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Efeito antitumoral da Hipericina encapsulada com Plurônico P-123 em modelo de neoplasia do epitélio mamário

Maringá
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Dissertação apresentada ao Programa de Pós-Graduação em Biociências e Fisiopatologia do Departamento de Análises Clínicas e Biomedicina, Centro de Ciências da Saúde da Universidade Estadual de Maringá, como requisito parcial para obtenção do título de Mestre em Biociências e Fisiopatologia
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"E aprendi que se depende sempre
De tanta, muita, diferente gente
Toda pessoa sempre é as marcas
Das lições diárias de outras tantas pessoas

E é tão bonito quando a gente entende
Que a gente é tanta gente onde quer que a gente vá
E é tão bonito quando a gente sente
Que nunca está sozinho por mais que pense estar"

Gonzaguinha

EPÍGRAFE

“Deus nunca disse que a jornada seria fácil, mas Ele disse que a chegada valeria a pena”

Max Lucado

Efeito antitumoral da Hipericina encapsulada com Plurônico P-123 em modelo de neoplasia do epitélio mamário

RESUMO

O câncer de mama é responsável por cerca de 25% dos casos novos de câncer que surgem a cada ano. Os tratamentos disponíveis na atualidade são limitados e apresentam elevadas taxas de insucesso. A terapia fotodinâmica (TFD) surge como uma nova opção, pois utiliza um composto fotossensibilizador (FS) que sob a ação da luz de determinado comprimento de onda na presença de oxigênio molecular (O_2) gera oxigênio singlete ($^1O_2$) e espécies reativas de oxigênio, responsável pela morte celular, atingindo alvos específicos sem provocar efeitos colaterais sistêmicos ou em tecidos sadios. A Hipericina (HIP) é um FS promissor para a TFD, porém apresenta baixa solubilidade em meio aquoso e fluídos biológicos, diminuindo seu potencial fotodinâmico. A encapsulação da HIP com copolímero P-123 (HIP/P-123) além de servir como veículo biotransportador, pode também acessar sítios biológicos específicos. Este trabalho teve como objetivo avaliar o efeito antitumoral da HIP encapsulada com copolímero P-123 em modelo de neoplasia do epitélio mamário. Para isto foram utilizadas as linhagens mamárias MCF-10A (normal) e MCF-7 (carcinoma ductal invasivo). A citotoxicidade da HIP/P-123 foi avaliada através do ensaio de exclusão por azul de tripan, na presença e na ausência de luz. Para determinar se a morte celular ocorreu por necrose ou apoptose, a marcação com Anexina–V e Iodeto de Propídeo foram realizadas. A migração celular foi determinada através do ensaio da ferida. A avaliação da citotoxicidade em longo prazo foi realizada por ensaio clonogênico. A interiorização e a localização subcelular da HIP/P-123 foram observadas em microscópio de fluorescência invertido. Os resultados obtidos demonstram que os danos em células MCF-7 iluminadas foram mais evidentes do que nas células MCF-10A, ocorrendo diminuição ou completa ausência de células MCF-7 viáveis nas maiores concentrações testadas. Na ausência de iluminação não ocorreu diminuição da viabilidade celular em ambas as linhagens testadas. O tratamento das células MCF-7 levou a diminuição na confluência e descolamento das células. A morte celular por necrose foi predominante na linhagem tumoral. O fechamento da ferida foi observado apenas nas células MCF-7 não tratadas, após o período de 72 horas. O potencial clonogênico foi reduzido de forma dose-tempo-dependente nos períodos avaliados (7 e 14 dias). A interiorização da HIP/P-123 foi maior nas células MCF-7. Este é o primeiro estudo que avaliou a HIP/P-123 em células MCF-7 e MCF-10A. Nossos resultados demonstraram que baixas doses do composto apresentaram efeitos antitumorais em células cancerígenas e baixos danos em células normais, apresentando-se como um promissor agente para tratamento de neoplasias mamárias.

Palavras-chave: Terapia Fotodinâmica. Hipericina. MCF-7. MCF-10A.

Antitumoral effect of hypericin encapsulated in Pluronic P-123 in mammary epithelial neoplasia model

ABSTRACT

Breast cancer accounts for about 25% of new cancer cases that arise each year. Currently available treatments are limited and have high failure rates. Photodynamic therapy (PDT) emerges as a new option, as it uses a photosensitizer (FS) compound that under the action of light of a certain wavelength in the presence of molecular oxygen (O_2) generates singlet oxygen ($^1O_2$) and reactive oxygen species, responsible for cell death, reaching specific targets without causing systemic side effects or in healthy tissues. Hypericin (HYP) is a promising FS for PDT, but presents low solubility in aqueous media and biological fluids, reducing its photodynamic potential. Encapsulation of HYP with copolymer P-123 (HYP/P-123) in addition to serving as a biotransporter vehicle, can also access specific biological sites. The aim of this study was to evaluate the antitumor effect of the encapsulated HYP with copolymer P-123 in a mammary epithelial neoplasia model. For this, mammary lines MCF-10A (normal) and MCF-7 (invasive ductal carcinoma) were used. The cytotoxicity of HYP/P-123 was assessed by the trypan blue exclusion assay, in the presence and absence of light. To determine if cell death occurred by necrosis or apoptosis, the labeling with Annexin-V and Propionic Iodide were performed. Cell migration was determined by the wound healing assay. The evaluation of long-term cytotoxicity was performed by clonogenic assay. The internalization and subcellular localization of HYP/P-123 were observed under inverted fluorescence microscopy. The results obtained demonstrate that damage in MCF-7 cells treated with HYP/P-123 was more evident than in MCF-10A cells, with a decrease or complete absence of viable MCF-7 cells at the highest concentrations tested. In the absence of irradiation there was no decrease in cell viability in both tested cell lines. Treatment of MCF-7 cells led to decreased confluence and detachment of cells. Necrosis cell death was predominant in the tumoral cell line. Wound closure was observed only in untreated MCF-7 cells after 72 hours. The clonogenic potential was reduced in a dose-time-dependent manner in the evaluated periods (7 and 14 days). The internalization of HYP/P-123 was higher in MCF-7 cells. This is the first study to evaluate HYP/P-123 in MCF-7 and MCF-10A cells. Our results demonstrated that low doses of the compound provided antitumor effects on cancer cells and low damage in normal cells, presenting as a promising agent for the treatment of breast neoplasms.

Keywords: Photodynamic therapy. Hypericin. MCF-7. MCF-10A.

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SUMÁRIO

CAPÍTULO I

CÂNCER, UM BREVE PANORAMA

Câncer é um conjunto de mais de 100 doenças caracterizadas pela interrupção dos mecanismos que regulam o crescimento e a divisão celular, além da habilidade das células cancerosas de invadirem tecidos [1]. A carcinogênese é caracterizada por um processo que compreende múltiplas etapas. Inicia-se com uma alteração genética em uma única célula, que se torna geneticamente instável e acumula sucessivamente outras alterações genéticas e epigenéticas [2].

O câncer é uma das principais causas de morte tanto em países economicamente desenvolvidos quanto em países subdesenvolvidos. Esta patologia deverá crescer em todo o mundo devido ao crescimento e ao envelhecimento da população, particularmente nos países menos desenvolvidos, nos quais residem cerca de 82% da população mundial. A adoção de comportamentos no estilo de vida que são conhecidos por aumentar o risco de câncer, como: tabagismo, má alimentação, sedentarismo e mudanças reprodutivas, aumentaram ainda mais as taxas desta doença em países economicamente menos desenvolvidos [3].

Estima-se que 14,1 milhões de novos casos e 8,2 milhões de mortes por câncer ocorreram no ano de 2012 ao redor do mundo (figura 1) [4]. Os tumores de pulmão e mama são os mais frequentemente diagnosticados (com exceção dos cânceres de pele não melanoma), e as principais causas de morte por esta patologia em homens e mulheres respectivamente. Outros cânceres diagnosticados com frequência em todo o mundo incluem os do fígado, estômago, bexiga e colo retal entre os homens e os de estômago, cervical, uterino e colo retal entre as mulheres [4,5].

No Brasil, as estimativas dos 10 tipos de câncer mais incidentes para o ano de 2016 estão indicadas na figura 2. O câncer de mama é o tipo de câncer mais incidente em mulheres, sendo considerado um grave problema de saúde pública uma vez que é a primeira causa de morte por câncer nas mesmas [6].

CÂNCER DE MAMA.

A maioria dos cânceres de mama são carcinomas, um tipo de câncer que começa nas células epiteliais. Quando o tumor inicia seu crescimento em células do tecido glandular são chamados de adenocarcinomas. Outros tipos de tumor podem ocorrer na mama, como os sarcomas, que começam nas células do músculo, gordura ou tecido conjuntivo [7].

O câncer de mama é o tipo mais frequentemente diagnosticado e a principal causa de morte por câncer entre as mulheres do mundo, com 1,7 milhões de casos e 521.900 mortes no ano de 2012 (figura 1) [4,5]. No Brasil, para o ano de 2016, são esperados 57.960 novos casos em mulheres. O câncer de mama também pode acometer os homens, sendo mais raro, atingindo cerca de 1% do total de casos desta doença [6].

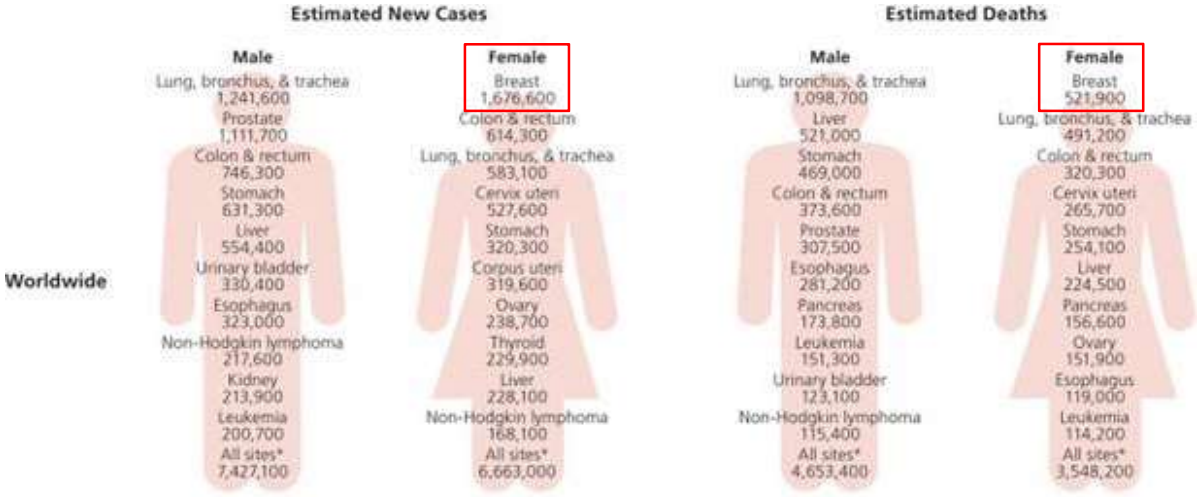

Figura 1: Estimativa dos novos casos de câncer no mundo e número de mortes no mundo por sexo [4].

Distribuição proporcional dos dez tipos de câncer mais incidentes estimados para 2016 por sexo, exceto pele não melanoma* (FIGURA 1)

Localização primária	casos novos	%	Homens / Mulheres	Localização primária	casos novos	%
Próstata	61.200	28,6%		Mama Feminina	57.960	28,1%
Traqueia, Brônquio e Pulmão	17.330	8,1%		Cólon e Reto	17.620	8,6%
Cólon e Reto	16.660	7,8%		Colo do Útero	16.340	7,9%
Estômago	12.920	6,0%		Traqueia, Brônquio e Pulmão	10.890	5,3%
Cavidade Oral	11.140	5,2%		Estômago	7.600	3,7%
Esôfago	7.950	3,7%		Corpo do Útero	6.950	3,4%
Bexiga	7.200	3,4%		Ovário	6.150	3,0%
Laringe	6.360	3,0%		Glândula Tireoide	5.870	2,9%
Leucemias	5.540	2,6%		Linfoma não Hodgkin	5.030	2,4%
Sistema Nervoso Central	5.440	2,5%		Sistema Nervoso Central	4.830	2,3%

* Números arredondados para múltiplos de 10

Figura 2: Distribuição proporcional dos dez tipos de câncer mais incidentes estimados para 2016 por sexo, exceto pele não melanoma [6].

