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EFEITO DA COMBINAÇÃO ENTRE RADIAÇÃO UVB E ÔMEGA3 E INTERAÇÃO ENTRE CISPLATINA E ÔMEGA 3 EM CÉLULAS DE MELANOMA

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Lista de Abreviaturas

AA - Ácido Araquidônico (Ômega-6)

ALA - Ácido α-linolênico (Ômega-3)

COX -Ciclooxigenase

CPt - Cisplatina

DHA - Ácido Docosahexaenóico (Ômega-3)

DUSP-6 - Fosfatase-6 de dupla especificidade

EPA - Ácido Eicosapentaenóico (Ômega-3)

ERCC1 - Reparo por Excisão Cross-Complementação Grupo 1

ERK - Quinase Regulada por Sinal Extracelular

LA - Ácido Linoleico

LOX -Lipooxigenase

LT -Leucotrienos

MAPK - Proteína Quinase Ativada por Mitógeno

MDR - Resistência a Múltiplas Drogas

NER - Reparo por Excisão de Nucleotídeo

Omega 3 PUFA - Ácidos Graxos Poliinsaturados ômega 3

PG - Prostaglandinas

Pgp - Glicoproteína P

PLA_2 -Fosfolipase A_2

TX -Tromboxanos

UVB -Radiação Ultrvioleta B

Resumo Geral

Mundialmente, a incidência de melanoma tem aumentado de forma preocupante, e uma terapia eficaz para a doença metastática ainda inexiste, dada a sua resistência intrínseca/extrínseca. Recentemente, ácidos graxos poliinsaturados ômega 3 (PUFA ômega 3) têm atraído um crescente interesse como promissores adjuvantes na prevenção e tratamento do câncer. Assim, as propostas deste trabalho foram avaliar o efeito do ácido α-linolênico (ALA) na exposição à radiação ultravioleta B (UVB) em uma linhagem melanocítica normal (Melan-a) e em uma linhagem melanocítica maligna (B16F10), investigar a atividade da glicoproteína P (Pgp), que é equívoca para o melanoma, e ainda analisar o efeito do tratamento combinado de cisplatina (CPt), e um PUFA ômega 3, ácido docosahexaenóico (DHA), em sensibilizar células de melanoma humano (WM266-4) e murino (B16F10) à ação antineoplásica da CPt. Também investigamos os efeitos do DHA em proteínas relacionadas à resposta de resistência à CPt no melanoma, ERCC1, DUSP6 e p-ERK. ALA apresentou um efeito inibitório na proliferação de células B16F10, e teve um mínimo efeito na Melan-a. ALA e UVB combinados mostraram, de uma forma dose e tempo dependente, uma ação fotodinâmica nas células B16F10 e um efeito fotoprotetor na Melan-a. Demonstramos que estes efeitos não tiveram a participação do processo de estresse oxidativo, uma vez que não foram observadas diferenças em 48 h após os tratamentos. Adicionalmente, ambas as células apresentaram uma expressiva atividade de Pgp. Mostramos ainda que o DHA apresentou um efeito inibitório e antiinvasivo em células de melanoma. DHA também reverteu os efeitos da CPt nas proteínas estudadas. Inclusive, outro PUFA ômega 3, o ácido eicosapentaenóico (EPA), foi também capaz de exercer efeitos similares na expressão de ERCC1, DUSP6 e p-ERK.

Palavras-chave: Ácido docosahexaenóico, Ácido eicosapentaenóico, Ácido α-linolênico, Ação Fotodinâmica, Fotoproteção, Quimiorresistência.

1. Introdução Geral

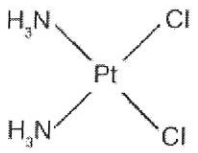
1.1 Melanoma e Principais Mecanismos de Resistência a Drogas

O câncer da pele é o mais frequente no Brasil, correspondendo a 25% de todos os tumores malignos registrados no país. Dentre eles, o melanoma representa 4% das neoplasias malignas cutâneas (INCA, 2012). As estimativas para o ano de 2016, válidas também para o ano de 2017, indicam a ocorrência de 3000 casos novos em homens e 2670 casos novos em mulheres (INCA, 2016). Neste sentido, a prevenção e o controle da progressão deste tipo de câncer são muito importantes, uma vez que representam um dos maiores desafios científicos e de saúde pública da nossa época. Embora o estágio inicial de desenvolvimento deste câncer da pele, ou seja, quando é restrito à epiderme ou derme superficial, seja curável, o prognóstico para indivíduos com invasão da derme é preocupante, apresentando uma taxa de sobrevivência de cinco anos em apenas 10% dos pacientes (Calviello & Serini, 2010).

O melanoma é uma desordem neoplásica originada pela transformação maligna de melanócitos normais. As características demográficas deste câncer da pele têm indicado a exposição à radiação ultravioleta (UV) como o principal fator etiológico. Numerosos estudos continuam sustentando esta relação, entre irradiação UV e melanoma. A radiação ultravioleta C (UVC) é efetivamente bloqueada por diferentes cromóforos, incluindo a camada de ozônio. A radiação ultravioleta B (UVB) (290-320 nm) está associada com a formação de eritema e indução do bronzeamento pela produção do pigmento melanina. Há dados consideráveis que apoiam seu papel etiológico no melanoma. Por outro lado, há algumas evidências sugerindo a radiação ultravioleta A (UVA) (320-400 nm). Estudos recentes têm permitido sugerir que a radiação UV causa este câncer por uma combinação de dano ao DNA, inflamação e supressão imune (De Vita *et al.*, 2011).

Atualmente, no mundo todo, o melanoma representa um dos cânceres mais comumente diagnosticados, e sua incidência tem aumentado de forma expressiva durante os últimos 30-40 anos (Howlader *et al.*, 2014). Apesar do progresso atingido recentemente em quimioterapia, tumores sólidos progressivos, incluindo o melanoma, ainda apresentam dificuldade no seu tratamento, e somente modalidades terapêuticas convencionais não têm sido suficientes para promover resultados clínicos satisfatórios a longo prazo (Lake & Robinson, 2005; Solyanik, 2010; Chapman *et al.*, 2011; Robert *et al.*, 2011). A resistência à quimioterapia tradicional, conhecida como drogas citotóxicas, normalmente causa esta falta de eficácia de resposta. A base para a resistência a drogas no melanoma tem sido principalmente relacionada à desregulação da apoptose, mecanismo de fuga do sistema imune, transporte de drogas e detoxificação alterados, e reparo de DNA intensificado, sendo este último investigado no nosso trabalho (Grossman & Altieri, 2001; Florea & Büsselberg, 2011; Galore-Haskel *et al.*, 2015). Desta forma, torna-se necessário a busca de estratégias que superem estas respostas, permitindo, assim, uma maior eficácia de drogas antitumorais utilizadas ultimamente (Siddiqui *et al.*, 2011; Johnstone *et al.*, 2014).

Mais recentemente, para o melanoma, tem sido descrito um aumento na expressão de genes de reparo de DNA, em resposta à ação de diferentes drogas antineoplásicas, e este achado pode estar relacionado a sua extrema resistência a quimioterápicos tradicionais que danificam o DNA. A cisplatina (CPt) é um agente quimioterápico que tem se mostrado efetivo contra muitos tipos de câncer, mas não no melanoma (Li & Melton, 2012). Este agente tem o potencial de induzir parada no crescimento e/ou apoptose na maioria dos tipos celulares tumorais e é considerado um dos mais eficientes quimioterápicos, comumente aplicado, utilizado para a terapia de cânceres humanos. Contudo, um desenvolvimento de resistência a drogas tem sido observado após o tratamento com este agente (Wang *et al.*, 2000).



Brabec & Kasparkova, 2005

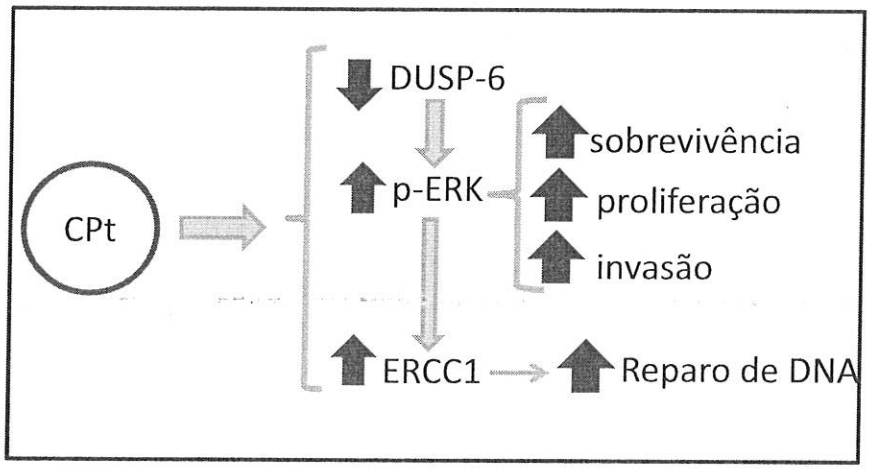
Fig. 1: Estrutura química da CPt

A maioria das alternativas na busca de novos métodos para o tratamento do câncer considera vias de sinalização importantes para o desenvolvimento desta doença, relacionadas ao crescimento, sobrevivência e migração celular. Por exemplo, no reparo por excisão de nucleotídeo (NER), as células tumorais desenvolvem estratégias que permitem a fuga da apoptose. Desta maneira, NER é conhecido como uma importante forma de resistência a drogas. A CPt é conhecida por formar adutos de DNA, promovendo, assim, uma intensificação de NER, o qual é capaz de conferir uma maior resistência a quimioterápicos que tem como alvo o DNA. NER aumenta particularmente pela superregulação de reparo por excisão cross-complementação grupo 1 (ERCC1), uma proteína essencial para este tipo de reparo, este pode ocorrer através da eliminação ou alteração de tais adutos (Stordal & Davey, 2007; Galluzzi *et al.*, 2012).

Neste sentido, a CPt tem sido descrita por aumentar a expressão de ERCC1 em diferentes tipos de câncer e, mais recentemente, também no melanoma (Kirschner & Melton, 2010; Li & Melton, 2012). De forma importante, Li & Melton têm demonstrado que a fosforilação de quinase regulada por sinal extracelular (ERK) é requerida para um aumento na expressão de ERCC1 em células de melanoma e que a CPt promove ainda a diminuição nos níveis de fosfatase-6 de dupla especificidade (DUSP-6), por estimular sua degradação e impedir sua transcrição (Li & Melton, 2012). Uma vez que DUSP-6 desfosforila ERK (Bermudez *et al.*, 2010), consequentemente, este fato poderia contribuir

para resultar na aberrante fosforilação de ERK e, desta forma, uma quimiorresistência aumentada observada em células de melanoma (Li & Melton, 2012). Assim, uma estratégia para aumentar a eficácia de quimioterápicos que danificam o DNA, amplamente utilizados, é a inibição de reparo de DNA (Madhusudan &Middleton, 2005).

Mecanismo de resistência à CPt em células de melanoma



Além disso, a via de sinalização da proteína quinase ativada por mitógeno (MAPK) é constitutivamente estimulada no melanoma (Oliveria *et al.*, 2006; Mirmohammadsadegh *et al.*, 2007) para regular anormalmente sobrevivência, proliferação e invasão celular (Hoshino *et al.*, 1999). O tratamento de linhagens celulares de melanoma com CPt tem sido mostrado por resultar em uma fosforilação aumentada de ERK, ao contrário do esperado (Mirmohammadsadegh *et al.*, 2007), indicando, assim, que a ativação de ERK estava associada com uma sobrevivência melhorada de células tratadas com CPt. Este achado sugere que combinando um agente capaz de inibir a fosforilação de ERK1/2 com a CPt, poderia superar a resistência a este quimioterápico observada em células de melanoma.

Além da relação das proteínas descritas anteriormente na resposta de resistência a drogas observada em células de melanoma, nosso trabalho também buscou considerar a atividade da glicoproteína P (Pgp) na contribuição para a resistência intrínseca no

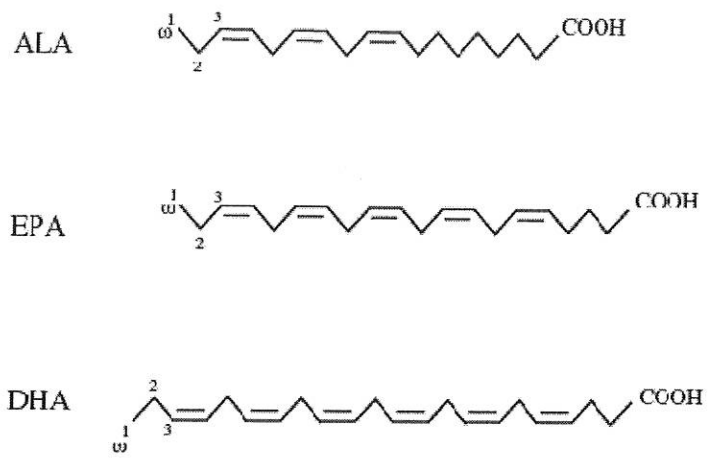
melanoma. O mecanismo melhor caracterizado responsável pela resistência a múltiplas drogas (MDR) envolve a expressão do produto do gene MDR1, a Pgp (Rumjanek *et al.*, 2001; Soengas & Lowe, 2003). As células que superexpressam Pgp apresentam uma concentração intracelular reduzida de drogas, que são substratos para este transportador. Esta bomba de efluxo de drogas é capaz de realizar a extrusão de uma ampla variedade de drogas não relacionadas estruturalmente, e como resultado, os níveis intracelulares destas substâncias não atingem uma concentração tóxica (Juliano & Ling 1976; Holohan *et al.*, 2013). A superexpressão de MDR1 tem sido associada com o insucesso na quimioterapia em muitos tipos de câncer, incluindo, rim, cólon e fígado, assim como leucemias e linfomas (Holohan*et al.*, 2013). Contudo, para células de melanoma, o envolvimento desta proteína ainda não é esclarecido, uma vez que estudos prévios têm demonstrado resultados contraditórios considerando a presença e funcionalidade da Pgp no melanoma, reforçando, desta forma, a necessidade de mais estudos.

1.2 Benefícios Potenciais de Ácidos Graxos Poliinsaturados Ômega 3 na Prevenção e Tratamento do Câncer

Atualmente, existem poucos componentes nutricionais que sejam tão amplamente reconhecidos como capazes de influenciar de forma benéfica em processos celulares desregulados, como os ácidos graxos poliinsaturados ômega-3 (PUFA ômega 3). Contudo, apesar da sua extrema popularidade e fácil disponibilidade comercial, muitas questões sobre as atividades biológicas destes compostos ainda permanecem não resolvidas. Em particular, há uma necessidade urgente de um melhor entendimento do seu possível papel como agente antitumoral (Calviello & Serini, 2010).

Sabe-se que há três principais PUFA ômega 3, os ácidos eicosapentaenóico (EPA; 20:5n-3) e docosahexaenóico (DHA; 22:6n-3), e o ácido α-linolênico (ALA; 18:3n-3) precursor de ambos. As mais ricas fontes de EPA e DHA são animais marinhos, em

contraste, o ALA é comumente encontrado em sementes e plantas (Pilkington *et al.*, 2011). Esta família de ácidos graxos tem sido demonstrada por promover imunidade antitumoral e inibir a iniciação do câncer, angiogênese tumoral, e metástase (Larsson *et al.*, 2004; Calviello *et al.*, 2007; Berquin *et al.*, 2008; Chapkin *et al.*, 2008; D'Eliseo & Velotti, 2016). Estas propriedades antineoplásicas têm sido confirmadas em muitos tipos celulares tumorais, incluindo células de melanoma (Moyad, 2005; Serini *et al.*, 2012). Os possíveis mecanismos pelos quais PUFA ômega 3 impedem o crescimento de células tumorais podem envolver uma diminuição na proliferação celular, indução de morte celular, ou uma combinação de ambos (Calviello & Serini, 2010).



Calviello & Serini, 2010

Fig. 2: Estrutura química dos principais PUFA ômega 3.

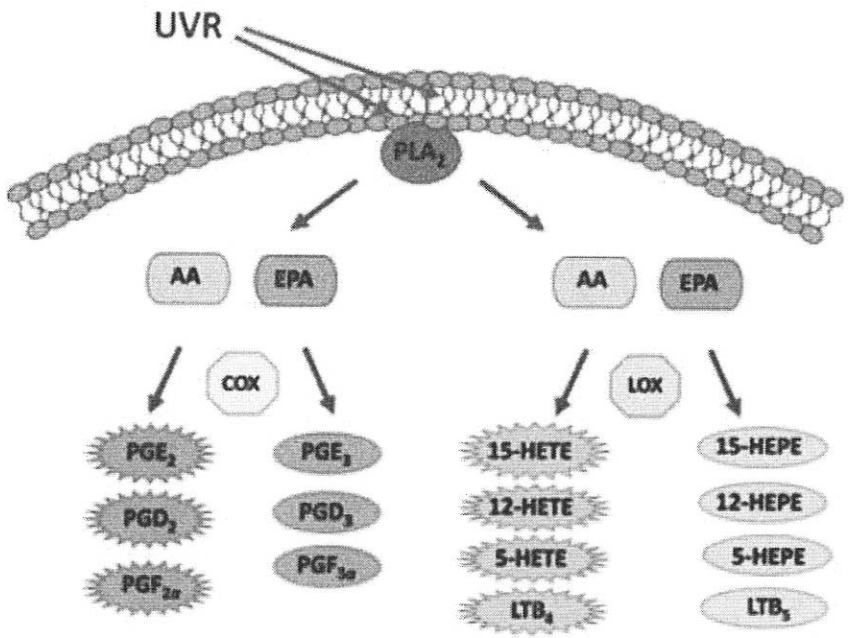
Muitos mecanismos de ação de PUFA ômega 3 na prevenção e tratamento do câncer têm sido propostos por estudos *in vitro* e *in vivo*. Estes compostos podem mediar os efeitos benéficos referidos anteriormente por afetar a expressão e/ou função de lipídios, proteínas e genes que regulam processos de proliferação e morte celular. Estes efeitos podem ser

explicados pelas alterações na composição lipídica das membranas e a função que ocorre quando estes compostos são incorporados nos tumores e em diversos modelos celulares utilizados (Biondo *et al.*, 2008; Calviello & Serini, 2010), já que a presença destes compostos na membrana plasmática resulta em propriedades físico-químicas únicas que afetam numerosas características de membrana. Dentre estas propriedades, podemos incluir permeabilidade, deformabilidade (Stillwell *et al.*, 1997), fluidez (Ehringer *et al.*, 1990), e mais recentemente, sabe-se também que influenciam na formação de microdomínios lipídicos (Shaikh *et al.*, 2001; Ma *et al.*, 2004; Schley *et al.*, 2007; Biondo *et al.*, 2008; Serini *et al.*, 2010).

Estas mudanças induzidas por PUFA ômega 3 na função da membrana podem alterar de forma significativa vias de sinalização importantes para o desenvolvimento do câncer. Por exemplo, levando a umadiminuição na expressão/atividade do fator de transcrição NF-kappaB em muitas células tumorais, permitindo, assim, a indução da apoptose. PUFA ômega 3 também podem inibir o crescimento de linhagens tumorais através da alteração no nível de ciclinas, quinases dependentes de ciclinas e proteína retinoblastoma (Calviello & Serini, 2010). Além disso, PUFA ômega 3 são altamente suscetíveis à peroxidação e é sugerido que a sua incorporação em fosfolipídios de membrana plasmática e mitocondrial possa sensibilizar as células tumorais ao ataque de espécies reativas de oxigênio, induzindo, dessa forma, um estresse oxidativo (Maziere *et al.*, 1999; Chapkin *et al.*, 2002). Os maiores efeitos dos produtos de peroxidação lipídica são a inibição de síntese de DNA, divisão e crescimento tumoral, além da indução de morte da célula tumoral (Galeotti *et al.*, 1986; Girotti, 1998).

