas frente aos modelos de células

tumorais de Ehrlich, RT2 (glioma murino) e T98 (glioma humano). Conduzimos também estudos *in vivo* de biodistribuição (por sacrifício e por imagens cintilográficas), de atividade antitumoral e de toxicidade das formulações em camundongos Swiss contendo o tumor sólido de Ehrlich. Os resultados desses estudos nos permitiram mostrar que houve uma correlação entre as propriedades físicas do radioisótopo com a sua atividade tumoral, e que, os lipossomas permitiram concentrar a radiação no modelo tumoral*in vivo* utilizado (Ehrlich). A estratégia de pesquisa utilizada para chegar a estes resultados baseou-se nos estudos abaixo descritos.

1 –Preparação do radioisótopo ^{159}Gd e avaliação preliminar da sua citotoxicidade *in vitro* frente ao modelo tumoral de Ehrlich (Figura 1).

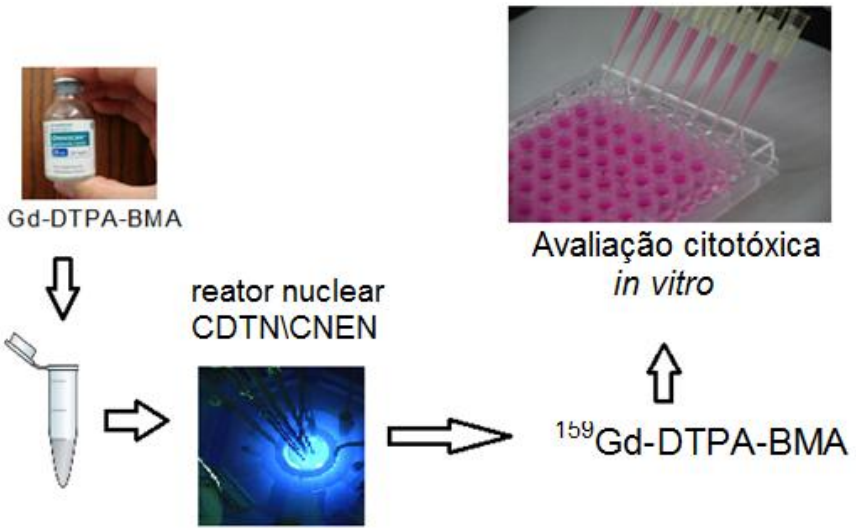

Figura 1 – Preparação do radioisótopo ^{159}Gd e avaliação preliminar da sua citotoxicidade *in vitro* frente ao modelo tumoral de Ehrlich.

Oradioisótopo ^{159}Gd foi obtido diretamente no reator nuclear da CDTN/CNEN (Belo Horizonte) a partir do complexo metálico Gd-DTPA-BMA (Omniscan®). Após irradiação; *(i)* quantificamos a atividade específica do radioisótopo obtido; *(ii)* certificamos que a integridade estrutural do complexo ^{159}Gd-DTPA-BMA foi mantida após o processo de irradiação, e, por fim; *(iii)* demonstramos que o complexo radioativo teve atividade citotóxica *in vitro* frente ao modelo tumoral de Ehrlich.

2 – Lipossomas radiomarcados com ^{159}Gd-DTPA-BMA: preparação,caracterização, perfil de liberação, avaliação citotóxica *in vitro*frente ao modelo de células tumorais RT2 (Figura 2).