TIPOS DE CÂNCER DE MAMA EM RELAÇÃO A SUA LOCALIZAÇÃO NO TECIDO MAMÁRIO.

Os tumores de mama são caracterizados de acordo com a morfologia das células quando observadas em microscópio. Existem 4 tipos principais:

- Carcinoma ductal *in situ*: também chamado de carcinoma intraductal, considerado não-invasivo ou pré-invasivo. As células que revestem o ducto alteram sua morfologia para células tumorais que crescem e preenchem o ducto, porém não rompem a parede do mesmo

espalhando-se para o tecido mamário adjacente. Também é considerado um pré-câncer, pois pode se tornar invasivo (figura 3) [7,8].

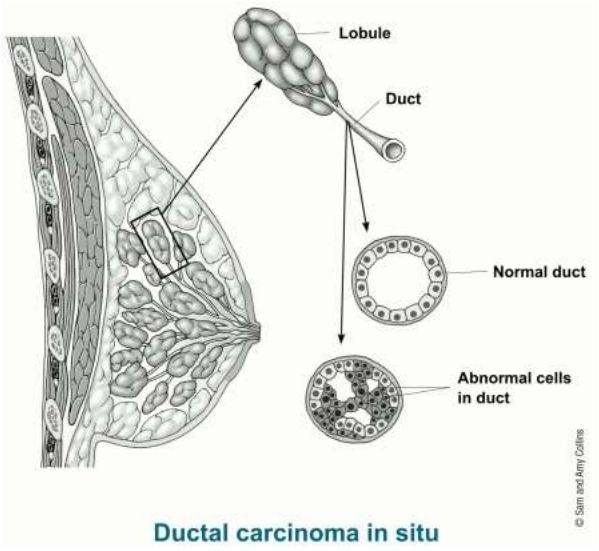

Figura 3: Esquema representando a localização do carcinoma ductal *in situ* [7].

- Carcinoma lobular *in situ*: Células cancerosas crescem e preenchem o lóbulo (glândula produtora de leite, localizada no final dos ductos), porém não crescem no tecido mamário adjacente (figura 4) [7,9,10,11].

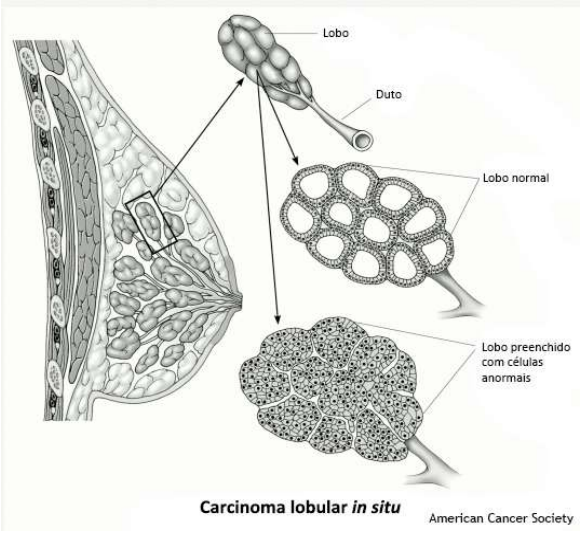

Figura 4: Esquema representando a localização do carcinoma lobular *in situ* [7].

- Carcinoma ductal invasivo ou infiltrativo: é o tipo mais comum de câncer de mama. Inicia em um ducto, rompe a parede desse ducto e cresce no tecido mamário adjacente [7,12].
- Carcinoma lobular invasivo: começa nos lóbulos. Assim como o carcinoma ductal invasivo pode se espalhar para outras partes do corpo. Cerca de 10% dos cânceres de mama invasivos correspondem ao carcinoma lobular invasivo, sendo então o segundo mais frequente [8,13].

CLASSIFICAÇÃO DAS CÉLULAS TUMORAIS MAMÁRIAS.

Os tumores invasivos também podem ser classificados com base na presença (positivo) ou ausência (negativo) da proteína chamada receptor de fator de crescimento epidérmico 2 (HER2) expressa pelas células cancerígenas ou ainda pela presença (positivo) ou ausência (negativo) de receptores hormonais. Os receptores hormonais frequentemente encontrados em células tumorais mamárias são os receptores de estrógeno (ER-positivo) e progesterona (PR-positivo). Cerca de dois terços dos casos apresentam no mínimo um destes receptores. A proteína promotora de crescimento mais observada é a HER2, ela é produzida devido à presença do gene HER2/*neu* nestas células, sendo então considerada HER2-positivo. Quando ela está presente, esses cânceres tendem a crescer e se espalhar de forma mais agressiva do que outros tumores de mama [7].

Sendo assim, as células tumorais mamárias são classificadas como: receptor de hormônios positivo, quando elas apresentam um ou os dois receptores, estrógeno e progesterona. Estas apresentam como característica o crescimento mais lento, respondem ao tratamento por terapia hormonal, são mais comuns em mulheres menopausadas. Isto não ocorre quando as células são negativas para receptores de estrógeno e progesterona, pois seu crescimento é mais rápido e não respondem ao tratamento hormonal estando presente em mulheres mais jovens e que ainda não entraram na menopausa [14,15]. Em relação à proteína HER2, também podemos classificar as células tumorais em HER2-positivo, quando esta proteína está aumentada ou possui copias extras do gene HER2/*neu* e HER2-negativo, quando as características citadas acima não estão presentes [16,17]. Uma última classificação ainda é considerada, quando as células não apresentam receptores hormonais e a proteína HER2 não está aumentada, o câncer é chamado de triplo negativo. Estes ocorrem com maior frequência em mulheres mais jovens e de origem africana, americana ou latina. Apresentam células tumorais que tendem a crescer e se espalhar mais rapidamente do que os outros tipos de câncer de mama [15].

TRATAMENTOS ATUALMENTE DISPONÍVEIS PARA O CÂNCER DE MAMA

Atualmente o tratamento do câncer de mama se restringe a dois tipos: locais e sistêmicos. A remoção cirúrgica do tumor e a radioterapia são tratamentos locais realizados independentemente do tipo e classificação celular do tumor [18]. Já os tratamentos sistêmicos são utilizados de acordo com as classificações descritas anteriormente. Para tumores classificados como ER-positivo e/ou PR-positivo a terapia hormonal é indicada e compreende duas classes principais, os moduladores dos receptores de estrógeno, que tem o tamoxifeno

como medicamento mais utilizado e os inibidores da aromatase, uma enzima que atua na produção de estrógeno e tem como representantes o letrozol e anastrozol [14,15]. A terapia com agentes progestacionais é uma terapia de segunda linha, uma vez que a utilização dos moduladores dos receptores de estrógeno e inibidores da aromatase demonstram maior sucesso [19]. Anticorpos monoclonais como o transtuzumab, pertuzumab, ado-transtuzumab emtansine dentre outros, tem como alvo a proteína HER2 e são utilizados com sucesso no tratamento de células tumorais mamárias que expressam essas proteínas [16,17]. Existem ainda as terapias quimioterápicas, cujos agentes agem independente da classificação das células pois são agentes citotóxicos, tendo como exemplo a doxorubicina, plaquitaxel, ciclofosfamida, etc [20].

TERAPIA FOTODINÂMICA, UMA MODALIDADE TERAPÊUTICA DE SUCESSO E CLINICAMENTE APROVADA.

O papel da luz já foi amplamente discutido em decorrência de suas funções vitais para a existência de vida na Terra. Os períodos de dia e noite são imprescindíveis para atividades necessárias à vida animal e vegetal, como a fotossíntese, que permite a planta produzir energia e oxigênio, e que só ocorre devido às alternâncias da presença de luz (dia e noite) [21]. Há 3500 anos os egípcios já haviam relatado o poder terapêutico da luz associada à ingestão de infusões naturais [22]. Na Alemanha, no ano de 1900, o estudante de medicina Oscar Raab, foi capaz de combater um protozoário, o *Paramecium*, utilizando acridina e luz solar [23]. Porém, somente nos séculos XIX e XX foi dispensada uma atenção maior a utilização da luz no combate às doenças, culminando no prêmio Nobel de medicina em 1903, por Niels Ryberg Finsen pelos resultados positivos alcançados no combate de *Lupus vulgaris* por luz UV (figura 5) [24,25].

A utilização da luz, associada ou não a compostos fotoquimicamente ativos, tem despertado interesse nos últimos anos, sendo a Terapia Fotodinâmica (TFD) uma modalidade amplamente estudada e considerada promissora no tratamento de diversas enfermidades como, por exemplo: infecções bacterianas, virais, fúngicas, desordens inflamatórias e alterações celulares benignas e malignas (câncer) [21, 26].

A TFD é composta por três componentes essenciais: luz em comprimento de onda adequado, um composto fotossensibilizador (FS) e oxigênio molecular (O_2) [25,27]. Após a administração de um FS, o mesmo frequentemente se acumula no tecido alvo. Se este tecido for iluminado por uma fonte de luz de comprimento de onda adequado, o FS será ativado comumente mediante sua transição entre o estado fundamental (S_0) e o estado excitado

singlete (S_1). Quando o FS retorna para seu estado S_0, ele libera energia a qual é absorvida pelo oxigênio molecular e substratos orgânicos, levando a formação de radicais livres (mecanismo do tipo I). Também, poderá levar a formação de espécies reativas de oxigênio (EROS), sendo o oxigênio singlete o principal (mecanismo do tipo II). Radicais livres e oxigênio singlete gerados via mecanismo I e II, respectivamente, são os responsáveis pelos danos celulares [28,29]. Os mecanismos do tipo I e II ocorrem simultaneamente (figura 6), e os danos celulares resultantes deste tipo de tratamento estão relacionados ao tipo de FS utilizado, a quantidade de células e oxigênio disponível, afinidade do FS pelas células, sua localização intra e extracelular e com a dose do FS e da luz administrada [25,27,30].

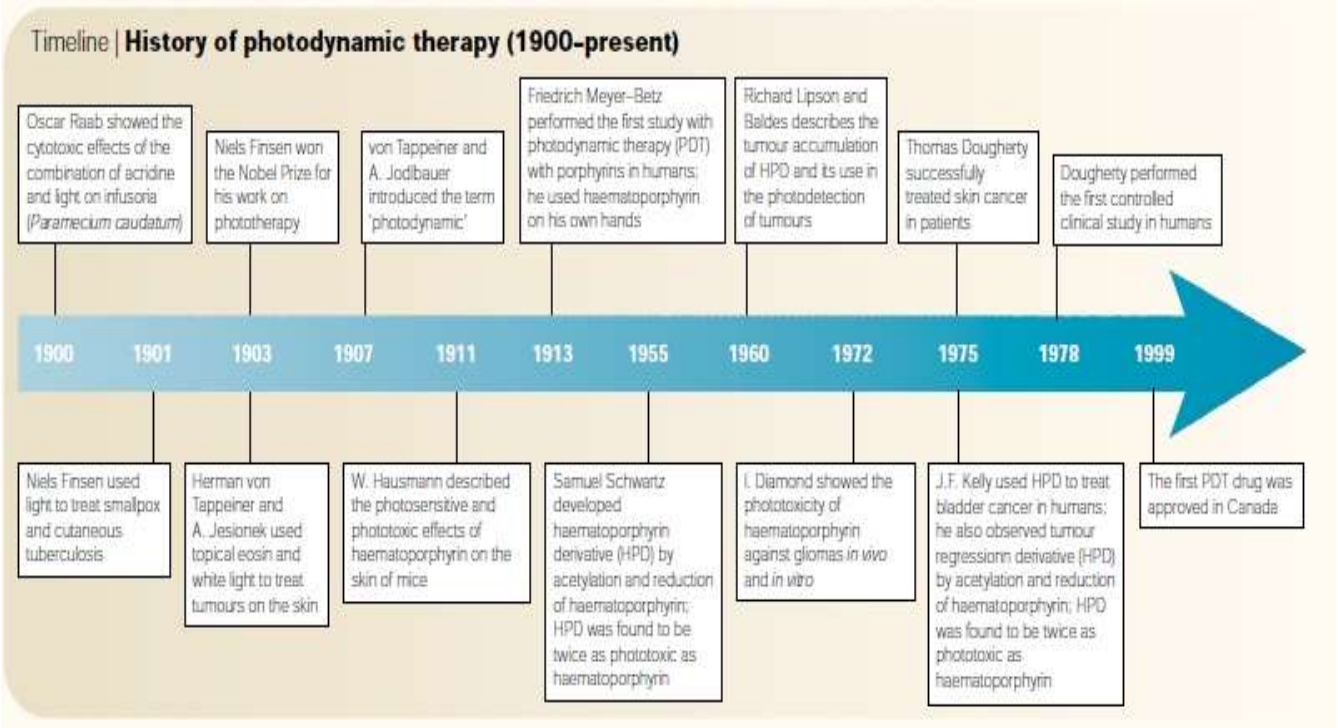

Figura 5: História da Terapia Fotodinâmica [27].