PUFA ômega 3 também são principalmente conhecidos por seus efeitos antiinflamatórios que são, em parte, relacionados à sua competição com PUFA ômega 6 como substratos para as enzimas ciclooxigenase (COX) e lipooxigenase (LOX), resultando

na formação de prostaglandinas e leucotrienos menos ativos.Seguindo uma série de estimulações celulares, o PUFA ômega 6, ácido araquidônico (AA), é liberado das membranas pela ação da fosfolipase A_2 (PLA_2), a qual é superregulada pela radiação UV, e metabolizado pelas enzimas COX e LOX a metabólitos oxigenados, prostaglandinas (PG), tromboxanos (TX) e leucotrienos (LT), coletivamente conhecidos como eicosanóides. Os eicosanóides derivados do AA são altamente bioativos, agindo em baixas concentrações. Eles têm o potencial de influenciar em eventos-chave de processos fisiológicos e patológicos, incluindo proliferação, sobrevivência e inflamação. A formação de produtos derivados do AA é normalmente controlada, mas em condições patológicas, como o câncer, quantidades excessivas são produzidas. EPA e DHA são capazes de induzir uma diminuição na produção de eicosanóides originados do AA e, dessa forma, reduzir todas as respostas moleculares relacionadas ao metabolismo oxidativo do AA (Calviello & Serini, 2010; Pilkington *et al.*, 2011; D'Eliseo & Velotti, 2016).



Pilkington *et al.*, 2011

Fig. 3: Metabolismo do EPA e AA a eicosanóides pelas enzimas COX e LOX.

Recentemente, existe um crescente interesse no potencial de PUFA ômega 3 como promissores fotoprotetores contra a carcinogênese da pele induzida pela radiação UV (Rhodes *et al.*, 2003; Black & Rhodes, 2016), uma vez que tem se tornado claro que a exposição a este estressor é contínua na vida cotidiana (Godar, 2005). Há muitas pesquisas relacionando os mecanismos que envolvem os efeitos fotoprotetores de PUFA ômega 3 de cadeia longa (EPA e DHA), por outro lado, poucos trabalhos descrevem esta atividade para PUFA ômega 3 de cadeia curta, como o ALA (Nicolaou *et al.*, 2011; Pilkington *et al.*, 2011).

Associações entre a ingestão de PUFA e o câncer da pele têm sido observadas em estudos caso-controle em humanos. Por exemplo, Hakim *et al.* (2000) encontraram que um maior consumo de PUFA ômega 3 estava associado com um menor risco de carcinoma celular e Kune *et al.* (1992) observaram uma relação inversa entre a ingestão de peixe e o risco de desenvolvimento do câncer da pele não melanoma.Alguns estudos epidemiológicos prévios têm descrito um potencial para um risco reduzido de melanoma com uma maior ingestão de peixe ou PUFA ômega 3 (Bain *et al.*, 1993; Millen *et al.*, 2004). Adicionalmente, a suplementação nutricional com óleo de peixe tem sido reportada por reduzir a sensibilidade ao eritema induzido pela radiação UV na pele humana (Orengo*et al.*, 1992; Rhodes *et al.*, 1994). Além disso, evidências sugerem que PUFA ômega 3 de cadeia longa são capazes de reduzir a inflamação induzida pela radiação UV na pele humana, e ainda podem conferir significativa proteção à fotoimunossupressão, fotocarcinogênese, fotoenvelhecimento e fotossensensibilidade (Rhodes *et al.*, 2003; Nicolaou *et al.*, 2011; Black & Rhodes, 2016). Além disso, EPA foi capaz de inibir a expressão de COX-2 na pele humana e reduzir os níveis de prostaglandina em queratinócitos humanos irradiados com UVB (Pupe *et al.*, 2002). Similarmente, os efeitos fotoprotetores de PUFA ômega 3 foram confirmados em um estudo com voluntários saudáveis usando 4 g de EPA purificado, em que aumentou a porcentagem de EPA nos fosfolipídios da epiderme, resultando em um aumento expressivo na dose mínima

eritematógena (Rhodes *et al.*, 2003). Com relação ao ALA, também tem sido demonstrada alguma proteção à radiação UV, reduzindo o eritema em associação com uma diminuição nos níveis de prostaglandina em camundongos (Nicolaou *et al.*, 2011).

Perguntas que se impõem diante dessas ideias são: Qual seria a resposta de uma linhagem já tumoral à combinação de PUFA ômega 3 e radiação UV? A proteção descrita até então para o composto ômega-3 em células não tumorais também é capaz de conferir proteção a células tumorais ou as desordens celulares que caracterizam o processo tumoral são suficientes para alterar as respostas em diferentes linhagens?

Além da importância de PUFA ômega 3 na prevenção de diferentes tipos de câncer, estes compostos também tem sido propostos como potenciais adjuvantes na quimioterapia. O uso de PUFA marinhos EPA e DHA, em um contexto terapêutico, é crescente em pacientes que recebem tratamento para muitos tipos de câncer (Vaughan *et al.*, 2013). Investigações em uma variedade de cânceres com diferentes agentes quimioterápicos têm demonstrado uma melhor eficácia da quimioterapia quando PUFA ômega 3 de cadeia longa são adicionados à dieta (Murphy *et al.*, 2011).

Estudos em células tumorais humanas, modelos animais e testes preliminares com indivíduos sugerem que a administração de EPA e DHA pode alterar toxicidade e/ou atividade de muitas drogas utilizadas para tratar o câncer. Múltiplos mecanismos são propostos para explicar como estes compostos modulam a resposta de células tumorais a drogas quimioterápicas. Relacionado a sua alta suscetibilidade à oxidação, tem sido proposto que PUFA ômega 3 possam causar um irreversível dano na célula tumoral através de um aumento na peroxidação lipídica. Estes compostos podem também aumentar a suscetibilidade da célula tumoral à apoptose por alterar a expressão ou função de proteínas apoptóticas, ou por modular a atividade de fatores de transcrição relacionados à sobrevivência, tais como, fator nuclear Kappa B. Alguns estudos sugerem que PUFA ômega

3 podem aumentar a captação de drogas ou mesmo intensificar a ativação de drogas (Biondo *et al.*, 2008).

Entre os principais PUFA ômega 3, o DHA tem sido sugerido como um adjuvante efetivo na quimioterapia, por apresentar propriedades antitumorais importantes (Merendino *et al.*, 2013). Dados na literatura sugerem que o DHA combinado com agentes antitumorais atua em diferentes níveis, incluindo, a sensibilização das células, a captação aumentada de drogas quimioterápicas, possibilitando, assim, a superação da resposta de resistência. A peroxidação lipídica aumentada é também sugerida por desempenhar um papel efetivo na combinação aditiva ou sinergística mediada pelo DHA com drogas anticâncer (Siddiqui et al., 2011). Adicionalmente, há algumas evidências indicando que o DHA ainda aumenta o efeito antimetastáticode drogas antitumorais (Yam *et al.*, 2001; Horia & Watkins, 2007; Bougnoux *et al.*, 2009).

Diante dessas bases, considerando a importância e o potencial de PUFA ômega 3 na prevenção e tratamento do câncer, estudamos o efeito do ALA em células melanocíticas normais e tumorais expostas à radiação UVB, além de verificar a atividade da Pgp nestas células. Avaliamos ainda a atividade antitumoral do DHA em combinação com a CPt em células de melanoma murino e humano. E ainda, analisamos os efeitos do DHA na expressão de proteínas relacionadas com a resposta de resistência à CPt no melanoma.

2. Objetivos

2.1 Objetivo Geral

Investigar uma possível atividade fotoprotetora e/ou antitumoral atribuída ao ALA em células melanocíticas murinas normais (linhagem celular Melan-a) e tumorais (linhagem celular B16F10) expostas à radiação UVB, além de verificar a atividade da Pgp nestas células. Adicionalmente, avaliar a atividade antineoplásica do DHA em combinação com a CPt em linhagens celulares de melanoma murino (B16F10) e humano (WM266-4). E ainda, analisar os efeitos do DHA na fosforilação de ERK e na expressão de ERCC1 e DUSP-6.

2.2 Objetivos Específicos

- Avaliar o efeito de diferentes concentrações de ALA na proliferação de linhagens celulares melanocíticas murinas tumoral (B16F10) e normal (Melan-a) a fim de construir uma curva dose-resposta para a linhagem B16F10 e aplicar as mesmas concentrações para a linhagem Melan-a;
- Avaliar o efeito de diferentes doses de radiação UVB na proliferação das linhagens celulares B16F10 e Melan-a, a fim de construir uma curva dose-resposta para a linhagem B16F10 e aplicar as mesmas doses para a linhagem Melan-a;
- Avaliar o efeito de diferentes combinações entre ALA e UVB a fim de verificar uma atividade antitumoral e/ou fotoprotetora nas linhagens celulares B16F10 e Melan-a;
- Avaliar o efeito de uma combinação entre ALA e UVB no ciclo e morte celular, e nos níveis de espécies reativas de oxigênio;
- Avaliar a atividade antitumoral do DHA em combinação com a CPt em células de melanoma humano (linhagem celular WM266-4) e murino (linhagem celular B16F10);
- Avaliar a atividade antiinvasiva do DHA em combinação com a CPt nas linhagens celulares WM266-4 e B16F10;

- Avaliar os efeitos do DHA e EPA em proteínas relacionadas com a resposta de resistência à CPt em células de melanoma (ERCC1, DUSP-6 e pERK).

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Interaction between Omega 3 and UVB Radiation: Photoprotective Effect in Normal and Tumoral Murine Melanocytes?

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Abstract

Recently, omega 3 polyunsaturated fatty acids (omega 3 PUFA) are attracting interest as potential adjuvants forcancer prevention and treatment. There is evidence about photoprotection in normal cells, but no previous studies have evaluated it in tumoral cells. Melanoma is notoriously resistant to chemotherapeutic agents and the involvement of P-glycoprotein (Pgp) in this process is unclear yet. So, this study investigated a photoprotective activity attributed to α-linolenic acid (ALA), in normal melanocytic cells (Melan-a) and a photodynamic action in tumoral melanocytic cells (B16F10) exposed to UVB radiation, as well as analysed the activity of Pgp. ALA exhibited an antiproliferative effect in B16F10 cells, and had minimal effect in Melan-a cells. ALA and UVB combined showed, in a dose and time dependent manner, a photodynamic action in B16F10 cells and a protective effect in Melan-a cells. ALA and UVB combined or UVB alone induced an accumulation of cell lines at S/G2/M phase. In addition, the combination and UVB alone induced cell death in 24h in both cells, and in 48 h, ALA attenuated this effect. Besides that, ALA did not alter ROS levels in both cells exposed to UVB radiation. Interestingly, both studied cell lines presented a high activity of Pgp.

Keywords: Melan-a cell line; B16F10 cell line; α-linolenic Acid; Photodynamic Action.

1. Introduction

Ultraviolet (UV) radiation is the main etiological agent of most types of skin cancer [1]. UV radiation is divided into three sections, each of which has distinct biological effects: UVA (320–400 nm), UVB (280–320nm), and UVC (200–280 nm). UVC is effectively blocked from reaching the Earth's surface by different chromophore, including the stratospheric ozone layer. UVA and UVB radiation both reach the Earth's surface in amounts sufficient to have important biological consequences to the skin and eyes [2]. More recently, UVA has been recognized by exerts strong procarcinogenic action. However, the UVB radiation is considered as the most powerful carcinogenic components of the UV solar radiation, even though their levels were much lower than those of UVA [3, 4].

Skin provides a protective barrier against environmental damage and is the primary target for UV radiation effects. Nowadays, skin cancer is the commonest form of cancer in white Caucasian populations, and the incidence continues to rise related to the tendency for greater recreational exposure to ambient UV radiation [5]. Moreover, importantly, there is enough scientific evidence that outdoor workers have an increased risk of developing work-related occupational skin cancer due to natural UV radiation exposure [6]. Basal cell carcinoma (BCC), arising from the basal epidermal layer, is the commonest skin cancer, followed by squamous cell carcinoma (SCC), derived from supra-basal keratinocytes, whereas malignant melanoma (MM), derived from melanocytes, is less common but carries a high mortality rate [7]. The poor prognosis of melanoma generally results from resistance to conventional chemotherapy, known as cytotoxic drugs [8, 9]. Traditionally, chemoresistance was attributed to many mechanisms, one of the most important is a failure of drug–target interactions involved in a reduction of the effective concentration of the drug, via enhanced drug efflux pumps, such as P-glycoprotein (Pgp) [10], that will be also considered in this study.

Importantly, topical sunscreens alone provide an insufficient measure to combat overexposure to UV radiation. Novel strategies of photoprotection are being searched as additional trials, with growing attention in the potential for systemic photoprotection through naturally obtained nutrients [11, 12]. Macronutrients such as omega 3 polyunsaturated fatty acids (omega 3 PUFA) are attracting interest as potential representatives for maintenance of skin health and treatment of skin disorders, particularly those influenced by solar UV radiation, including cancer, sunburn, photosensitivity and photoageing [13, 14].

In this sense, it is important to regard that human skin exhibits active metabolism of fatty acids and, as demonstrated by the historic studies of Burr and Burr [15], is strongly dependent on dietary consumption of fats. Linoleic acid (LA; 18:2 omega−6) and α-linolenic acid (ALA; 18:3 omega−3) are the precursor compounds of two families of PUFA with contrasting significance for skin health. LA and ALA cannot be synthesised by mammals and, consequently, are considered as essential fatty acids, as well as their elongated and desaturated PUFA derivatives arachidonic acid (AA; 20:4 omega−6), eicosapentaenoic acid (EPA; 20:5 omega−3) and docosahexaenoic acid (DHA; 22:6 omega−3). LA is the most abundant PUFA in human epidermis (approximately 12% of total fatty acids). Arachidonic acid is the second most abundant fatty acid in human epidermis (up to 3% of total fatty acids), whilst EPA and DHA usually appear to be minor constituents (each contributing less than 1% of total fatty acids) [16].

Therefore, it is necessary to consider that alterations in the modern western diet have expressively changed the balance of omega 3/omega 6 PUFA, consequently, decreasing the content of EPA and DHA in human cells [17]. This condition could have influenced to a more predisposed skin phenotype to damage by UV radiation. Thus, the nutritional interventionof omega 3 PUFA could reestablish the omega 3/omega 6 PUFA rate to a healthier condition [18] and improve the natural skin defenses against the damaging effects of UV radiation [19].

Omega 3 PUFA are multi-active mediators that may promote photoprotection through a number of mechanisms, such as, alterations in membrane fluidity, modification of signal transduction, transcription factor activation, modulation of oxidative stress, and production of bioactive lipid mediators. While studies continue into mechanisms involving the protective effects of long-chain omega 3 PUFA (EPA and DHA), attention is developing into the potential effects of short-chain omega 3 PUFA, like α-linolenic (ALA, 18:3n-3) acid, also found in the human diet [20]. Dietary compounds acting within skin cells to regulate biological responses to UV radiation has the potential to provide a safe and continuous systemic strategy to UV radiation protection, additionally to the use of physical measures [21, 22].

Additionally, the expression of multidrug transporter, P-glycoprotein (Pgp), is under the control of NFkappaB transcription factor [23, 24]. As the activity of this factor can be modulated by omega 3 PUFA, it is possible that regulation of NFkappaB expression in a tumor could decrease the expression of Pgp, leading to a decreased efflux of chemotherapy drugs in tumor cells [25]. Moreover, it was recently demonstrated in colon cancer cells, the rate of cholesterol synthesis is directly related to the production of the phenotype known as MDR (multidrug resistance) [26, 27]. The causes of MDR phenotype are various but mostly are related to the overexpression of drug transporters of the ABC family, which can make the extrusion chemotherapy [28]. Although the resistance process is multifactorial, overexpression of Pgp, also known as transporter ABCB1, is the best studied mechanism [29]. However, the MDR phenotype can be reversed in the presence of omega 3 PUFA, since this compound acts in the negative regulation of endogenous cholesterol synthesis [30], therefore, demonstrating another potential benefit of these dietary compounds in cancer chemoresistance.

On these bases, the aim of this study was analyse the activity of the resistance protein, Pgp, in normal melanocytic cells (Melan-a) and tumoral melanocytic cells (B16F10) as well as to investigate a possible photoprotective activity attributed to ALA in Melan-a cells and a photodynamic action in B16F10 cellsexposed to UVB radiation.

2. Materials and Methods

2.1 Cell culture conditions

The Melan-a cell line (murine normal melanocyte) was obtained from the Cell Biology Laboratory of the Translational Research Centre in Oncology from Cancer Institute of São Paulo. The cell line B16F10 (murine malignant melanocyte) was obtained from cell bank of Rio de Janeiro. The cell lines were maintained in DMEM, supplemented with sodium bicarbonate (0.2 g / L), L-glutamine (0.3 g / L) and HEPES (3 g / L) with 10% fetal bovine serum and 1% antibiotic (penicillin [100 U/ml] and streptomycin [100 μg/ml]) and antimycotic (amphotericin [0.25 μg/ml]) in culture flasks at 37°C. Additionally, for Melan-A cell line was added 200 nM of Phorbol Myristate Acetate (PMA) in order to stimulate cell growth.

2.2 Treatment of cell lines with omega 3 PUFA ALA

The Melan-a cells ($4x10^5$ cells/mL) and B16F10 cells ($2x10^5$ cells/mL) were incubated for 24 h for adhesion in 96-well culture plates containing DMEM medium at 37°C. The cells were treated in medium containing different concentrations of omega 3 PUFA ALA (7.5, 15, 30 and 60 μM), and control cells received the same quantity of vehicle used, ethanol (0.05%). After treated, the cell lines were incubated at 37°C for 24, 48 and 72h, and one of the plates was used for immediate reading.

2.3 Treatment of cell lines with UVB radiation

The Melan-a cells ($4x10^5$ cells/mL) and B16F10 cells ($2x10^5$ cells/mL) were incubated for 24 h for adhesion in 96-well culture plates containing DMEM medium at 37°C. After this time, the medium was removed and added 200 µL of PBS to expose the cells to UVB radiation, in determined doses (0.005, 0.01, 0.05, 0.10 and 0.20 J/cm^2). For this purpose, it was used a UVB lamp 115, 30 W (with emission peak at 313 nm). Once irradiated, the cells were again placed in culture medium and incubated for 24, 48 and 72 h, with one of the plates used for immediate reading.

2.4 Pre-treatment of cell lines with omega 3 PUFA ALA and subsequent exposure to UVB radiation

The B16F10 cells ($2x10^5$ cells/mL) and Melan-a cells ($4x10^5$ cells/mL) cells were incubated for 24 h for adhesion in 96-well culture plates containing DMEM medium at 37°C. The cells were pretreated in a medium containing 7.5 µM or 30 µM of ALA. After 24 h, the medium was removed and added to 200 µL of PBS and the cells were exposed to 0.01 J/cm^2 or 0.005 J/cm^2 of UVB, respectively. After treated, the cell lines were incubated at 37°C for 24, 48 and 72h, with one of the plates used for immediate reading.