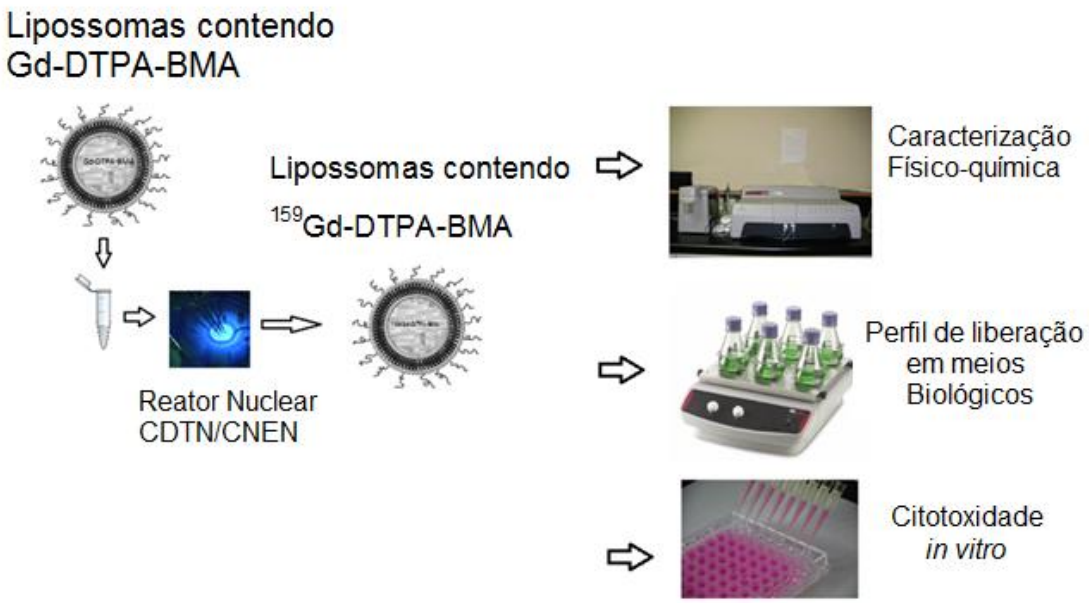

Figura 2 – Preparação, caracterização, perfil de liberação e avaliação citotóxica *in vitro* de lipossomas radiomarcados com ^{159}Gd-DTPA-BMA frente ao modelo de células tumorais RT2 .

Com o objetivo de diminuir o manuseio de substâncias radioativas,primeiramente preparamos no laboratório os lipossomas contendo o complexo não radioativo Gd-DTPA-BMA. Em seguida, irradiamos estes lipossomas no reator nuclear e verificamos que:*(i)*eles mantiveram suas características físico-químicas e morfológicas após até 8 horas de irradiação; *(ii)*o perfil de liberação em meios biológicos do radioisótopo encapsulado nos lipossomas foi do tipo prolongado, o que poderá minimizar seus efeitos colaterais; e, finalmente que; *(iii)*a atividade citotóxica *in vitro*do complexo radioativo foi amplificada frente às células RT2, demonstrando que as vesículas modificam significativamente a forma de apresentação do complexo radioativo às células tumorais.

Os lipossomas contendo o complexo radioativo ^{159}Gd-DTPA-BMA apresentaram diâmetro médio de 100 nm, baixo índice de polidispersividade e potencial Zeta negativo. Para serem utilizados no tratamento do câncer estes parâmetros apresentam significativa importância. Alguns autores têm demonstrado que o diâmetro médio de nanoestruturas influencia a sua biodistribuição. Por exemplo, nanoestruturas com diâmetro maior que 300nm e menor que 70nm são rapidamente depurados da corrente sanguínea por células do sistema mononuclear fagocitário (SMF) (Gref *et al.,* 1994). O índice de polidispersividade baixo é requisito fundamental para o emprego intravascular de nanoestruturas evitando-se assim o risco de embolia. O potencial Zeta reflete o potencial elétrico existente entre uma nanoestrutura e o meio o qual se encontra disperso. Nanoestruturas contendo potenciais Zeta significativamente positivos ou negativos sofrem menos agregações e fusões entre si, devido ao aparecimento de repulsão eletrostática. Além disso, a carga superficial de nanoestruturas mostra ser uma importante propriedade regulatória farmacocinética. Por exemplo, estudos mostram que lipossomas catiônicos ou aniônicos ativam o sistema de complemento através da via clássica ou alternativa, conduzindo assim a uma opsonização de proteínas séricas sanguíneas e consequente fagocitose por células do SMF (Chonn *et al.*, 1991). Outros estudos mostram que lipossomas catiônicos possuem reduzido tempo de circulação na corrente sanguínea, afetando diretamente na biodistribuição entre a microvasculatura e interstício tumoral, impactando sensivelmente na sua absorção tumoral (Campbell *et al.*,

2002). Os resultados obtidos mostram que os lipossomas preparados neste trabalho apresentam características físico-químicas adequadas para acumulação tumoral.

3 – Lipossomas radiomarcados com ^{159}Gd: citotoxicidade *in vitro* frente às células tumorais de Ehrlich e biodistribuição em camundongos Swiss contendo o tumor de Ehrlich (Figura 3).