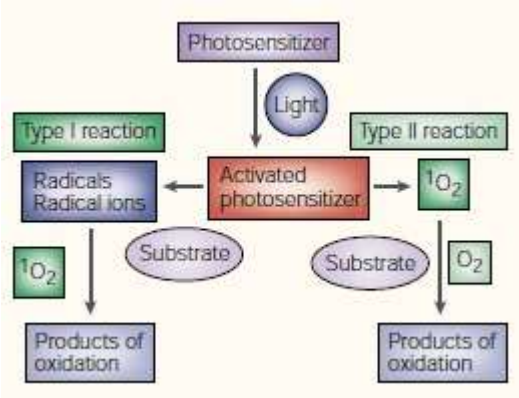

Figura 6: Mecanismos tipo I e II na terapia Fotodinâmica [27].

TERAPIA FOTODINÂMICA NO TRATAMENTO DO CÂNCER

A seletiva retenção do fotossensibilizador nos tecidos neoplásicos e a ativação da droga por irradiação “in situ” fazem com que a TFD ofereça vantagens em relação à quimioterapia convencional e a radioterapia, utilizadas no tratamento do câncer, pois combinam uma mínima toxicidade sistêmica com uma alta seletividade fotodinâmica de destruição das células tumorais. A TFD tem sido amplamente utilizada no tratamento de lesões de pele que são fáceis de irradiar [31], como é o caso de ceratoses actínicas, carcinoma basocelular, doença de Bowen e melanoma [32,33,34]. Contudo, não está limitada apenas ao câncer de pele e tem se tornado uma modalidade terapêutica altamente aceita e promissora para o tratamento de vários tipos de tumor. O desenvolvimento de lasers potentes combinados com fibras óticas flexíveis, tem tecnicamente tornado viável a aplicação clínica desta terapia em tecidos mais profundos [31].

A TFD leva principalmente a morte celular por necrose e/ou apoptose (figura 7), porém sua ação não se limita somente ao efeito citotóxico sobre as células neoplásicas, mas também promove danos na vascularização do tumor e ativação de uma resposta imune contra as células alvo [35,36].

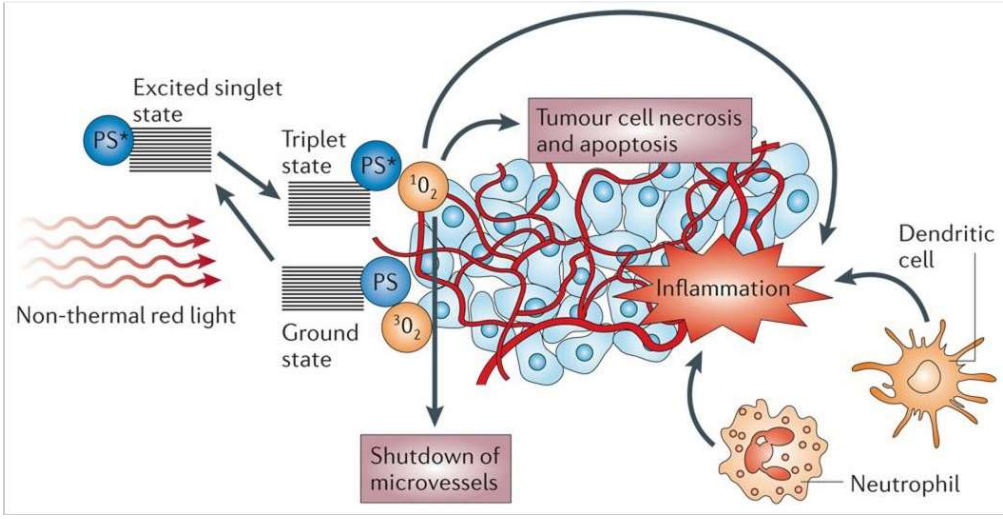

Figura 7: Mecanismos de ação da Terapia Fotodinâmica nos tumores [36].

HIPERICINA, UM FOTOSSENSIBILIZADOR PROMISSOR PARA USO NA TFD

Hipericina (4,5,7,4’,5’,7’-hexahidroxil-2,2’-dimetilnaftodiantrona) (figura 8) é um composto natural, biossintetizado por algumas espécies do gênero *Hypericum*, podendo também ser sintetizada quimicamente a partir de uma antraquinona precursora, a emodina antrona [31,37]. A Hipericina (HIP) foi primeiramente isolada da espécie *Hypericum perforatum* L., comumente conhecida como Erva de São João, que apresenta uma ampla

aplicação farmacológica, sendo utilizada como antidepressivo, antimicrobiano, anticancerígeno, anti-inflamatório, cicatrizante e etc [38].

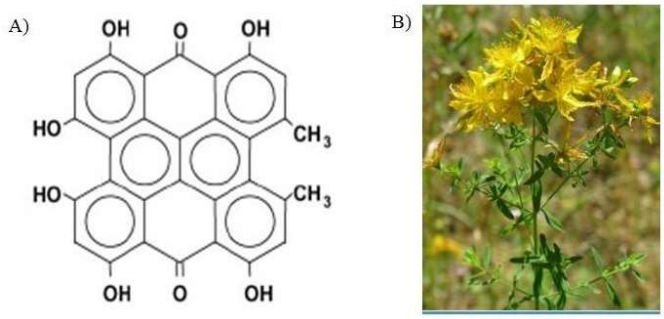

Figura 8: A) Estrutura química da Hipericina B) Ilustração da planta Erva de São João [31].

As propriedades fototerapêuticas da HIP foram primeiramente reconhecidas estudando casos de hipericismo em animais. Hipericismo foi um termo utilizado após observações de danos cutâneos causados pela fotosensitividade após a ingestão de grandes quantidades de plantas do gênero *Hypericum* e exposição à luz [39].

A HIP é um fotossensibilizador poderoso encontrado na natureza. Ela exibe uma brilhante fluorescência vermelha, produz altas taxas de oxigênio singlete e radicais livres, com baixa toxicidade na ausência de luz, acumulando-se preferencialmente em tecidos neoplásicos [31,40,41,42]. Os radicais livres e o oxigênio singlete gerados são responsáveis por induzir a morte por necrose, apoptose, morte associada à autofagia e até mesmo morte celular imunogênica. O mecanismo do tipo II é o responsável pela ativação da morte celular imunogênica, sendo a combinação da TFD com HIP um promissor agente para a imunoterapia, pois provoca a translocação e secreção de DAMPs (padrões moleculares associados a danos) pelas células tumorais, que são capazes de ativar o sistema imune pela ligação a receptores específicos, aumentando a resposta imune antitumoral [43,44]. O efeito inibitório da HIP contra enzimas relacionadas à regulação da sobrevivência e proliferação celular também colaboram para seu efeito antitumoral eficaz [45].

Sendo um composto altamente hidrofóbico, sua dissolução pelos veículos farmacêuticos mais utilizados é dificultada, porém a conjugação da HIP com polímeros solúveis em água ou a encapsulação com carreadores nanoparticulados como as micelas são uma maneira eficiente de superar problemas relacionados à administração sistêmica, o que torna possível a utilização de baixas concentrações da droga, diminuindo a possibilidade de fotossensibilidade cutânea e dificultando a auto-agregação do fotossensibilizador, pois na forma agregada seu potencial fotodinâmico é diminuído [46,47].

Um trabalho realizado pelos pesquisadores do Núcleo de Pesquisa em Sistemas Fotodinâmicos do Departamento de Química da Universidade Estadual de Maringá, foi à otimização da síntese da HIP a partir da emodina e posterior encapsulação em micelas utilizando o copolímero P-123® fabricado pela BASF (Alemanha). Sua composição em tri-bloco PEO (polietilenoglicol) - PPO (polipropilenoglicol) – PEO (polietilenoglicol) permite que o composto hidrofóbico, no caso a HIP, permaneça aprisionado na parte hidrofóbica PPO e a parte hidrofílica PEO do copolímero permite a dispersão da micela no solvente hidrofílico desejado [48]. Essas micelas já demonstraram ser eficientes como sistemas de entrega para drogas antitumorais, acumulando-se preferencialmente nos tecidos neoplásicos, sendo biocompatíveis e altamente estáveis [49,50].

HIPERICINA, ESTUDOS PRÉ-CLÍNICOS E CLÍNICOS COMPROVANDO SUA EFICÁCIA NA TFD

Uma revisão publicada no ano de 2016 resume todos os trabalhos realizados atualmente sobre a utilização da HIP como composto FS na terapia fotodinâmica. Além de muitos estudos *in vitro*, utilizando células tumorais e estudos pré-clínicos em ratos e camundongos, três estudos clínicos em humanos já foram realizados [51].

Em 1997, Alecu *et. al.* testaram uma injeção de HIP intralesional com posterior fotoativação com luz visível em onze pacientes com carcinoma celular basal e oito pacientes com carcinoma celular escamoso, observando que a HIP foi efetiva no tratamento de ambas as desordens de pele, com diminuição do tamanho do tumor, facilitando a geração de um novo epitélio na superfície das lesões. No tecido saudável circundante a lesão, não ocorreu necrose nem perda celular, com fraco eritema em apenas cinco pacientes [32].

Kacerovská *et.al.* no ano de 2008 tratou pacientes com queratose actínica, doença de Bowen e carcinoma celular basal com o sistema HIP-TFD, com significativa redução dos casos tratados após seis meses, em especial nos pacientes com doença de Bowen em que ocorreu completa remissão histológica em 80% dos pacientes [33].

No terceiro estudo clínico, em 2010, Rook *et. al.* testaram a HIP-TFD para o tratamento de desordens de pele como a micose fungóide, que é um tipo de linfoma cutâneo de células T e a psoríase cutânea (não tumoral), obtendo resultados promissores para ambas as doenças [34].

JUSTIFICATIVA

O câncer de mama ainda permanece com grande incidência mundial e constitui-se como importante problema de saúde pública, causando significativa morbidade e mortalidade feminina. É o mais incidente em mulheres, excetuando-se os casos de pele não melanoma, representando 25% do total de casos de câncer no mundo em 2012, com aproximadamente 1,7 milhão de casos novos naquele ano. É a quinta causa de morte por câncer em geral (522.000 óbitos) e a causa mais frequente de morte por câncer em mulheres. A taxa de mortalidade por câncer de mama ajustada pela população mundial apresenta uma curva ascendente e representa a primeira causa de morte por câncer na população feminina brasileira, com 12,66 óbitos/100.000 mulheres em 2013. As opções de tratamento para obter efeitos citotóxicos em células neoplásicas e evitar, portanto, o avanço do câncer de mama ainda são limitados e apresentam elevadas taxas de insucesso. Assim ainda existe a necessidade de buscar novos fármacos para o tratamento desse tipo de câncer. Nesta perspectiva, diversos estudos já demonstraram a atividade da HIP após a fotoativação, provocando danos celulares em concentrações muito pequenas sobre diversas linhagens de tumor. Porém, não existem estudos que avaliem a atividade antitumoral *in vitro* da HIP encapsulada com o copolímero P-123 (HIP/P-123) em células de carcinoma ductal invasivo de mama.

OBJETIVOS

GERAL

Avaliar o efeito antitumoral da HIP encapsulada no copolímero P-123 em modelo de neoplasia do epitélio mamário, a fim de auxiliar na busca de novas opções de tratamento para o câncer de mama.

