UNIVERSIDADE FEDERAL DO PARANÁ

LUCAS AUGUSTO LOPES GENEZ

# RELAÇÃO DA VIMENTINA E AUTOFAGIA COM A MORTE DE CÉLULAS MELAN-A E B16-F10 TRATADAS COM RBAc-PDT

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências - Bioquímica, Setor de Ciências Biológicas da Universidade Federal do Paraná, como requisito parcial à obtenção do Título de Mestre em Ciências - Bioquímica.

Orientadora: Profª. Drª. Glaucia Regina Martinez

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**TERMO DE APROVAÇÃO**

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Dissertação aprovada como requisito parcial para obtenção do grau de Mestre no curso de Pós-Graduação em Ciências-Bioquímica, Setor de Ciências Biológicas, Universidade Federal do Paraná, pela sequinte banca examinadora:

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“A ciência nunca resolve um problema
sem criar pelo menos outros dez”.

(George Bernard Shaw)

## RESUMO

O câncer de pele do tipo melanoma apresenta alta taxa de mortalidade e baixa resposta frente aos tratamentos atualmente disponíveis. Desta forma, estudos procuram compreender melhor os mecanismos celulares envolvidos na resistência e na elevada agressividade das células de melanoma para desenvolver novas estratégias terapêuticas. A terapia fotodinâmica (PDT) envolve a geração de oxigênio molecular singlete ($^1O_2$) por um fotossensibilizador (PS) irradiado com luz em um comprimento de onda específico, desencadeando respostas celulares que podem levar à destruição do tumor. Entretanto, a efetividade da PDT em melanomas é baixa. Anteriormente, foi mostrado usando análise proteômica, que os níveis de vimentina eram alterados em melanócitos e células de melanoma tratadas com $^1O_2$. A vimentina é uma proteína do citoesqueleto que é geralmente superexpressa em carcinomas e parece estar relacionada com o crescimento e a metástase de tumores. Sendo assim, o objetivo deste trabalho foi melhor entender a influência da vimentina no processo de morte celular em melanócitos (Melan-A) e células de melanoma (B16-F10) após o tratamento com $^1O_2$ gerado pela PDT, usando como PS o Rosa Bengala Acetato (RBAc). A viabilidade foi avaliada pela metabolização do MTT, pela marcação com cristal violeta (CVS) e pela captação de vermelho neutro (NRU). Os resultados mostraram que ambas as linhagens apresentaram uma diminuição da viabilidade (MTT e CVS) com 5,0 $\mu g.mL^{-1}$ de RBAc, 18 horas após terem sido irradiadas com LED (526 nm; 1,5 $J/cm^2$). A análise pelo método NRU mostrou um efeito inverso aos resultados do CVS e MTT; esse padrão é um forte indicativo de morte das células por autofagia. Para confirmar, foi analisada a expressão de proteínas relacionadas ao processo autofágico, Beclin-1 e LC3 por *western blotting*. Em ambas as linhagens celulares, a PDT promoveu diminuição dos níveis de Beclin-1 e da relação LC3 II/LC3 I na análise feita imediatamente ao término da irradiação. Após 18 horas, o nível de Beclin-1 foi restaurado e houve um aumento na relação LC3 II/LC3 I em ambas as linhagens. Em B16-F10, o efeito da PDT ocasionou aumento na relação vimentina fosforilada na serina 56/vimentina, 18 horas após a PDT. No caso das células Melan-a, não tivemos variação na relação vimentina fosforilada serina 56/vimentina, mostrando que essas duas linhagens tem alterações diferentes no citoesqueleto após a PDT. No presente estudo, nós demonstramos a indução de autofagia após 18h de RBAc-PDT em melanoma e melanócitos. Mostramos ainda que ambas as linhagens celulares têm diferentes alterações na expressão de proteínas relacionadas à autofagia e citoesqueleto e que existem diferenças quando a análise é feita imediatamente após a PDT ou 18h depois. Desta forma, este estudo proporciona uma melhor compreensão da relação da morte celular com alterações de proteínas do citoesqueleto após PDT em melanócitos e células de melanoma.



Palavras-chave: melanoma, fosforilação, rosa bengala, terapia fotodinâmica e vimentina.

## ABSTRACT

Melanoma is a type of skin cancer with high mortality rate and low response to the currently available treatments. Thus, studies look for a better understanding about the cellular mechanisms involved in resistance and high aggressiveness of melanoma cells in order to develop new therapeutic strategies. Photodynamic therapy (PDT) involves the generation of singlet molecular oxygen ($^1O_2$) when a photosensitizer (PS) is irradiated with light in a specific wavelength which triggers cellular responses that can lead to tumor destruction. However, the effectiveness of PDT in melanomas is low. Previously, proteomic analysis, showed that vimentin levels were altered in melanocytes and melanoma cells treated with $^1O_2$. Vimentin is a cytoskeletal protein that is overexpressed in cancers and generally appears to be related to the growth and metastasis of tumors. Thus, the main objective of this study is the investigation about the influence of vimentin in the process of cell death in melanocytes (Melan-a) and melanoma cells (B16-F10) after treatment with $^1O_2$ generated by PDT, using Rose Bengal Acetate (RBAc) as PS. The viability was assessed by MTT metabolism, by crystal violet (CVS) labeling and neutral red uptake (NRU). The results showed that both cell lines showed reduced viability (MTT and CVS) with 5.0 µg.mL-1 RBAc, 18 hours after being irradiated with LED (526 nm, 1.5 J/cm$^2$). The NRU analysis showed an inverse effect to the CVS and MTT results; this pattern is a strong indicator of cell death by autophagy. To confirm, we analyzed the expression of proteins related to autophagic process, beclin-1 and LC3 by western blotting. In both cell lines, PDT caused a reduction of beclin-1 levels and in the relation LC3 II/ LC3 I in the analysis performed immediately at the end of irradiation. After 18 hours, the level of beclin-1 was restored and an increase in the relation LC3 II/LC3 I was observed in both cell lines. To B16-F10, the effect of PDT resulted in an increase in the relation vimentin phosphorylated at serine 56/vimentin, 18 hours after PDT. For Melan-a cells, no variation in the relation vimentin phosphorylated serine 56/vimentin was observed, indicating that these two cell lines have different alterations in the cytoskeleton after PDT. In the present study, we demonstrated the induction of autophagy after 18h of RBAc-PDT in melanoma and melanocytes. We also show that both cell lines have different changes in protein expression related to autophagy and cytoskeleton and that there are differences when the analysis is done immediately after PDT or 18h later. Thus, this study provides a better understanding of cell death related proteins cytoskeletal changes after PDT in melanocytes and melanoma cells.



Keywords: melanoma, phosphorylation, photodynamic therapy, rose bengal, vimentin.

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## LISTA DE ABREVIATURAS E SIMBOLOS

**AAU** – unidades arbitrárias de autofagia

**Atgs** – genes relacionados à autofagia

**ATP** – adenosina trisfosfato

**BECN1** – anticorpo anti-BECN1 monoclonal de rato

**BSA** – albumina de soro bovino

**B16-F10** – células metastáticas de melanoma murino

**CVS** – coloração cristal violeta

**$DHPNO_2$** - endoperóxido da N,N´-di(2,3-dihidroxipropil)-3,3´(1,4-naftilideno) dipropanamida

**DMSO** – dimetilsulfóxido

**DNA** – ácido desoxirribonucléico

**EDTA** – ácido etilenodiamino tetra-acético

**EMT** – transição epitélio-mesenquimal

**FBS** – soro fetal bovino

**HBSS** – solução balanceada de Hanks

**IF** – filamentos intermediários

**kDa** – unidade de peso molecular protéico

**LC3** – anticorpo anti-LC3 policlonal de coelho

**LED** – diodo emissor de luz

**MELAN-A** – células de melanócitos murino

**MTT** – brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difenil tetrazólio

**NR** – corante vermelho neutro

**NRU** – captação de vermelho neutro

**PAK1** – proteína quinase ativada por p21

**PBS** – tampão fosfato-salino

**PCD** – programa de morte celular

**PDT** – terapia fotodinâmica

**PMA** – acetato miristato de forbol

**PMSF** – fenilmetilsulfonilflúor

**PS** – droga fotossensibilizadora

**PKC** – proteína quinase C

**RB** – rosa bengala

**RBAc** – rosa bengala acetato

**$RIPA^+$** – tampão de lise

**RNA** – ácido ribonucléico

**RNAm** – ácido ribonucleico mensageiro

**ROS** – espécies reativas de oxigênio

**Ser56** – serina 56

**TRIS** – tris(hidroximetil)aminometano

**UVR** – radiação ultravioleta

$^{1}O_{2}$ – oxigênio molecular singlete

**SUMÁRIO**

# 1. INTRODUÇAO

## 1.1. MELANOMA

O melanoma é uma neoplasia maligna originada nos melanócitos, células especializadas em produzir pigmentação e encontradas nos olhos, no cabelo e na pele (CHEN *et al.*, 2006).

Embora o melanoma seja o tipo menos prevalente de câncer de pele (representa menos de 5% dos casos), é responsável por cerca de 80% do total de mortes por este tipo de tumor. O aumento da incidência e mortalidade do melanoma é um problema de saúde pública que vem crescendo em muitos países (KOH *et al.*, 1992, MENDES *et al.*, 2010). Nos Estados Unidos, por exemplo, a *American Cancer Society* (ACS) registrou a ocorrência de 76.000 casos de melanoma, o tipo mais grave dos cânceres de pele, e ainda, responsável por mais de 9.000 dos mais de 12.000 mortes por câncer de pele a cada ano (ACS, 2015). No Brasil, o Instituto Nacional do Câncer (INCA) estimou a ocorrência de 6.230 casos igualmente distribuídos entre os sexos, no ano de 2012 (INCA, 2014).

Fácil de ser visualizado na superfície da pele, o melanoma é um dos tipos de câncer mais facilmente detectados e curados em estágios iniciais (MITCHELL e LESLIE, 2013). A detecção e o tratamento precoce contribuem para a redução da mortalidade por melanoma (KOH *et al.*, 1992).

O melanoma surge de uma lesão denominada nevo benigno, que são melanócitos que sofreram uma alteração no controle de crescimento celular, adquirindo um crescimento uniforme e limitado ao longo da membrana basal. Nesta fase, as lesões possuem uma coloração regular e geralmente são planas ou pouco elevadas. Em seguida, ocorre a fase de crescimento radial (RGP), etapa onde as células evoluem para malignidade, adquirindo um crescimento radial ou superficial não invasivo, proliferando-se por toda a epiderme, e não somente ao longo da lâmina basal. Aqui as lesões são caracterizadas por um crescimento aberrante e pela assimetria, bordas e colorações irregulares que servem como marcadores clínicos para o diagnóstico. Essas lesões são conhecidas como nevos displásicos. A fase de crescimento vertical (VGP) é caracterizada pela capacidade das células tumorais de penetrar a membrana basal, atingindo a derme. Por último, a metástase é a fase na qual as células se desprendem do tumor original, podendo crescer em tecidos mais profundos da pele e atingir a corrente sanguínea e, assim, colonizar outros órgãos. A alta letalidade é devido à capacidade angiogênica, invasiva e metastática das células de melanoma

(GOVINDARAJAN *et al.*, 2007, MILLER e MIHM, 2006). Essas etapas estão ilustradas na figura 1.