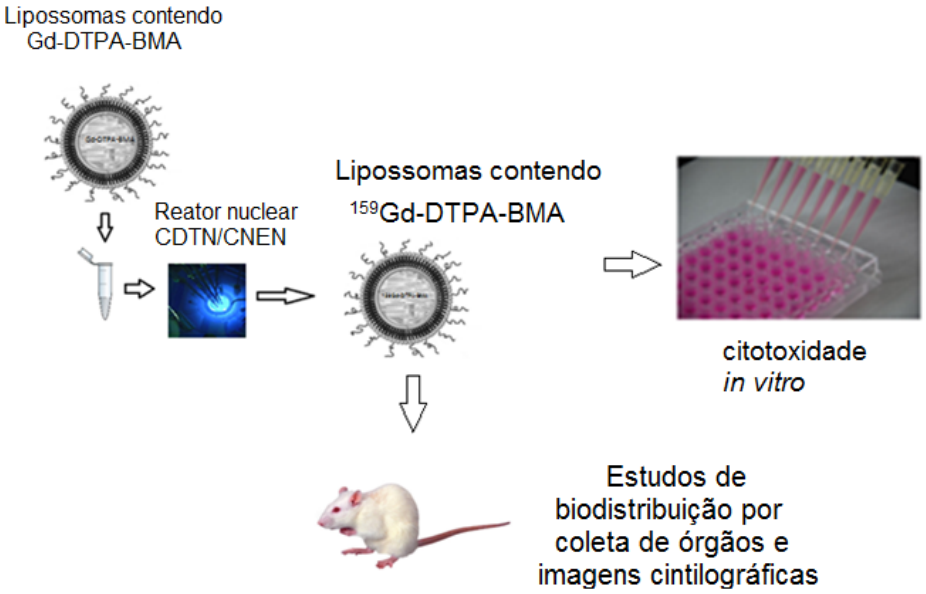

Figura 3 – Citotoxicidade *in vitro* frente às células tumorais de Ehrlich e biodistribuição em camundongos Swiss contendo o tumor de Ehrlich de lipossomas radiomarcados com ^{159}Gd.

Os resultados da atividade antitumoral *in vitro* contra as células do modelo tumoral de Ehrlich revelaram que ^{159}Gd-DTPA-BMA livre possui atividade citotóxica cerca de 95 vezes maior que Gd-DTPA-BMA livre (não radioativo). Para os lipossomas os resultados revelaram que a formulação radioativa (^{159}Gd-SpHL) incrementou em cerca de 16 vezes a atividade citotóxica da formulação não radioativa (Gd-SpHL). Entretanto, quando comparamos a atividade citotóxica de ^{159}Gd-SpHL e ^{159}Gd-DTPA-BMA livre, observamos que a formulação lipossomal permitiu um incremento na atividade

citotóxica em cerca de 58 vezes (1,4mM para 0,024mM). Estes resultados estão de acordo com os estudos realizados por Carvalho Júnior e colaboradores (2007b) que demonstraram também um significativo aumento da atividade citotóxica do fármaco cisplatina após sua encapsulação em lipossomas pH-sensíveis também constituídos por DOPE, CHEMS e DSPE-PEG_{2000}.

Em seguida, fizemos estudos buscando identificar o comportamento *in vivo* das formulações em camundongos Swiss contendo o tumor de Ehrlich. Baseado em trabalhos descritos na literatura (Gosselin & Lee, 2002; Gabizon *et al.*, 2003), onde o ácido fólico foi imobilizado na superfície de lipossomas, com o objetivo de melhorar a vetorização de fármacos terapêuticos às células tumorais, investigamos a biodistribuição de duas formulações lipossomais contendo o complexo radioativo ^{159}Gd-DTPA-BMA, que se diferiram apenas pela imobilização de folato na superfície de uma delas. Os resultados nos mostraram que as formulações sem folado (^{159}Gd-SpHL) e com folato (^{159}Gd-FTSpHL) desempenharam um importante papel na modificação do perfil de biodistribuição do complexo radioativo, permitindo um aumento de mais de 82 vezes na acumulação do complexo radioativo no tecido tumoral quando comparado com o radioisótopo livre. Entretanto, observamos que as formulações também incrementaram a acumulação do complexo radioativo nos tecidos hepáticos e esplênicos. Estes resultados estão de acordo com os estudos realizados por Carvalho Júnior e colaboradores (2007b) que demonstraram também uma significativa acumulação de lipossomas de mesma constituição nas células do fígado e baço. Esta acumulação nos causou preocupação, principalmente porque foram encontrados relevantes quantidade do isótopo radioativo no tecido hepático, principalmente a partir do emprego da formulação contendo folato. Estudos bioquímicos e hematológicos foram feitos adicionalmente e estão descritos no capítulo 4. ^{159}Gd-SpHL apresentaram uma redução da captura renal em relação aos ^{159}Gd-FTSpHL, o que é desejável para pacientes que sofrem de insuficiência renal crônica evitando um potencial risco de desenvolver fibrose nefrogênica sistêmica (Thomsen, 2006).