ESPECÍFICOS

1 – Determinar as taxas de morte celular decorrentes do tratamento com HIP/P-123 em células neoplásicas mamárias MCF-7 e em células epiteliais mamárias normais MCF-10A;

2 – Diferenciar o estado de morte celular entre necrose, apoptose e senescência em células neoplásicas mamárias MCF-7;

3 – Avaliar o efeito da HIP/P-123 no processo de migração de células neoplásicas mamárias MCF-7, quantificado por ensaio de migração do tipo ensaio da ferida;

4 – Determinar a citotoxicidade em longo prazo da HIP/P-123 em células neoplásicas mamárias MCF-7, através do ensaio clonogênico;

5 – Observar alterações morfológicas em células neoplásicas mamárias MCF-7, após o tratamento com HIP/P-123;

6 – Visualizar a interiorização da HIP/P-123 em células epiteliais mamárias normais MCF-10A e em células neoplásicas mamárias MCF-7.

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CAPÍTULO II

Artigo: Antitumoral effect of photodynamic therapy against breast cancer cells utilizing pluronic P-123 delivery of hypericin (4, 5, 7, 4', 5', 7'-hexahydroxy-2' 2'-dimethylnaphthodianthone)

Antitumoral effect of photodynamic therapy against breast cancer cells utilizing pluronic P-123 delivery of hypericin (4, 5, 7, 4', 5', 7'-hexahydroxy-2' 2'-dimethylnaphthodianthone)

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Abstract

Hypericin (4, 5, 7, 4', 5', 7'-hexahydroxy-2' 2'-dimethylnaphthodianthone) is a promissing photosensitizer with high fluorescence quantum yield, but has as main problem the lipophilic nature, which tends to form aggregates in aqueous enviroment. The encapsulation of Hypericin (HYP) with pluronic P-123 have improved it solubility. In this work were evaluated the antitumoral effects via photodynamic therapy (PDT) of HYP encapsulated with pluronic P-123 (HYP/P-123) in human breast cancer cell line (MCF-7) in comparison with human breast normal cells (MCF-10A). Damage in MCF-7 cells were more evident than in MCF-10A cells, there is a reduction or complete absence of viable MCF-7 cells in the highest concentrations tested. In the absence of illumination does not occur decreased in cell viability. Treatment of MCF-7 cells resulted in a decrease attachment and the confluence of the cells. The necrotic cell death was prevalent in the tumor cells. The wound closure was observed only in MCF-7 cells untreated after 72 hours. The clonogenic potential was reduced in a dose-time-dependent manner in the periods evaluated (7 and 14 days). The internalization of HYP/P-123 was higher in MCF-7 cells. Our results showed that low doses of the compound presented antitumor effects in cancer cells and low damage to normal cells presenting as a promising agent for the treatment of breast tumors.

Keywords: Hypericin; Photodynamic therapy; Breast cancer; Pluronic P-123; MCF-7; Antitumoral; Cell death pathway.

1. Introduction

Breast cancer is currently the second most frequent type of cancer in the world and were estimated in 2012, 1.67 million of new cases of these cancer diagnosed (25% of all cases) [1]. Aging appears as the main risk factor but other factors are well established, like the woman's reproductive life (early menarche, first pregnancy after 30 years of age, use of oral contraceptives, late menopause and hormone replacement) and familiar components as changes in genes regulation, the hormonal metabolism and DNA repair [2,3].

The treatments currently available are divided into two groups: Local, comprising radiation therapy and surgical procedures and systemic, using chemotherapy, hormone therapy and biological therapy. However, when evaluating the mortality rate from breast cancer adjusted by the world's population, it presents an upward curve, being the most frequent cause of cancer death in women in less developed regions [1,4,5,6,7]. Thus, it appears that the available treatments today are limited and have high failure rates, requiring the constant search for new treatments.

From this perspective, photodynamic therapy (PDT) emerges as a new modality, as it has shown success in treating different kinds of diseases with proliferating cells and may reach specific targets without causing systemic side effects or in healthy tissues [8,9]. Is a relatively new therapy using a photosensitizer compound which under the action of light of a given wavelength in the presence of molecular oxygen (O_2) generates singlet oxygen ($^1O_2$), responsible for cell death. PDT antitumoral effects are related to citotoxic effects in cancer cells, damage in vasculature of the tumor and conduction to a inflamatory process that leads to a imune responce [9].

Hypericin (4, 5, 7, 4', 5', 7'-hexahydroxy-2' 2'-dimethylmes-*o*-naphthodianthone) is a napthodianthrone compound found in the *Hypericum perforatum* L. species [10]. Hypericin (HYP) has been explored in PDT for presenting high quantum yield values of fluorescence

and high ability to generate reactive oxygen species (ROS) after activation with light [11]. It is widely used as antidepressant, but other therapeutic uses like antiviral and antineoplasic also have been reported [12, 13]. The main problem in the progression of HYP–PDT to a clinical practice is the lipophilic nature of HYP which tends to form aggregates in aqueous environment. This aggregation significantly diminishes its photosensitizing efficacy [14] and can be overcome either by synthesis of water-soluble forms of HYP or by encapsulating hydrophobic HYP into appropriate drug carriers [15]. The encapsulations of hydrophobic molecules using amphiphilic blocks of copolymers, like pluronics, have improved the solubility of formulations with doxorubicin [16], hidroclorotiazide [17] and carbamazepine [18], which are already available in the market. In this sense, the pluronics have shown to be safe and it has been used even for parenteral solutions [19].

Although its photodynamic effect alone is known, to the best of our knowledge, the antitumoral activity of HYP encapsulated with P-123 (HYP/P-123) in the breast cancer cells have not yet conducted. So, in order to assist the searches for new treatment options for these cancer type, the present work aim to evaluate the antitumoral effects of HYP encapsulated with P-123 in MCF-7 human breast cancer cell line in comparison with MCF-10A human breast normal cells.

2. Material and methods

2.1. Photosensitizer

2.1.1. Synthesis and characterization of hypericin

HYP was synthesized by Research Nucleus in Photodynamic System (NUPESF) of the Universidade Estadual de Maringá (UEM)/Paraná State/Brazil through the following steps. First, emodin antrhaquinone (180 g) was extracted from the bark of *Rhamnus frangula* L. and purified by cromatography on column of silica gel (elution with trichloromethane/ethyl

acetate (*a*), *a* = 0 to 15% v/v). The crude product was recrystallized from a mixture of trichloromethane/hexane (80:20 v/v) to give 2.16 g (1.2%) of orange crystals. The reduction of emodin antrhaquinone to emodin antrhone was achieved dissolved 1.00 g emodin in 45 mL acetic acid and 5.00 g tin dichloride dihydrate dissolved in 40 mL concentrated hydrochloric acid were added and the solution was refluxed by 1 hour under vigorous stirring. After the reflux period, the solution was cooled and poured in 250 mL water and neutralized with 20% sodium carbonate solution. The precipitate is filtered and dried in vacuum (yield 0.98 g; 97%). Protohypericin was achivied by oxidative dimerization of emodin antrhone based on the procedure described in the patent of Mazur et al. [20]. To a solution of 1.00 g emodin anthrone in 22.0 mL of pyridine:piperidine (10:1 v/v) were added 2.00 g of pyridine N-oxide and 0.05 g of ferrous (II) sulfate monohydrate and the mixture was heated in the dark at 100ºC by 1 hour under vigorous stirring and air atmosphere. After cooling, the solvent was concentrated to approximately 5.0 mL and the solution was neutralized with 3.0% hydrochloric acid solution under stirring. The precipitate so formed is filtered off and dry under vacuum (yield 0.52 g; 52%). HYP was then obtained by irradiating with visible light at room temperature a solution of 0.50 g protohypericin in 500 mL acetone. The reaction was monitored by UV-Vis until the maximum absorbance in λ_{max} = 596 nm. After evaporation of the solvent, the crude product was purificated by size-exclusion chromatography using ethanol/water (80:20 v/v) as eluent and recrystallization from hexane to yield 0.49 g (98.0%) of a crystalline blue-dark solid.

2.1.2. NMR Experiments

Nuclear magnetic resonance (NMR) experiments was performed on the *Bruker AVANCE III HD* spectrometer (Bruker, USA) at 500 MHz equipped with direct field gradient

and probe of 5 mm, at constant temperature of 25º C. The spectra was referenced to tetramethyl silane (TMS) peak and processed using the *Bruker TopSpin 3.1* software.

2.1.3. Preparation of hypericin-loaded pluronic P-123 micelles (HYP/P-123)

The thin-film hydration method [21] was performed for incorporation HYP in P-123 copolymeric micelles. The solution stock for HYP and P-123 (Pluronic® P-123 (EO_{20}-PO_{65}-EO_{20}, MW=5750 $gmol^{-1}$; Sigma-Aldrich, USA) was first prepared in ethanol. Then, in a round-bottom flask both are added and the ethanol was evaporated by rotary evaporation at 50ºC to obtain a thin film. Residual solvent in the achieved solid matrix was removed under vacuum in desiccator for 12 h. After, the dried film was hydrated with distilled water at 50ºC under stirring for 2 h. The resulting formulation was lyophilized and hydrated when necessary.

2.2. Light Source

The light source was a device with 66 units of light-emitting diode (LED), emitting white light, 3.15 J/cm^2 at a range of 450 to 750 nm wavelength.

2.3. Cells culture

The two cell lines included in the study were: MCF-7, human breast invasive ductal carcinoma; and MCF-10A, human breast normal cell (non-tumorigenic control cells). MCF-7 line was acquired from Cell Bank of Rio de Janeiro/Brazil, and was maintained in a 75 cm^2 culture flask in DMEM/F-12 (Gibco, USA) supplemented with 20% fetal bovine serum (FBS, Gibco, USA), 1% of penicillin/streptomycin (Gibco, USA) and 1% of Amphotericin B solution (Sigma-Aldrich, USA) at 37 °C in a humidified atmosphere with 5% CO_2. MCF-10A cells were kindly donated by Dr. Marcelo Gialuisi Bonini (University of Illinois at Chicago,

USA) and maintained in a 75 cm^2 culture flask in DMEM/F-12 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 10 µg/mL of Insulin Solution Human (Sigma-Aldrich, USA), 20 ng/mL Human Epidermal Growth Factor (hEGF, Sigma-Aldrich, USA), 0.5 µg/mL Hydrocortisone (Sigma-Aldrich, USA), 1% of penicillin/streptomycin (Gibco, USA) and 1% of Amphotericin B solution (Sigma-Aldrich, USA) at 37 °C in a humidified atmosphere with 5% CO_2.

2.4. HYP/P-123 treatment

HYP/P-123 was dispersed in sterile distilled water at a concentration of 100 µmol/L HYP/8,8x10^{-5} mol/L P-123 and stored at 2°C. After reaching sub-confluence (70%–80% confluency), the cells were exposed to stock HYP/P-123 solution newly diluted in sterile distilled water (0.4–1.4 µmol/L hypericin and 0.35 – 1.25x10^{-6} mol/L P-123) for 30 minutes in absence of light. Following, cells treated were illuminated for 15 minutes, and again incubated in absence of light for 30 minutes. Cells treated with DMEM/F-12 or P-123 alone (1.25x10^{-6} mol/L) were used as negative controls in all assays.

2.5. Cellular viability assays

Cells viability was determined by trypan blue exclusion staining assay [22]. MCF-7 and MCF-10A cells were seeded in 24-well tissue culture plates at a density of approximately 2.5×10^5 cells/mL. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. After 24 hours, they were treated with a concentration range (0.4–1.4 µmol/L HYP and 0.35–1.25x10^{-6} mol/L P-123) and illuminated as described above (2.4). Next, the culture medium was removed and the cells were washed with phosphate-buffered saline (PBS), and 200 µL of 0.25% trypsin/EDTA solution (Gibco, USA) was added to detach the cells from the plate. The cells viability was assessed by counting live versus dead cells using standard trypan blue

(Gibco, USA) on a hemocytometer in an inverted microscope (EVOS FL Cell Imaging System, Life Technologies, CA, USA). The results were expressed as a percentage of the control cells, which was considered 100% of cell viability, and the data are shown as the mean values ± standard deviation (SD) of three independent experiments in triplicate.