2.5 Viability assay

Cell viability was assessed by the method 3-4,5-dimethylthiazol-2-yl, 2,5-diphenyltetrazolium (MTT) immediately, 24, 48 and 72 h after exposure [31].

2.6 Activity of Pgp in Melan-a and B16F10 cells

Analysis of the activity of Pgpwas investigated using the fluorescent probe Rhodamine 123 (Rho) in an efflux assay. In brief, $4x10^5$ cells in DMEM were incubated with

300 ng/ml Rho 123 for 1 h at 37°C. This incubation was performed in the presence or absence of Pgp inhibitor, verapamil (VP) 5 µM. After the incubation, cells were washed and re-incubated with or without Pgp inhibitor for 1 h in medium, in the absence of the dye, to allow its extrusion. After these time, cells were washed with phosphate-buffered saline (PBS) and dye accumulation was assessed immediately by fluorometer.

2.7 Analysis of ROS levels

After treatment using 7.5 µM of ALA and 0.01 J/cm^2 of UVB, B16F10 and Melan-a cells were incubated with CMH2DCFDA (5 µM) in culture medium for 30 min at 37°C. After this time, the cells were centrifuged, washed with PBS and it was added PBS again for 30 min at 37°C. Data were acquired in an Attune NxT Flow Cytometer.

2.8 Cell cycle analysis using propidium iodide

After treatment using 7.5 µM of ALA and 0.01 J/cm^2 of UVB, B16F10 and Melan-a cells were trypsinized, centrifuged, washed with PBS and then were fixed in 70% ethanol for at least 2 h, washed twice in PBS (4 min, 2000 rpm), and then incubated with 200 μL of a propidium iodide (PI) solution containing 20 μg/mL of PI, 200 μg/mL RNAse A, and 0.1% Triton v/v in PBS. After 30 min of incubation in the dark, data were acquired in an Attune NxT Flow Cytometer. Samples were analysed, and the results were expressed in the percentage of cells in the G0/G1, S, and G2/M phases and hypodiploid cells.

2.9 Statistical Analyses

Data from experiments regarding the evaluation of cellular viability and Pgp analysiswere presented as mean ± standard error, analyzed using ANOVA with Tukey's post-test. For flow cytometry, data were analysed using one-way ANOVA followed by

Bonferroni post hoc tests using GraphPad Prism 4.0 software. Differences were considered significant when $P < 0.05$.

3. Results

3.1 Treatment with omega 3 PUFA ALA

After treatment with different concentrations of ALA, it was observed that B16F10 cell line was more sensitive than Melan-a cell line.Our results showed an inhibition of proliferation for B16F10 with 60 µM of ALA in 24 h, in 48 h for the concentrations of 15 µM, 30 µM and 60 µM and in 72 h for all the concentrations (7.5 µM, 15 µM, 30 µM and 60 µM) compared to control cells (Figure 1A).On the other hand, Melan-a demonstrated a decreasing of proliferation only 72 h after the treatment with 60 µM of ALA (Figure 1B).

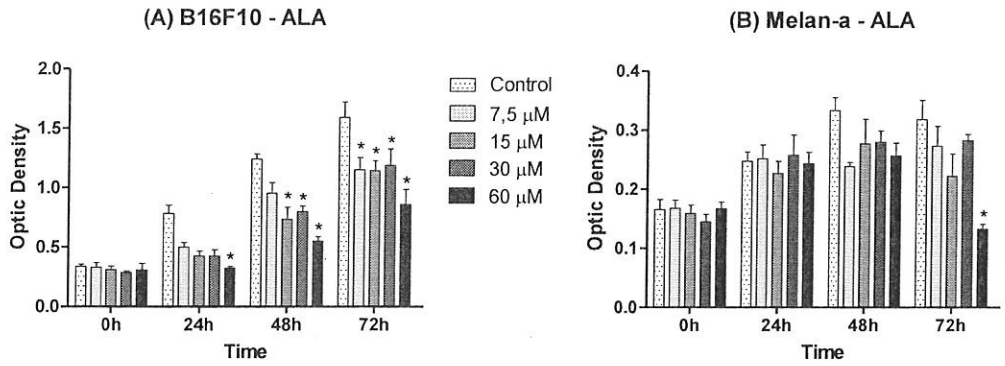

Figure 1: B16F10 (A) and Melan-a (B) cell lines treated with different concentrations of ALA (7.5 µM, 15 µM, 30 µM, 60 µM). Optical density evaluated by MTT assayimmediately, 24 h, 48 h and 72 h after treatment. Data presented as mean ± standard error, ($p \leq 0.05$). (*) Significant difference in relation to control within each time.

3.2 Exposure to UVB radiation

The both cell lines had similar response to this treatment. B16F10 cell line showed an inhibition of cell proliferation for the dose of 0.01 J/cm^2 in 48 h and 72 h. It was also

demonstrated that cell viability was significantly decreased at the higher dose tested (0.20J/cm^2) in 24 h, and this effect was maintained until 72 h. In addition, a loss of cell viability was observed for the doses of 0.05 J/cm^2 and 0.10 J/cm^2 in 48 h and 72 h (Figure 2A). For Melan-a cell line, a similar effect was observed. Although, the dose of 0.005 J/cm^2 induced a decreasing of proliferation after 72 h (Figure 2B).

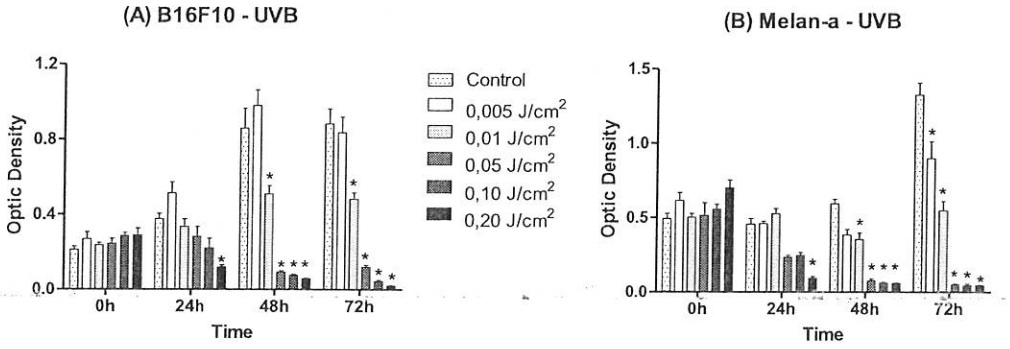

Figure 2: B16F10 (A) and Melan-A (B) cell lines exposed at different doses to UVB radiation (0.005 J/cm^2, 0.01 J/cm^2, 0.05 J/cm^2, 0.10 J/cm^2, 0.20 J/cm^2), respectively. Optical density evaluated by MTT assay immediately, 24 h, 48 h and 72 h after treatment. Data presented as mean ± standard error ($p \leq 0.05$). (*) Significant difference in relation to control within each time.

3.3 Combination of omega 3 PUFA ALA and UVB radiation

For this analysis, it was firstly chosen the combination of 7.5 µM of ALA + 0.01 J/cm^2 of UVB radiation. For B16F10 cells, it was observed an inhibition of cell proliferation in 24 and 48 h in the combination treatment. This effect was suggested to be related to UVB radiation, since the result of this treatment is similar to those produced by the interaction. Nevertheless, the effect of the combination in 72 h was similar with that produced by the factors alone (Figure 3 A). For Melan-a cell line, it was demonstrated an inhibition of

proliferation only at 48 h in the treatments using both combination and the factors alone. However, this effect was reverted in 72 h (Figure 3 B).

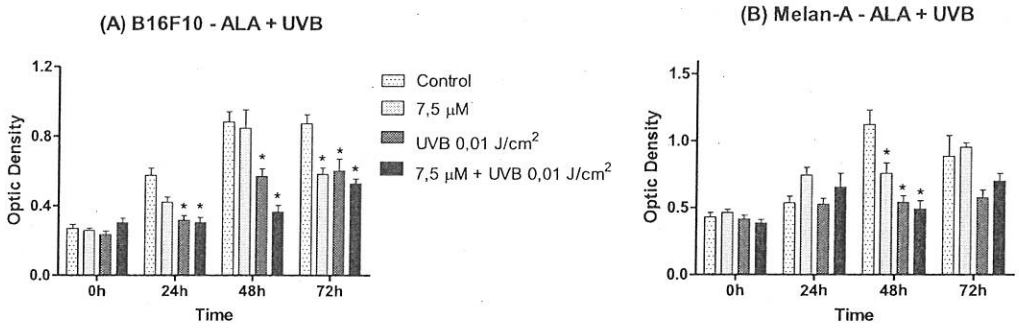

Figure 3: Comparison of three independent tests in B16F10 (A) and Melan-a (B) cell lines. Treatment with 7.5 µM of ALA; Exposure to 0.01 J/cm^2 of UVB radiation and the interaction between these factors. Optical density evaluated by MTT assay immediately, 24 h, 48 h and 72 h after treatment. Data presented as mean ± standard error ($p \leq 0.05$). (*) Significant difference in relation to control within each time.

3.4 Activity of Pgp in Melan-a and B16F10 cells

The assay with the fluorescent dye Rho showed that both cell lines present activity of Pgp, although there was a significant accumulation of the dye in B16F10 cells treated with Rho alone compared to control cells. However, the inhibitor of Pgp, VP, decreased the activity of the transporter, since the Rho accumulation was significantly higher than in the cells treated only with Rho in both cell lines, showing the presence and activity of Pgp, as this fluorescent dye is a substrate for Pgp (Figures 4 and 5). In the present study, the retention of Rho was modified by VP, indicating that the measurements reflected dye efflux due to Pgp activity.

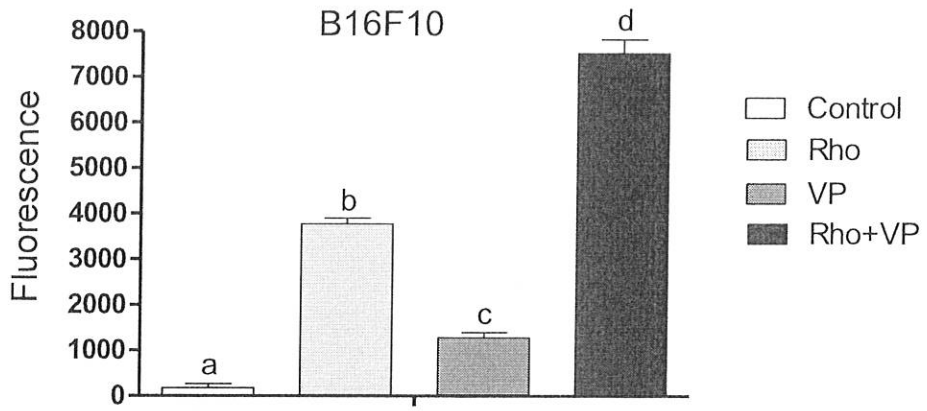

Figure 4: Analysis of Pgp in B16F10 cell line by Rhodamine 123 (Rho) protocol using Verapamil (VP). Reading performed in fluorometer (485/590 nm). Data presented as mean ± SE, different letters represent significant difference among treatments.

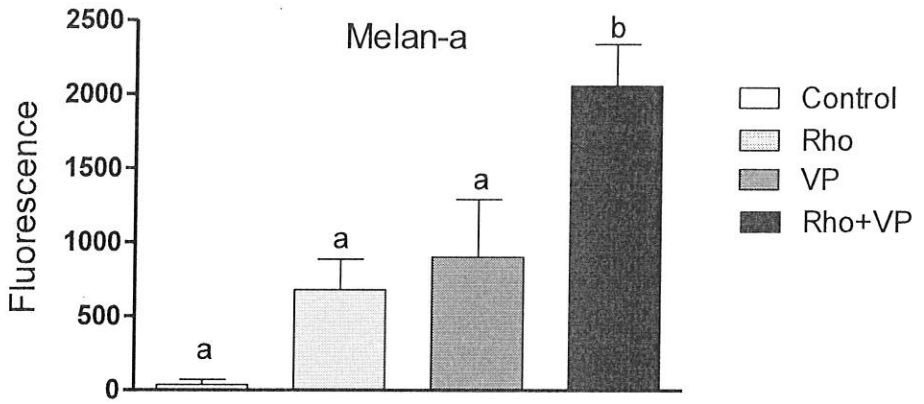

Figure 5: Analysis of Pgp in Melan-a cell line by Rhodamine 123 (Rho) protocol using Verapamil (VP). Reading performed in fluorometer (485/590 nm). Data presented as mean ± SE, different letters represent significant difference among treatments.

3.5 Photoprotective effect of ALA for B16F10 cells

In order to study the effect of ALA in B16F10 cell line, the cells were seeded in a twelve-well plate and were incubated in culture medium containing or not ALA (7.5 μM) for 24 h. After this period, the cells were exposed to UVB radiation (0.01 J/cm^2). Then, the cells

were observed under an inverted microscopy and photographed (20x Optical Zoom) in 24 and 48 h after treatment. According to the figure 6, it is possible to observe an apparent photoprotective effect of ALA against UVB radiation in 48 h, since it is evident that in the treatment using UVB alone there are more floating cells.

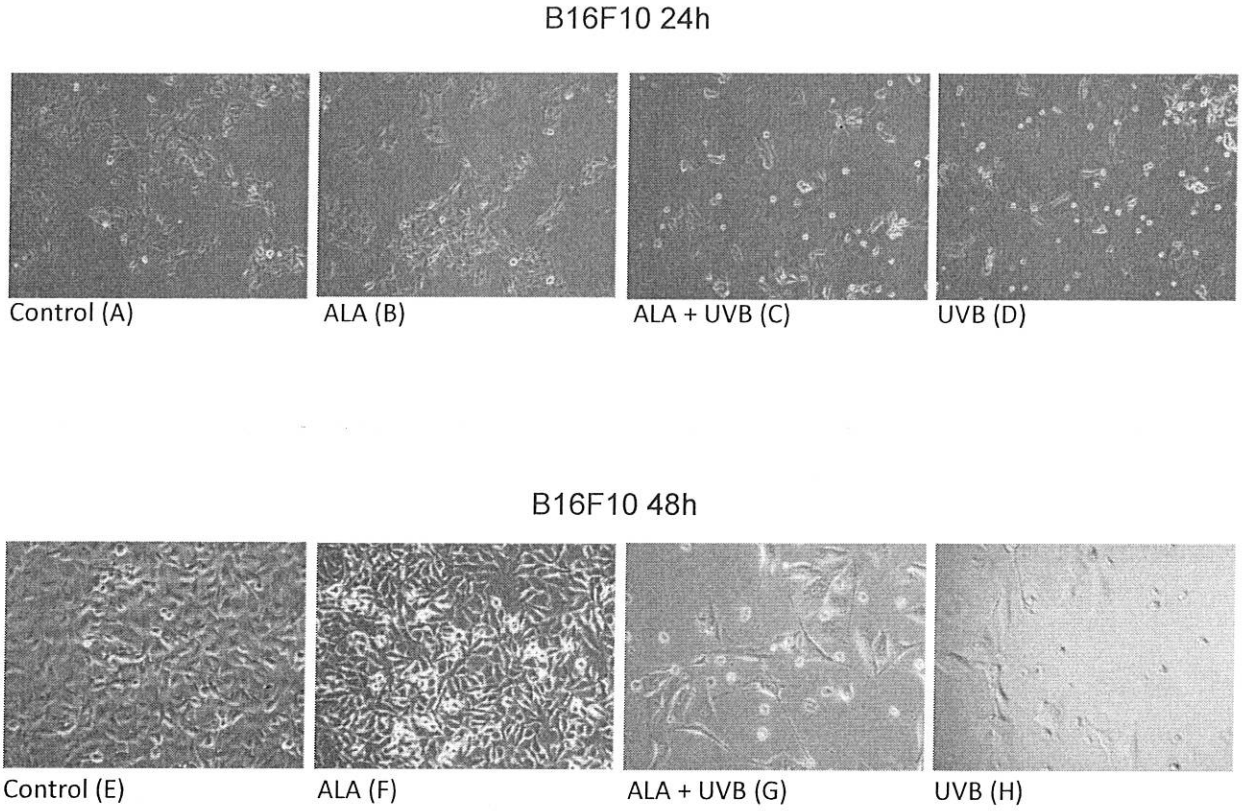

Figure 6: B16F10 control cells (A) B16F10 cells treated with 7.5 µM of ALA (B), combination between ALA and UVB (C) and 0.01 J/cm2 of UVB (D) in 24h. The same treatments were tested in 48h (E), (F), (G) and (H).

3.6 Evaluation of ROS levels in Melan-a and B16F10 cells

In order to analyse ROS levels in Melan-a and B16F10 cell lines in response to the treatments with ALA and UVB radiation, the cells were seeded in a twelve-well plate and were incubated in culture medium containing or not ALA (7.5 µM) for 24 h. After this period,

the cells were exposed to UVB radiation (0.01 J/cm^2) and then the cells were counted and incubated with CMH2DCFDA to measure the ROS levels. The addition of ALA did not alter ROS levels in both cell lines (figures 7 and 8). In relation to the treatments using UVB, it was demonstrated an increasing in ROS levels for Melan-a cells in the first 24 h of treatment (figure 7 A) and a decreasing in ROS levels for B16F10 cell line (figure 8 A). However, these effects are not observed in 48 h, since there is no difference in ROS levels among treatments for B16F10 and Melan-a cell lines (figures 7 B and 8 B).

Melan-a 24h (A) Melan-a 48h (B)

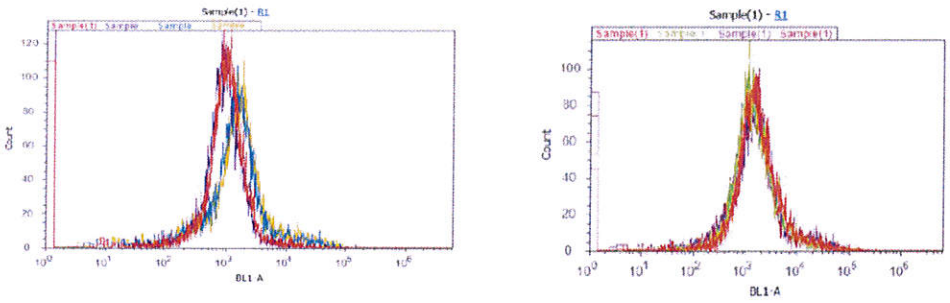

Figure 7: Level of ROS in Melan-a 24h (A) and in 48h (B), measured by CMH2DCFDA fluorescence. The cells were treated with 7.5 µM of ALA, 0.01 J/cm^2 of UVB and the combination between ALA and UVB. Data were obtained by flow cytometry. In the figure A, Red represents control cells; Purple represents ALA; Blue represents ALA + UVB and Yellow represents UVB. In the figure B, Red represents the control cells; Green represents ALA; Purple represents ALA + UVB and Dark red represents UVB.