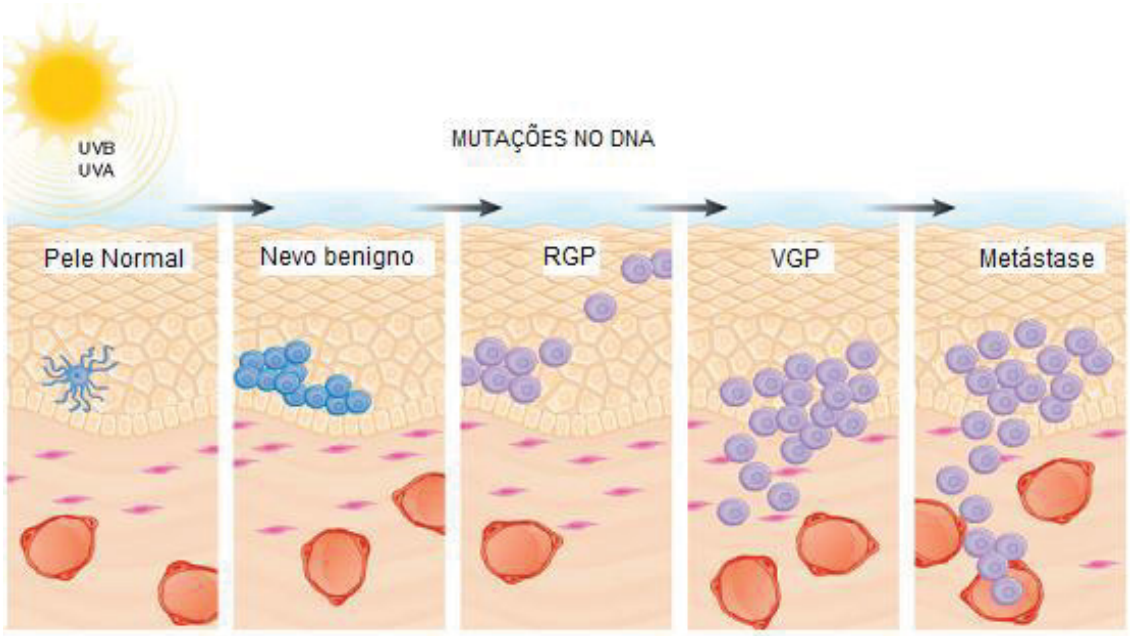



FIGURA 1 – ETAPAS DE INICIAÇÃO E PROGRESSÃO DO MELANOMA.
FONTE: modificado (ZAIDI, DAY e MERLINO, 2008).
Nota: A figura mostra um modelo proposto da iniciação e progressão do melanoma humano, no qual o melanócito após irradiação UV pode sofrer mutações genéticas e se transformar em células cada vez mais invasivas até chegar na fase metastática. RGP ("*radial growth phase*") e VGP ("*vertical growth phase*").

A espessura do tumor é o maior indicador de prognóstico. Tumores finos podem ser tratados facilmente com cirurgia simples, já que a mortalidade está associada com o aumento da profundidade do tumor (MITCHELL e LESLIE, 2013).

O desenvolvimento do melanoma é multifatorial, uma vez que inúmeros fatores biológicos, culturais, geográficos e o descuido com a proteção solar estão envolvidos. Dentre os fatores estão: a cor da pele, o comportamento com relação à exposição à radiação ultravioleta e a predisposição genética (ACS, 2014; MITCHELL e LESLIE, 2013). A exposição à radiação ultravioleta é um elemento importante no desenvolvimento do melanoma (MITCHELL e LESLIE, 2013).

Visando diminuir a mortalidade por melanomas, estudos procuram compreender melhor os mecanismos celulares envolvidos na resistência e na elevada agressividade das células de melanoma para desenvolver novas estratégias terapêuticas.

## 1.2. TRATAMENTO

O sucesso no tratamento do melanoma depende muito de um diagnóstico precoce. Nos estados Unidos, a *American Cancer Society* recomenda uma análise cuidadosa da pele por um médico como parte de uma rotina de *check up* relacionada ao câncer (ACS, 2013). A USPSTF (*US Preventive Services Task Force*) recomenda que os médicos permaneçam alertas para lesões de pele com características malignas, observadas no contexto de exames físicos de rotina. Os médicos têm a opção de usar a regra ABCDE, muitas vezes recomendada para orientar os exames a olho nu de lesões pigmentadas: Assimetria, Bordas irregulares, variabilidade de Cor, Diâmetro geralmente maior que 6 mm e Evolução ou qualquer alteração no tamanho, forma, cor, elevação ou outra característica, ou qualquer novo sintoma, como sangramento, coceira ou crostas. Nem sempre as cinco características estarão presentes, mas sempre mais de uma o estará (RIGEL *et al*., 2005)

Em 80% dos casos, nos quais a doença está menos avançada, a remoção cirúrgica da massa tumoral, juntamente com uma parte do tecido circundante, já é o suficiente para a eliminação do melanoma (MILLER e MIHM, 2006).

Terapias adjuvantes tem sido utilizadas como pós-operatórias para melanoma maligno. Para melanoma avançado, o Interferon-α (IFN-α) é o mais utilizado, porém sua eficácia é duvidosa, e a interleucina-2 (IL-2), com baixa eficácia e alta toxicidade (TARHINI e AGARWALA, 2006).

Os agente quimioterápicos mais comuns para o tratamento de melanoma é a Dacarbazina (DTIC), carmustina (BECENUN), o paclitaxel (Taxol), temozolomida e cisplatina, porém mostraram ser efetivos em menos de 10% dos casos de melanoma metastático (TARHINI e AGARWALA, 2006). Além da quimioterapia, a radioterapia também pode ser empregada como complemento à intervenção cirúrgica (ACS, 2013). As consequências da quimioterapia e da radioterapia para os pacientes são notórias: queda dos pêlos, deficiência imunológica, náuseas, dores, vômitos, etc. Devido a estas limitações quanto à eficácia e efeitos colaterais, novas estratégias terapêuticas ou meio para o qual as drogas cheguem de forma mais específica aos seus alvos têm sido pesquisadas. O melhor conhecimento das vias de sinalização que se encontram desreguladas no melanoma tem permitido o desenvolvimento de novos fármacos e processos terapêuticos que auxiliam no seu tratamento.

Avanços vêm sendo conquistados em prol de uma terapia alternativa para o tratamento do melanoma localizado como a terapia fotodinâmica (PDT).

## 1.3 TERAPIA FOTODINAMICA

O mecanismo de ação da PDT envolve a geração de oxigênio molecular singlete ($^1O_2$), definido como oxigênio molecular eletronicamente excitado, o que o torna altamente reativo frente às biomoléculas. Sua formação ocorre por um fotossensibilizador irradiado com luz em um comprimento de onda específico. O fotossensibilizador passa para um estado excitado, podendo transferir sua energia para o oxigênio, levando à formação de ROS, tal como de $^1O_2$. Esse processo desencadeia mudanças em eventos celulares, resultando na destruição do tumor por meio da apoptose ou mecanismos não apoptóticos, como autofagia e necrose, ou a combinação dos três mecanismos (CHEN *et al.*, 2008; FERNANDEZ-GUARINO *et al.*, 2007; DINI, 2010).

Fotossensibilizadores são drogas não-tóxicas ou corantes administrados de forma local ou sistêmica. A sua eficiência depende de suas propriedades físico-químicas, bem como sua pureza, sua localização específica nas células neoplásicas, tempo de residência longo o suficiente dentro das células, curto tempo de intervalo entre as administrações da droga, seu acúmulo em células tumorais, e ativação no comprimento de onda com ótima penetração tecidual (PANZARINI, 2011).

Segundo Dini *et al.* (2010), um importante fotossensibilizador que poderia ser utilizado é o Rosa Bengala. Porém, devido a sua característica aniônica, torna-se difícil sua entrada e acumulação nas células tumorais. A adição do grupo acetato, foi uma alternativa para facilitar a entrada do RB nas células a fim de torná-lo mais hidrofóbico para que possa produzir ROS intracelularmente a partir da irradiação por luz verde, variando entre 530-560nm (LUTTRULL, 1988).

A destruição do tumor na PDT é multifatorial, ou seja, ocorre por destruição direta das células tumorais, danos à vascularização e por rápido recrutamento e ativação de células de defesa (KORBELIK, 2006).

É sabido que as células tumorais podem ser mais sensíveis às espécies reativas de oxigênio (ROS) (FANG, SEKI e MAEDA, 2009). É nesse ponto que a quimioterapia convencional atua, porém está associada a efeitos colaterais inesperados ou sistêmicos, já que possui pequena seletividade celular. Já a PDT, possui efeito terapêutico superior do que a quimioterapia já que envolve a administração de uma droga que não só se localiza preferencialmente em células malignas, mas também é ativada por luz, levando à formação de ROS, em especial o $^1O_2$, e consequentemente à morte celular (PANZARINI, 2011). Porém, no caso do melanoma, o tratamento não é satisfatório e ainda depende de quimioterapia ou cirurgias para retirada do tumor (FOUNDATION SKIN CANCER, 2008).

Comparando com os tradicionais tratamentos farmacológicos e cirúrgicos, a PDT apresenta vantagens importantes: trata-se de uma terapia não invasiva, não exige anestesia local ou geral, pode ser usada no tratamento de lesões superficiais localizadas ou generalizadas, apresenta baixa toxicidade na ausência de radiação, pode ser administrado no local do tumor e pode ser usada em conjunto com outros tipos de tratamentos (PANZARINI, INGUCIO e DINI, 2011).

Por outro lado, uma das desvantagens da PDT é o aparecimento de células resistentes, que por sua vez, são responsáveis por processos metastáticos e recidivos (PERONA e SANCHEZ-PEREZ, 2004).

## 1.4 OXIGENIO MOLECULAR SINGLETE E MORTE CELULAR

A presença de antioxidantes e espécies reativas de oxigênio é regulada continuamente pelo organismo. O equilíbrio entre esses dois fatores é rigidamente controlado e extremamente importante para a vitalidade celular e funções bioquímicas já que o desequilíbrio que resulte em um aumento de ROS tende a promover um estresse oxidativo, podendo levar ao dano (KOHEN e NYSKA, 2002).

Segundo estudos realizados por Chen *et al.* (2008),foram encontradas alterações protéicas como diminuição da atividade da citrato sintase, da vimentina, da GRP78 e aumento da expressão das proteínas HSP86 e TRAP1 após o tratamento com oxigênio molecular singlete (MB-PDT), provocando graves alterações celulares como alteração do potencial de transmembrana da mitocôndria, levando a liberação de citocromo c e apoptose por via intrínseca caspase-9/caspase-3. Danos nos ácidos nucleicos, mitocôndrias, lisossomos, membrana plasmática e o citoesqueleto, são as principais consequências causadas pelo estresse oxidativo, podendo comprometer a viabilidade celular, levando à morte (DALLE-DONNE *et al.*, 2006).

Em células de hepatoma humano HepG2, foi comprovada a morte celular por meio do $^1O_2$, que induziu a liberação de citocromo c da mitocôndria, porém não houve ativação das caspases e de outros eventos seguintes esperados do processo de morte celular por apoptose, como fragmentação do DNA, degradação das proteínas do núcleo e do citoesqueleto e a formação de corpos apoptóticos caracterizando, portanto, uma morte celular por apoptose atípica (OTSU *et al.*, 2005). Por sua vez, estudos da ação do $^1O_2$ em células de melanoma ainda não estão completamente elucidados.