The IC_{30} (concentration that inhibited cell growth by 30% compared to not treated controls), IC_{50} (concentration that inhibited cell growth by 50% compared to not treated controls) and IC_{90} (concentration that inhibited cell growth by 90% compared to not treated controls) values were obtained by nonlinear regression analysis of the data using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).

To evaluate the dark toxicity (cytotoxic effect of the HYP/P-123 without irradiation), tripan blue exclusion assay was performed in the same way as described in above, however without the lighting stage of treatment.

2.6. Cells morphologic analysis

MCF-7 cells were seeded in a density of $5x10^4$ cells/mL in 12-well tissue culture plates and cultured at 37°C in a humidified atmosphere with 5% CO_2. After reaching sub-confluence, they were treated with IC_{50} value of HYP/P-123 and illuminated as described above (2.4). Not treated (NT) cells were used as negative controls. The growth and morphology of cells were observed immediately after treatment with an inverted microscope (EVOS FL Cell Imaging System, Life Technologies, CA, USA).

2.7. Clonogenic assay

To determine the long-term growth suppression effects of HYP/P-123 photodynamic treatment, the clonogenic assay was performed [23]. MCF-7 cells were seeded in 6-well tissue culture plates at a density of 600 cells/well. After 24 h, cells were exposed to IC_{30} and IC_{50} of

HYP/P-123 values and illuminated as described above (2.4). Following, cells were incubated in ideal conditions for 7 and 14 days (medium was changed after 7 days for cells that were exposed for 14 days). The recovery ability of colonies was evaluated. The colonies formed from MCF-7 cell line were stained with crystal violet solution (Sigma-Aldrich, USA) after fixation with methanol (Synth, BRAZIL) and counted manually. In each case results are expressed as survival colonies, which were obtained by dividing the number of colonies that arose after cell treatment by the number of cells seeded and plate efficiency (PE: number of colonies formed by untreated cells/number of cells seeded) multiplied by 100.

2.8. Analysis of cells senescence

Cell senescence was evaluated using β-galactosidase [24]. Cell line MCF-7 was seeded in a 24-well tissue culture plates in a density of $5x10^4$ cells/mL and cultured at 37 °C in a humidified atmosphere with 5% CO_2. After 24 hours, cells were treated with IC_{50} and IC_{90} values of HYP/P-123 and illumination. Then, cells were washed twice in PBS and fixed in solution containing 0.5% glutaraldehyde (Synth, BRAZIL) for 15 minutes. The fixation solution was removed by washing the cells twice with PBS containing $MgCl_2$ (Synth, BRAZIL), and then X-Gal staining solution (1 mg/mL X-gal Invitrogen, USA; 0.12 mM $K_3Fe(CN)_6$ Synth, USA; 0.12 mM $K_4Fe(CN)_6$ Synth, USA; 1 mM $MgCl_2$ Synth, USA in PBS) was added. Cells were then incubated at 37 °C in a CO_2 free environment for 4 hours. Doxorubicin was used at a concentration of 5 μg/mL as positive control. The percentage of cells stained with blue cytoplasm was considered senescent and determined after counting three random fields of 100 cells each. Data are shown as the mean value ± SD of three independent experiments in triplicate.

2.9. Analysis of cells death pathway

Apoptosis and necrosis assays were performed by Annexin V-FITC/Propidium Iodide (PI) using a previously described protocol with some modifications [25]. Briefly, $2.5x10^5$ MCF-7 cells/mL were seeded overnight in 24-well tissue culture plates and cultured at 37 °C in a humidified atmosphere with 5% CO_2. These cells were treated with IC_{50} and IC_{90} values of HYP/P-123 and illumination. After treatment, cells in the supernatant and the adherent cells were washed with PBS and binding buffer (10 mM HEPES, pH 7.5, containing 140 mM NaCl and 2.5 mM $CaCl_2$) and stained with 1 μg/mL of FITC-conjugated Annexin-V for 15 minutes and 40 μg/mL of PI for 5 minutes. Camptothecin 20 μM and Digitonin 80 μM were used as positive controls for apoptosis and necrosis, respectively. Cells without treatment with HYP/P-123 were used as negative controls. Each sample was analyzed by an inverted fluorescence microscope (EVOS FL Cell Imaging System, Life Technologies, CA, USA) to distinguish the apoptotic (green fluorescence) and necrotic cells (red fluorescence). Fluorescence intensity was measured using ImageJ software (v1.48, public domain, National Institute of Health, USA) [26].

2.10. Wound-healing migration assay

Wound-healing assays were performed as previously described [27]. MCF-7 cells ($2.5x10^4$ cells/mL) were seeded in 6-well tissue culture plates and cultured at 37 °C in a humidified atmosphere with 5% CO_2. The confluent monolayer of cells was then mechanically scratched with a blue pipet tip (1000 μL), and cell debris was removed by washing with PBS. Then, the wounded monolayer was treated with HYP/P-123 (IC_{30} and IC_{50} values) and illumination. Culture medium was used as negative control. Cells migration into the scratched region was recorded using an inverted microscope (EVOS FL Cell Imaging

System, Life Technologies, CA, USA) at 0, 24, 48 and 72 hours. The results were calculated by comparing the wound closure after 24, 48 and 72 h to initial measurement.

2.11. Internalization of H/P-123 in breast cell lines.

MCF-7 and MCF-10A cell lines were seeded in a 24-well tissue culture plates at a density of approximately $2.5x10^4$ cells/mL. Cells were allowed to attach overnight at 37°C in a humidified atmosphere with 5% CO_2. After 24 hours, cells were incubated for 30 minutes with 50 μmol/L HYP and $4.4x10^{-6}$ mol/L P-123. To display fluorescence emitted by HYP inside the cell an inverted fluorescence microscope with RFV (red) filter (EVOS FL Cell Imaging System, Life Technologies, CA, USA) was used.

2.12. Subcellular distribution of H/P-123

MCF-7 and MCF-10A cell lines were seeded and incubated as above (item 2.11). After 24 hours of incubation, cells were incubated for 1 hour with subcellular organelle probes specific for mitochondria (Mitotracker®, Invitrogen, USA) and for endoplasmic reticumum (ER-tracker®, Invitrogen, USA) and for 30 minutes with 50 μmol/L HYP and $4.4x10^{-6}$ mol/L P-123. To display fluorescence emitted by HYP and stained cell organelles an inverted fluorescence microscope with RFP (red) and GFP (green) filters (EVOS FL Cell Imaging System, Life Technologies, CA, USA) was used.

2.13. Statistical analysis

Data were expressed as the means ± standard deviation (SD) of at least three independent experiments. Significant differences among means were identified using analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test, except for viability experiments, where Student's *t*-distribution was used for data analyses. The data were

analyzed using Prism 6.0 software (GraphPad, San Diego, CA, USA). Values of $P < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. HYP synthesis and characterization

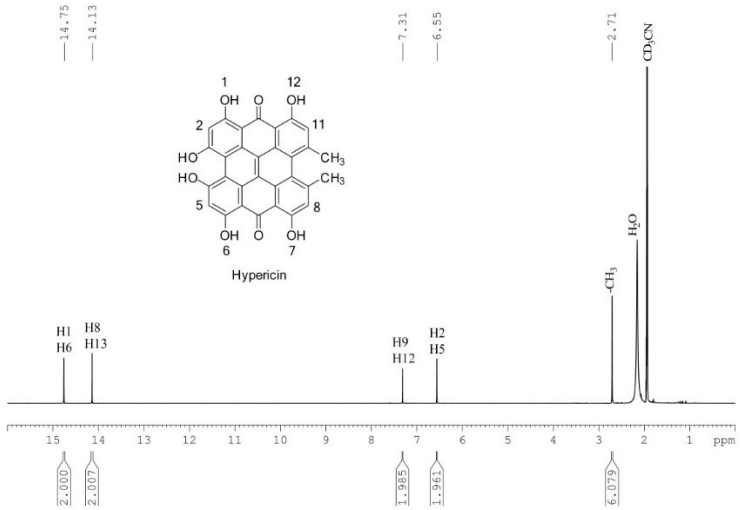

Figure 1. The nuclear magnetic resonance spectrum (^{1}H NMR) of HYP. The spectrum of purified substance was acquired in 500 MHz nuclear magnetic resonance spectrometer. In CD_3CN at 25°C.

The ^{1}H NMR spectrum shown in figure 1 further confirms the molecular structure and purity of the synthesized HYP. [H-NMR (500 MHz, CD_3CN at 25° C): δ = 14.75 (s, 2H, OH-1 e OH-6), 14.13 (s, 2H, OH-7 e OH-12), 7.31 (s, 2H, *Ar*-H8 e *Ar*-H11), 6.55 (s, 2H, *Ar*-H2 e *Ar*-H5), 2.71 (s, 6H, *Ar*-CH_3) ppm; UV-Vis (EtOH) λ_{max} = 392, 480, 513, 552 e 596].

3.2. Cells viability post HYP/P-123 treatment

To study the photodynamic effect of HYP/P-123 on tumor cells as well as on normal cells, we treated a breast cancer cell line, MCF-7 and a normal breast cell line, MCF-10A. The cytotoxicity test was performed in the absence and presence of light (figures 2.A and 2.B). As indicated in figure 2.B, HYP/P-123 exerted dose-dependent cytotoxic effects on

MCF-7 and MCF-10A cell lines in presence of light. Damage in tumor cells MCF-7 in the presence of light was more evident than in normal cells MCF-10A, where it was observed the decrease or complete absence of MCF-7 viable cells in the largest concentrations tested (0.8 – 1.4 µmol HYP). HYP alone was described for had a higher affinity for tumor cells than for normal cells [28,29]. After systemic administration a 16 times higher concentration of HYP was found in tumor tissue than in surrounding healthy tissue [29].

When evaluating the cytotoxicity of HYP/P-123 system in the absence of light, there was no decrease in cells viability (figure 2.A) in both lines. P-123 polymer did not show cytotoxic effect on cells when tested in the absence (figure 2.A) or presence (figure 2.B) of light. In both cell lines no treated (NT) with HYP/P-123 but exposed to the action of light, the cells viability didn´t changed (figure 2.A).