B16F10 24h (A) B16F10 48h (B)

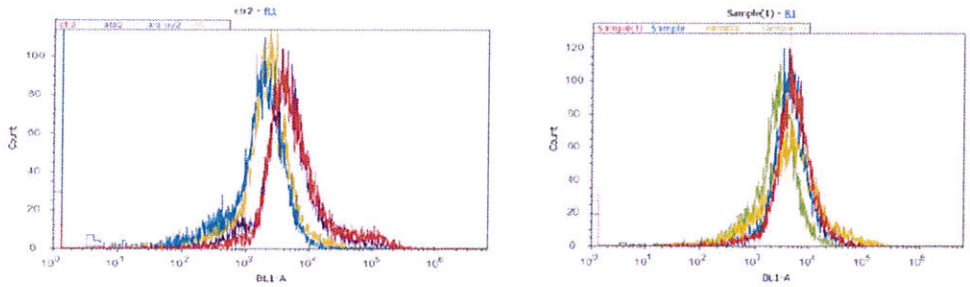

Figure 8: Level of ROS in B16F10 in 24h (A) and in 48h (B) measured by CMH2DCFDA fluorescence. The cells were treated with 7.5 µM of ALA, 0.01 J/cm^2 of UVB and the combination between ALA and UVB. Data were obtained by flow cytometry. In the figure A, Red represents control cells; Purple represents ALA; Blue represents ALA + UVB and Yellow represents UVB. In the figure B, Red represents the control cells; Blue represents ALA; Yellow represents ALA + UVB and Green represents UVB.

3.7 Cell cycle and cell death analyses

Because of the cytostatic effectobserved for ALA, it was analysed whether this fatty acid could be regulating the cell cycle or influencing on cell death process. Therefore, it was conducted cell cycle and cell death studies.

B16F10 and Melan-a cell lines were exposed to ALA alone (7.5 µM), UVB alone (0.01 J/cm^2) and the combination with ALA and UVB. It was demonstrated that for B16F10, the treatments using ALA + UVB and UVB alone showed that the number of cells at the S/G2/M of the cell cycle was increased in 24 h and 48 h (figure 9). For Melan-a, it was not observed significant differences among the treatments in 24 h. Although, in 48 h, it was possible to observe that the treatments using ALA + UVB and UVB alone were able to increase the number of cells at the phase S/G2/M (figure 10).

In relation to cell death, it was demonstrated that the addition of ALA in combination with UVB and UVB alone induced an increasing in the number of hypodiploid cells in B16F10 (figure 11) and Melan-a (figure 12) cells in 24h. In 48h, it is possible to observe a significant difference between the treatments using ALA + UVB and UVB, demonstrating that ALA was able to attenuate the effect of UVB for both cell lines (figures 11 and 12).

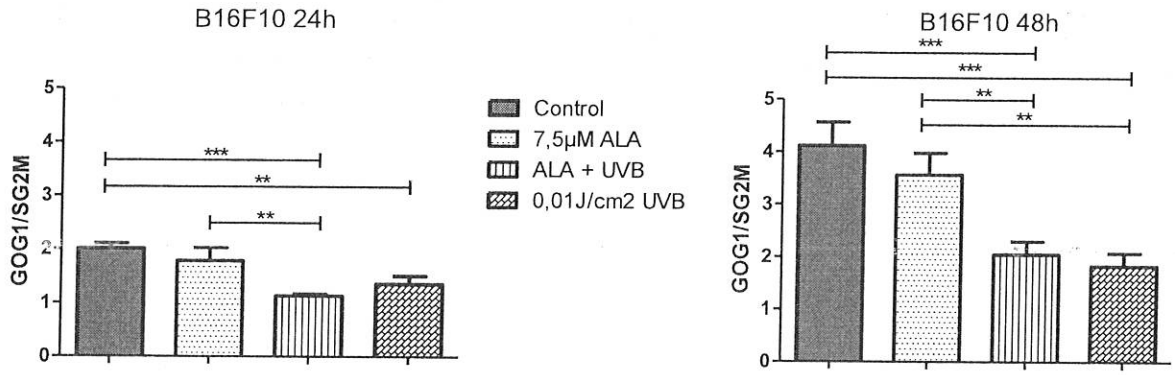

Figure 9: Cell cycle analysis for B16F10 treated with 7.5 μM of ALA alone, combination between ALA and UVB and 0.01 J/cm^2 of UVB in 24h and 48h. Data presented as mean ± standard error ($p \leq 0.05$). (*) represents significant difference among all treatments.

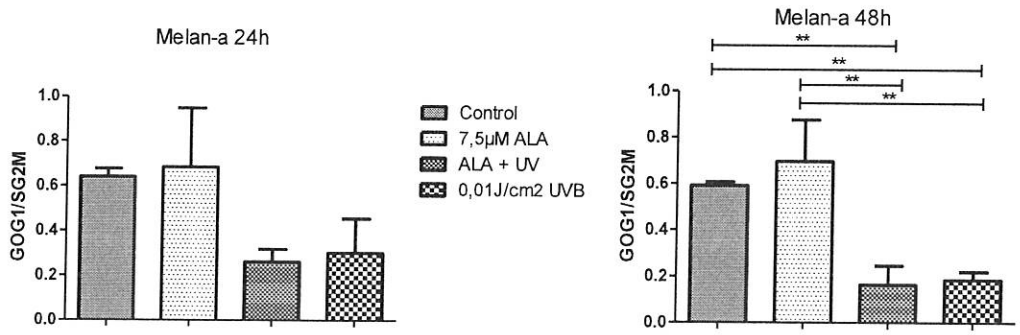

Figure 10: Cell cycle analysis for Melan-a treated with 7.5 µM of ALA alone, combination between ALA and UVB and 0.01 J/cm^2 of UVB in 24h and 48h. Data presented as mean ± standard error ($p \leq 0.05$). (*) represents significant difference among all treatments.

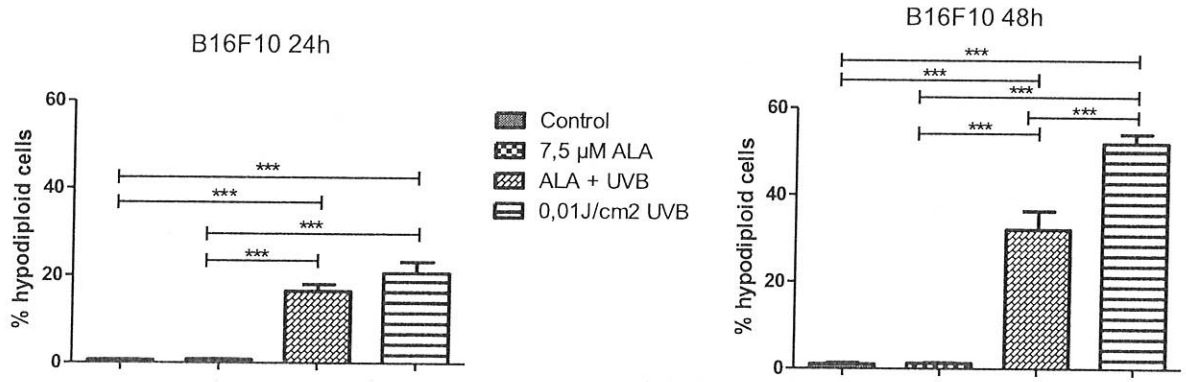

Figure 11: Cell death analysis by percentage of hypodiploid cells for B16F10 treated with 7.5 µM of ALA alone, combination between ALA and UVB and 0.01 J/cm^2 of UVB in 24h and 48h. Data presented as mean ± standard error ($p \leq 0.05$). (*) represents significant difference among all treatments.

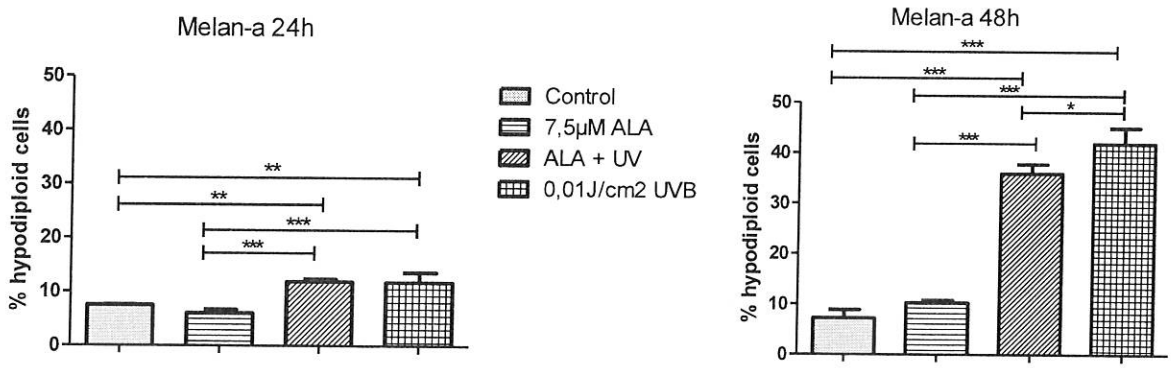

Figure 12: Cell death analysis by percentage of hypodiploid cells for Melan-a treated with 7.5 µM of ALA alone, combination between ALA and UVB and 0.01 J/cm^2 of UVB in 24h

and 48h. Data presented as mean ± standard error ($p \leq 0.05$). (*) represents significant difference among all treatments.

3.8 Sensitivity to photodynamic action between ALA and UVB in B16F10 and Melan-a cells

In order to demonstrate a possible photodynamic action, it was also tested another combination of ALA and UVB, a higher concentration of ALA, 30 µM, and a lower dose of UVB, 0.005 J/cm^2. After combined treatment, it was observed that the effect was not protective. B16F10 cell line showed a proliferation inhibition effect after 24 h of combined treatment, indicating a photodynamic action (Figure 13 A).

Nevertheless, Melan-a cell line showed a lower sensitivity to this combination when compared to the tumor cell, since the proliferative inhibitory effect was evident only at 72 h. This result suggests a photoprotective effect of ALA until 48h in these cells (Figure 13 B).

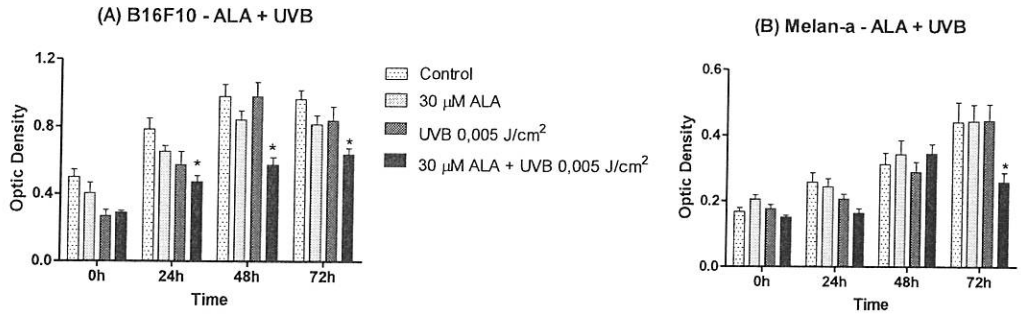

Figure 13: Comparison of three independent tests in B16F10 (A) and Melan-a (B) cell lines: Treatment with 30 µM of ALA; Exposure to 0.005 J/cm^2 of UVB radiation and the interaction between ALA 30 µM and 0.005 J/cm^2 of UVB radiation. Optical density evaluated by MTT assay immediately, 24, 48 and 72h after treatment. Data presented as mean ± SE ($p \leq 0.05$). (*) Significant difference in relation to control regarding each time.

4. Discussion

In the present study, we showed that ALA exhibited an antiproliferative effect on the murine melanoma B16F10 cells and had minimal effect on the normal cells as measured by MTT reduction assay, suggesting an antitumoral activity. Our data show that the inhibitory effect of ALA in melanoma cells did not correlate with exposed concentrations, but with time exposition. Although the molecular mechanisms that participate for the biological benefits of omega 3 PUFA are not completely understood, there is significant evidence from animal tumors and human cell lines that providing these dietary agents will increase apoptotic and other death pathways and decrease cell growth [32, 33]. Besides that, there is a growing body of data demonstrating that dietary fat is able to influence the development and progression of many cancers [32, 34, 35, 36, 37], including melanoma [38, 39, 40], with omega 3 PUFA having suppressive activity. Several molecular mechanisms related to the potential benefit of omega 3 PUFA in influencing carcinogenesis have been suggested. These mechanisms include suppression of eicosanoid biosynthesis derived from an omega 6 PUFA, AA, which results in altered immune response to cancer cells and modulation of inflammation, cell proliferation, apoptosis, metastasis, and angiogenesis; influences on transcription factor activity, gene expression, and signal transduction, which leads to changes in metabolism, cell growth, and differentiation; increased or decreased production of free radicals and reactive oxygen species (ROS) [33, 37].

There is considerable evidence for membranes rich in omega 3 PUFA to become cells more susceptible to the production of ROS. On the other hand, there is also significant evidence that these compounds are still able to reduce tumour cell growth by decreasing the intracellular oxidative stress [32]. In relation to UVB radiation, it is known that UVB-induced apoptosis has been demonstrated to be mediated by DNA damage, through producing photoproducts including cyclobutane pyrimidine dimmers and 6-4 photoproducts

(6-4PPs), death receptor activation, and the production of ROS [41, 42]. However, no changes in ROS levels have been demonstrated in the treatments using ALA for both cell lines, Melan-a and B16F10. On the other hand, in relation to treatments using UVB, it was demonstrated an increasing in ROS levels during the first 24 h of treatment for Melan-a cells and a decreasing in ROS levels for B16F10 cell line. For Melan-a cells, it was expected this response, since UVB is able to generating ROS. While for B16F10, it is possible to suggest that the effect of ROS depletion could be related to the amount of melanin present in these cells that is is capable of protect against oxidative damage. Although, in 48 h there are no differences in ROS levels among treatments for Melan-a andB16F10 cells. This response could be associated to the dose used in the experiment. Since it is well established that the main target of UVB is DNA [43], the obtained results suggest that the dose was not enough to cause a significant increase in ROS levels or that an increasing in the activity of antioxidant proteins [44] could have been sufficient to inhibit oxidative stress.

In addition, this work also demonstrated that in relation to UVB radiation, Melan-a and B16F10 cells presented a similar sensitivity in the higher tested doses, showing a cell death effect. Importantly, the most cytotoxic type of solar radiation, that reach the Earth's surface, corresponds to UVB radiation, since DNA bases directly absorb incident photons within this narrow wavelength range [43]. In order to prevent DNA damage from leading to skin carcinogenesis, the cells of the skin present mechanisms, such as growth arrest followed by DNA repair, and, as demonstrated in this study, cell death [45, 46].

In relation to cell cycle, studies have reported that omega 3 PUFA can slow or arrest the growth of cancer cells by affecting cyclins [32]. In this work, it has not been observed an effect on cell cycle in the treatment using ALA alone, on the other hand, it was demonstrated an accumulation of cells at the S/G2/M phase in the treatment using ALA in combination with UVB radiation and UVB alone. Interestingly, Albino *et al.* [47] studying the effects of

DHA on growth of melanoma cell lines, observed that this omega 3 PUFA increased the percentage of SK-Mel-110 (resistant cell line) in S phase. In addition, Khan *et al.* [48] also demonstrated that DHA inhibited the growth of FM3A mouse mammary cancer cells by arresting their progression from the late-G1 tothe S phase of the cell cycle. At present, little is known about the effects of fatty acids on cell cycle control [47]. Regarding the effect of UVB radiation on cell cycle, Bolognia *et al.* [49] also exposed melanoma cells to UVB (20-30 mJ/cm^2) and they showed that the cells accumulated at the G2/M phase of the cell cycle, according to this study. Although cell cycle arrest induced by UV irradiation is generally recognized, the mechanism which regulates arrest remains obscure and may be dependent on UV wavelength (UVA, B or C) and on the cell type analyzed. In melanocytes and melanoma cells, for instance, accumulations in G1, S and G2/M phases have been described [50]. However, G2 cell cycle arrest seems to be characteristic of the response of normal human melanocytes and melanoma cells to UVB [49, 51, 52]. Moreover, initiation of the G2 phase arrest in the basal layer cells may represent more than a protective checkpoint response to cellular damage [51]. The most important defense mechanisms that protect human skin against UV radiation involve not only active repair mechanisms, but also melanin synthesis [53]. Since αMSH expression increases after UV radiation, and the binding of αMSH and tyrosinase activity are increased in G2 phase not only for normal cells, but also for arrested melanoma cells, these effects co-operate to increase the sensitivity of irradiated cells to αMSH and stimulate melanin synthesis [51]. Therefore, these authors corroborate the obtained results in this study that demonstrated for both cell lines, a higher percentage of cells at the S/G2/M phase, allowing suggest that, after treatment with UVB radiation, the most cells are in G2 phase.

Recently, an increasing interest exists in the promising possibility of omega 3 PUFA as nutritional factors with the potential to reduce carcinogenesis induced by UV radiation [7, 12, 14, 54], since it has become clear that the main portion of UV radiation exposure takes

place not during special occasions as vacations but in everyday life [55]. In the present study, it was observed that ALA, in a low concentration (7.5 μM), was able to protect Melan-a and B16F10 cells against UVB radiation (0.01 J/cm^2) in 48 h after treatment, since ALA was able to attenuate the cell death effect of UVB. Interestingly, it was also observed a photodynamic action in B16F10 cells, when treated with 30 μM of ALA and 0.005 J/cm^2 of UVB from 24h, while for Melan-a cells, this effect was showed just in 72h after treatment. These data are very important, regarding B16F10 is a tumoral cell. Nowadays, there is evidence about photoprotection exhibited by omega 3 PUFA in normal cells, but no previous studies have evaluated whether this effect is present in tumoral cells too. Previous studies have reported that omega 3 PUFA have potential to protect the skin from UV radiation damage through a number of mechanisms, including alterations in membrane fluidity, modification of signal transduction, transcription factor activation, modulation of oxidative stress, production of bioactive lipid mediators, that mediate inflammatory and immune responses and inhibition of certain genotoxic markers of UVR-induced DNA damage, such as UVR- induced cutaneous p53 [12, 14].

Another important consideration is still the unknown of the exact mechanisms related to drug resistance in the studied cell models. The MDR phenotype is known as the best studied mechanism that involves the overexpression of Pgp on cell membranes [10]. Although, for the cell lines used in this study, the involvement of this protein is unclear yet. In this work it was observed a strong activity of this resistance protein in Melan-a and B16F10 cells. A few previous studies have reported contradictory results regarding the presence and functionality of Pgp in malignant melanoma. For instance, Berger *et al.* [56] investigated this issue on thirty tree cell lines established from primary and metastatic lesions of human malignant melanoma and they demonstrated that immunocytochemically, 33% of the cell lines stained positive for Pgp. This result correlated with Pgp-radioimmunometric (antibody-binding) assay [56]. Differently of the present work, the

expression of Pgp was studied in primary and metastatic lesions of melanoma as well as in melanoma cell lines, but no significant melanoma-specific Pgp upregulation could be detected [57]. Moreover, Ichihashi and Kitajima [58] also showed that none of the analysed cases of melanoma expressed Pgp. These studies, in which malignant melanoma were not found to express Pgp, indicate that further verification is necessary.

In this sense, the results of the present work are very important since there is no doubt about the activity of Pgp. So, this finding may be contributing to relate a complementary protective mechanism against toxic agents in malignant melanoma. The result of this study may be associated with the resistance of melanoma cells to a broad range of anticancer drugs and of normal melanocytes to toxic melanin intermediates and metabolites [59].