Apesar das formulações empregadas no presente estudo terem recebido o recobrimento com polímeros hidrofílicos (PEG e PEG-folato) elas foram acumuladas

nos tecidos hepático e esplênico. Os lipossomas, segundo os resultados de caracterização físico-química, apresentaram potencial Zeta significativamente negativo o que propicia a ativação do sistema de complemento e conseqüente captura por células do SMF, amplamente presente nestes órgãos (Chonn *et al.*, 1991). A formulação contendo folato foi captada em maior quantidade no tecido hepático e em menor extensão no baço, o que pode ser justificado pela presença e ausência de receptores de folato nas células dos respectivos órgãos.

4 –Atividade antitumoral e toxicidade *in vivo* de lipossomas radiomarcados com ^{159}Gd-DTPA-BMA frente ao modelo tumoral de Ehrlich (Figura 4).

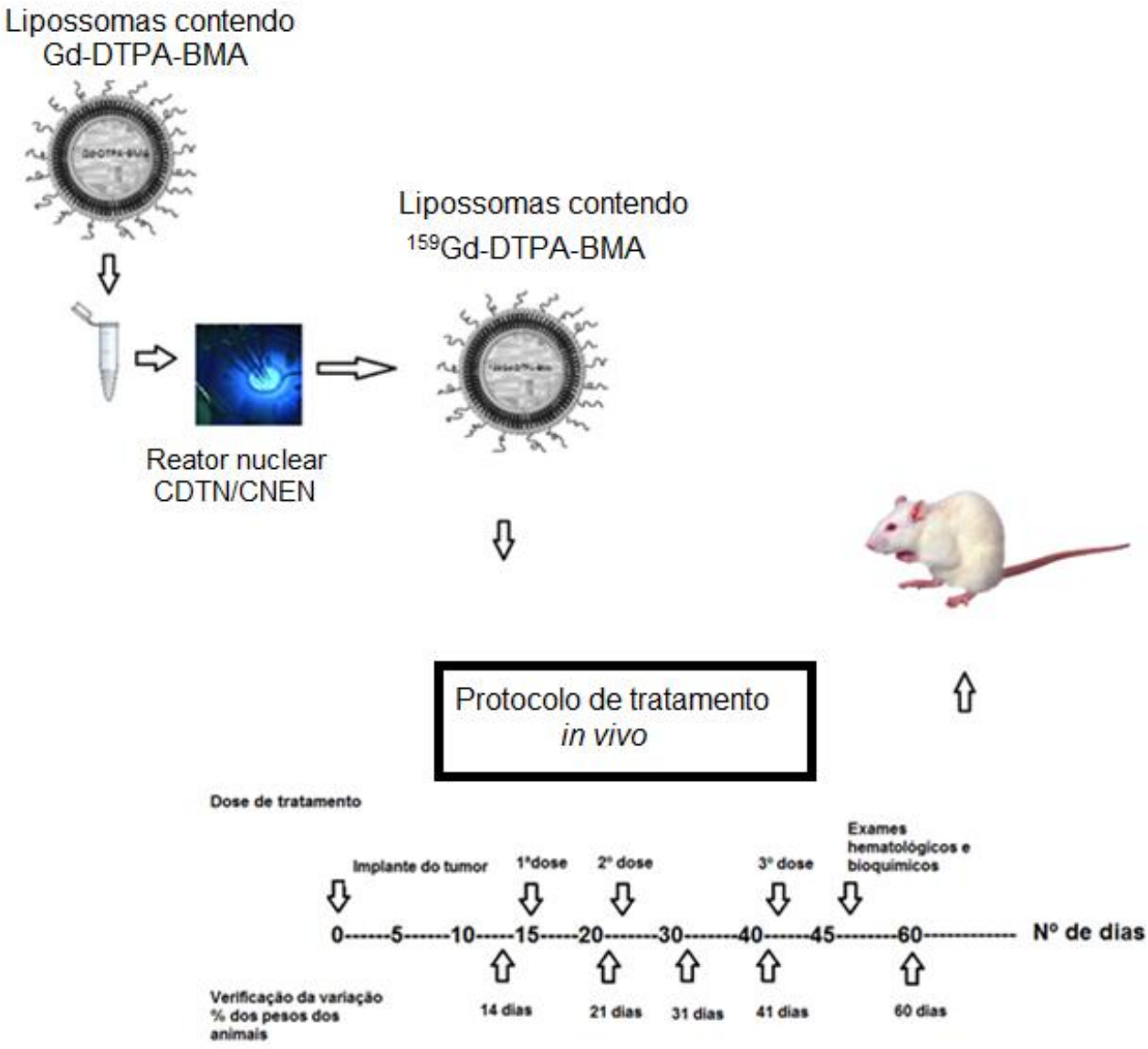

Figura 4 – Atividade antitumoral e toxicidade *in vivo* de lipossomas radiomarcados com ^{159}Gd-DTPA-BMA frente ao modelo tumoral de Ehrlich.

Os estudos da atividade antitumoral *in vivo* revelaram que os animais submetidos ao tratamento com as formulações radioativas apresentaram menor ganho de volume e peso em relação aos animais dos grupos controle, além de um aumento de sobrevida. Estes resultados sugerem que a radiação ionizante desempenhou uma importante atividade citotóxica contra as células do tumor sólido. Nos resultados de biodistribuição apresentados no capítulo 3, foi verificado um maior acúmulo de lipossomas recobertos com folato no tumor, comparado aos