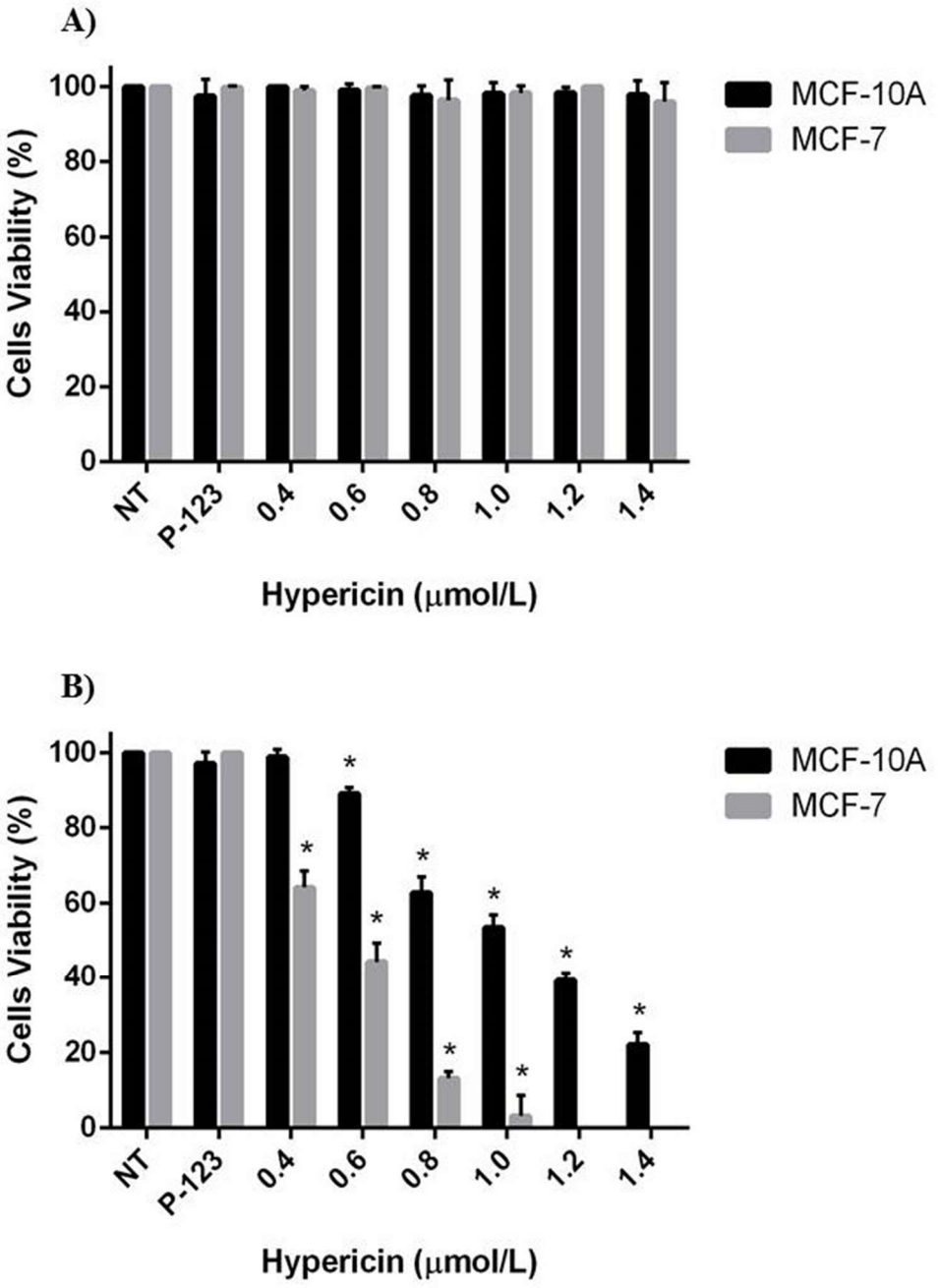

Figure 2. Cytotoxic effects of HYP/P-123 (μmol/L) on breast cancer cell line (MCF-7) and normal human breast cell line (MCF-10A). A) Graph indicating that viability of breast MCF-7 and MCF-10A after exposure to HYP/P-123 (0.4–1.4 μmol/L of hypericin) in absence of light did not change. B) Graph indicating the viability of breast MCF-7 and MCF-10A cells post treatment with HYP/P-123 (0.4– 1.4 μmol/L) in presence of light. This data shows a significant decrease in MCF-7 viability more than in MCF-10A. The * show statistically significant ($P < 0.05$) when cells treated with HYP/P-123 were compared with not treated cells (NT).

The Table 1 shows IC_{30}, IC_{50} and IC_{90} values obtained from trypan blue dye exclusion assay to cells viability (figure 2.B). These dose-response graph shows a significant decrease in the percentage of cell viability of breast cancer cell line MCF-7 compared to not treated (NT) cells in presence of light. Once again, these data highlighted the selective effect of HYP/P-123 on cancer cells, similar to studies in other human cancer types with HYP alone, that reported its low intrinsic toxicity and differential effects in normal versus cancer cells [29].

The cytotoxic effect of HYP/P-123 showed significant reduction from the lowest concentration tested (0.40 µmol/L; P=0.0001), which not occurred in normal breast cells MCF-10A, where the reduction of cell viability was only significant from the concentration of 0.60 µmol/L (P=0.0004) (figure 2.B).

Table 1 IC_{30}, IC_{50} and IC_{90} values of HYP/P-123 determined according to the cells viability obtained in figure 2.B.

	MCF-7	**MCF-10A**
IC_{30}	0.40 µmol/L HYP – $0.35x10^{-6}$mol/L P-123	0.89 µmol/L HYP – $0.80x10^{-6}$mol/L P-123
IC_{50}	0.66 µmol/L HYP – $0.55x10^{-6}$mol/L P-123	1.49 µmol/L HYP – $1.30x10^{-6}$mol/L P-123
IC_{90}	1.20 µmol/L HYP – $1.05x10^{-6}$mol/L P-123	2.68 µmol/L HYP – $2.35x10^{-6}$mol/L P-123

IC: inhibitory concentration; IC_{30}: concentration that inhibited cell growth by 30% compared to not treated controls; IC_{50} : concentration that inhibited cell growth by 50% compared to not treated controls; IC_{90}: concentration that inhibited cell growth by 90% compared to not treated controls; HYP/P-123: hypericin incorporated in P-123 copolymeric micelles; MCF-7: human cell line derived from breast invasive ductal carcinoma; MCF-10A: human breast normal cell line.
Each line represents the mean ± standard deviation (SD) of three separate experiments in triplicate.

HYP has a higher affinity for tumor cells than for normal cells, as described by Chen et. al. [29]. This explains the low phototoxicity effect HYP/P-123 within MCF-10A cells, as well as higher IC values for MCF-10A cell line, showing less action on normal breast epithelial cells.

3.3. Cells morphology post HYP/P-123 exposure

Moreover, the cells cytotoxicity induced by HYP/P-123 exposure in presence of light was verified by microscopic observation (figures 3.A and 3.B). Treatment of MCF-7 cells with IC_{50} value promoted changes in cell morphology as seen in the figure 3. There was an increase in the detachment, retraction of cytoplasmic expansion and the confluence of cells decreased. These cells have the characteristic growth in adhesion.

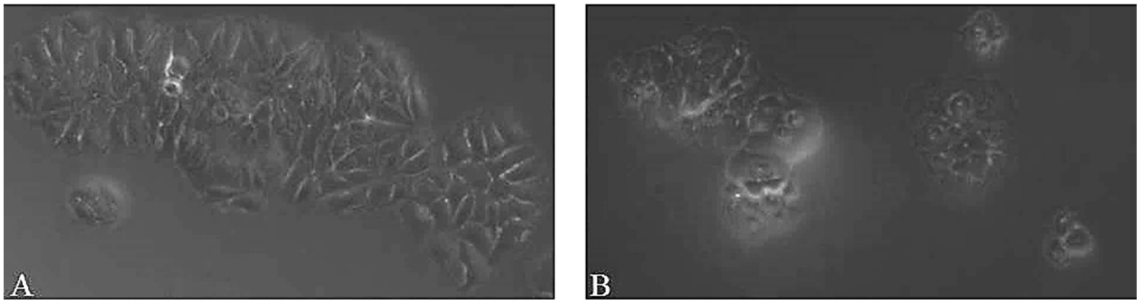

Figure 3. Differential effects on MCF-7 cells morphology post HYP/P-123 treatment in presence of light. Cell photomicrographs were taken in 20x magnification. A) Cell not treated with HYP/P-123; B) Cells post treated with IC_{50} value of HYP/P-123. Can be observed an increased cells detachment, retraction of cytoplasmic expansion and a decreased cells confluence.

3.4. Long-term cytotoxicity post HYP/P-123 exposure

In the clonogenic assay, the clonogenic potential was reduced by HYP/P-123 in a dose-time-dependent manner in the presence of light (figure 4.A and 4.B). Although there was a recovery that promoted the growth of colonies, a decrease in the amount and size of the circumference of the colonies was observed. Comparing cells exposed with IC_{30} and with IC_{50} of HYP/P-123 with NT cells, a lower percentage of colonies recovered in times of 7 and 14 days post exposure ($P<0.0001$ and $P<0.0001$, respectively). These results indicated that HYP/P-123 exposure in the light presence exerted long-term dose-dependent phototoxicity effects on MCF-7, which reflects the decrease in cell proliferation.

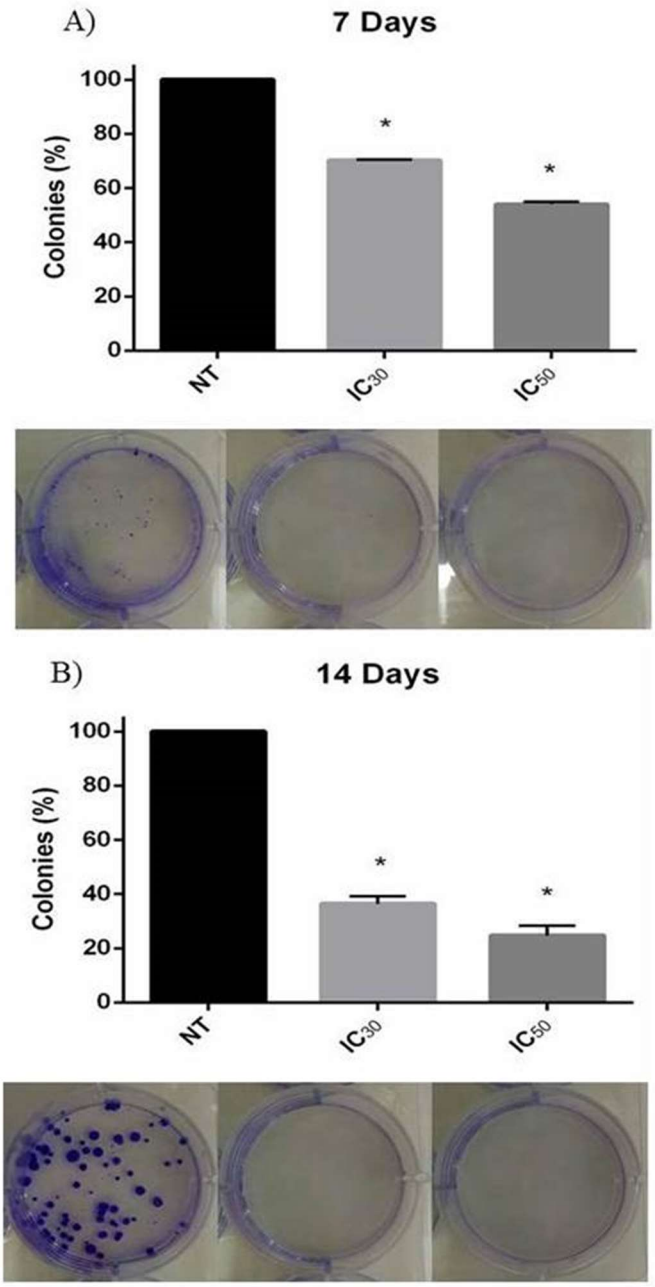

Figure 4. The effect of activated HYP/P-123 treatment in presence of light in long-term cytotoxicity (clonogenicity) of MCF-7 cells at 7 and 14 days. Graphics in A and B indicates that the recovery of colonies diminishes with increasing times and dose of exposure. The * show statistical significance when comparing treated cell groups (IC_{30} and IC_{50}) with not treated cells (NT), within 7 and 14 days. Data are shown as the mean values ± standard deviation (SD) of three independent experiments in triplicate. Photos in A and B indicate that treatment of MCF-7 cells with HYP/P-123 in presence of light reduced colony formation in 7 and 14 days.

3.5. Cells senescence post HYP/P-123 exposure

Cells senescence was determined by β-galactosidase staining, a biomarker for senescence in mammalian cells which exhibit lysosomal-galactosidade activity [24]. Senescence was not observed after MCF-7 cells treatment with HYP/P-123 (IC_{50} and IC_{90}). Therefore, we showed that HYP/P-123 was not effective as a senescence inducer in MCF-7 cells.

3.6. Cells death pathway post HYP/P-123 exposure

As described above, HYP/P-123 photodynamic treatment induces a significant decrease in MCF-7 cells viability. To determine the type and extent of cell line death, we initially analyzed whether HYP/P-123 photodynamic treatment could induce apoptosis in MCF-7 cells via an Annexin V/PI assay using fluorescence imaging. Annexin V staining detects the translocation of phosphatidylserine from the inner to the outer cell membrane during early apoptosis (green fluorescence), and PI can enter the cell during necrosis, late-stage apoptosis and dead cells (red fluorescence) [25].

The figure 5 shows that HYP/P-123 induced predominant death by necrosis once MCF-7 cells treated at the both IC_{50} and IC_{90} concentrations had similar fluorescence intensity to the fluorescence of the positive control PI (necrosis). Still, apoptotic cells detected only in MCF-7 cells treated at the concentration of IC_{50} but not of IC_{90}.