Em um estudo utilizando melanócitos murinos (Melan-a) e células de melanoma murino (B16-F10) após o tratamento com endoperóxido ($DHPNO_2$), gerador de $^1O_2$, foi observada a diminuição na viabilidade somente de células de melanoma, mostrando um mecanismo de resposta diferenciado dos melanócitos frente a essa

espécie reativa. De acordo com esse estudo, em melanócitos, houve diminuição dos níveis de vimentina, porém não houve alteração nos níveis de RNA mensageiro, indicando uma possível degradação da proteína, sem mecanismo de aumento da expressão. No caso do melanoma, em relação ao controle, os níveis protéicos de vimentina não foram alterados pelo tratamento com $^1O_2$, porém o tratamento pode ter causado diminuição da transcrição, já que houve diminuição nos níveis de RNA mensageiro, o que poderia estar relacionado com a diminuição da viabilidade dessas células (CUNHA, 2012).

## 1.5 VIMENTINA

Frequentemente usado como marcador de desenvolvimento de células e tecidos, a vimentina é um dos membros mais conhecidos da família de filamentos intermediários do citoesqueleto. Estudos recentes mostraram a participação da vimentina em diversas funções, muitas vezes relacionada com a organização de proteínas que estão envolvidas com a adesão, migração e sinalização celular (IVASKA *et al.*, 2007). Filamentos de vimentina também atuam no processo de endocitose, no correto posicionamento de endossomos e lisossomos e na maturação de autofagossomos (STYERS *et al.*, 2004; HOLEN *et al.*, 1992).

A vimentina é conhecida também por manter a integridade celular e promover resistência contra o estresse. Além disso, é superexpressa em vários tipos de cânceres epiteliais, incluindo o melanoma, indicando um acelerado crescimento tumoral, invasão e um prognóstico ruim. E ainda, é um dos melhores indicativos de EMT (transição epitélio-mesenquimal) em carcinomas, demonstrando um importante papel na migração celular e consequentemente na invasão tumoral (DAUPHIN *et al.*, 2013), apesar disso, sua função em eventos mediados pelo processo de EMT não estão completamente explicados.

A vimentina também está relacionada com a sinalização celular (Figura 2). A vimentina interage com Erk fosforilada, evitando a sua desfosforilação (PERLSON *et al*., 2006). Interage também com a família de proteínas 14-3-3, que regulam a progressão do ciclo celular, apoptose e transdução de sinais (TZIVION, LUO e AVRUCH, 2000); além disso, AKT1 se liga a forma fosforilada da vimentina e a protege da proteólise por meio de caspases, levando ao aumento de migração celular e capacidade invasiva das células (ZHU *et al*., 2011); Vimentina fosforilada também poderia interagir com Scrib, proteína envolvida na migração celular, evitando sua degradação e consequentemente promovendoa migração celular e o aumento da capacidade invasiva das células (PHUA, HUMBERT e HUNZIKER 2009). Por outro lado,

a vimentina fosforilada aumentaria a migração celular regulando a proteína Axl envolvida na haptotaxis, um tipo de migração celular (VUORILUOTO *et al.,* 2011).

Foi proposto que as características especiais de cada membro da família de filamentos intermediários (IF) estão relacionadas com as regiões N- e C- terminal. Uma explicação plausível seria a habilidade dessas regiões interagirem com várias estruturas e moléculas sinalizadoras. A fosforilação possui um papel muito importante na regulação da dinâmica das proteínas, modulando a organização das redes de IF, bem como suas distribuições (OMARY *et al.*, 2006). A montagem e a função da vimentina são reguladas por um complexo padrão de fosforilação envolvendo sítios e quinases específicas para diferentes estágios celulares, como na mitose, na diferenciação celular e sob estresse (IVASKA *et al.*, 2007).

A vimentina sofre rápida fosforilação e desfosforilação influenciando sua montagem em filamentos proteicos ou na desmontagem em subunidades tetraméricas. Muitos sítios de fosforilação são controlados pela proteína quinase C (PKC). Estudos confirmaram que o transporte de integrinas está diretamente ligado à fosforilação da vimentina pela PKC, permitindo, portanto, o retorno de integrinas endocitadas à membrana plasmática, num processo de reciclagem, tornando-se fundamental para a migração celular (IVASKA *et al.*, 2005).

Em células de musculo liso, as mudanças na regulação da fosforilação da vimentina estão implicados na migração e contração celular. A PAK1, também chamada de p21 quinase-ativada, é considerada reguladora da vimentina por fosforilação do sitio Ser-56. Os autores sugeriram que se esse sítio fosse mutado, por exemplo, impediria a desmontagem da vimentina e a migração celular por meio da estimulação da PAK1 (LI *et al.*, 2006).

A fosforilação da vimentina no sítio Ser-56 em células de músculo liso regula a reorganização estrutural dos filamentos de vimentina durante a estimulaçao com serotonina (5-HT) (TANG *et al.*, 2005). O rearranjo de outros filamentos intermediários e da vimentina é importante para a adesão e migração celular, como por exemplo, na adesão e migração dos linfócitos atraves do endotélio (NIEMINEN *et al.*, 2006).

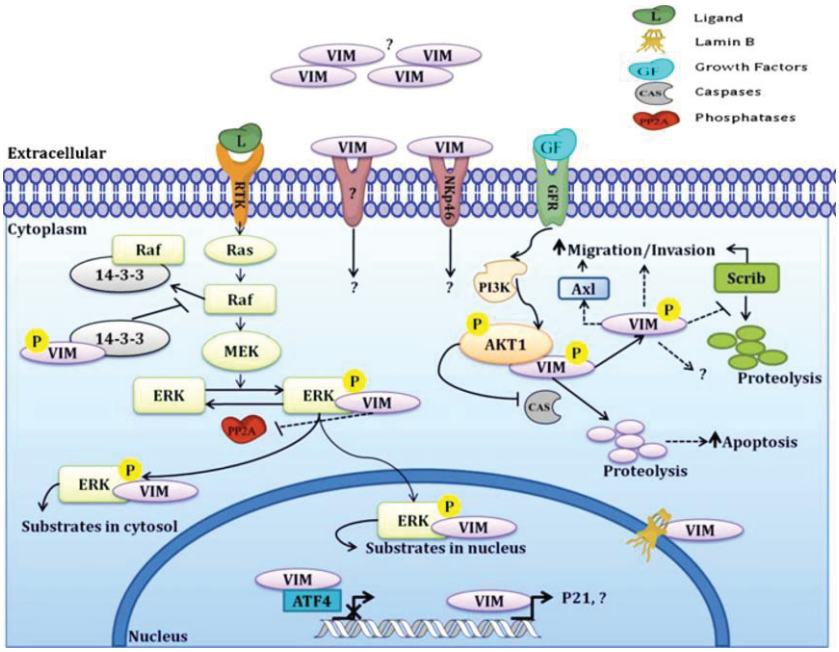



FIGURA 2 – PAPEL DA VIMENTINA NA SINALIZAÇÃO CELULAR.
FONTE: SATELLI e LI, 2011.
Nota: A figura mostra o envolvimento da vimentina na formação de complexos com moléculas sinalizadoras.

## 1.6 AUTOFAGIA EM PDT

Tendo em vista a alta mortalidade causada por melanomas, se faz necessário aprofundar as pesquisas sobre a terapia fotodinâmica como uma estratégia terapêutica para esse tipo de tumor.

Em estudos envolvendo PDT e fibroblastos embrionários, cuja apoptose foi bloqueada pela deficiência de BAX e BAK (proteínas pró-apoptóticas) ou inibindo a proteína pró-caspase 3, uma caspase efetora, foi observada morte celular cujas características estruturais e bioquímicas se diferenciavam da apoptose ou necrose. Os autores verificaram morte por autofagia (WHITACRE, 2002).

A autofagia é diferenciada pela presença do sistema de auto-digestão lisossomal, pela presença de vacúolos autofágicos, na qual organelas e proteínas deformadas ou agregadas serão degradadas e pela ausência de cromatina condensada. Todo o processo é dependente de ATP e requer proteínas do citoesqueleto para movimentação do sistema vacuolar (CODOGNO e MEIJER, 2004; DINI *et al.*, 2010).

O fator mais comum que pode induzir a autofagia é a falta de nutrientes, mas também se pode ativar a autofagia por meio da hipóxia, no estresse do reticulo endoplasmático, pela falta de energia celular, por estímulos hormonais e por agentes farmacológicos (BURSCH, 2008).

A autofagia na terapia fotodinâmica possui um papel controverso. Esse processo pode agir como supressor tumoral em alguns estudos, na qual o estímulo à autofagia por meio da PDT representa uma alternativa na prevenção da progressão tumoral e, consequentemente, da metástase, ao liberar proteases lisossomais, levando à morte celular (KESSEL, 2012). Uma das proteínas clivadas é a Bcl-2, considerada anti-apoptótica e inibidora de autofagia (KIM *et al.*, 1999). Indicando um papel importante na indução de morte pela terapia.

Por outro lado, a auto-digestão pode degradar organelas com dano oxidativo e agregados de proteínas induzidos pela fotossensibilização, uma vez que o aumento do estresse oxidativo provocado por danos persistentes à proteínas e organelas, leva à execução de programas de adaptação celular, promovendo crescimento do tumor (KESSEL, 2012). Ainda, uma autofagia insuficiente pode não mais fornecer nutrientes para o metabolismo celular, ou prevenir a acumulação de proteínas e organelas defeituosas favorecendo a malignização das células (KESSEL, 2012).

A etapa de iniciação da autofagia é caracterizada pelo desenvolvimento de uma estrutura inicial denominada fagóforo, composta de uma membrana lipídica dupla, que inicia o processo de envolvimento de organelas citoplasmáticas e proteínas (Figura 3). Nessa fase, alguns membros da família Atg (Atg5, 6, 7, 10, 12 e 16) estão envolvidos, sendo os três principais Atg6 (beclin-1), 5 e 12. A seguir, na etapa de sequestramento, há o englobamento final dos componentes celulares, resultando na formação do autofagossomo. As proteínas envolvidas nesse processo são os membros 3,4 e 7 da família de genes Atg, além da proteína LC3 (BEHRENDS *et al.*, 2010).

Beclin 1 é uma proteína que tem função de recrutar Atgs para a formação do autofagossomo. Essa proteína possui um papel regulatório da autofagia (SOENGAS, 2012).

A proteína LC3 é um importante marcador de autofagia, uma vez que sofre uma modificação pós-traducional (clivagem), resultando na forma LC3I (citosólica), e essa por sua vez, quando o processo autofágico é ativado, sofre clivagem e uma lipidação por Atg7 e Atg3, sendo convertida à LC3II, que se liga à membrana do autofagossomo. É devido a essas alterações que esta associada a importância da LC3 no desenvolvimento do processo autofágico (KABEYA, Y., *et al.*, 2000; WU, J., *et al.*, 2006).

Logo após, ocorre o processo de fusionamento, onde o autofagossomo se une com um lisossomo resultando no autofagolisossomo, a estrutura funcional da autofagia. Nessa etapa, há a desestabilização e degradação da proteína LC3II pelo

ambiente ácido e enzimas lisossomais (WANG, C.W. e KLIONSKY, D.J. 2003; BEHRENDS *et al*., 2010).