lipossomas não recobertos. Entretanto, a partir do protocolo experimental adotado, não foi possível observar diferença estatisticamente significativa entre os resultados dessas duas formulações em termos de atividade antitumoral, indicando que a imobilização de folato na superfície dos lipossomas teve baixa relevância no tratamento empregado.

Como no capítulo anterior verificamos que houve uma acumulação do complexo radioativo nos tecidos hepático e esplênico, conduzimos estudos analíticos com o objetivo de melhor esclarecer a possível toxicidade nestes órgãos. Os resultados da função renal mostraram que os níveis plasmáticos de uréia e creatinina, em comparação com o grupo controle tratado com solução de NaCl (0,9% p/v), não apresentaram diferenças estatísticas e assim, deduzimos que as funções tubulares e glomerulares dos animais tratados não foram alteradas pelas formulações. Na avaliação da função hepática dos camundongos tratados com a formulações contendo ou não folato, os resultados não revelaram alterações significativas em todos os parâmetros investigados exceto pelos valores aumentados de alanina aminotransfersase (ALT) e aspartato aminotransferse (AST) que são denominadas de alterações de natureza aguda nos hepatócitos. Entretanto, estudos mais detalhados a cerca dessa citotoxicidade deverão ser conduzidos com o intuito de melhor conhecer a extensão dessas alterações.

5– Apoptose mediada por caspase-3 e efeitos anticâncer p53 dependente do complexo ^{159}Gd-DTPA-BMA sob células de glioblastoma murino (Figura 5).