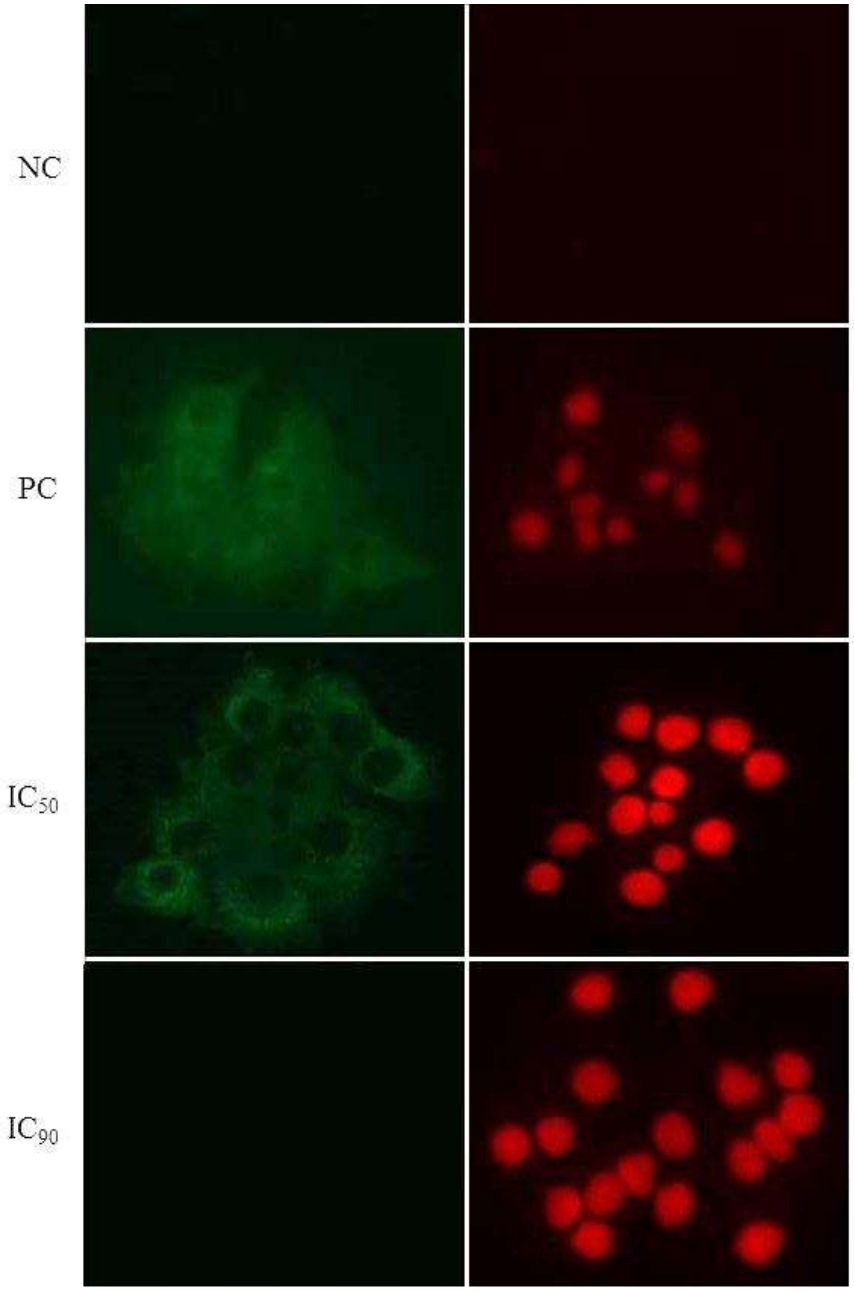

Figure 5. Cells death pathway post MCF-7 cells treatment with HYP/P-123 and illumination. Representative figures of cancer cell line MCF-7 exposed to activated HYP/P-123 (IC_{50} and IC_{90}) stained with the apoptosis marker Annexin V (green fluorescence) and necrosis marker propidium iodide (PI) (red fluorescence). Camptotecin 20 μM and Digitonin 80 μM were used as positive controls (PC) for apoptosis and necrosis, respectively, and cells not treated (NT) with HYP/P-123 were used as negative controls. 20x magnification.

In the figure 6, the histogram show the quantification of fluorescence as a mean % of Annexin V/PI positive MCF-7 cells treated with HYP/P-123 (IC_{50} and IC_{90}). MCF-7 cells showed mean of PI positive cells % of approximately 100% (IC_{50} $P<0.0001$ and IC_{90}

P<0.0001). For Annexin V, positive cell % was approximately 60% and was detected only in MCF-7 cells treated with IC_{50} of HYP/P123 (P<0.0001).

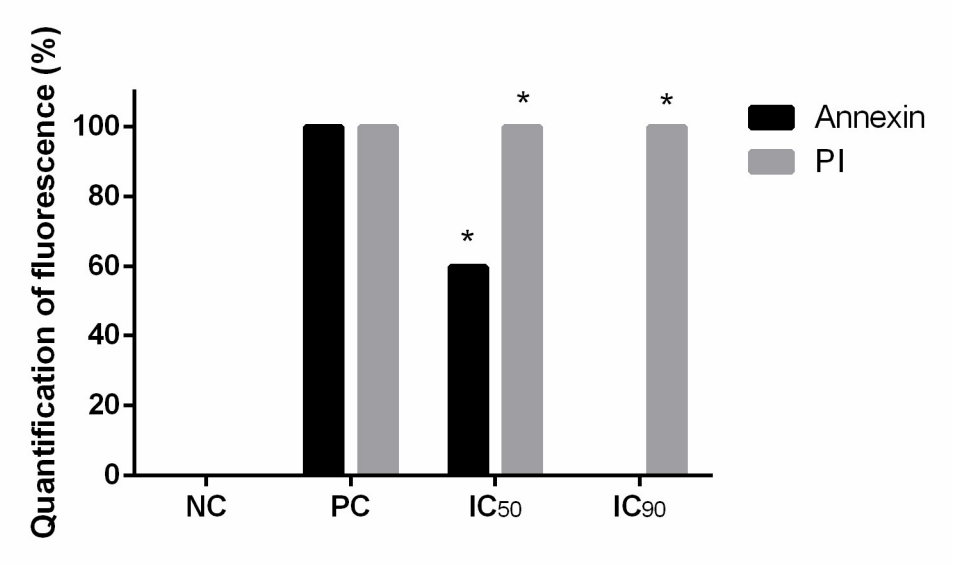

Figure 6. Histogram show mean % annexin-positive and propidium iodine (PI)-positive cells treated with HYP/P-123 (IC_{50} and IC_{90}) under irradiation. Camptotecin 20 μM and Digitonin 80 μM were used as positive controls (PC) for apoptosis and necrosis, respectively, and cells not treated (NT) with HYP/P-123 were used as negative controls. Data are shown as the means ± SD of three independent experiments in triplicate. *$P < 0.05$ vs. NT was considered significant.

Our results are consistent with other studies, as follows. In human and murine cancer cell lines, was observed that a shift from apoptotic to necrotic cell death can be markedly produced by increasing the concentration of HYP and/or the light dose applied to sensitize the cells [30,31,32]. Necrosis was described by Mikes et. al. as predominant pathway of death in the human colon adenocarcinoma HT-79 line, after treatment with HYP alone [33]. Lavie et al showed the change in apoptotic pathway for necrosis, when they treated promyelocytic leukemia HL-60 cells by increasing the extracellular concentration of HYP [34]. The same was observed when MCF-7 cells were treated with IC_{50} and IC_{90}. Death by apoptosis is also evident in treatment with IC_{50}, but when increased the concentration of HYP/P-123 to IC_{90}, was only observed death by necrosis.

3.7. Cells migration post H/P-123 exposure

The wound-healing assay showed that HYP/P-123 (IC_{30} and IC_{50})/illumination inhibited MCF-7 cells migration in all times tested ($P<0.0001$ in 24 hours, $P<0.0001$ in 48 hours and $P<0.0001$ in 72 hours). Total wound closure only was observed in NT cells within 72 hours (figure 7), showing the capacity of the HYP/P-123 in preventing the migration of tumor cells, decreasing its ability to form metastases. Furthermore, they confirm the selectivity of H/P-123 action on tumor cells equal to HYP alone, since only NT cells were able to migrate and promote full confluence [29]. These data suggest that HYP/P-123 can exert its anti-tumorigenic effect not only via its influence on the cytotoxicity of tumorigenic MCF-7 cells but also via an effect on their motility, and thus their invasive properties.

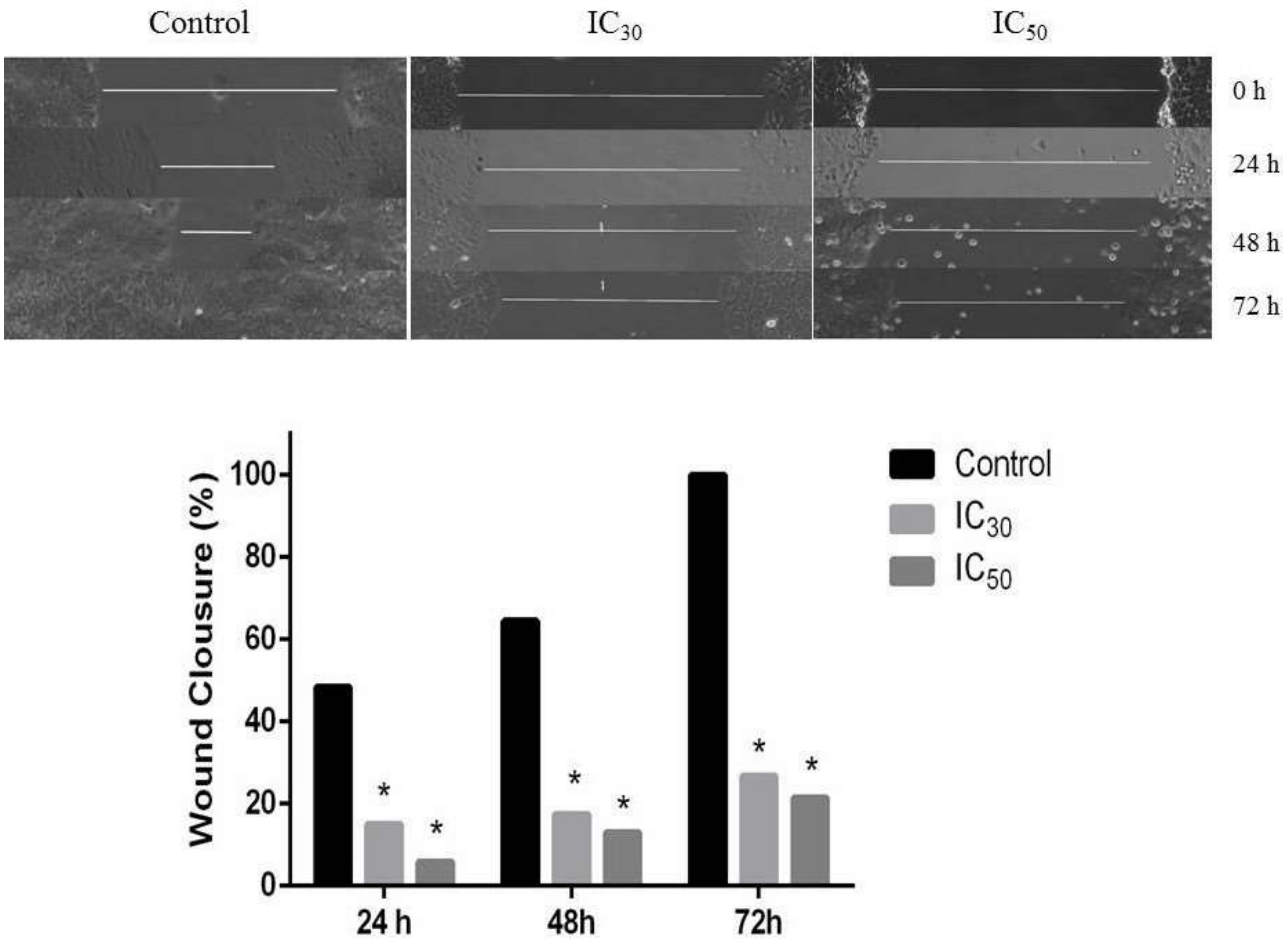

Figure 7. Cell migration analysis using the wound-healing assay. MCF-7 cells were tested in 6-well plates (2.5 x 10^4 cells/mL) after scratching in the absence (negative control-NT) and presence of HYP/P-123 (IC_{30} and IC_{50})/illumination. The results were calculated by comparing the wound closure after 24, 48 and 72 h with the measurement at the initial time, and data are shown as the mean values ± SD of three independent experiments in triplicate. *$P < 0.05$ vs. NT was considered significant.