Em um estudo envolvendo Beclin 1, Wang e colaboradores (2012), investigaram se a serina quinase Akt, frequentemente aumentada em casos de câncer, inibiria a autofagia regulando diretamente o maquinário autofágico independentemente da mTOR. É sabido que a Akt possui muitos alvos envolvidos na tumorigênese, e um dele é a mTOR, um potente inibidor autofágico. Resultados mostraram que Akt inibe a autofagia independente de mTOR, fosforilando Beclin 1, uma proteína pró-autofágica, que por sua vez interage com proteínas 14-3-3. De acordo com Ivaska *e colaboradores* (2007), as proteínas da família das 14-3-3, interagem com as proteínas do citoesqueleto, em especial a vimentina. Nessa interação, a vimentina age como suporte para que a 14-3-3 possa estimular a via de sinalização de mTOR, regulando a síntese de proteínas e crescimento celular. Essa interação é regulada negativamente pela falta de nutrientes e inibição de Akt. Portanto, Akt promove interação da Beclin-1 com a vimentina por meio da fosforilação de Beclin-1 e a geração de sítios de ligação com a 14-3-3.

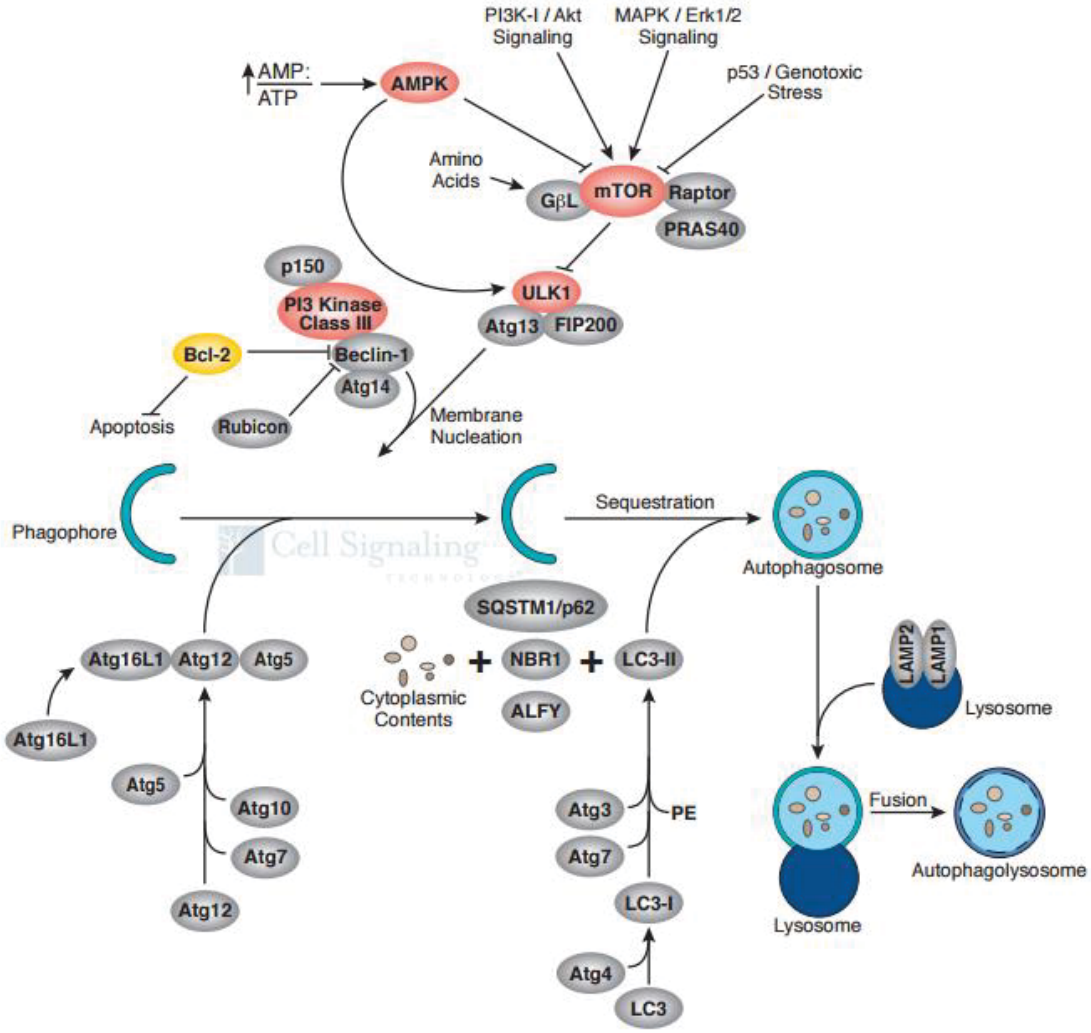



FIGURA 3 – VIAS DE SINALIZAÇÃO DE CONTROLE DA AUTOFAGIA
FONTE:http://www.cellsignal.com/common/content.jsp?id=pathways-autophagy
Nota: A figura mostra as principais proteínas envolvidas na modulação e execução do processo autofágico.

## 2 JUSTIFICATIVA

Tendo em vista os níveis de mortalidade causados por melanoma e a ineficiência de algumas terapias, cada vez mais se tem buscado compreender os mecanismos de morte celular para que o desenvolvimento de novas estratégias terapêuticas.

Por estar superexpressa em carcinomas e ser relacionada com o crescimento e a metástase de tumores, a vimentina pode ser vista como alvo de terapia do câncer. Para tanto, faz-se necessário a realização de estudos críticos para avaliar a sua função específica na resposta do tumor a agentes indutores de morte celular.

Portanto, este estudo destinou-se ao entendimento da relação da vimentina e autofagia no processo de morte celular após o tratamento com oxigênio molecular singlete (RBAc-PDT) em melanócitos e células de melanoma.

# 3 OBJETIVO

## 3.1 Objetivo geral

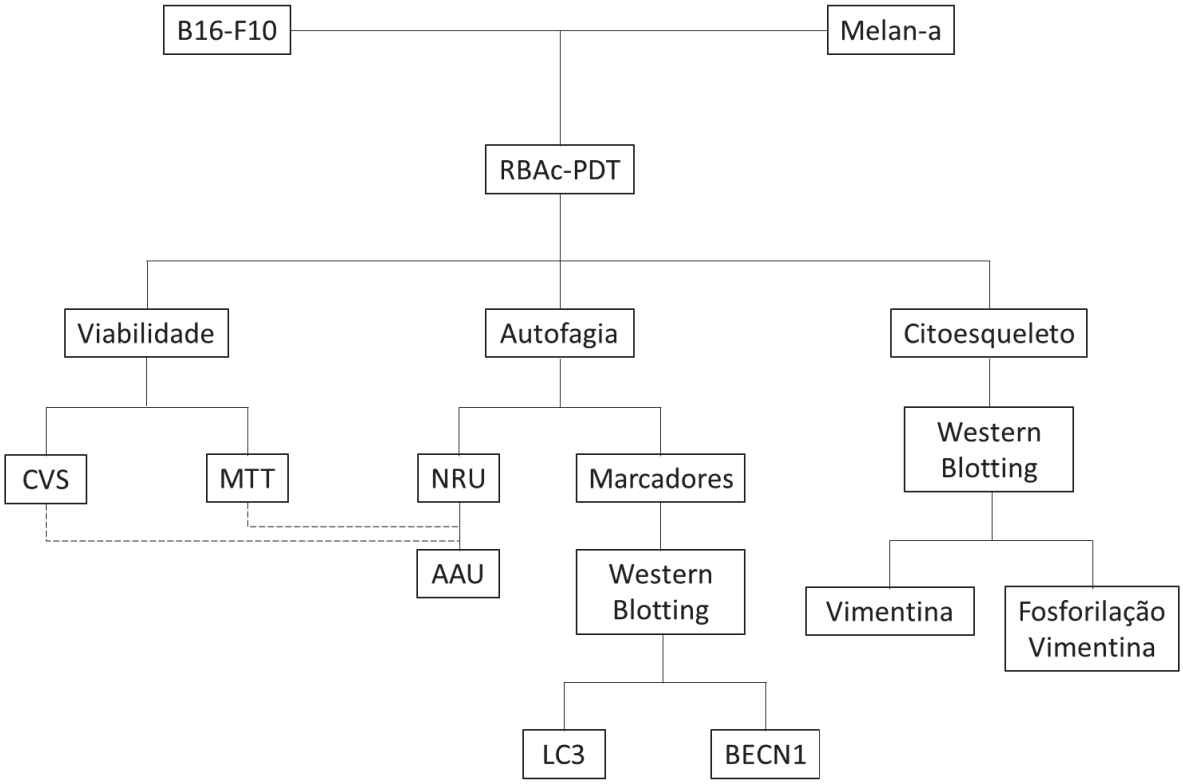
Este trabalho teve por objetivo avaliar os efeitos do oxigênio molecular singlete em relação aos níveis de vimentina em melanócitos Melan-a e células de melanoma murino B16-F10.

## 3.2 Objetivos Específicos

Para compreensão da relação da vimentina com a morte celular em células de melanoma e melanócitos, após o tratamento *in vitro* com Rosa Bengala e fotossensibilização, pretendeu-se avaliar:

- Viabilidade celular
- Os níveis de vimentina e alterações na sua fosforilação
- Ocorrência de autofagia verificando alterações nos níveis de marcadores deste processo como LC3 e Beclin-1.

# 4 ESTRATÉGIA EXPERIMENTAL




**Legenda**

**CVS:** coloração cristal violeta

**MTT:** brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difenil tetrazólio

**NRU:** captação de vermelho neutro

**AAU:** unidades arbitrárias de autofagia

**BECN1:** anticorpo anti-BECN1 monoclonal de rato

**LC3:** anticorpo anti-LC3 policlonal de coelho

## 5. ARTIGO CIENTÍFICO

Manuscrito preparado para submissão na revista "*Photochemistry and Photobiology*"

Vimentin alterations in Autophagy induced by RBAc-PDT in Melan-a and B16-F10 Cells

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ABSTRACT
Cutaneous malignant melanoma incidence is increasing in many parts of the world. When surgery is not effective, chemotherapy and radiation therapy can also be used in addition to surgery. Photodynamic therapy (PDT) is a non-invasive treatment that requires the exposure of cells or tissue to a photosensitizing drug (PS) inducing cell death by the efficient induction of apoptotic as well as non-apoptotic mechanisms, such as necrosis and autophagy, or a combination of all three mechanisms. Rose Bengal is an important photosensitizer with high efficiency of singlet oxygen generation, which may cause alteration of organelles like mitochondria and the cytoskeleton by photodamage. However, the exact reason whereby PDT doesn't work in human melanoma is still matter of debate. To understand the effects of singlet molecular oxygen on vimentin, we investigated the induction of autophagy in Melan-a and B16-F10 cells photosensitized with Rose Bengal Acetate (RBAc). After incubation with Rose Bengal Acetate (0.5, 2.0, 5.0 $\mu g.mL^{-1}$), B16-F10 and Melan-a cells were irradiated for 15 min (green LED - 1.5 $J/cm^2$ as the total light dose). Induction of autophagy were observed in both cell lines after irradiation. Autophagy was measured with the AAU strategy and Western Blotting for the BECN1 and LC3 II proteins. In fact, B16-F10 and Melan-a cells showed autophagic death post-PDT, and different expression of vimentin and its phosphorylated form, suggesting that vimentin filaments are required for endosomal-lysosomal vesicle transport and positioning of endosomes and lysosomes, and could be correlated with autophagy.

Keywords: Melanoma, Phosphorylation, Photodynamic Therapy, Rose Bengal, Vimentin.

### 5.1. Introduction

Cutaneous malignant melanoma incidence is increasing in many parts of the world. It represents the third most common skin cancer type. Exposure to the ultraviolet radiation (UVR), biological factors (like skin color and genetics), cultural, geographical and carelessness with sunscreen, are involved in melanoma development. Atypical nevi preceeds melanoma and may undergo into radial growth or non-invasive growth, which, due to genetic changes, may acquire invasive, angiogenic, and metastatic capabilities, leading to recurrence, metastasis, and death [1,2,3].