5. Conclusions

The present work demonstrate that the tumoral cell line is more sensitive than the normal cell line in relation to the combined treatments with ALA and UVB radiation. Moreover, in a dose and time dependent manner, this interaction showed a significant photodynamic action in the tumoral cell line and a protective effect in the normal cell line. It was still demonstrated that these effects did not have the participation of altered ROS levels. Finally, it was possible to show that both studied cell lines presented activity of Pgp.

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Effect of Docosahexaenoic Acid on Cisplatin Chemoresistance in Murine and Human Melanoma cells

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Abstract

Even though melanoma is a highly aggressive cancer whose incidence is continuously growing, an effective therapy for the metastatic form of this cancer is still lacking. In most cases, chemotherapy is ineffective for the intrinsic/extrinsic resistance exhibited by melanoma cells. In particular, although the chemotherapeutic agent cisplatin (CPt) is highly effective against a wide range of cancers, melanoma appears resistant to it.There is a continuous search for new therapeutic approaches, and one possibility under scrutiny is the identification of agents that, combined with CPt, may revert the resistance to it. In this work, we have examined the potential of combined treatment of CPt and omega-3 polyunsaturated fatty acids (omega-3 PUFA) in sensitizing melanoma cells *in vitro* to the antineoplastic action of CPt. Plenty of experimental *in vitro* and *in vivo* studies have contributed to support the antineoplastic properties of omega-3 PUFA in tumor cells of different origin, including melanoma. In particular, docosahexaenoic acid (DHA) exerts powerful antitumor activity, and has been considered a potential adjuvant in chemotherapy of several kinds of cancer. We demonstrated that DHA sensitized the melanoma cells to the growth inhibitory and anti-invasive effect of CPt. We analyzed the effects of DHA on the expression of proteins (ERCC1, pERK and DUSP6) related to CPt chemoresistance in melanoma cells, and observed that DHA reverted the effects of CPt on ERCC1, DUSP6 and p-ERK. Also eicosapentaenoic acid (EPA) exerted similar effects on ERCC1, p-ERK and DUSP6 expression. Overall, the findings suggest that the combination of CPt with a dietary supplementation with omega-3 PUFA could represent a new potential adjuvant therapeutic strategy for overcoming CPt resistance in melanoma.

Keywords: Omega-3 PUFA; cisplatin; combined treatments; chemoresistance; melanoma cell lines.

1. Introduction

Despite the progress made in cancer chemotherapy, solid tumors are still difficult to treat, and the conventional therapeutic modalities alone result not sufficient to provide satisfactory long-term clinical results (Lake and Robinson, 2005; Solyanik, 2010; Robert *et al.*, 2011; Chapman *et al.*, 2011). This lack of an adequate response is generally ascribable to the development of drug resistance. The basis for it has been mainly related to many mechanisms, including dysregulation of apoptosis, altered drug transport, detoxification, as well as enhanced DNA repair (Grossman and Altieri, 2001; Florea and Büsselberg, 2011). Therefore, great deal of effort has been put in searching strategies to overcome drug resistance and enhance the efficacy of the currently used anticancer drugs (Siddiqui *et al.*, 2011). In particular, attention has been focused on nucleotide excision repair (NER), an enzyme complex involved in DNA repair and considered involved in some forms of drug resistance (Basu and Krishnamurthy, 2010). An essential protein in NER complex is the Excision Repair Cross-Complementation group 1 (ERCC1), which is able to repair DNA adducts by eliminating or changing them (Stordal and Davey, 2007; Galluzzi *et al.*, 2012).

One of the drugs currently used against a wide range of cancers is Cis-diamminedichloroplatinum (commonly named Cisplatin, CPt). It mainly acts by inducing growth arrest and/or apoptosis as a result of its ability to induce DNA damage. Even though CPt is considered one of the most efficient chemotherapic drugs, the development of resistance is often observed after treatment with it (Wang *et al.*, 2000). One possible strategy to enhance the efficiency of currently accessible DNA damaging agents, such as CPt, is inhibiting cellular DNA repair system (Madhusudan and Middleton, 2005).

Among solid tumors, melanoma represents one of the most commonly diagnosed cancers, and its incidence has massively increased worldwide during the last 30-40 years (Howlader *et al.*, 2014). Treatment of metastatic melanoma is extremely difficult, due to the

high resistance of this cancer to traditional cytotoxic chemotherapeutics (Li and Melton 2012). In particular, CPt is not used in current treatment of metastatic melanoma, where the overexpression of repair genes renders it extremely resistance to both chemo- and radio-therapy (Kauffmann, 2008).

CPt has been reported to increase ERCC1 expression in different cancers, and more recently, also in melanoma (Kirschner and Melton, 2010; Li and Melton, 2012). This may represent an important route through which the CPt treatment may enhance the already existing intrinsic resistance of melanoma cells to other DNA damaging agent. Importantly, Li and Melton have shown that ERK phosphorylation is required for the enhanced ERCC1 expression in melanoma cells and that CPt decreases the levels of the tyrosine-phosphatase dual-specificity phosphatase-6 (DUSP-6), by stimulating its degradation and impeding its transcription (Li and Melton, 2012). Since DUSP6 de-phosphorylates ERK (Bermudez *et al.*, 2010), it may be contributing to result in the aberrant ERK phosphorylation and, thereafter, the improved chemoresistance reported for these cells (Li and Melton, 2012). Besides that, the phosphorylation of the mitogen-activated kinase protein (MAPK) signaling pathway is constitutively stimulated in melanoma (Oliveria *et al.*, 2006; Mirmohammadsadegh *et al.*, 2007) to abnormally regulate cell survival, proliferation and invasion (Hoshino *et al.*, 1999). In this sense, the treatment of melanoma cell lines with CPt has been shown not to cause the expected decrease in extracellular signal-regulated kinase (ERK) phosphorylation (Mirmohammadsadegh *et al.*, 2007). Instead, it resulted in increased phosphorylation, suggesting that ERK activation was associated with enhanced survival of CPt-treated cells. This finding suggests that combining agents able to inhibit ERK1/2 phosphorylation with CPt could overcome melanoma CPt resistance.

A number of epidemiological and clinical human studies have shown the potential preventive role that omega 3 polyunsaturated fatty acids (omega-3 PUFA), may exert in different types of cancer (Reddy, 2004; Terry *et al.*, 2004). Omega-3 PUFA are dietary

compounds present at high levels in fish, and several experimental *in vitro* and *in vivo* studies have contributed to support their antineoplastic activity in different kinds cells of cancer cells (Calviello *et al.*, 2006), including melanoma (Serini *et al.*, 2012). Moreover, the consumption of omega-3 PUFA, and particularly DHA, was demonstrated to be able to inhibit not only the development of cancers, but also their progression. In fact, these dietary compounds are able to inhibit the metastatic activity of murine and human tumor cells *in vivo* (Broitman and Cannizzo, 1992; Rose *et al.*, 1995; Yam *et al.*, 1997; Gleissman *et al.*, 2011).

The most represented omega-3 PUFA in fish oil and in our cell membranes is docosahexaenoic acid (DHA). This compound is known for its anti-inflammatory, anti-proliferative, pro-apoptotic, anti-angiogenetic, anti-invasive, and anti-metastatic properties (Yam *et al.*, 2001; Horia and Watkins, 2007; Bougnoux *et al.*, 2009). Moreover, it was hypothesized that DHA could have the potential to act as an effective adjuvant in cancer chemotherapy (Merendino *et al.*, 2013). An important mechanism often invoked to explain the ability of DHA to enhance the efficacy of anticancer drugs is the ability of DHA to increase lipid peroxidation and oxidative stress. In addition, many intracellular targets were found to play an essential role in enhancing the sensitivity of cancer cells to antineoplastic agents (Siddiqui *et al.*, 2011). Finally, in some instance, it was shown that DHA combined with antitumoral agents may induce sensitization to chemotherapic drugs, by enhancing their uptake in cancer cells, thus allowing to overcome the drug resistance. Another possibility still not investigated is that omega-3 PUFA, and DHA in particular, may enhance the sensitivity of tumor cells to antineoplastic DNA-damaging drugs by modulating the expression of proteins involved in the repair of DNA.

On these bases, in this work we analyzed in murine and human melanoma cells the effects of DHA on some molecular target/pathways that have been involved in the resistance of melanoma to the antineoplastic action of CPt (ERCC1 and DUSP6 expression, and ERK phosphorylation). Moreover, we have treated melanoma cells with DHA and CPt

combinations with the purpose to evaluate if the addition of this fatty acid was able to enhance the sensitivity of melanoma cells to the antineoplastic activity of CPt.

2. Materials and methods

2.1 Cell lines and treatments

The Melan-a cell line (murine normal melanocyte) was obtained from the Cell Biology Laboratory of the Translational Research Centre in Oncology from Cancer Institute of São Paulo/ Brazil. The B16F10 cell line (murine malignant melanocytes) was obtained from cell bank of Rio de Janeiro/ Brazil. The cell lines were maintained in DMEM, supplemented with sodium bicarbonate (0.2 g/L), L-glutamine (0.3 g/L) and HEPES (3 g/L).The primary WM115 and the metastatic WM266-4 melanoma cell lines (a kind gift of Dr M.C. Failla, Instituto Dermopatico dell'Immacolata, Rome, Italy) were grown in Eagle's minimum essential medium containing 2 mM glutamine, non-essential aminoacids and Na-pyruvate. All cells were supplemented with 10% fetal bovine serum and maintained at 37°C in a humidified atmosphere containing 5% CO_2. DHA and EPA were purchased from Sigma-Aldrich (Sigma, St Louis, MO, USA) and were added to the culture medium from an absolute ethanol stock solution. DHA (10 and 30μM) and EPA (30 μM) were used in the experiments with B16F10 and WM266-4 cells. Control cells were treated with the same amount of vehicle (ethanol) alone, and the final ethanol concentration never exceeded 0.5% (vol/vol).CPtwas obtained by Sigma-Aldrich (Sigma, St Louis, MO, USA) and it was diluted in a solution of NaCl (0.9%).

2.2 Analysis of cell growth and viability

B16F10 and WM266-4 cells were seeded in a 24-well culture plate (3×10^4 cells/mL). After 24h, culture medium was removed and replaced with fresh culture medium containing or not increasing concentrations of CPt (1, 2.5 and 5 μM) alone or in combination with DHA (10 μM). At different time points (24-72 h), cells (three wells for each culture condition) were

trypsinized, washed in phosphate buffered saline (PBS, pH 7.4), centrifuged and counted by a Neubauer hemocytometer chamber. Cell viability was analyzed by the Trypan blue dye exclusion method.

2.3 Wound healing assay *in vitro*

The invasive ability of melanoma cells was evaluated by the Wound Healing assay *in vitro*. For this purpose, disposable silicon inserts (Ibidi, Munchen, Germany) were used. These inserts consist of two chambers separated by a 500 µm width silicon sept. The inserts were put into 12-well multiwell culture plates and 70 µl of B16F10 and WM266-4 melanoma cell suspensions (at the concentration of 3 x 10^5 cells/ml) were put into each chamber of the silicon inserts. Culture medium not containing cells (1.3 ml) was put in the wells outside the silicon inserts. After 24 h, the silicon inserts were removed and cell monolayers separated by a 500 µm cell free gap were obtained. Samples were photographed (representing the time 0) and then cell culture medium was replaced by fresh culture medium without serum containing or not DHA (10 µM) and CPt (1µM) given alone and/or in combination. Melanoma cell migration was analyzed at the indicate time points (24, 48 and 72 h) by evaluating the residual cell free area in the wells. The cultures were observed under a light microscope and photographed (10x Optical Zoom) to save images, that were then analyzed by the TScratch software (developed by the group of Dr. Koumoutsakos (CSE Lab), at the ETH, Zurich, Switzerland) [Gebäck, T., Schulz, M.M.P., Koumoutsakos, P. & Detmar, M. A novel and simple software tool for automated analysis of monolayer wound healing assays. *BioTechniques* (2008).]

2.4 Western Blot analysis

Whole cell extracts were prepared as described previously (Serini *et al.*, 2012). Briefly, cells were lysed in ice-cold lysis buffer (1 mM $MgCl_2$, 350 mM NaCl, 20 mM HEPES, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM $Na_4P_2O_4$, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin,1.5 mM leupeptin, 20% glycerol, 1% NP-40) for 30 min at 4°C and centrifuged for 10 min at 12000g to remove cell debris.80µg of proteins were separated on a 10% sodium dodecyl sulfate polyacrylamide gel and electroblotted on a nitrocellulose membrane. The membrane was blocked with 5% dried milk (wt/vol) for 1 h at room temperature and then incubated overnight at 4°C with specific antibodies to ERCC1 (FL-297: sc-10785, Santa-Cruz Biotechnology, Santa Cruz, CA, USA), DUSP-6 (EPR129Y: ab76310, Abcam, Eugene, CA, USA) p-ERK (E-4: sc-7383, Santa-Cruz Biotechnology) and total ERK (K-23: sc-94, Santa-Cruz Biotechnology). As loading controls, the blots were reprobed with anti-α-actinin (B-12: sc-166524, Santa Cruz Biotechnology), anti-β-actin (AC40: A-4700, Sigma-Aldrich). Following incubation with secondary mouse (for p-ERK, α-actinin and β-actin) or rabbit (for ERCC1, DUSP6, total ERK) antibodies, the immunocomplexes were visualized using the enhanced chemiluminescence detection system (Amersham) and quantitated by densitometric analysis.

2.5 Statistical analysis

Data were expressed as the means ± SD and analyzed by One-Way Analysis of Variance (ANOVA) followed by Tukey's test. Differences were considered significant when $P < 0.05$.

3. Results

3.1 Cell Growth

Human WM266-4 and murine B16F10 melanoma cells were exposed to increasing concentrations of CPt (1-5 μM) alone or in combination with 10 μM DHA. Figure 1 shows that CPt inhibited the growth of human WM266-4 and murine B16F10 melanoma cells in a dose- and time-dependent way. The addition of DHA sensitized human WM266-4 melanoma cells to the growth inhibitory effect of CPt, and the sensitizing effect was evident after 24h - 48h of treatment, when DHA was combined with the lowest concentration (1 μM) of CPt (Figure 1). In particular, we observed that the growth inhibitory effect of CPt was enhanced by 35% at 24h by the contemporaneous administration of DHA (Figure 2). This finding is worth noticing, since suggests that the high and health-threatening CPt doses used in cancer therapy to obtain a considerable growth inhibitory could be potentially substituted by lower doses in combination with a dietary supplementation with DHA.

Similar results were obtained also by treating the murine B16F10 cell line for 24-72 h (Figure 3). In this case, however, DHA alone was more efficient than 1 μM CPt in inhibiting cell growth (almost two fold efficient) (Figure 4). Nevertheless, the CPt/DHA combined treatment resulted significantly more efficient than the treatments with each of the two compounds administered alone.

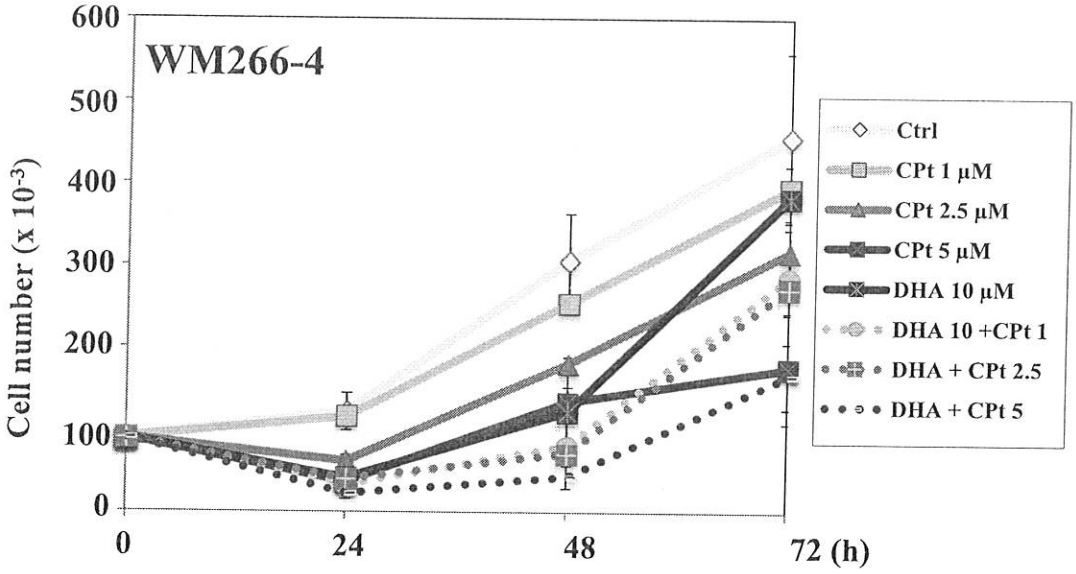


Figure 1: Effect of Cisplatin and DHA on the growth of WM266-4 melanoma cells. Cells were treated for 24-72 h with increasing concentrations of cisplatin (1-5 µM) alone and in combination with DHA (10 µM). Data are the means ± SD of three different experiments.

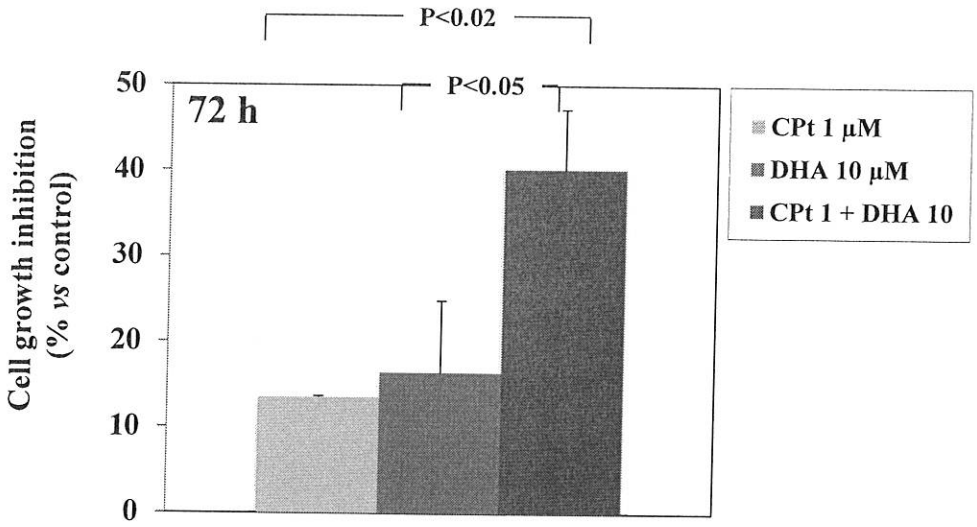


Figure 2: Effect of the combination cisplatin/DHA on the growth of WM266-4 cells. Cells were treated with 1 µM cisplatin, 10 µM DHA and their combination for 24 h. Data are the means ± SD of three different experiments. Significant differences were evaluated by One-Way ANOVA, followed by Tukey's test.