3.8. Internalization and subcellular distribution of HYP/P-123

The presence of intracellular HYP/P-123 was observed through the emitted fluorescence visualized in inverted fluorescence microscope with RFV (red) filter, as can be observed in figure 8. It is possible to observe that in the tumorigenic MCF-7 cells (figure 8.A) HYP/P-123 acumulates in plasma membrane more than in plasma membrane of MCF-10A cells (figure 8.B), justifing more cell death observed in tumoral cells. The plasma membrane is also an important target, contributing to a more effective cell death because the produced singlet oxigen leads to membrane rupture or membrane lipid peroxidation [35,36]. Still, the

entry of HYP in the cells was facilitated by the P-123 which acts as a delivery system and makes it available for transport across the cytoplasmic membrane or the target binding site [37].

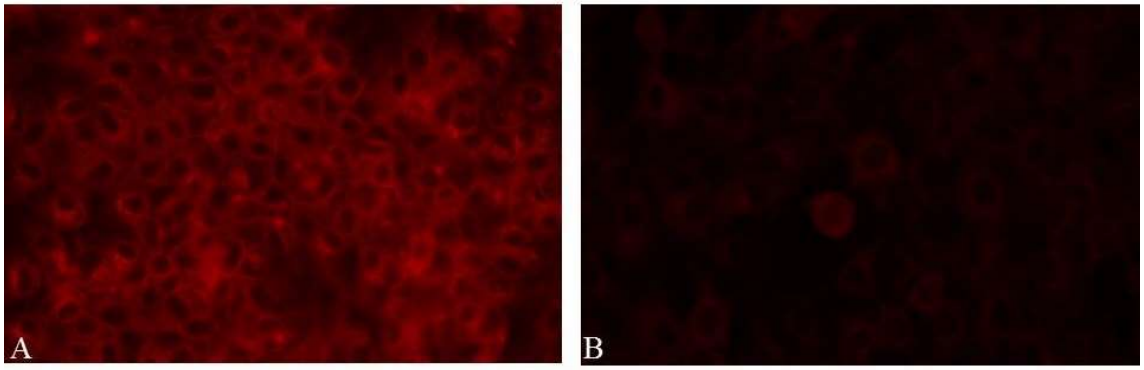

Figure 8. MCF-7 (A) and MCF-10A (B) cells shows a red fluorescence inside result of the internalization of HYP/P-123. 20x magnification.

After exposure of the MCF-7 and MCF-10A cells with HYP/P-123 for 30 minutes followed by Mitotracker® and ER-tracker® for 1 hour, the subcellular distribution was observed through the emitted fluorescence visualized in inverted fluorescence microscope with RFP (red) and GFP (green) filters, as can be observed in figures 9 and 10. H/P-123 was found to be localized in the mitochondria and endoplasmic reticulum (ER) in both cell lines. The accumulation of HYP in ER and mitochondria was also observed by Galanou et. al. [38]. The subcellular localization in these two compartments was previously proposed as primary targets for the phototoxicity of HYP and the action of HYP on these two organelles may be related to the destruction of SERCA2, a protein from the ER, impairing the homeostasis of intracellular Ca^{+}, requiring an aid from the mitochondria that is damaged due to increased Ca^{+} load. This mechanism added to the damage caused by phototherapy in mitochondria may be responsible for the increase in cell death [38,39,40].

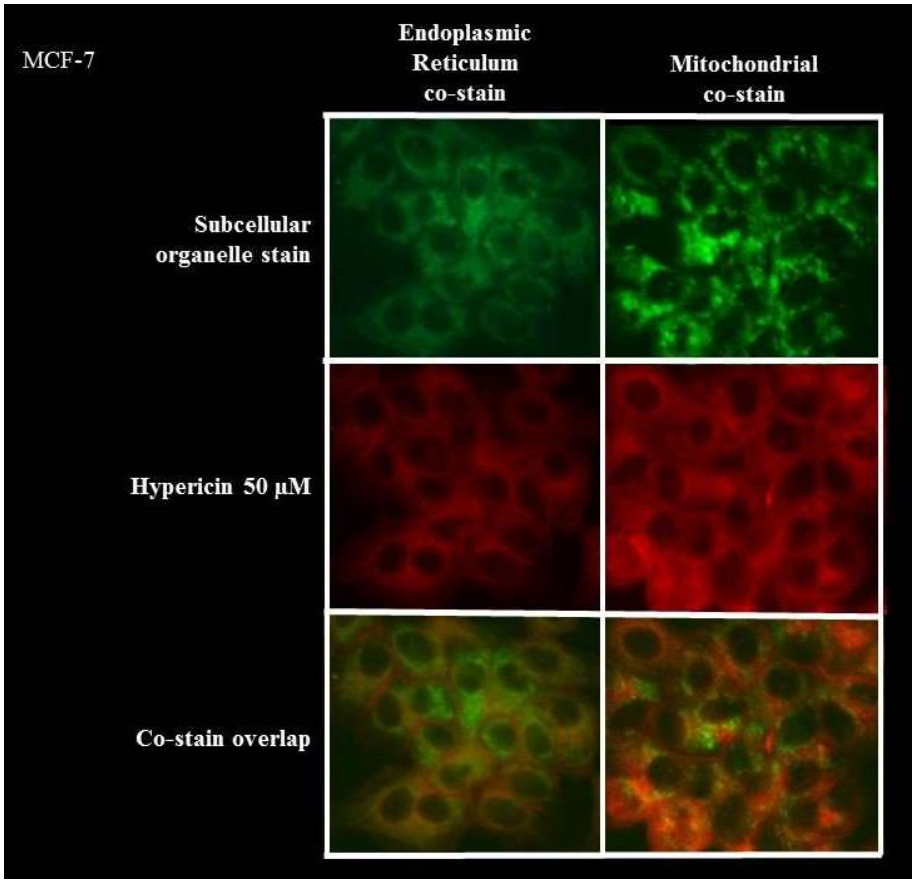

Figure 9. Fluorescence microscopy of tumorigenic MCF-7 cells incubated with HYP/P-123 for 1 hour. Cells were costained with Mitotracker® (specific probe for mitochondria) and ER-tracker® (specific probe for endoplasmic reticulum).

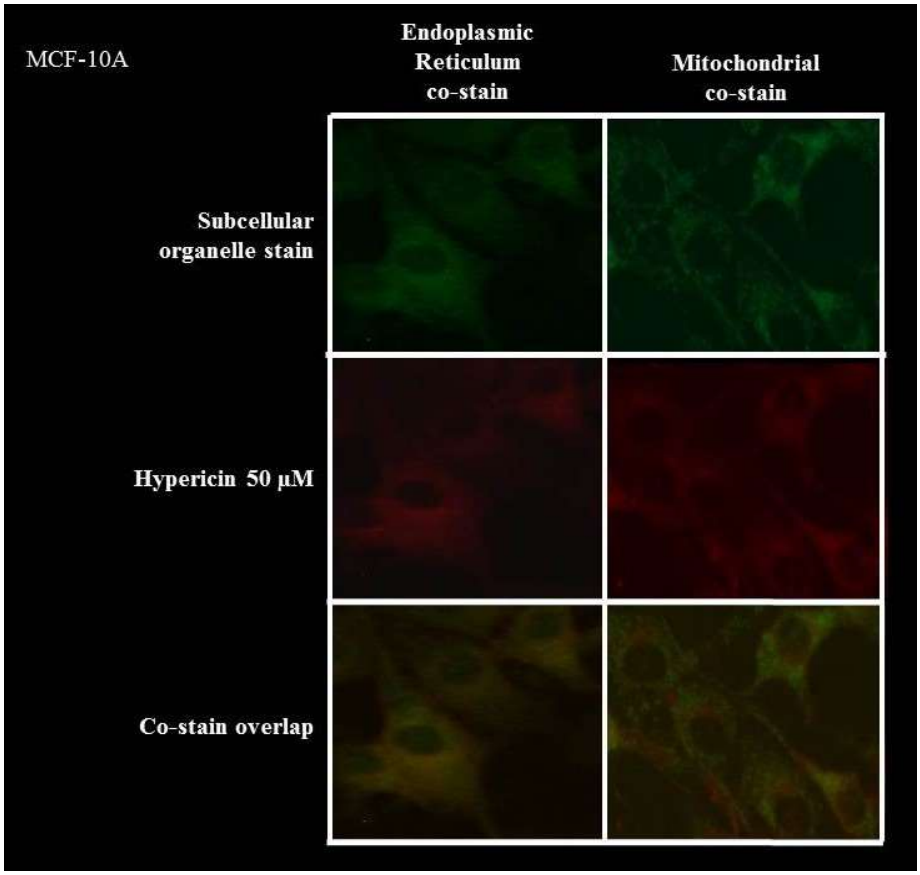

Figure 10. Fluorescence microscopy of normal breast MCF-10A cells incubated with HYP/P-123 for 1 hour. Cells were costained with Mitotracker® (specific probe for mitochondria) and ER-tracker® (specific probe for endoplasmic reticulum).

The figures 9 and 10 showed that the fluorescence intensity of Mitotracker®, ER-tracker® and HYP/P-123 in the mitochondria, ER and both organelles was much higher in tumorigenic MCF-7 cells (figure 9) than in normal MCF-10A cells (figure 10). This data justifies again the higher rates of cells death observed in tumoral cells. Together, these results further support the successful delivery of HYP by P-123 and clearly confirm the presence of HYP/P123 in the cytoplasm of cells and the subcellular lozalization of HYP/P123.

4. Conclusion

In the present study, we evaluated the antitumoral effects of HYP encapsulated with pluronic P-123 in MCF-7 human breast cancer cell line in comparison with MCF-10A human breast normal cells. Together, our results demonstrated that HYP/P-123 is a photodynamic

agent that had a selective time- and dose-dependent inhibitory effect on breast cancer cells, but low damage in breast normal cells. These findings are important for the success of an antitumor therapy while it depends on greater selectivity of action and fewer systemic effects, reducing damage to healthy tissues and side effects.

Additionally, HYP/P-123 inhibited cancer cells migration and thereafter invasion. Encapsulation of HYP with the copolymer P-123 facilitated entry in the tested tumor cells once was possible to visualize its fluorescence in the cytoplasm of MCF-7 cells with greater intensity than in the MCF-10A cells. Thus, P-123 acts as a delivery system for HYP and makes it available for transport across the cytoplasmic membrane or the target binding site.

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CAPÍTULO III

CONCLUSÕES

A utilização da HYP encapsulada com o copolímero P-123® como fotossensibilizador na terapia fotodinâmica demonstrou que:

1 – Baixas doses do composto apresentaram efeitos antitumorais em células tumorais mamárias e baixos danos em células mamárias normais;
2 – A interiorização da HYP/P-123 foi maior em células tumorais mamárias do que em células mamárias normais;
3 – O tratamento impediu a migração das células tumorais mamárias evitando assim a possível formação de metástases;
4 – Foi constatada a diminuição do potencial clonogênico após o tratamento, diminuindo a capacidade de proliferação celular;
5 – A HYP/P-123 apresentou-se como um agente promissor no tratamento deste modelo de neoplasia do epitélio mamário.

PERSPECTIVAS FUTURAS

Como os resultados obtidos na linhagem tumoral MCF-7 foram promissores, novos estudos devem ser realizados abrangendo mais linhagens tumorais mamárias, assim como estudos em animais, para melhor explorar o potencial fotodinâmico da HIP.

Formulações farmacêuticas também devem ser desenvolvidas para o composto a fim de possibilitar possíveis estudos clínicos em humanos.

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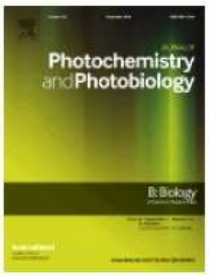

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