Melanoma treatment success depends on early diagnosis. In 80% of cases, surgical removal of the tumor with a part of the surrounding tissue is enough for

melanoma elimination. However, when surgery is not effective, chemotherapy and radiation therapy can also be used in addition to surgery [4,5]. Melanoma is resistant to some traditional types of chemotherapy and radiotherapy, leading to advances in favor of an alternative therapy for the treatment of the localized melanoma: photodynamic therapy (PDT) [6].

PDT is a non-invasive treatment to both cancer and non-oncological disorders. It requires the exposure of cells or tissue to a photosensitizing drug (PS) and its subsequent activation by light at specific wavelengths, inducing reactive oxygen species (ROS) generation, mainly singlet oxygen ($^1O_2$). ROS are involved in the cell death by the efficient induction of apoptotic, as well as, non-apoptotic mechanisms, such as necrosis and autophagy, or a combination of all three mechanisms [7].

Autophagy is a catabolic and essential process of selective degradation and recycling of cellular components. The initial step of autophagy is the development of the phagophore, which involves cytoplasmic organelles and proteins [8]. Some members of Atg family are involved in this step, but the Atg6 (beclin-1) is of particular importance since it has a regulatory role in autophagy, recruiting other members of Atg family to autophagosome formation [9]. The next step is the maturation process in which LC3 is an important protein involved, LC3 I is converted to LC3 II that binds to autophagic membrane vesicles. For this reason, it has been used as an autophagic marker [8]. After that, the autophagosome fuses to lysosome, where cytoplasmic contents are digested and recycled.

Rose Bengal (RB) is an important PS with high efficiency of intersystem crossing and a high quantum yield of $^1O_2$ generation [10]. It has been reported that RB irradiation in a cellular model, may cause alteration of organelles like mitochondria and the cytoskeleton by photodamage [11,12].

One of the major constituents of the cell cytoskeleton is the Intermediate filaments (IFs). Among the IF proteins, one of the most widely expressed and highly conserved proteins of this family is vimentin, a 57 kDa protein, that has gained much importance as a hallmark of mesenchymal-like conversion of epithelial cells and appears to be one of the best indicators of EMT (epithelial–mesenchymal transition) in carcinomas [13,14]. This process is characterized by the expression of vimentin in epithelial cells, which normally express only keratin, resulting in cell shape alterations and increased motility (involved in physiological or pathological processes requiring epithelial cell migration) [13].

A major problem in cancer management is metastasis, the recurrent migration and invasion of tumor cells into the vasculature and tissues. Moreover, vimentin-positive cells in primary tumours could be more metastatic [13]. Classically,

diagnosis of melanoma has relied on the presence of several protein markers, including vimentin [15,16]. Also, vimentin phosphorylation is related with functional consequences, including disassembly regulation and structural reorganization of vimentin, cell migration, cell contraction, adhesion and traffic of integrin [15,16].

In a study using Melan-a and B16-F10 cells after treatment with endoperoxide ($DHPNO_2$), a singlet oxygen generator, a decrease only in melanoma cell viability was observed, showing a differentiated response of melanocytes towards this reactive species. According to this study, in melanocytes, there was a decrease in levels of vimentin, however, there was no change in RNAm levels, indicating a possible protein breakdown. In the case of melanoma, vimentin levels were practically the same, however, the treatment may have caused a transcription decrease, since there was a decrease in RNAm levels, which could be related to the reduction in viability of this cells [17].

This study aims to evaluate the effects of RB-PDT in relation to vimentin and its phosphorylation levels in Melan-a and B16-F10 cells in order to stablish a relationship with the process of autophagy.

## 5.2. Material and Methods

### 5.2.1. Chemicals and Reagents

Dimethyl Sulfoxide (DMSO), Phosphate Buffered Saline (PBS), Hanks Balanced Salt Solutions (HBSS), Culture Medium RPMI 1640 from Cultilab. Ethylenediaminetetraacetic Acid (EDTA), TRIS (tris(hydroxymethyl) aminomethane), MTT (Thiazolyl Blue-Tetrazolium-Bromide), Tween 20 and 2-D Quant Kit were purchased from GE Healthcare, FBS (Fetal Bovine Serum) from Gibco® – Life Technologies, Gentamicin from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA), PMA (Phorbol Myristate Acetate), ECL Select™ Western Blotting Detection Reagent was purchased from GE Healthcare Amersham Biosciences. Pierce® ECL Western Blotting Substrate were purchased from Thermo Scientific. All other reagents were commercial products of the highest available purity and the solutions were prepared with ultrapure water from Gehaka equipment (Brazil).

### 5.2.2. Culture Medium

The medium used in the study was RPMI 1640 which was reconstituted with ultrapure water and sterilized by filtration through a cellulose acetate-nitrate membrane (MF-Millipore™ Membrane Filters). For the experiments, the medium was supplemented with 7.5% fetal bovine serum (FBS), 20 $mmol.L^{-1}$ (4-(2-hydroxyethyl)-1-

piperazineethanesulfnic (HEPES) and 8 mmol.$L^{-1}$ $NaHCO_3$ to adjust the pH to 7.4 and gentamicin (40 mg.$mL^{-1}$). For Melan-a lineage, 200 nM of mitotic inducer phorbol 12-myristate 13-acetate (PMA) was added to the medium so that these cells proliferate in parallel with the tumorigenic strain B16-F10.

5.2.3. Rose Bengal Acetate (RBAc) Photosensitizer Synthesis

The synthesis of Rose Bengal Acetate (RBAC) was performed as [18], previously synthesized by [19]. A stock solution (10 mg.$mL^{-1}$) was obtained by diluting RBAc in dimethyl sulfoxide, and dilutions to the final concentration of 0.5 , 2.0 and 5.0 µg.$mL^{-1}$ were performed directly in the culture medium at the time of incubation.

5.2.4. Cell lines and cell treatment

The cell lines used in this work were the cells of murine melanocytes Melan-a and murine metastatic melanoma B16-F10, which were kindly donated by Professor Dr. Roger Chammas, College of Medicine, University of São Paulo.

Exponentially growing B16-F10 and Melan-a cells were seeded at $2x10^4$ cells/well in a 96-well-microtiter culture dish for 24 h to adhesion. After that, cells were treated with RBAc, the incubation for 120 min was selected as the most appropriate according to [19]. Before irradiation, the culture medium was replaced with Hanks Balanced Salt Solutions (HBSS), and the cells named “LED” were irradiated for a total dose of 1.5 J/$cm^2$ for 15 min using as an innovative green light source a light emitting diode, LED (526 nm). The respective controls underwent the same process, however, were kept all the time in the dark. To evaluate cell viability immediately after the end of the treatment, the HBSS was removed and the viability assays was performed. To evaluate cell viability 18 hours after the end of irradiation, the HBSS was replaced with culture medium and the cells were maintained in a 37 °C incubator at a moist atmosphere of 5% $CO^2$, in the dark.

5.2.5. Cell viability assays

To evaluate the cell viability after treatment with RBAc and irradiation exposure, three assays were used: MTT, *Crystal Violet Staining* (CVS) and *Neutral Red Uptake* (NRU).

5.2.5.1. Viability assay by MTT reduction

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasolium bromide (MTT) assay (Sigma-Aldrich), was performed according to the method described by [20]. It is a soluble compound that, once internalized, is reduced by mitochondrial enzymes

(succinate dehydrogenase and cytochrome c oxidase) resulting in a purple colored product. Briefly, following the RBAc incubation and irradiation, to each well was added 0.2 ml medium containing 0.5 mg MTT/ ml, and the microplate was incubated at 37 $^{o}$C for 3 h. At the end of the incubation period, the medium with MTT was removed, and 0.2 ml of DMSO was added to each well. Living cells were determined by MTT dye reduction. The plate was shaken on the microplate shaker to dissolve the blue MTT-formazan that is directly associated with cell viability and it can be determined spectrophotometrically at 550 nm on a microplate reader. The viability was normalized to the absorbance values of untreated cells of the control group "dark" (100% viability).

5.2.5.2. Viability assay by CVS

The Crystal Violet assay, is a cytochemical staining assay based on the fact that only viable cells remain attached to the culture surface. After each well was washed with PBS, the cells attached to the bottom of the plate were fixed with methanol and stained with 0.2% crystal violet solution. The dye which, in turn, binds to DNA, was eluted by 50% (v/v) ethanol – 0.05 mol.L$^{-1}$ sodium citrate. The absorbances were measured at 540 nm in a microplate reader [21]. The viability was normalized to the absorbance values of untreated cells of the control group "dark" (100% viability).

5.2.5.3. Viability assay by NRU

The Neutral Red Uptake assay was based on the method of [22], wherein the Neutral Red (NR) cationic dye accumulates in lysosomotropic membranes. To each well, it was added 0.2 ml of medium containing 50 mg NR/ml. The dye diffuses across the cell membrane, being trapped in lysosomes of viable cells due to change of load caused by acid lysosomal pH. The plate was put in the incubator for further 2 h for the uptake of the vital dye crystals. Thereafter 0.1 ml 1% acetic acid solution containing 50% ethanol was added to extract the dye from the cells. After cell lysis by extracting solution, the diluted dye was quantified by absorbance measuring on microplate reader at 550 nm. In cases of autophagy induction, there is a higher incorporation of Neutral Red by lysosomotropic autophagic vacuoles (autolysosomes), whose survival rates obtained by MTT assays and CVS allows the calculation of an arbitrary autophagy units (AAU) [23].

5.2.6. Principle of AAU strategy for measuring autophagic cell death *in vitro*

To measure autophagy *in vitro,* the detection strategy of autophagic vacuoles was used for AAU variable (Autophagic Arbitrary Units) [23]. This strategy was based on labeling of autophagic vacuoles by neutral red lisossomotropic dye, using the neutral red assay.

According to this approach, during the induction of death by autophagy there is a dye accumulation in autolysosomes or late autophagic vacuoles (fusion between autophagosome and lysosome at the final stage of the autophagic process) compared to the untreatred control group, which has the highest frequency of viable lysosome. Therefore, due to higher incorporation and neutral red retention in late autophagic vacuoles, there is an overestimation of cell survival rate. In addition, at this rate normalized by the mean of cell survival rates estimated by MTT and CVS assays, is obtained the AAU variable, which when higher than 1.0, is significantly correlated and linearly with autophagic cell death.

Therefore, to calculate the AAU variables, the NRU survival rate was normalized to the mean of the MTT and CVS survival rates according to this formula:

$$\mathrm{AAU} = \left\{ \frac{X_1}{\left(X_2 + X_3\right)/2} \right\}$$

Where $X_1$,$X_2$ and $X_3$ were respectively the survival rates measured by NRU, CVS and MTT assays.