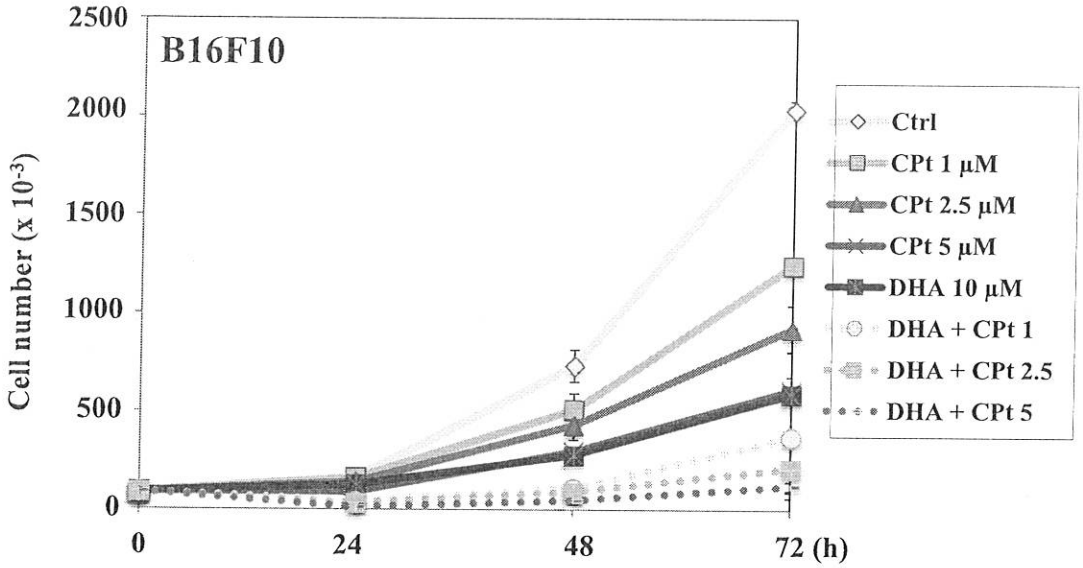


Figure 3: Effect of cisplatin and DHA on the growth of B16F10 melanoma cells. Cells were treated for 24-72 h with increasing concentrations of cisplatin (1-5 µM) alone and in combination with DHA (10 µM). Data are the means ± SD of three different experiments.

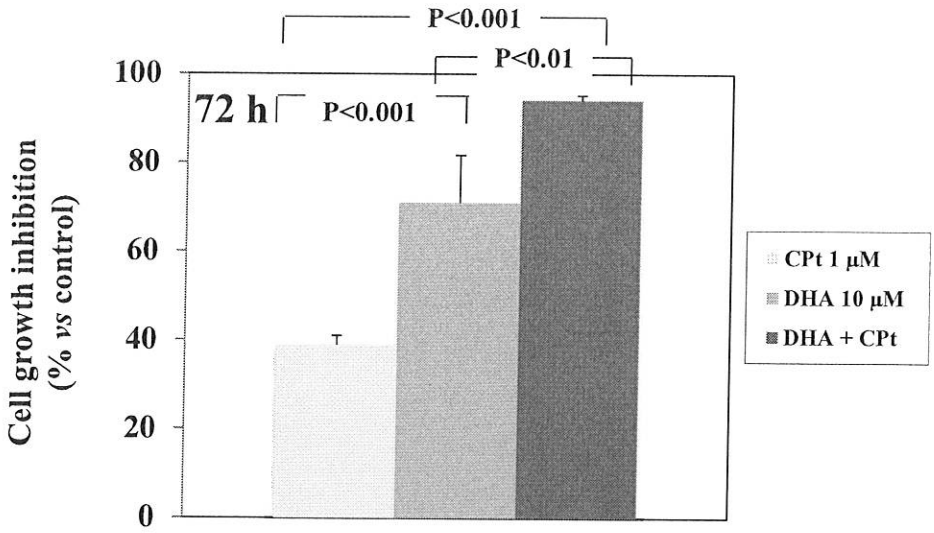


Figure 4: Effect of the combination cisplatin/DHA on the growth of B16F10 cells. Cells were treated with 1 µM CPt, 10 µM DHA and their combination for 24 h. Data are the means ± SD of three different experiments. Significant differences were evaluated by One-Way ANOVA, followed by Tukey's test.

3.2 Effect of CPt and DHA on melanoma cell migration *in vitro*

In order to analyze how DHA and CPt may affect the migration and the invasive potential of melanoma cells, we performed experiments of wound healing assay *in vitro*. Human and murine melanoma cells were treated with CPt 1 μM, DHA 10 μM and their combination. In control conditions, the cell-free area (area not occupied by growing cells, see under Materials and Methods) decreased rapidly, and at 72 h it almost completely disappeared. On the other hand, in the presence of either CPt or DHA a conspicuous portion of cell-free area still remained at each time point, demonstrating the ability of the two compounds to inhibit melanoma cell migration *in vitro*. Interestingly, in the human WM266-4 and murine B16F10 melanoma cells the inhibition was even more evident when the two compounds were administered in combination (Figures 5-6).

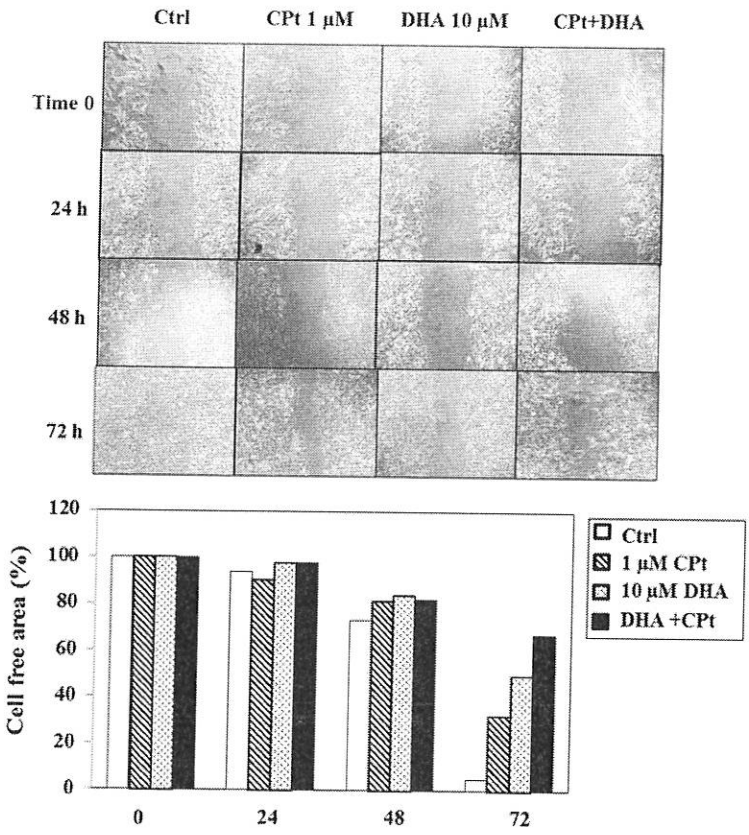


Figure 5: Effect of cisplatin and DHA on the migration of WM266-4 melanoma cells. Migration has been evaluated by the Wound healing assay *in vitro* (for further details, see

Materials and Methods section). In the histogram, the percentages of cell free areas in the culture wells are presented. Data are the means of two different experiments. In the upper panel, representative images of one of the experiments are shown.

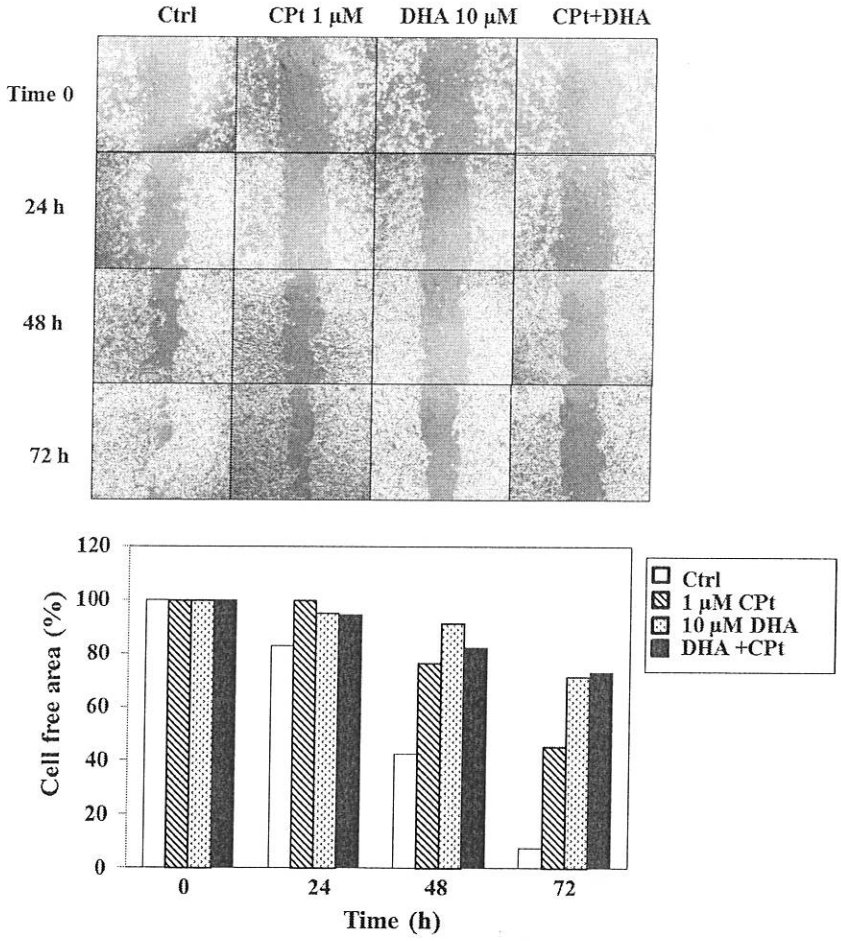
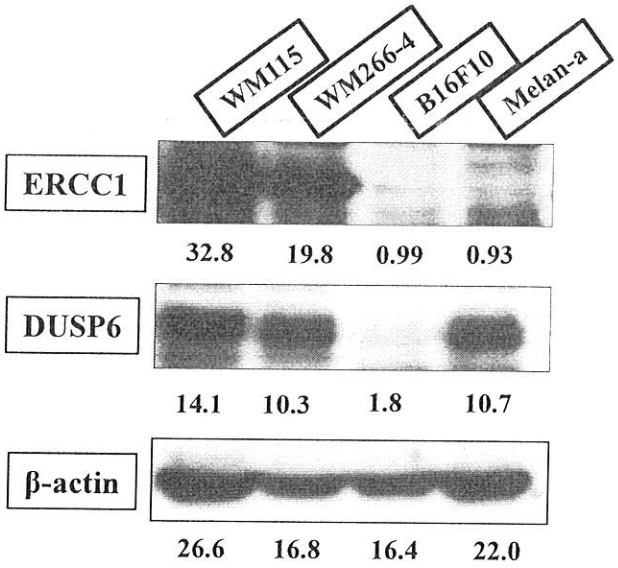


Figure 6: Effect of cisplatin and DHA on the migration of B16F10 melanoma cells. Migration has been evaluated by the Wound healing assay *in vitro* (for further details, see Materials and Methods section). In the histogram, the percentages of cell free areas in the culture wells are presented. Data are the means of two different experiments. In the upper panel, representative images of one of the experiments are shown.

3.3 Effect of CPt and DHA on ERCC1 and DUSP6 protein expression and ERK phosphorylation in melanoma cells

First of all, we examined the basal levels of ERCC1 and DUSP-6 in different cell lines of melanocytic origin (WM115, WM266-4 and B16F10 melanoma cell lines and the normal melanocytic Melan-a cell line). B16F10 expressed the lowest levels of ERCC1 and DUSP-6, while WM115 and WM266-4 expressed comparable and high levels of these proteins. Melan-a cells express low levels of ERCC1 and high levels of DUSP-6, as expected for a normal cell line (Figure 7).



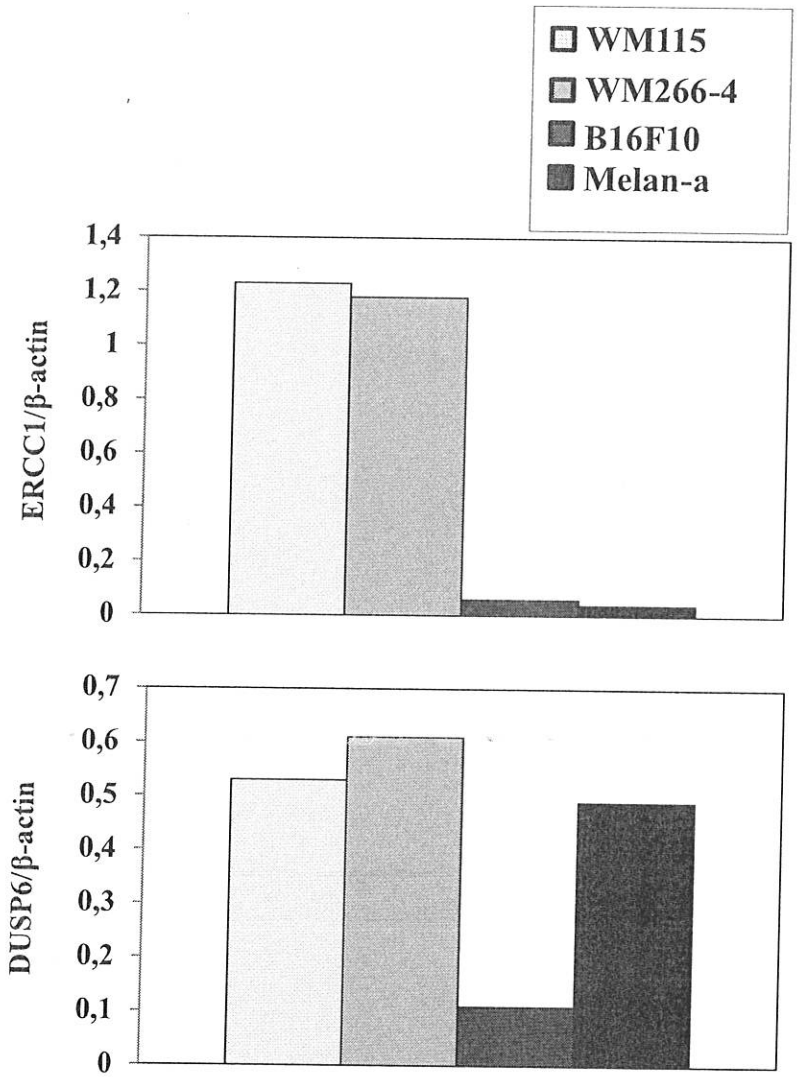


Figure 7: Basal levels of ERCC1 and DUSP-6 expression in WM115, WM266-4, B16F10 and Melan-a cell lines. Cells were seeded in 100 mm Petri dishes (3 x 10^5 cells/ml) and trypsinized at confluence. Data in the histograms are the means of two different experiments. In the upper panel representative Western Blot experiments are shown.

On the basis of the observation that CPt is able to enhance ERCC1 and reduce DUSP-6 expression in various cancer cells, including melanoma (Kirschner and Melton, 2010; Li & Melton, 2012), we evaluated if these results would be confirmed also in WM266-4 and B16F10 cell lines used in this study. We treated WM266-4 and B16F10 cells with 1-5

μM CPt for 24 h and confirmed that CPt was able to increase ERCC1 and decrease DUSP-6 in a dose-dependent manner in both WM266-4 and B16F10 cell lines (Figures 8 and 9).

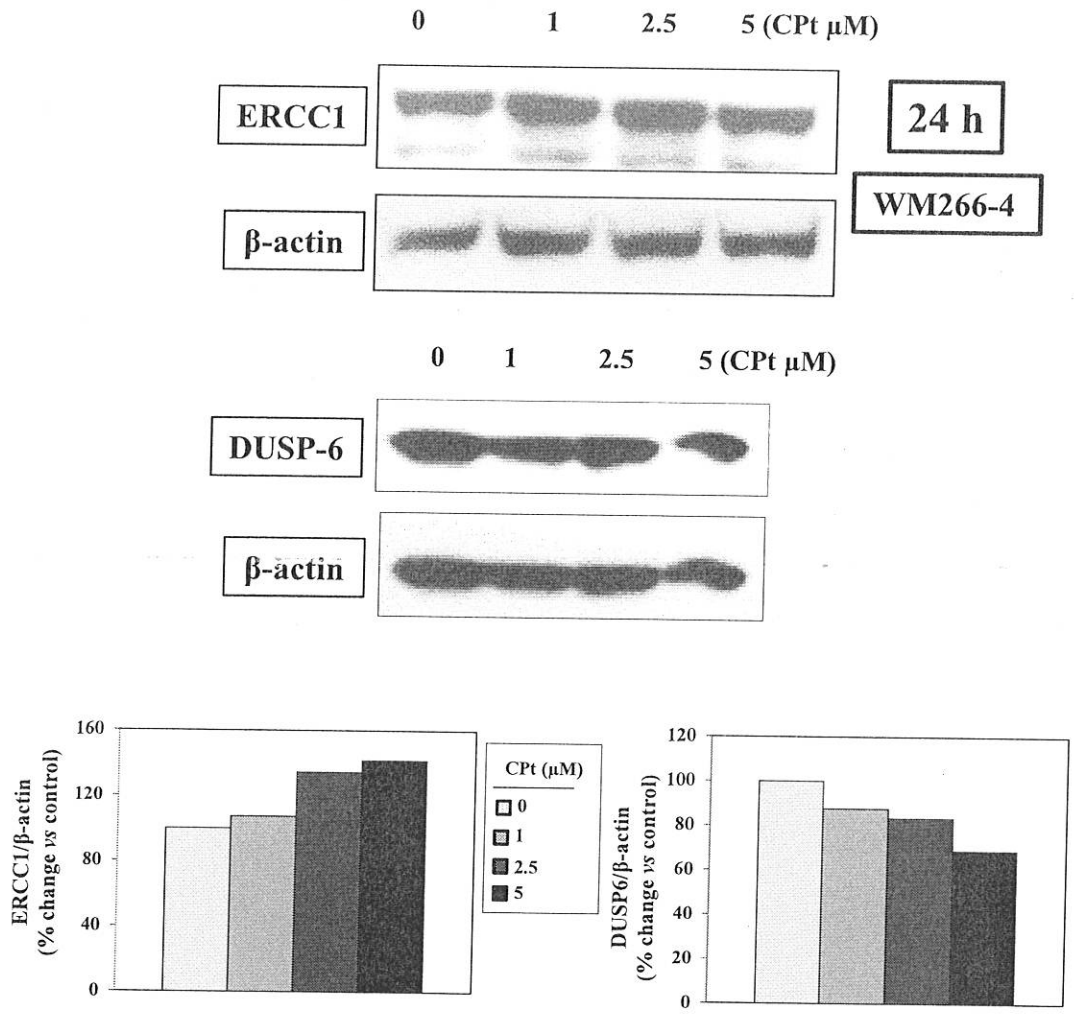


Figure 8: Effect of cisplatin on ERCC1 and DUSP-6 expression in WM266-4 melanoma cells. Cells (3 x 10^5 cells/ml) were treated with increasing concentrations of cisplatin (1, 2.5 and 5 μM) for 24 h. Data in the histograms are the means of two different experiments. In the upper panel representative Western Blot experiments are shown.