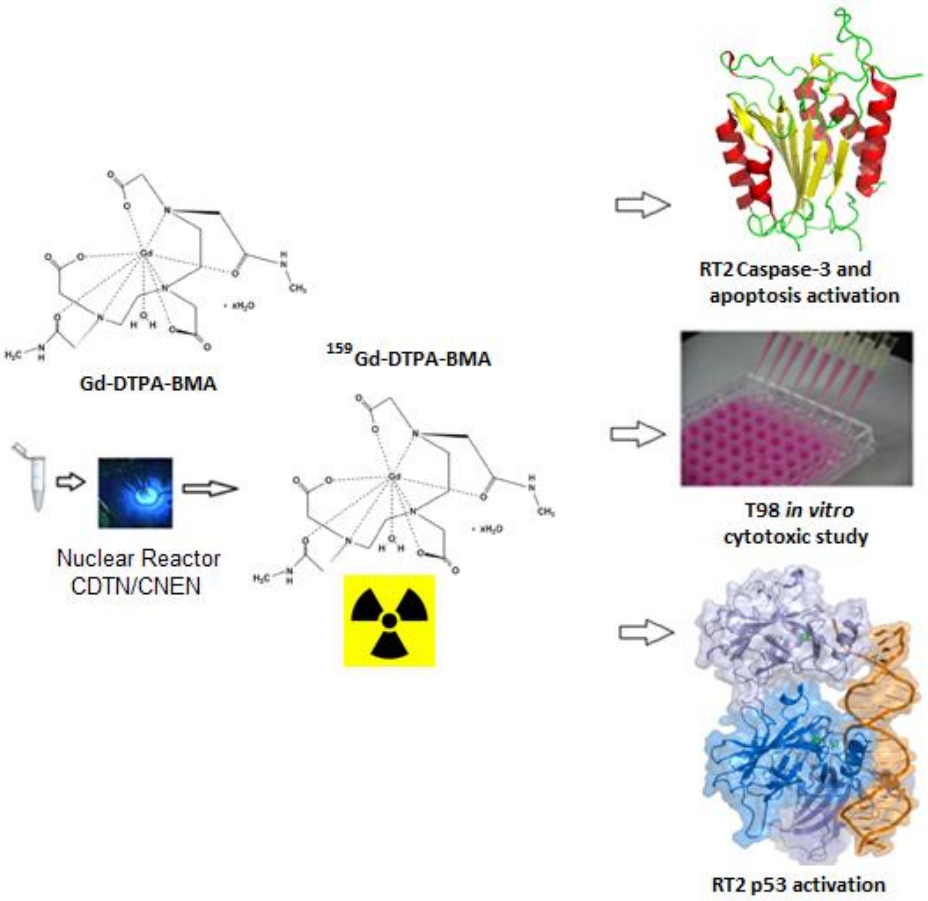

Figura 5– Apoptose mediada por caspase-3 e efeitos anticancerígenos p53 dependente do complexo ^{159}Gd-DTPA-BMA sob células RT2.

A apoptose é um importante mecanismo de morte celular associado ao emprego terapêutico da radiação ionizante(Chan *et al.*, 1999; Chu *et al.*, 2004).Por suspeitarmos que o mecanismo citotóxico de ^{159}Gd-DTPA-BMA, nas células RT2,estaria envolvido com a apoptose, assim como constatado no capítulo 3 para células de Ehrlich, conduzimos dois estudos distintos. No primeiro, verificamos quena presença do complexo radioativo, as células de RT2 apresentaram um elevação na concentração da

enzima caspase-3, que está diretamente ligado ao processo apoptose (Choi *et al.*, 2004; Brown & Attardi, 2005;Zambetti, 2005) No segundo estudo, verificamos a atividade citotóxica do complexo radioativo e não radioativo frente as células tumorais T98. Este modelo tumoral apresenta uma mutação no gene TP53, o que impede que a proteína p53 seja atuante (códon 337) (Aghi *et al.*, 2008). Os resultados deste último estudo revelaram que não há diferença estatística na citotoxicidade entre o complexo radioativo e o não radioativo. Estes resultados nos fazem supor que a proteína p53 pode estar relacionada à atividade citotóxica mediada pela radiação ionizante nas células de Ehrlich e RT2. A associação destes resultados com os encontrados no capítulo 3 nos fez concluir que a apoptose, provavelmente, foi o principal mecanismo mediador da ação citotóxica do complexo radioativo. Entretanto, estudos mais detalhados devem ser conduzidos com o intuito de melhor elucidar os mecanismos de morte das células tumorais, tais como investigações citomorfométricas que podem ser capazes de confirmar a presença e dimensionar a extensão de lesões apoptóticas. Além disso, a detecção de outras enzimas caspases, identificará a origem mitocondrial ou nuclear do processo apoptótico.

CONCLUSÃO GERAL

Através dos resultados obtidos, verificamos que o radioisótopo ^{159}Gd pode ser preparado por meio da técnica de ativação neutrônica em reator nuclear com uma atividade específica adequada às pretensões de tratamento do câncer.

A estabilidade do complexo Gd-DTPA-BMA após o processo irradiação foi verificada e confirmada através de duas metodologias analíticas distintas (FT-IR e HPLC), sendo os resultados obtidos consonantes.