5.2.7. Protein Extraction and *Western Blotting*

Melanoma cells and melanocytes were plated and treated. After treatment time, 0.5 mL of protein extraction buffer RIPA$^{+}$ supplemented with protease inhibitors (100 mM Tris (pH 7.6), 1% Triton X-100, 150 mM NaCl, 1.5x10$^{-8}$ mM Aprotinin, 0.2 mM PMSF, 10 mM $Na_3VO_4$, 100 mM NaF, 10 mM $Na_4P_2O_7$ and 4 mM EDTA) was added to the culture plate. The samples were homogenized until become very fluid. After an incubation for 30 minutes at 4$^{o}$C, the samples were centrifuged at 13,000 x g for 10 min at 4 ° C to remove cellular fragments. The supernatant was removed and quantified by the 2D Quant kit from GE Healthcare Amersham Biosciences, and then aliquots of the same protein concentration (20 μg) were submitted to gel electrophoresis at 12% polyacrylamide-SDS-PAGE. Then the proteins were transferred from the gel to the PVDF membrane (AmershamHybond ™ -P, GE Healthcare) by wet transfer method. The membrane containing the proteins of the samples was blocked in 5% (m/v) BSA solution in TBST 1X (50 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.1% (v/v) Tween 20) for 1 hour. Once blocked, the membrane was incubated with primary antibody against the molecule of interest. And then, the membrane was incubated with secondary antibody. For the revelation of blotting, a chemiluminescent substrate of peroxidase (Sigma) was used. To evaluate cell death by autophagy was used BECN1 and LC3 antibodies, and for analysis

of vimentin and your phosphorylation was used Vimentin (R28) and p-Vimentin S56 antibodies (Cell Signaling).

5.2.8. Statistical Analysis

The data were analyzed with the Prisma software, using one-way ANOVA followed by Tukey's test, as indicated in the legend.

## 5.3. Results

5.3.1 Cell viability after irradiation in the presence of RBAc

Using RBAc and the irradiation chamber to generate $^1O_2$, assessment of cell viability was performed by three different colorimetric methods: MTT, Crystal Violet and Neutral Red. In all these three assays, cell viability was assessed immediately after exposure to radiation as well as 18 hours after the end of irradiation.

5.3.1.1 MTT

First, in B16-F10, in relation to data obtained immediately after irradiation (0 h), it was observed that RBAC in the absence of irradiation or also called "DARK" group, did not show cytotoxicity at the three administered concentrations (0.5, 2.0 and 5.0 $\mu g.mL^{-1}$) >Figure 1A<. However, after irradiation or also called "LED" group, a significant reduction of viability (14%) was observed at the highest concentration (5.0 $\mu g.mL^{-1}$) >Figure 1B<. In the groups analyzed 18 hours after irradiation (18 h), we observed that the RBAc on DARK group did not show cytotoxicity in any of the three administered concentrations >Figure 1A<. However, in irradiated groups, the reduction of viability at the concentration of 5.0 $\mu g.mL^{-1}$ RBAc was intensified (40%) >Figure 1B<. We can also observe that RBAc without irradiation and the irradiation in the absence of RBAC showed no cytotoxicity.

In Melan-a cells, in relation to data obtained immediately after irradiation, the group DARK and LED showed no cytotoxicity in all the three administered concentrations of RBAc >Figure 1C and 1D<. Also, in the group DARK 18h, RBAc showed no cytotoxicity, but in the LED group 18h, in the concentration 5.0 $\mu g.mL^{-1}$, we can observe that the viability decreased 20% >Figure 1D<.

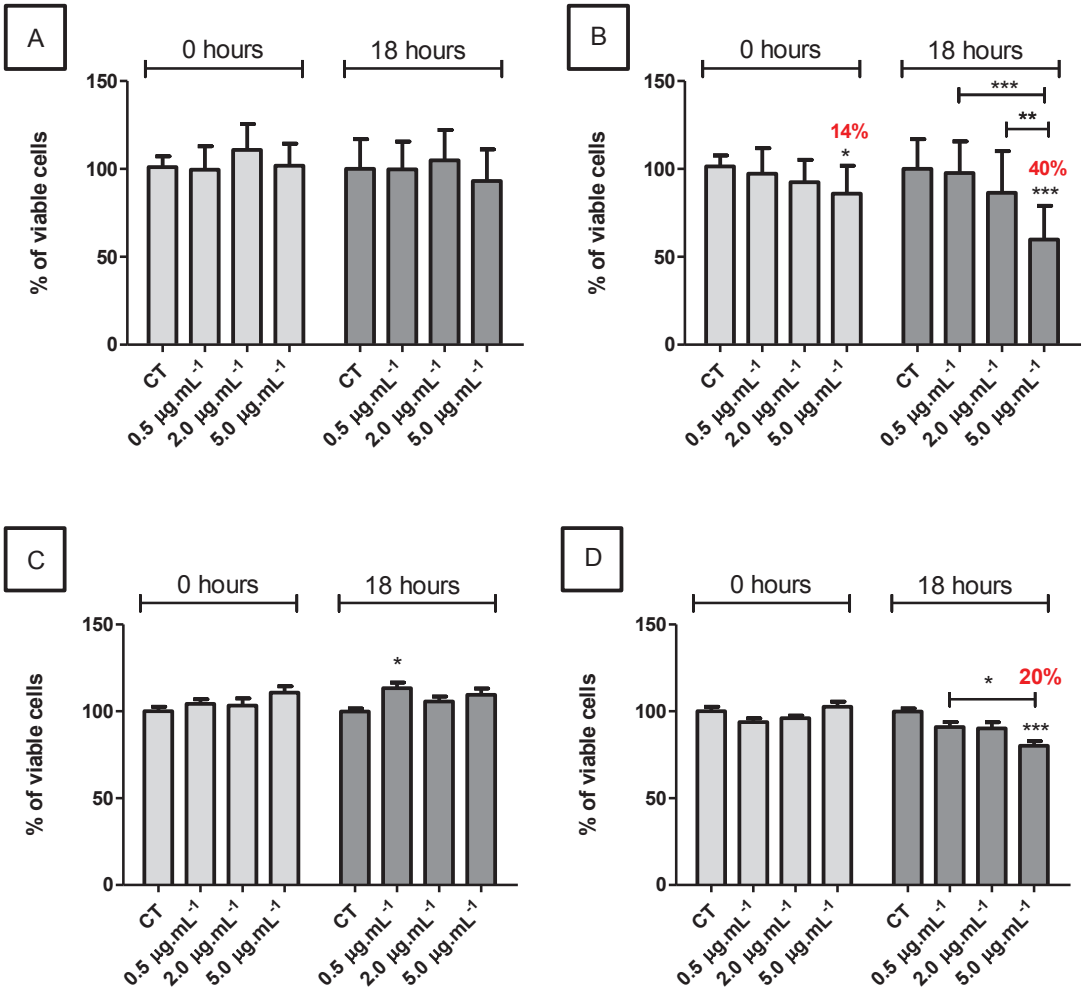


**Fig. 1**. Cell viability measured immediately and 18 hours after irradiation in B16-F10 and Melan-a cells by MTT. (A) MTT DARK group B16-F10. (B) MTT LED group B16-F10. (C) MTT DARK group Melan-a. (D) MTT LED group Melan-a. The cells (2 x $10^4$cells/well) were plated in 96-well plates and after 24 hours, they were incubated in the presence of 0.5, 2.0 and 5.0 µg.mL$^{-1}$ RBAc for 2 h. Then, the medium was removed and replaced by HBSS and the cells were irradiated for 15 min. Cell viability was assessed immediately after irradiation and also 18 hours after the end of irradiation by MTT method. The data represent the mean ± SD from three experiments performed in triplicate (* p <0.05; ** p <0.01; *** p <0.001).

5.3.1.2. Crystal Violet Staining

Regarding to the data obtained immediately after irradiation (0 h) in cell line B16-F10, it was observed that RBAc in the absence of irradiation (DARK group) and in the presence of irradiation (LED group), did not show cytotoxicity at the three administered concentrations (0.5, 2.0 and 5.0 µg.mL$^{-1}$) >Figure 2A and 2B<. However, 18 hours after the end of irradiation in the DARK group, there was a significant reduction (28%) in the cell viability with 0.5 µg.mL$^{-1}$ RBAc >Figure 2A<. On the LED group, 18

hours after the end of irradiation, a significant reduction of viability 27%, 19% and 21% was observed respectively at 0.5 , 2.0 and 5.0 µg.mL$^{-1}$ of RBAc >Figure 2B<.

In Melanocytes, the same pattern was observed at 0 h in DARK and LED groups: no cytotoxic activity. But, 18 hours after irradiation, both groups showed decrease on viability in all concentrations. DARK group had significant decrease of 18%, 10% and 12% in the respective concentrations of 0.5, 2.0 and 5.0 µg.mL$^{-1}$ of RBAc. In LED group, the decrease was 20%, 35% and 36%, respectively.

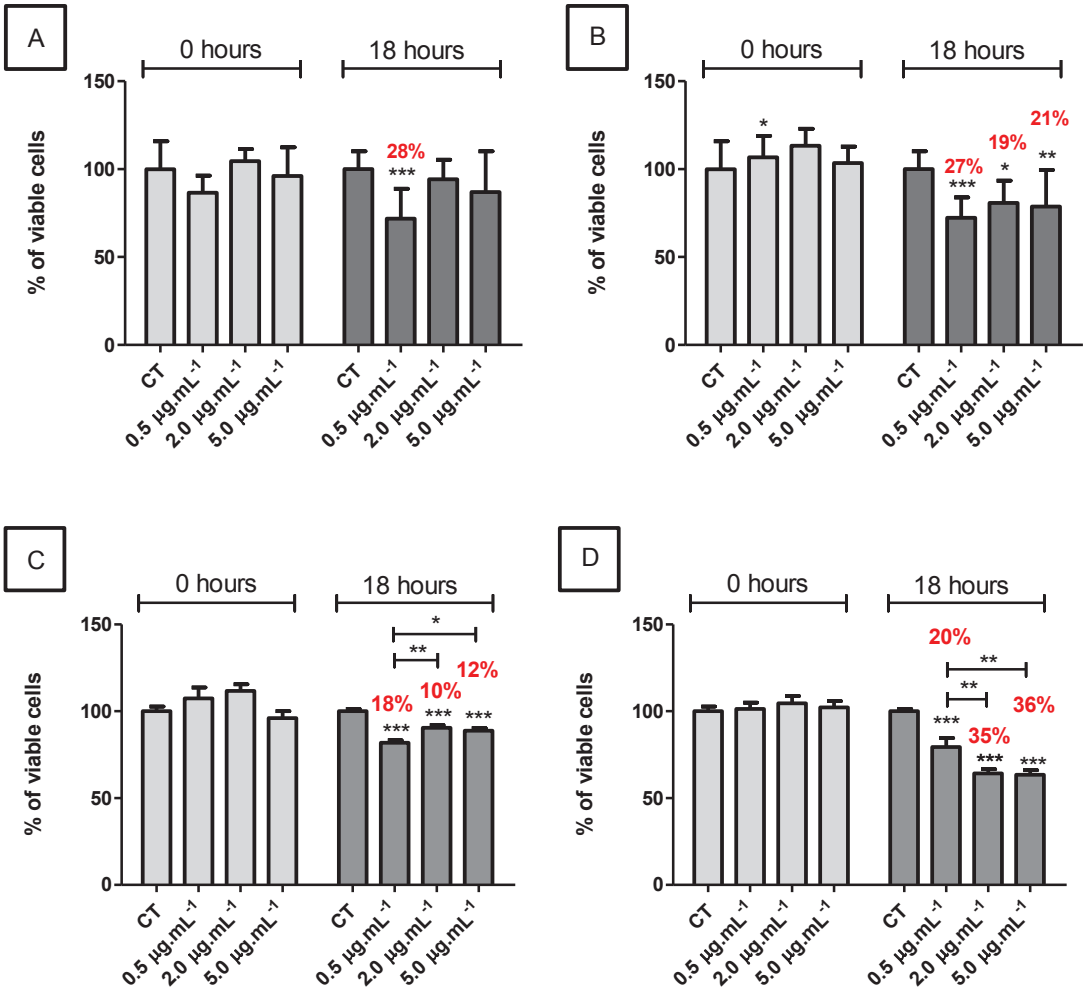


**Fig. 2**. Cell viability measured immediately and 18 hours after irradiation in B16-F10 and Melan-a cells by CVS. (A) CVS DARK group B16-F10. (B) CVS LED group B16-F10. (C) CVS DARK group Melan-a. (D) CVS LED group Melan-a. The cells (2 x $10^4$cells/well) were plated in 96-well plates and after 24 hours, they were incubated in the presence of 0.5, 2.0 and 5.0 µg.mL$^{-1}$ RBAc for 2 h. Then, the medium was removed and replaced by HBSS and the cells were irradiated for 15 min. Cell viability was assessed immediately after irradiation and also 18 hours after the end of irradiation by CVS method. The data represent the mean ± SD from three experiments performed in triplicate (* p <0.05; ** p <0.01; *** p <0.001).