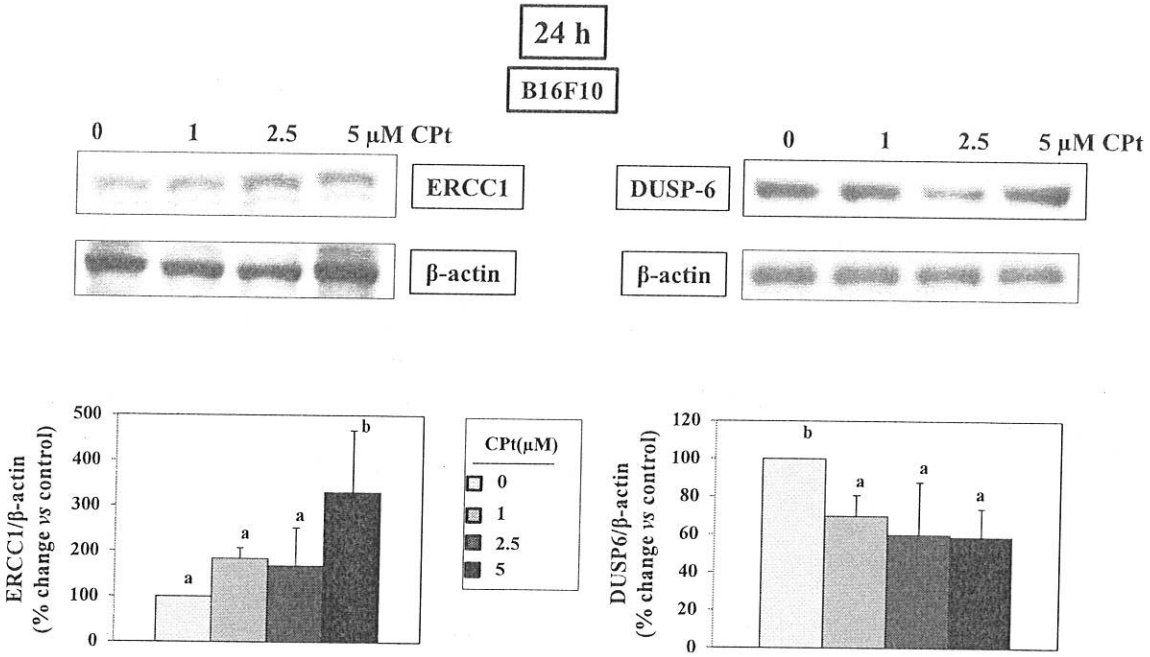


Figure 9: Effect of cisplatin on ERCC1 and DUSP-6 expression in B16F10 melanoma cells. Cells (3 x 10^5/ml) were treated with increasing concentrations of cisplatin (1, 2.5 and 5 µM) for 24 h. Data in the histograms are the means ± SD of three different experiments. Values not sharing the same superscript are significantly different ($p<0.05$, One-Way ANOVA, followed by Tukey's test). In the upper panel representative Western Blot experiments are shown.

Moreover, we demonstrated that the decrease of DUSP-6 expression induced by CPt 1 µM in both WM266-4 and B16F10 cells was time-dependent (Figure 10).

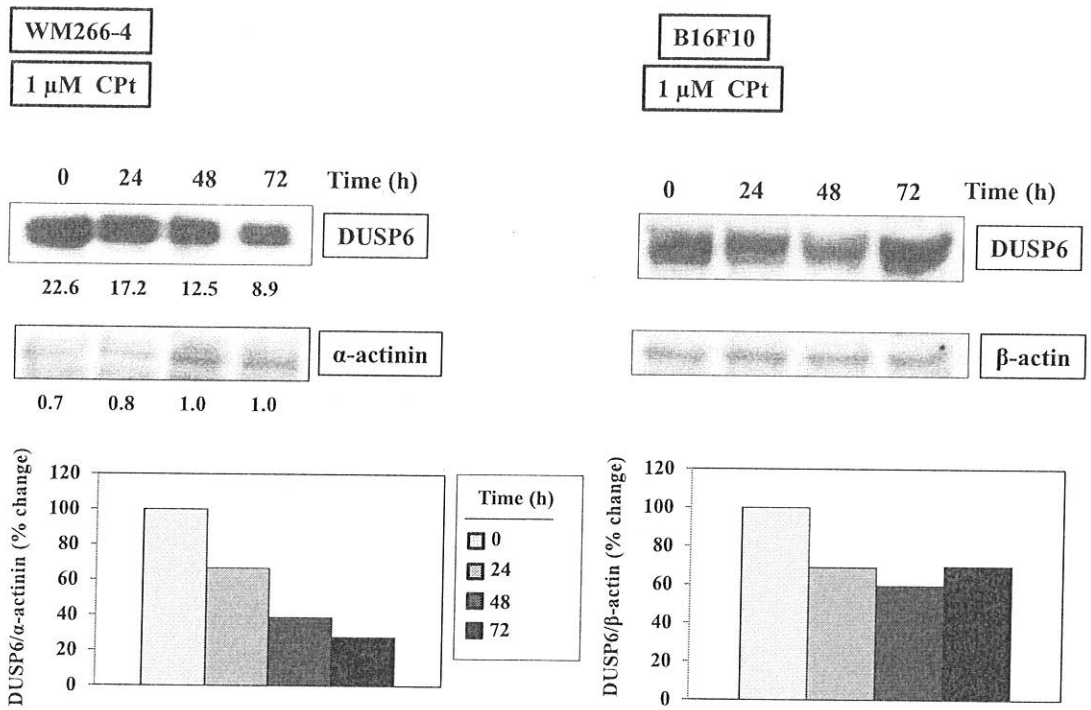
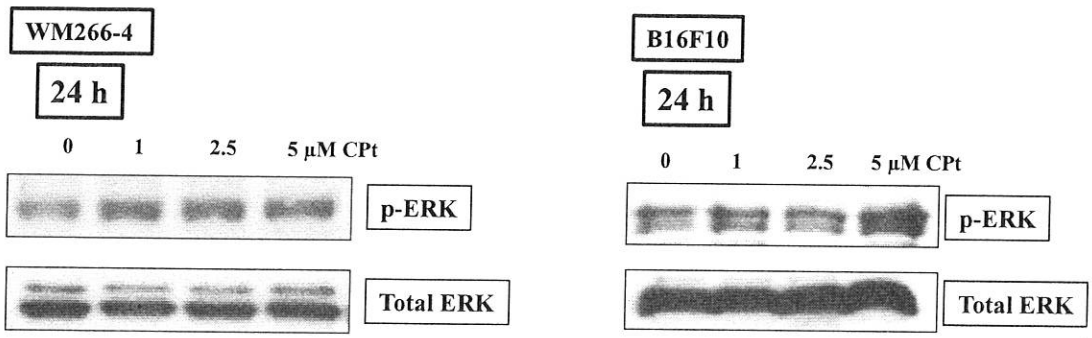


Figure 10: Time-dependent effect of cisplatin on DUSP-6 expression in WM266-4 and B16F10 melanoma cells. Cells (3 x 10^5/ml) were treated with 1 μM cisplatin for increasing periods of time (24-72 h). Data in the histograms are the means of two different experiments. In the upper panel, representative Western Blot experiments are shown.

Furthermore, we evaluated the effect of increasing concentrations of CPt on ERK phosphorylation. We demonstrated that CPt increased the phosphorylation of ERK in a dose-dependent manner both in WM266-4 and B16F10 cell lines (Figure 11).



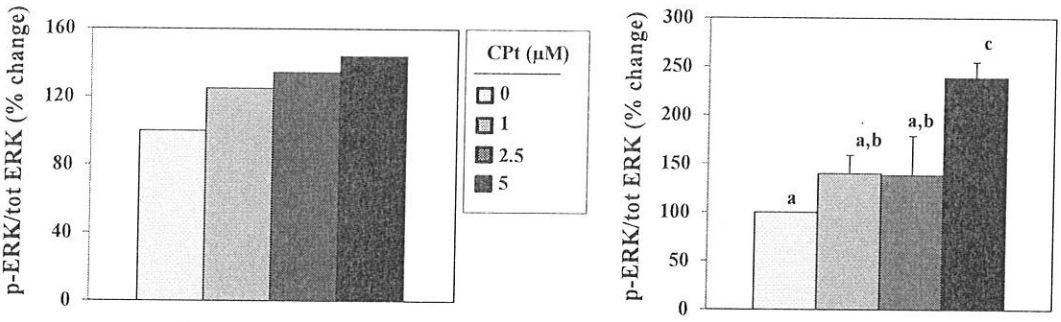


Figure 11: Effect of cisplatin on ERK phosphorylation in WM266-4 and B16F10 melanoma cells. Cells (3 x 10^5/ml) were treated with increasing concentrations of cisplatin (1, 2.5 and 5 μM) for 24 h. Data in the histograms are the means of two different experiments (WM266-4 cells) and the means ± SD of three different experiments (B16F10 cells). Values not sharing the same superscript are significantly different ($p<0.05$, One-Way ANOVA followed by Tukey's test). In the upper panel, representative Western Blot experiments are shown.

On the basis of the results so far obtained, next we investigated if and how the addition of DHA could modify the effects of CPt on ERCC1 and DUSP-6 expression and ERK phosphorylation. In order to perform these experiments, it was chosen B16F10 cells, since we obtained more evident results using this cell line. We observed that the treatment of B16F10 melanoma cells with 30 μM DHA for 24 h was able to revert both the increase in ERCC1 expression and the decrease DUSP-6 expression induced by CPt 10 μM (Figure 12).

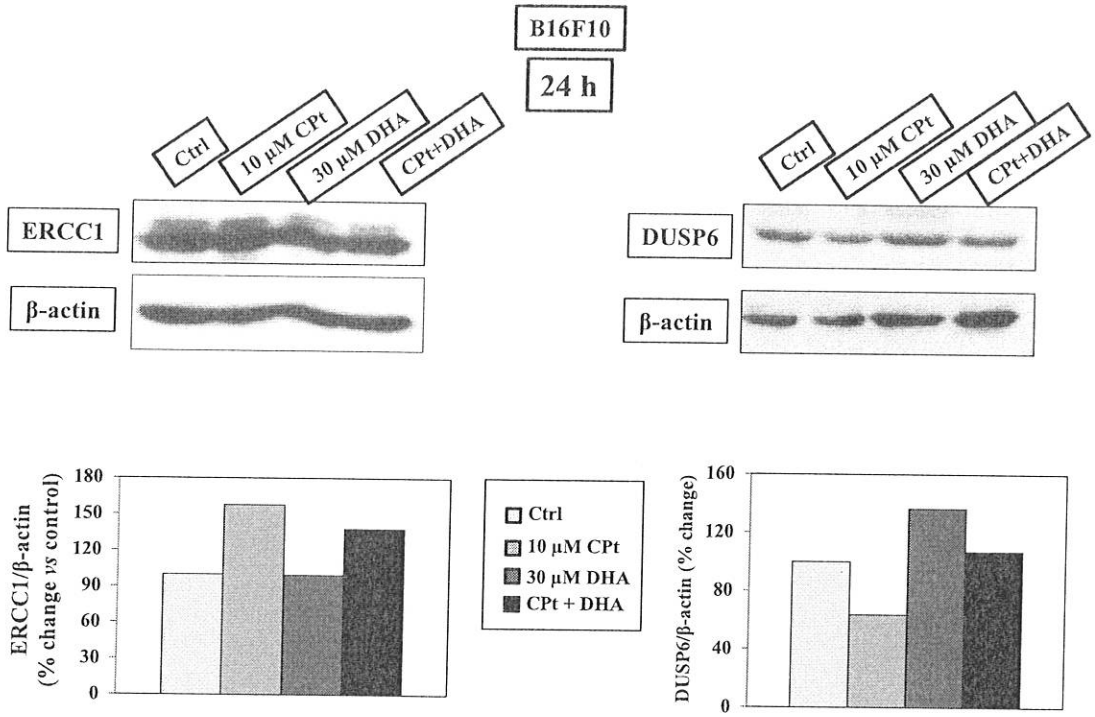


Figure 12: Effect of DHA and cisplatin on ERCC1 and DUSP-6 expression in B16F10 melanoma cells. Cells (3 x 10^5/ml) were treated with 10 μM cisplatin and 30 μM DHA, alone and in combination, for 24 h. Data are the means of two different experiments. In the upper panel, representative Western Blot experiments are shown.

Moreover, we demonstrated that similarly to DHA, also 30 μM EPA was able to revert the increasing effect of CPt on ERCC1 expression. Correspondingly, we observed that either EPA or DHA were able to revert the decreasing effect of CPt on DUSP-6 expression (Figure 13).

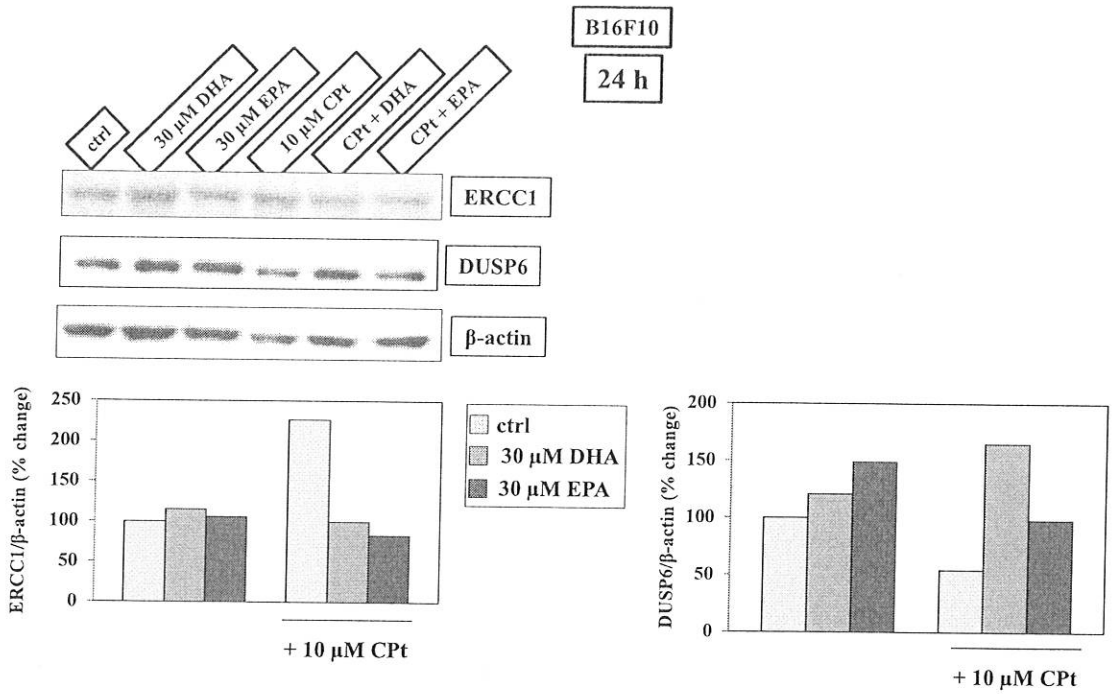
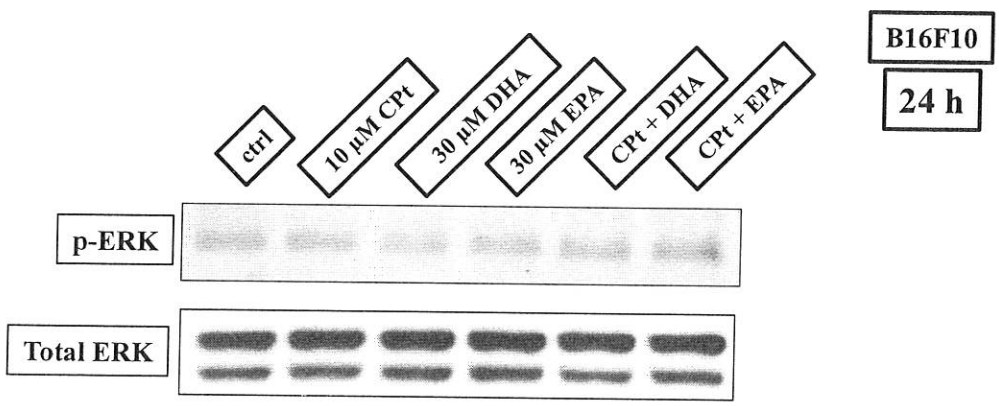


Figure 13: Effect of cisplatin, DHA and EPA on ERCC1 and DUSP-6 expression in B16F10 melanoma cells. Cells (3 x 10^5/ml) were treated with 10 μM cisplatin, 30 μM DHA and/or 30 μM EPA, alone and in combination for 24 h. Data in the histograms are the means of two different experiments. In the upper panel, representative Western Blot experiments are shown.

In agreement, we also observed that either DHA or EPA (30 μM) were able to revert the inducing effect of CPt on ERK phosphorylation in B16F10 cells after 24 h of treatment (Figure 14).



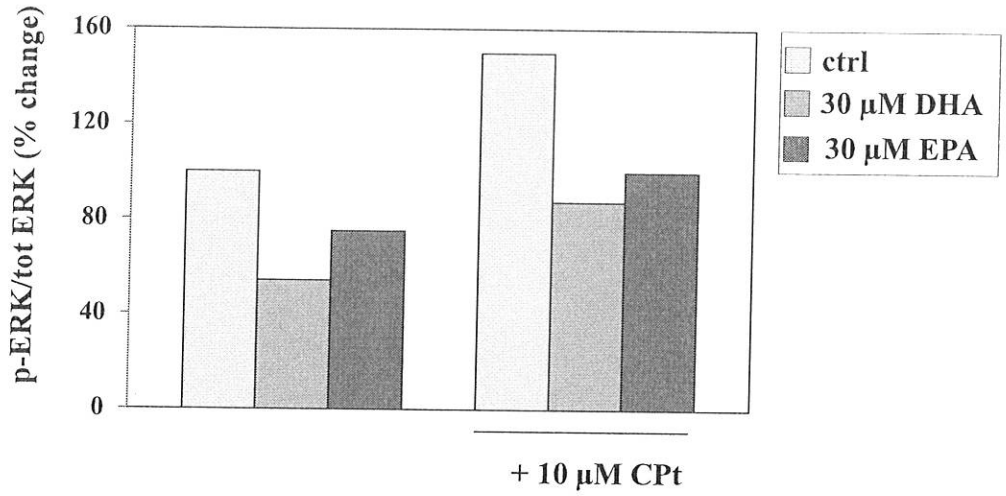


Figure 14: Effect of cisplatin, DHA, and EPA on ERK phosphorylation in B16F10 melanoma cells. In the histogram, data are the means of two different experiments. In the upper panel, representative Western Blot experiments are shown.

4. Discussion

In the present work, we demonstrated that DHA sensitizes human WM266-4 and murine B16F10 melanoma cells to the growth inhibitory and anti-invasive effect of CPt.

A number of studies have investigated the role of omega-3 PUFA in cancer prevention, cancer treatment, and cachexia. In particular, the growth-inhibiting effect of this fatty acid was reported by plenty of studies performed in a variety of cancer cells (Calviello *et al.*, 2004; Calviello *et al.*, 2007 a; Serini *et al.*, 2008; Serini *et al.*, 2012, Calviello *et al.*, 2006, Calviello *et al.*, 2007 b; Slagsvold *et al.*, 2010; Serini *et al.*, 2014; D'Eliseo and Velotti, 2016). There are plenty of findings demonstrating that as omega-3 PUFA become incorporated in tumor membranes, these may modify their physical and chemical properties (Stillwell *et al.*, 2005; Serini *et al.*, 2010; Serini *et al.*, 2014; Calviello *et al.*, 2006). Among these alterations, deep changes have been described in cell membrane fluidity and degree of unsaturation, altered lipidic and proteic components (such as carriers, enzymes,

receptors, adapter proteins) of membrane microenvironments (lipid rafts) involved in signal transduction (Serini *et al.*, 2010). Moreover, an interesting omega-3 PUFA property that has been described and that could make them a useful anticancer agent is to be specifically cytotoxic towards cancer cells, while sparing normal cells (D'Eliseo and Velotti, 2016).