Os lipossomas pH sensíveis recobertos ou não folato foram obtidos com sucesso pela metodologia descrita. Os resultados da caracterização físico-química mostraram que as vesículas submetidas ao processo de irradiação com nêutrons tinham um diâmetro médio em torno de 110 nm, potencial Zeta negativo de -50 ± 5 mV e baixo índice de polidispersividade. A caracterização morfológica e a espectroscopia de correlação de fótons mostraram resultados consonantes na manutenção da integridade estrutural dos lipossomas antes e após o processo de irradiação com nêutrons.

Os estudos da atividade antitumoral*in vitro* e as concentrações inibitórias de 50 % demonstraram que o emprego da radiação ionizante e o uso dos lipossomas potencializaram em muitas vezes a ação citotóxica do complexo Gd-DTPA-BMA contra os modelos tumorais de células RT2 e de Ehrlich. O emprego dos lipossomas permitiu uma modificação na forma de apresentação do radioisótopo às células tumorais, o que pode ser comprovado pelo aumento da citotoxicidade em todos os testes realizados em que a formulação lipossomal foi empregada, aumentado significativamente a atividade citotóxica inclusive da formulação não radioativa.Estudos mais detalhados em relação à entrega e a retenção do complexo de gadolínio nas células tumorais e bem como a sua interação com as células tumorais podem melhor elucidar os mecanismos envolvidos na ação citotóxica contra as células tumorais estudadas.

A metodologia adotada para a confecção dos lipossomas permitiu uma encapsulação do complexo em torno de 20 ± 2 %. Esta taxa de encapsulação permitiu administrar por dose 236 mg/Kg de animal, o que foi considerado adequado as pretensões de

tratamento. A adição do aminoácido L-lisina à formulação não mostrou um aumento significativo na taxa média de encapsulação. Assim, optamos pela não utilização em função dos custos.

A adição do DSPE-PEG-Folato a formulação lipossomalconduziu a uma maior absorção nos rins e fígado e a uma menor acumulação esplênica conforme dados publicados na literatura. Entretanto, observamos uma significativa diferença na absorção pelo tecido tumoral, aumentando em cerca de 3 vezes a absorção em relação à formulação sem folato. Assim, acreditamos que as células do tumor sólido de Ehrlich expressam em sua superfície receptores para ácido fólico, justificando assim o aumento da absorção.

Os estudos da cinética de liberação de Gd-DTPA-BMA e Gd-DTPA-BMA-L-Lisina nos meios biológicos DMEM e plasma de rato a 70% revelaram que mesmo após 24 horas, menos de 3% do conteúdo dos lipossomas foi liberado para os meios biológicos, indicando assim que as formulações apresentam uma relevante segurança de utilização do radioisótopo ^{159}Gd em estudos *in vivo*.

Os estudos de biodistribuição revelaram resultados coerentes com os encontrados na literatura, onde o complexo ^{159}Gd-DTPA-BMA apresenta baixa afinidade pelos tecidos investigados e tendo sua eliminação tipicamente renal. As formulações apresentaram um perfil de biodistribuição bastante distinto ao do complexo livre, acumulando-se principalmente no fígado, baço, rins. Os resultados revelaram ainda, que ocorreu acumulação extremamente baixa do complexo livre no tumor sólido de Ehrlich, inviabilizando assim qualquer tentativa terapêutica a partir do complexo radioativo livre. Entretanto, as formulações permitiram um expressivo aumento dessa acumulação no tecido tumoral, viabilizando assim estudos *in vivo* da atividade antitumoral em camundongos.

Os resultados da atividade antitumoral *in vivo* revelaram que os animais tratados com as formulações radioativas tiveram menores ganhos de peso e volume tumoral, acompanhados de um importante aumento na sobrevida. Estudos citomorfométricos se encontram em andamento para elucidar os mecanismos envolvidos na ação citotóxica do complexo radioativo.

As análises hematológicas foram utilizadas para estudar a toxicidade radioinduzida das formulações e revelaram alterações em parâmetros relacionados à toxicidade aguda em hepatócitos. Adicionalmente, os resultados revelaram alterações transitórias na medula óssea.

O conjunto dos resultados apresentados nesta tese nos leva a concluir que é grande a potencialidade da utilização de lipossomas radiomarcados com gadolínio para o tratamento do câncer, porém, é necessário que sejam feitos estudos complementares visando diminuir, por exemplo, a toxicidade hepática da formulação. Uma grande limitação em fazer estes estudos é a ausência do ^{159}Gd no mercado.

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