Considering the viability rates obtained here, the concentration of 5.0 $\mu g.mL^{-1}$ of RBAC was chosen for subsequent experiments, since after 18 h of treatment, more than 60% of cell viability was still maintained for both cell lines, which represented a good population of cells to perform further experiments.

5.3.1.3. Neutral Red Uptake

NR incorporates into lysosomes of viable cells. However, autophagic vacuoles accumulation lead to an increase in NR incorporation in autolysosomes. Immediately after photosensitizing B16-F10 cells with light dose of 1.5 $J/cm^2$ in the presence of RBAC (5.0 $\mu g.mL^{-1}$), the value measured by survival assay neutral red was 109%, not corresponding to those measured by MTT (86%) and CVS (103%) assays >Table 1<. According to >Figure 3A<, RBAc 5 – 0 h LED had a significant value of AAU (1.2 ± 0.2), suggesting autophagic process. The DARK group did not show increase of NR labelling.

After 18 hours from the end of irradiation of B16-F10 cells in the presence of RBAc (5.0 $\mu g.mL^{-1}$), the survival rates by NRU assay was 93%, not corresponding to those measured by MTT (60%) and CVS (79%) assays >Table 1<. According to >Figure 3C<, RBAc 5 – 0 h LED had a significant value of AAU (1.4 ± 0.4) suggesting autophagic process. The DARK group did not show increase of NR labelling.

| GROUPS | MTT Survival (%) | SD | NRU Survival (%) | SD | CVS Survival (%) | SD |
|---|---|---|---|---|---|---|
| CT 0 h DARK | 100 | 7 | 100 | 9 | 100 | 16 |
| RBAc 5 - 0 h DARK | 102 | 13 | 100 | 7 | 96 | 16 |
| CT - 0 h LED | 100 | 6 | 100 | 4 | 100 | 5 |
| RBAc 5 - 0 h LED | 86 | 16 | 109 | 11 | 103 | 9 |
| CT 18 h DARK | 100 | 17 | 100 | 5 | 100 | 10 |
| RBAc 5 - 18 h DARK | 93 | 18 | 97 | 5 | 87 | 23 |
| CT - 18 h LED | 100 | 5 | 100 | 6 | 100 | 9 |
| RBAc 5 - 18 h LED | 60 | 19 | 93 | 5 | 79 | 21 |

**Table 1**. The viability assays and their values immediately after (0 h) and 18 hours after irradiation (18 h) of B16-F10. The table shows the survival rates by three different viability methods. The data represent the mean ± SD from three experiments performed in triplicate.

On the other hand, in Melan-a cell line, immediately after photosensitizing (0 h) in the presence of RBAC (5.0 $\mu g.mL^{-1}$), the value measured by survival assay

neutral red was not overestimated (101%), corresponding to those measured by MTT (103%) and CVS (102%) assays >Table 2<. And also, the value of AAU (1.0 ± 0.1) variable wasn't higher than 1.0 >Figure 3B<. Only RBAc DARK 0 h had a decrease in NRU values (83%) compared to MTT (111%) and CVS (96%) >Table 2< and a decrease in AAU (0.8 ± 0.2).

Differently from what was observed in 0 hour for Melan-a cells, 18 hours after the end of irradiation, the survival rates in RBAc 5 – 18 h LED was overestimated by NRU assay (105%), not corresponding to those measured by MTT (80%) and CVS (63%) assays >Table 2<. The value of AAU of RBAc 5 – 0 h LED (1.5 ± 0.2) was significantly higher than control >Figure 3D<.

| GROUPS | MTT Survival (%) | SD | NRU Survival (%) | SD | CVS Survival (%) | SD |
|---|---|---|---|---|---|---|
| CT 0 h DARK | 100 | 9 | 100 | 12 | 100 | 9 |
| RBAc 5 - 0 h DARK | 111 | 13 | 83 | 10 | 96 | 14 |
| CT - 0 h LED | 100 | 6 | 100 | 10 | 100 | 8 |
| RBAc 5 - 0 h LED | 103 | 10 | 101 | 14 | 102 | 12 |
| CT 18 h DARK | 100 | 6 | 100 | 9 | 100 | 5 |
| RBAc 5 - 18 h DARK | 110 | 13 | 96 | 5 | 89 | 5 |
| CT - 18 h LED | 100 | 6 | 100 | 6 | 100 | 13 |
| RBAc 5 - 18 h LED | 80 | 9 | 105 | 10 | 63 | 9 |

**Table 2**. The viability assays and their values immediately after (0 h) and 18 hours after irradiation (18 h) of Melan-a. The table shows the survival rates by three different viability methods. The data represent the mean ± SD from three experiments performed in triplicate.

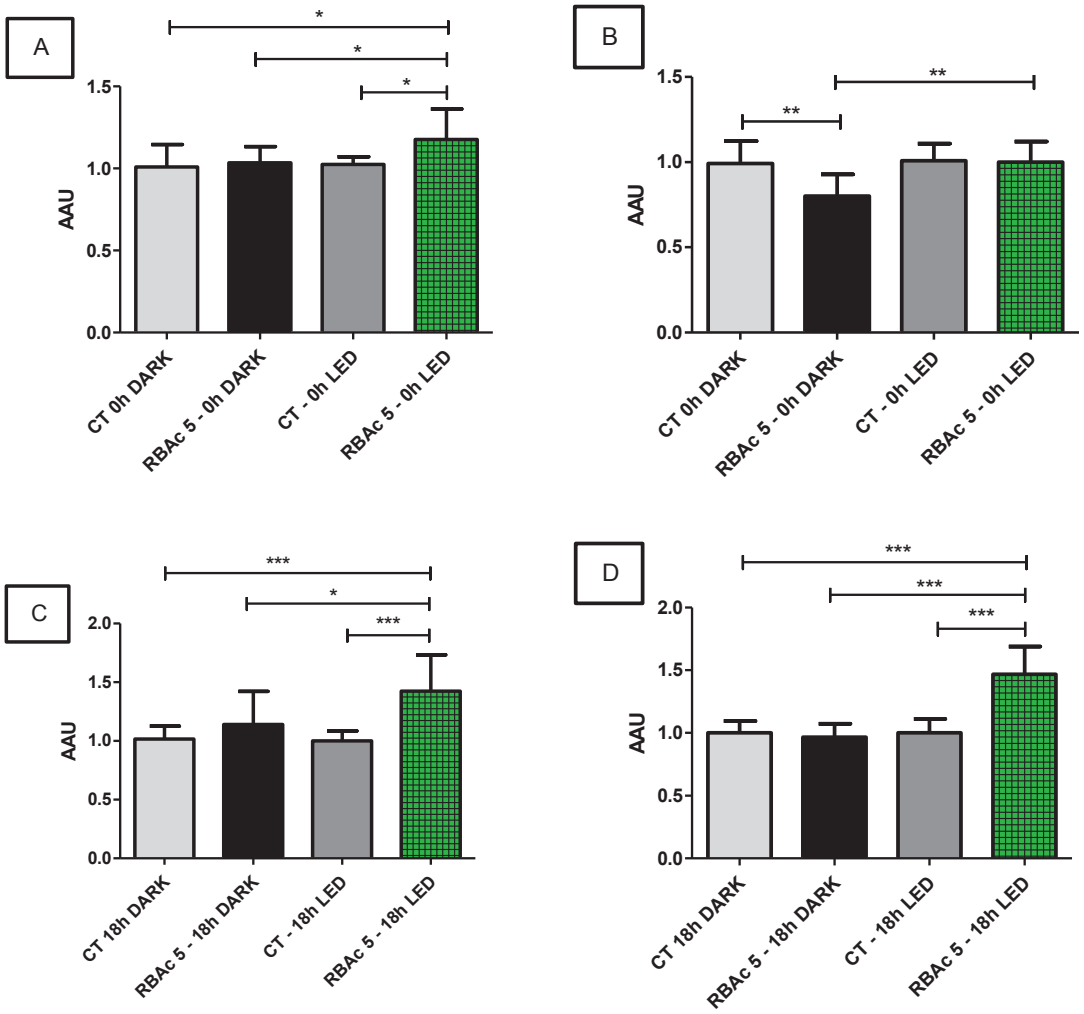


**Fig. 3**. AAU variable relation with dark control and RBAc treatment groups with or without irradiation. The graphic shows the AAU values calculated from the MTT, CVS, and NRU survival rates and your relation with untreated and treated groups, immediately and 18 hours after irradiation from untreated and treated cells with 5.0 μg.mL$^{-1}$ RBAc. (A) 0 hours B16-F10, (B) 0 hours Melan-A, (C) 18 hours B16-F10 and (D) 18 hours Melan-a. AAU average was calculated from three independent experiments. The data represent the mean ± SD from three experiments performed in triplicate.

5.3.2. Analysis of autophagic markers, vimentin and vimentin phosphorylation by *Western Blotting*

The Western blotting assay was performed to evaluate and confirm autophagy after the photosensitization of cells and correlate this dying mechanism with the cytoskeletal vimentin protein and its phosphorylation.

In order to analyze the expression of BECN1 comparing to the control group DMSO DARK 0 h (untreated cells without irradiation), we can observe, in both cell lines

>Figure 4A-4B< , that immediately after irradiation, the expression of BECN1 decreased in RBAc LED 0 h. The expression was restored 18 hours after the end of irradiation in B16-F10 cells. However, in Melan-a, the expression increased and was higher than control DMSO DARK 18 h.

The levels of LC3 II, in RBAc LED 0 h, had a decrease in B16-F10 cells wheras in Melan-a the expression increased. At 18 hours, the expression is restored in B16-F10, and, in Melan-a, the levels kept higher. Analyzing the relation LC3 II/LC3 I, B16-F10 and Melan-a had no higher value compared to control group at 0 hour in RBAc LED. However, at 18 hour, both cell lines showed higher levels of LC3 II/LC3 I in RBAc LED group.

About vimentin expression, in B16-F10, no alteration was observed in any condition. In Melan-a, RBAc LED, had an increase at 0 hour, but 18 hours later, it was normalized.

The other target of investigation was the phosphorylation of vimentin, p-vimentin S56. In B16-F10, at 0 hour, no difference between the groups was observed; at 18 hours, RBAc LED and DMSO LED increased the expression. In Melan-a, at 0 hour, RBAc LED decreased p-vimentin S56 levels, also led to a decrease in the others control groups. At 18 hours, RBAc LED decreased the phosphorylation and the group with just RBAc increased more than the others.

In a relation p-vimentin/vimentin, in B16-F10, an increase was observed in RBAc LED at 18 hours. In Melan-a, this relation has not changed.