A recent study conducted in the same laboratory where this work was performed, demonstrated that 30 μM DHA was able to inhibit cell growth and induce the differentiation of the human primary WM115 and metastatic WM266-4 melanoma cell lines, originally derived from the same patient (Serini *et al.*, 2012). In the present study, we have treated the cells with 10 μM DHA, and have confirmed that also at this lower concentration DHA exerts a growth-inhibitory effect, both in the human WM266-4 melanoma cell line used before, and also in the highly metastatic murine B16F10 melanoma cell line. In both the previous and the present work, we have used concentrations of DHA that do not alter cell viability more than 5-10 %, as evaluated in preliminary experiments performed with the trypan blue dye exclusion assay (data not shown). Doing so, we have avoided high DHA concentrations that may induce oxidative stress, lipid peroxidation, and cause massive cytotoxicity (Zajdel *et al.*, 2013), and that may preclude the study of specific action of DHA on molecular targets involved in cell growth and invasion.

Moreover, we have now also shown that 10 μM DHA was able to inhibit the migration of the human and murine melanoma cells in a time-dependent manner. This finding is of value since melanoma cells possess high invasive and metastatic potentials (Jemal *et al.*, 2010), and their active migration is considered essential for invasion and metastasis (van Zijl *et al.*, 2011). This observation corroborates previous findings showing the anti-invasive and anti-metastatic activities of omega-3 PUFA in *in vitro* and *in vivo* animal models of cancer. In particular, Denkins *et al.* (2005) showed that DHA decreased the *in vitro* invasion of 70W human melanoma cell line. The ability of omega-3 PUFA to inhibit invasion and metastasis *in vivo* was reported for different kinds of tumors, including breast and lung

cancer. For instance, in one of the pioneer animal studies performed *in vivo* with omega-3 PUFA, Rose *et al.* (1994) reported that the dietary supplementation with these fatty acids to nude mice injected with human MDA-MB-435 breast cancer cells significantly suppressed both the primary tumor growth rate and the occurrence and severity of lung metastases.The anti-metastatic activity of DHA was also observed *in vivo* by Yam *et al.* (1997) in the model of Lewis Lung Carcinoma (3LL) metastases in C57BL/6J mice. Interestingly, in the same experimental model, these authors (Yam *et al.*, 2001) found that a dietary supplementation with fish oil combined with the treatment with CPt significantly reduced metastasis formation. Concerning this point, it is important to underline that, currently, there is great interest for the possible role of omega-3 PUFA as adjuvants in cancer therapy (Slagsvold *et al.*, 2010; Merendino*et al.*, 2013). Combinations of antineoplastic drugs with omega-3 PUFA treatment have been suggested by many authors as potential strategies to enhance the clinical outcome of cancer patients (Laviano *et al.*, 2013; D'Eliseo and Velotti, 2016). In particular, plenty of *in vitro* and animal studies suggest that combining DHA with other anticancer agents often improves efficacy of anticancer drugs and radiations (for comprehensive reviews, describing these studies see: Calviello *et al*, 2009; Siddiqui *et al.*, 2011). In line with this hypothesis, in the present study we have also investigated the effect of DHA in combination with CPt, a chemotherapeutic DNA-damaging agent widely used in the therapy of many solid tumors, but known to be not effective in metastatic melanoma treatment (Li and Melton, 2012). We have observed that the combination of CPt with DHA had a strong inhibitory effect on cell growth and invasion in human and murine melanoma cells.

Even though CPt is a widely used anticancer drug, cancer cells often develop resistance toward it. In particular, metastatic melanoma shows an intrinsic chemoresistance to CPt. An increased expression of a number of DNA repair genes has been reported in melanoma (Li and Melton, 2012), and this alteration has been hypothesized to contribute to the extreme resistance that this tumor shows towards CPt (Helmbach *et al.*, 2001).

Accordingly, we found a high basal expression of the DNA-repairing protein ERCC1 in our melanoma cell lines. Moreover, Li and Melton [2012] reported that CPt induces the expression of ERCC1 and of the other DNA-repairing protein XPF. In agreement, in the present study we found that CPt was able to further increase the levels of ERCC1 protein in human and murine melanoma cells in a dose-dependent manner. Moreover, the same authors [Li and Melton 2012] demonstrated that in these cells MAPK ERK is necessary for the increase of ERCC1 expression taking place. The MAP-kinase signaling pathway has been reported to be constitutively activated in melanoma (Oliveria *et al.*, 2006), where it is involved in the regulation of cell survival, proliferation and invasion (Hoshino *et al.*, 1999). It has been observed that an increased phosphorylation of the MAP kinase ERK can be induced in melanoma cells by CPt (Mirmohammadsadegh *et al.*, 2007). In keeping, we have observed that CPt was able to increase ERK phosphorylation in a dose-dependent manner in both the melanoma cell lines. Moreover, it was previously demonstrated that CPt was able to reduce the levels of the tyrosine-phosphatase dual-specificity phosphatase-6 (DUSP6), by inducing its degradation and inhibiting its transcription (Li and Melton 2012). We confirmed this finding also in our cellular models, where we observed that CPt significantly reduced DUSP6 expression in dose-and time-dependent manner. Since DUSP6 is able to de-phosphorylate ERK (Bermudez *et al.*, 2010), it was previously hypothesized that it could contribute to induce the abnormal ERK phosphorylation seen in melanoma cells, that, in turn, may increase ERCC1 expression, and thus be probably responsible of the chemoresistance observed in these cancer cells (Li and Melton 2012).

In the same laboratory where the present work has been performed, it was previously demonstrated that DHA was able to reduce the phosphorylation of different MAP kinases, including ERK, in different kinds of human cancer cells (Calviello *et al.*, 2004, Serini *et al.*, 2008). The same authors also reported that DHA induced apoptosis in lung adenocarcinoma cells through the de-phosphorylation of ERK, and that this effect was related to the DHA-

induced increased expression of another MAP Kinase Phosphatase, namely DUSP3 (also known as MKP-1) (Serini *et al.*, 2008). In the present study, we found that DHA was able to revert the effects of CPt on DUSP6 protein expression in murine and human melanoma cells. This fatty acid reduced also ERK phosphorylation and ERCC1 expression, suggesting that the modulation of the DUSP6/p-ERK/ERCC1 pathway could be strictly related to the ability of DHA to sensitize melanoma cells to the antineoplastic effects of CPt. Further experiments of DUSP6 silencing are in progress in the laboratory in order to directly relate the DHA induction of DUSP6 expression to the growth-inhibiting and anti-invasive effect of this fatty acid in melanoma cells. Comparable modifications of the DUSP6/p-ERK/ERCC1 pathway were obtained also when CPt was used in combination with the other long-chain omega-3 PUFA EPA, thus suggesting that the ability to modify this signaling pathway involved in melanoma CPt resistance may be common to the entire omega-3 PUFA class.

5. Conclusions

The results of the present study demonstrate the ability of DHA to sensitize melanoma cells to the growth-inhibitory and anti-invasive effect exerted *in vitro* by CPt. They also permit to suggest that DHA may exert its beneficial effect by modulating the DUSP6/p-ERK/ERCC1 signaling pathway. The data shown are of extreme value since demonstrate that DHA is potentially able to revert the resistance of melanoma cells to the anti-neoplastic agent CPt. They suggest the potential use of this combination in the therapy of metastatic melanoma, thus offering a possible way to improve the efficacy of Cpt in this kind of tumors, as well as to reduce the deleterious side-effect often associated to high doses of this antineoplastic drug.

Acknowledgments

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3. Discussão Geral

Atualmente, o melanoma é considerado um dos mais preocupantes tipos de câncer, uma vez que sua incidência tem aumentado rapidamente no mundo todo e ainda não existe uma terapia efetiva para a doença metastática (Li & Melton, 2012). A quimioterapia não é efetiva na maioria dos casos devido à resistência intrínseca/extrínseca observada em células de melanoma (Helmbach *et al.*, 2001, Holohan *et al.*, 2013). Em nosso trabalho, foi possível demonstrar uma significativa atividade de Pgp em células de melanoma, linhagem B16F10. Células melanocíticas normais também apresentaram uma atividade expressiva desta proteína. Resultado relevante, uma vez que poucos trabalhos têm descrito a atividade desta proteína em células normais e tumorais de melanócitos, evidenciando assim, um mecanismo de resistência importante. Outro mecanismo também conhecido por participar da resposta de quimiorresistência no melanoma, em resposta ao tratamento com o quimioterápico CPt, é a superexpressão de proteínas de reparo do DNA, como por exemplo a proteína ERCC1 (Basu & Krishnamurthy, 2010; Li & Melton, 2012). De fato, neste trabalho demonstramos uma elevada expressão basal desta proteína em células de melanoma humano, linhagens WM115 e WM266-4, e por outro lado, uma baixa expressão em células de melanoma murino, linhagem B16F10. Além disso, a exposição ao quimioterápico CPt foi capaz de aumentar a expressão de ERCC1 nas linhagens WM266-4 e B16F10 de maneira dose dependente. Também observamos uma elevada expressão basal de DUSP-6 nas linhagens celulares WM115 e WM266-4 e uma baixa expressão na linhagem B16F10. Ainda demonstramos que o tratamento com CPt diminuiu a expressão desta proteína nas linhagens WM266-4 e B16F10 de maneira dose e tempo dependente. O tratamento com CPt também foi capaz de aumentar a fosforilação de ERK em ambas as linhagens, WM266-4 e B16F10. Desta forma, estratégias que possam reverter o fenótipo de resistência de

células tumorais ou sensibilizar células tumorais ao tratamento com CPt necessitam ser exploradas (Wang *et al.*, 2000).

Neste trabalho, pela primeira vez, foi investigado o potencial de PUFA ômega 3, DHA e EPA, em aumentar a sensibilidade de células de melanoma a um quimioterápico conhecido por danificar o DNA, através da modulação da expressão de proteínas envolvidas no reparo de DNA. Importantemente, demonstramos que ambos os PUFA ômega 3 foram capazes de reverter o mecanismo de resistência à CPt, através da diminuição de ERCC1 e pERK e aumento de DUSP-6. Atualmente, há um crescente interesse de PUFA ômega 3 como adjuvantes na terapia do câncer (Slagsvold *et al.*, 2010; D'Eliseo & Velotti, 2016). Em particular, muitos estudos *in vitro* e *in vivo* sugerem que PUFA ômega 3, especialmente o DHA, em combinação com outros agentes anticâncer frequentemente aumentam a eficácia de drogas antitumorais (Siddiqui *et al.*, 2011; Laviano *et al.*, 2013; D'Eliseo & Velotti, 2016), em concordância com nossos resultados. Além disso, em contraste com terapias tradicionais, PUFA ômega 3 parecem causar citotoxicidade seletiva direcionada às células tumorais com pouca ou nenhuma toxicidade às células normais (D'Eliseo & Velotti, 2016). Este efeito benéfico do EPA e DHA tem sido atribuído às suas propriedades antiinflamatória, antiproliferativa, pró-apoptótica, antiangiogenética, antiinvasiva e antimetastática, que tornam estes compostos potenciais adjuvantes na quimioterapia (Merendino *et al.*, 2013). Nesta linha de pensamento, nosso trabalho também evidenciou que o tratamento combinado de CPt e DHA foi capaz de exercer um efeito antiproliferativo e antiinvasivo nas linhagens celulares WM266-4 e B16F10. É de extrema importância considerar que, mesmo usando uma baixa concentração de CPt (1 μM), em combinação com o DHA, foi possível obter um efeito significativamente maior do que usando os compostos sozinhos. Este resultado é de grande valia uma vez que sugere uma possibilidade promissora do uso de baixas concentrações de CPt com DHA com o propósito

de obter o mesmo efeito de concentrações maiores da droga e, assim, evitar os efeitos colaterais deste quimioterápico.

Concordando com a literatura, nossos trabalhos demonstraram que, de fato, PUFA ômega 3 apresentam uma atividade antiproliferativa importante em células de melanoma murino e humano. Uma vez que o ALA, em diferentes concentrações, mostrou um efeito de inibição de proliferação na linhagem B16F10 e teve mínimo efeito na linhagem Melan-a, sugerindo uma atividade antitumoral. Resultado importante, uma vez que um agente terapêutico necessita ser tóxico às células tumorais sem danificar as células normais, ou causando menores danos. Além disso, o DHA também mostrou um efeito inibitório no crescimento das células B16F10 e WM266-4. De fato, muitos estudos experimentais usando PUFA ômega 3 têm evidenciado uma atividade inibitória no crescimento de uma variedade de linhagens celulares tumorais (Calviello *et al.*, 2004; Calviello *et al.*, 2007 a; Serini *et al.*, 2008; Calviello *et al.*, 2006, Calviello *et al.*, 2007 b; Slagsvold et al., 2010; D'Eliseo & Velotti, 2016), incluindo células de melanoma (Serini *et al.*, 2012; Serini *et al.*, 2014).

Atualmente, terapias combinadas com produtos naturais têm se mostrado promissoras em vários tipos de câncer, em especial, no melanoma (da Rocha *et al.*, 2001; Mishra & Tiwari, 2011; AlQathama & Prieto, 2015). As terapias combinadas merecem destaque uma vez que já foi demonstrado que células de melanoma podem adquirir resistência ao tratamento quimioterápico. Como descrito anteriormente, alguns autores mostraram que células de melanoma foram resistentes ao tratamento com CPt, pois esta droga induz a expressão de proteínas de reparo do DNA (Kirschner & Melton, 2010; Li & Melton, 2012). Em nosso trabalho demonstramos que a combinação entre CPt e PUFA ômega 3 foi capaz de vencer a resistência a esse quimioterápico.

Em relação ao tratamento com a radiação UVB, nossos resultados demonstraram que a radiação foi citotóxica nas maiores doses tanto para a linhagem tumoral quanto para a não tumoral. Em contrapartida, a combinação de UVB e ALA para a linhagem Melan-a, na menor dose de ALA, foi fotoprotetora; entretanto, para a maior dose de ALA, foi possível demonstrar o processo de ação fotodinâmica em ambas as linhagens, com maior sensibilidade para a linhagem tumoral. Assim, ALA apresentou uma atividade fotoprotetora, além de um efeito de ação fotodinâmica dose e tempo dependente nas linhagens celulares Melan-a e B16F10. A radiação UV tem sido descrita como um agente capaz de induzir a expressão de COX-2, uma proteína usualmente não detectável na maioria dos tecidos epiteliais normais. Sua superexpressão está relacionada a um papel estabelecido na carcinogênese (Dempke *et al.*, 2001; Sobolewski *et al.*, 2010). Neste sentido, tem sido demonstrado que PUFA ômega 3 são capazes de reduzir a superexpressão de COX-2 em diferentes tipos de células tumorais (Calviello *et al.*, 2004; Calviello *et al.*, 2007 b; Lim *et al.*, 2009). Assim, o efeito de fotoproteção observado neste trabalho poderia estar relacionado a este mecanismo.

Estudos prévios têm reportado que PUFA ômega 3 têm o potencial de proteger a pele da radiação UV através de muitos mecanismos, incluindo alterações na fluidez da membrana, modificação da transdução de sinal, ativação de fator de transcrição, produção de mediadores lipídicos bioativos, que medeiam respostas inflamatória e imune e inibição de certos marcadores genotóxicos de dano ao DNA induzido pela radiação UV, como a p53 induzida pela radiação UV e modulação do estresse oxidativo (Pilkington *et al.*, 2011; Black & Rhodes, 2016). Contudo, não evidenciamos alteração nos níveis de ROS nos tratamentos com ALA para ambas as linhagens celulares estudadas, Melan-a e B16F10. Em relação ao UVB, demonstramos uma diminuição nos níveis de ROS nas primeiras 24 h de tratamento apenas para a linhagem B16F10. Sugerimos que este efeito de diminuição de ROS em 24 h esteja relacionado à quantidade de melanina presente nestas células, capaz de proteger

contra danos oxidativos. Contudo, este efeito não foi observado em 48 h. Esta resposta poderia estar relacionada às doses de UVB utilizadas no experimento. Uma vez que é bem estabelecido que o principal alvo da radiação UVB é o DNA (Ichihashi *et al.*, 2003), os resultados sugerem que a dose não foi suficiente para causar um aumento significativo nos níveis de ROS ou que um aumento na atividade de proteínas antioxidantes (Black *et al.*, 2008) poderia ter sido suficiente para inibir um estresse oxidativo.

Naturalmente, as células da pele apresentam mecanismos de prevenção de dano de DNA, que possa levar à carcinogênese, tais como parada do crescimento e morte celular. Além disso, como observado neste estudo, ALA apresentou um efeito benéfico de inibição de proliferação em células de melanoma, desta forma, foi relevante analisarmos se este PUFA ômega 3 poderia estar regulando o ciclo celular ou influenciando no processo de morte celular. Nossos resultados mostraram um aumento no número de células na fase S/G2/M do ciclo celular em 24 e 48 h para a linhagem B16F10 nos tratamentos usando ALA + UVB e UVB sozinho. Este efeito foi também evidenciado para a linhagem Melan-a em 48 h. Este efeito possivelmente tenha sido observado apenas nos tratamentos com UVB, uma vez que parada do ciclo celular em G2 parece ser característica de resposta de células melanocíticas normais e malignas à radiação UVB (Bolognia *et al.*, 1994; Pavey *et al.*, 2001; Marrot *et al.*, 2005). Desta forma, estes autores corroboram os resultados obtidos neste trabalho que demonstrou para ambas as linhagens celulares, uma maior porcentagem de células na fase S/G2/M, permitindo sugerir que, após o tratamento com a radiação UVB, a maioria das células estão na fase G2. Em relação à morte celular, foi demonstrado que a adição de ALA em combinação com UVB e UVB sozinho induziram um aumento no número de células hipodiplóides nas linhagens B16F10 e Melan-a em 24 h. E em 48 h, foi possível observar uma diferença significativa entre os tratamentos usando ALA + UVB e UVB,

demonstrando que ALA foi capaz de atenuar o efeito do UVB em ambas as linhagens celulares, confirmando, assim, um processo de fotoproteção.

Finalmente, destacamos a importância deste estudo, uma vez que evidenciamos não só um efeito de fotoproteção atribuído ao ALA, como também um processo de ação fotodinâmica, dose e tempo dependente em células melanocíticas normais e tumorais. Ainda demonstramos que estes efeitos não tiveram a participação de alteração nos níveis de ROS. Interessantemente, mostramos que ambas as linhagens, B16F10 e Melan-a apresentaram atividade de Pgp, ressaltando um mecanismo de resistência importante. Também confirmamos a habilidade do DHA em sensibilizar células de melanoma ao efeito inibitório no crescimento e antiinvasivo exercido *in vitro* pela CPt. Além disso, nossos resultados ainda atestam que o DHA pode exercer seu efeito benéfico por modular a via de sinalização DUSP-6/p-ERK/ERCC1. Os dados mostrados podem ser de extremo valor, uma vez que demonstramos que o DHA é potencialmente capaz de reverter a resistência de células de melanoma ao agente antineoplásico CPt, sugerindo, desta forma, o uso potencial desta combinação na terapia do melanoma metastático, permitindo uma possível estratégia para melhorar a eficácia da CPt neste tipo de tumor, assim como, uma redução de efeitos colaterais frequentemente associados a altas doses desta droga antineoplásica.

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