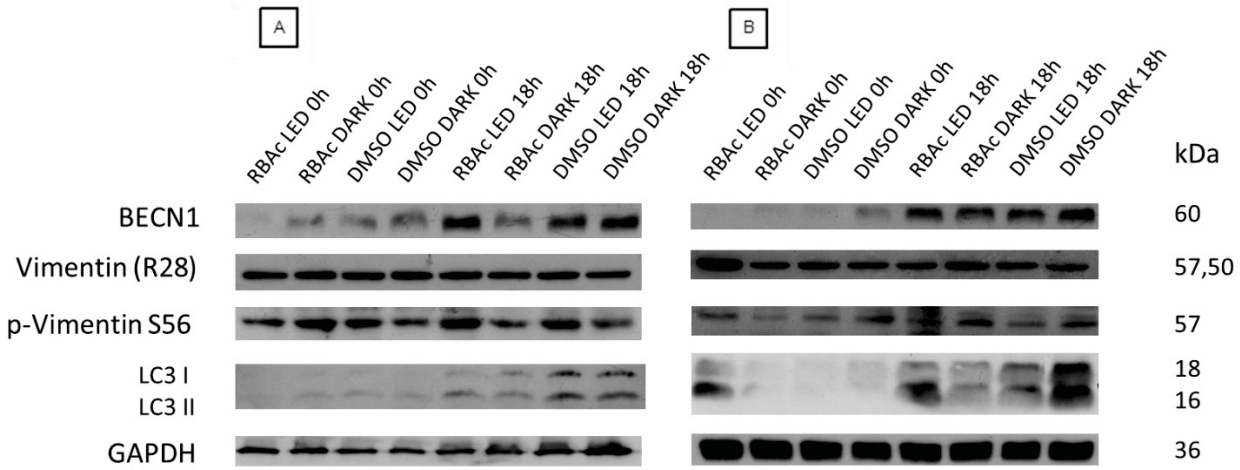


**Fig. 4**. Autophagy and vimentin phosporylation. All blots shown (A-B) are representative of two experiments immediately and 18 hours after irradiation from untreated and treated cells with 5.0 $\mu g.mL^{-1}$ RBAc. (A) B16-F10 (B) Melan-a.

## 5.4 Discussion

Rose Bengal acetate (RBAc) could have a practical application in PDT, because it has a high efficiency of singlet oxygen generation. Also, the addition of an acetate group improves its ability to cross cell membranes, (increasing its hydrophobicity) and accumulation into cells. Inside the cells, RB molecules are restored by cytoplasmic esterases that recognize and split the acetate group. Back to non-acetylated form, there is a reduction of permeability through cell membranes, leading to drug efflux reduction and consequent accumulation within the cell. RB redistributes through the cytoplasm, inducing multiple organelle photodamage after irradiation [24,18]. In this paper, the effects of singlet molecular oxygen in relation to levels of vimentin in Melan-A and B16-F10 cells were investigated after cell photodamage by RBAc irradiated with LED.

Under our experimental conditions, the viability was evaluated by MTT, CVS and NRU. First, B16-F10 and Melan-a cells had similar results in viability tested by MTT. Both lines showed significant higher decrease in viability at 5.0 $\mu g.mL^{-1}$ of RBAc 18 hours after had been irradiated >Figure 1<. In case of CVS method, both cells showed significant decrease in all three concentrations of RBAc 18 hours after had been irradiated, and also at time 0 hour, Melan-a showed significant decrease in all three concentrations of RBAc without irradiation, showing that Melan-a was more sensitive to the treatment when evaluated by adherence maintenance >Figure 2<. Comparing the differences in the decrease of viability at 18 hours LED and DARK in Melan-a cells, we can see that this difference is proportional to the decrease in B16-F10 18 hours LED (around 20% lower).

Autophagic detection analysis was initially performed from NR dye incorporation profile compared to CVS and MTT results [23]. This strategy is based on the increased neutral red uptake in case of autophagic death, because this dye has an affinity for acidic vacuoles, which are present in greater quantity in autophagic process. Therefore, when evaluating the percentage of neutral red survival assay, we may observe an overestimated value. When relating the neutral red survival value with CVS and MTT, the AAU (Autophagy Arbitrary Units) variable was obtained. In which, values above 1.0 indicate death by autophagy [23] and the higher this value, the greater the correlation with PCD (Programmed Cell Death) type II (autophagy).

After photosensitization of B16-F10 and Melan-a cells treated with RBAc, the NRU assay values were overestimated >Figure 3<, not corresponding to the CVS and MTT values. According to [23] this difference is due to the greater NR incorporation by autophagic acid vacuoles (autolisosomes) in cells that receive irradiation compared to the control group with no irradiation, in which NR incorporation is only perfomed by

available lysosomes. Therefore, when the NRU levels correlate inversely with CVS and MTT results, is an indication of cell death by autophagy.

To confirm autophagy, we used BECN1 and LC3 II immunoblotting. In both cell lines, BECN1 showed a decrease in the expression in RBAc LED 0 h compared to others control groups. We can say that RBAc with LED altered beclin-1 levels, which is not compatible with AAU index observed to B16-F10 RBAc LED 0 h >Figure 3A< that suggest autophagy according to [23]. We suggest that beclin-1 suffered an alteration in the structure immediately after irradiation, probably resulting in oxidation or phosphorylation, preventing the recognition by the antibody, causing a decrease in its detection by western blotting or maybe, beclin-1 was cleaved by caspases as demonstrated in a study of apoptosis, destroying its pro-autophagic activity [25]. Furthermore, no paper was found specifically correlating beclin-1 expression immediately after PDT treatment [26]. Interestingly, 18 hours after the treatment of cells, BECN1 levels were restored in B16-F10, wheras Melan-a levels of BECN1 increased. This overexpression is usually associated to autophagy [27] and it is in accordance to AAU variable 1.5 ± 0.2 >Figure 3D<. Therefore, we can say that expression of beclin-1 is time-dependent as observed by [28], that used human epithelial carcinoma cell line (A431) and malignant melanoma cell line (G361) treated with pheophorbide a (Pa) and irradiated with LED and observed that beclin-1 increased with time (6-24h).

When autophagic process is activated, LC3 I is cleaved and converted to LC3 II, which associates to autophagic membrane vesicles [11]. The treatment plus irradiation had the same effect on cell lines in the relation of expression of LC3 II/LC3 I. Melan-a and B16-F10 had no higher relation than the control at 0 hour, however, 18 hours after irradiation, the relation LC3 II/LC3 I increased, suggesting autophagic process, which agrees with AAU values.

In Melan-a, at 0 hour, autophagy is not happening according to BECN1 levels and LC3 II/LC3 I relation. At 18 hour, the level of BECN1 was restored, then the phagophore can be formed and LC3 II could bind in autophagosome continuing the process. This is in accordance to AAU 1.0 to 0 h, AAU 1.5 to 18 h and cell viability results. In B16-F10, analyzing the BECN1/LC3 levels, we can suggest that the cells had their viability decreased due to other routes of death, not only autophagy, maybe apoptosis [29]. The AAU levels were higher than 1.0, because NRU method assessed the viability according to the capacity of lysosomes accumulate NR and we already reported that PDT causes photodamage in organelles which is degraded *via* the endosomal-lysosomal system [7]. In fact, our results showed a significant and strong correlation between AAU levels and autophagic cell death in Melan-a. In B16-F10, the analyses of BECN1 and LC3 II/LC3 I were not compatible with AAU values at 0 hour >Figure 3A< that had

indicated occurrence of an autophagic process. However, according to [29], in HeLa cells with RBAc-PDT, autophagy was induced while apoptosis happens – mitochondria lost their potential membrane and release cytocrome-c. Autophagy occurred faster than apoptosis (8-12h) and disappeared faster as well. We suggest that immediately after photosensitization, the cells died by apoptosis since autophagy did not happened due to beclin-1 cleavage [25]. Autophagy was started, hours later, due to elimination of damaged mitochondria, Golgi, ER or cytoskeleton by oxidation. On the other hand, at 18 hour, the relation LC3 II/LC3 I showed occurrence of autophagic process that is compatible with AAU value. Therefore, in Melan-a cells, we can say that the main cell death process was autophagy, in the case of B16-F10 cells, autophagy was occurring along with another type of programmed cell death, this difference can be attributed as a distinction between the two cell lines.

In relation to vimentin, it has been reported by [11,12] that after photoactive RB was restored, it is distributed dynamically throughout the cytoplasm and may cause alteration of organelles like mitochondria and the cytoskeleton. Thereafter, we can suggest that in B16-F10, PDT effects caused an increase in the phosphorylation of vimentin, indicating vimentin dismantling on tetrameric subunits [30]. It has been shown that vimentin interacts with 14-3-3 in a phosphorylation-dependent manner, 14-3-3 is a protein family that is involved in many functions, such as apoptosis, by limiting the ability of 14-3-3 to interact with target molecules like Bad, a apoptosis promoting factor. Therefore, the association with 14-3-3 shows that vimentin could be involved in the modulation process requiring 14-3-3 [15].

In the case of Melan-a cells, we did not observed any increase in the phosphorylation of vimentin >Figure 4<. When we compare phosphorylation levels between the two cell lines, it is evident that Melan-a cells have more total vimentin expression in comparison to the phosphorylated form.

It had already been reported by [13] that vimentin is overexpressed in many types of cancer, including melanoma, indicating accelerated tumor growth, invasion and poor prognosis. Nevertheless, after RBAc-PDT, Melan-a cells leads to cell death mainly by autophagy (18 h) agreeing with levels of LC3 II/BECN1 >Figure 4< and AAU value (1.5 ± 0.2). In melanoma cells, the death occurred not only by autophagy but also by another program cell death, according to [29], since LC3 II/BECN1 levels were not high but AAU levels suggested autophagic process. We could correlated this to vimentin phosphorylation and its association with 14-3-3 protein family.

However, the relationship of phosphorylation of vimentin and autophagy is not fully elucidated, it is necessary other studies with different cells, to establish a pattern,

in order to use it as a target for other therapies. Furthermore, in this case, the values of autophagy markers were not compatible with the AAU strategy in B16-F10 at 0 hour, making it necessary to review other cell death pathways, as well autophagy, when using this method.

## 5.5 Conclusions

The fact that cancer cells can die through different mechanisms is a relevant clue that facilitates the choice and development of new anticancer therapies.

In the current study, we have demonstrated the induction of autophagy after RBAc-PDT in melanoma and melanocytes, as consequence of photodamage. We showed that both cell lines have different behaviors towards PDT, and this can be considered as a positive factor in the perspective of an application of RBA-PDT in other types of cells. In addition, this study provides a better understanding of cell death related to cytoskeleton proteins post-PDT.

## 5.6 Acknowledgments

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## 6. CONCLUSÕES

Nesse estudo, demonstramos a indução de autofagia após RBAc-PDT em melanoma e melanócitos usando o índice AAU como indicativo do processo autofágico. Entretanto, a correlação com os marcadores Beclin-1 e LC3 ocorreu mais diretamente em relação à linhagem Melan-a. Os resultados na razão vimentina/vimentina fosforilada para as células B16-F10 são mais concordantes com a relação dessa proteína e a autofagia, mas nas células Melan-a essa relação é menos evidente. Dessa forma, mostramos que ambas as linhas celulares têm diferentes mecanismos de resposta frente à PDT, apesar do resultado final em relação à perda de viabilidade ser semelhante entre elas. Assim, este estudo permite uma melhor compreensão da morte celular relacionada com as proteínas do citoesqueleto pós-PDT em função da célula ser tumoral ou não.

## 7. REFERÊNCIAS

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