Universidade Federal Do Rio Grande Do Sul
Programa de Pós-Graduação em Genética e Biologia Molecular

Tese de Doutorado

AVALIAÇÃO DOS MECANISMOS DE AÇÃO DO CO-TRATAMENTO DOS AGENTES BEVACIZUMAB E ERLOTINIB E DO DITELURETO DE DIFENILA VISANDO A APLICAÇÃO DESTES AGENTES NA TERAPIA ANTITUMORAL

Cristiano Trindade

Porto Alegre, 2017

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Tese submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular como requisito parcial para a obtenção do grau de Doutor.

Cristiano Trindade

Orientador: João Antonio Pêgas Henriques

Porto Alegre, 19 de julho de 2017

"Para que levar a vida tão a sério

Se ela é uma incansável batalha

Da qual jamais sairemos vivos"

Bob Marley

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No final desta etapa, são muitos os amigos e colegas a quem gostaria de agradecer o auxílio, estímulo e companheirismo. Meus sinceros agradecimentos a todos que contribuíram direta ou indiretamente, para realização e concretização deste trabalho.

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(pipeta) calibrada no laboratório hahahaha Gio, você sabe que tenho uma grande admiração pelo seu trabalho, obrigado por tudo que você fez a apoiou comigo.

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ESTRUTURA DA TESE

O presente trabalho está dividido na seguinte forma: Introdução Geral, Objetivos (gerais e específicos), três capítulos escritos na forma de artigos científicos, Discussão Geral, Conclusões, Perspectivas, Referências Bibliográficas e Anexo I e II.

A Introdução Geral contempla um breve comentário sobre as estatísticas, epidemiologia do câncer e os diferentes processos envolvidos no processo de caircinogênese, uma síntese dos tipos de quimioterápicos utilizadas no tratamento do câncer e algumas novas terapias antitumorais que estão em desenvolvimento. Dando sequência, a introdução aborda brevemente estudos sobre os compostos de telúrio, principalmente o ditelureto de difenila.

O Capítulo I descreve os resultados do tratamento com os agentes Bevacizumab e Erlotinib utilizando diferentes modelos de estudo de câncer colorretal. Neste Capítulo, buscou-se identificar as principais vias celulares ativadas em resposta ao co-tratamento de ambos agentes. Estes resultados deram origem a um manuscrito que será submetido à revista *Clinical Cancer Research.*

No Capítulo II, estão ilustrados os resultados obtidos com o estudo do Ditelureto de Difenila (DTDF) em linhagens de fibroblastos de Hamster chinês (V79). Utilizando diferentes mutágenos como agentes tóxicos, o pré tratamento em baixas concentrações de DTDF conferiu efeito protetor, possivelmente por sua atividade antioxidante. Estas observações foram publicadas na revista M*utagenesis.*

O Capítulo III é uma mini-revisão com dados sobre o DTDF. Nessa revisão, são abordados os principais resultados sobre os efeitos protetores e antitumorais do DTDF em diferentes modelos biológicos.

A Discussão Geral contempla os comentários sobre os resultados apresentados nos três Capítulos e a sua importância para a contribuição científica deste estudo. Após, estão descritas as Conclusões e as Perspectivas geradas por este trabalho, as Referências Bibliográficas utilizadas na elaboração desta Tese.

Como parte da formação, em anexo esta um artigo relacionado publicado durante o período do Doutorado, do qual sou primeiro autor.

RESUMO

O objetivo deste trabalho foi investigar os mecanismos de ação da combinação dos quimioterápicos bevacizumab e erlotinib em modelos de câncer coloretal *in vitro* e *in vivo* e o potencial antigenotóxico, antimutagênico e antiproliferativo do ditelureto de difenila *in vitro*, visando a aplicação destes agentes na terapia antitumoral. As combinações de bevacizumab com uma pequena molécula inibidora de EGFR, erlotinib, são significativamente mais ativas que o bevacizumab sozinho em modelos CRC independente da presença de *KRAS* mutado. Nossos resultados mostram que o tratamento com bevacizumab provocou níveis aumentados de VEGFR1, VEGFR2 e EGFR fosforilados ativos em todos modelos de celulares, bem como nas células endoteliais associadas ao tumor que são atenuadas na presença de erlotinib e que erlotinib regula a secreção de anfiregulina e TGF-alfa *in vitro* como *in vivo* independentemente do status *KRAS* e sensibilidade ao bevacizumab*,* aumentando assim a atividade antitumoral. Ditelureto de difenila (DTDF) é um composto orgânico de telúrio que demonstrou atividades antioxidantes interessantes, tanto *in vitro* como *in vivo*. No presente estudo, DTDF não foi citotóxico e genotóxico em concentrações variando de 0,01 a 0,1 μM. O pré-tratamento com DPDT por 2h nestas concentrações mostrou propriedades antioxidantes, antigenotóxicas e antimutagênicas contra peróxido de hidrogênio (H_2O_2), *tert*-butil hidroperóxido (*t*-BOOH), metil metanossulfonato (MMS) ou radiação ultravioleta (C) em células V79. Os nossos resultados demonstraram que as células tratadas com o DTDF nas concentrações de 0,01, 0,05 e 0,1 μmol não aumentaram os níveis de TBARS e produção de ROS. Entretanto, observou-se um aumento da intensidade dos ROS e na atividade da enzima superóxido dismutase (SOD) após o tratamento com o DTDF, sugerindo um efeito pró-oxidante desse composto. Além disso, DTDF mostrou propriedades antitumorais em várias linhagens celulares de câncer. Em conjunto, nossos resultados elucidam aspectos importantes do mecanismo de ação dos agentes bevacizumab, erlotinib e DTDF, destacando as atividades promissoras dessas drogas que, empregadas como agentes únicos ou em associação com quimioterápicos, podem contribuir para melhoramento dos protocolos de terapia antitumoral.

ABSTRACT

This study investigated the mechanisms of action of the combination of chemotherapeutics bevacizumab and erlotinib in colorectal cancer models *in vitro* and *in vivo* and the antigenotoxic, antimutagenic and anti-proliferative potential of diphenyl ditelluride, aiming the application of these agents in antitumor therapy. The combinations of bevacizumab with a small molecule EGFR-inhibitor, erlotinib, are significantly more active than bevacizumab alone in CRC models with different *KRAS* status and bevacizumab sensitivity. Our results show that bevacizumab treatment provoked increased levels of active, phosphorylated VEGFR1, VEGFR2 and EGFR in all tumor models as well as in the tumor-associated endothelial cells which is attenuated in the presence of erlotinib and that erlotinib down-regulates the secretion of amphiregulin and TGF-alpha *in vitro* as *in vivo* irrespective of *KRAS* status, increasing the antitumor activity. Diphenyl ditelluride (DPDT) is an organotellurium compound that demonstrated interesting antioxidant activities both in vitro and in vivo. In the present study, DPDT was not cytotoxic and genotoxic at concentrations ranging from 0.01 to 0.1 μM. The pre-treatment for 2h with DPDT showed antioxidant, antigenotoxic and antimutagenic properties against hydrogen peroxide (H_2O_2), *t*-butyl hydroperoxide (t-BOOH), methyl methanesulphonate (MMS) or ultraviolet (UV)C radiation in V79 cells. Our results demonstrated that DPDT-treated cells at concentrations of 0.01, 0.05 and 0.1 μmol did not change TBARS levels and ROS generation. However, the observed increased intensity of ROS foci and increased superoxide dismutase activity (SOD) following DPDT treatment suggest pro-oxidative effect of this compound. Furthermore, DPDT showed anticancer properties in several cancer cell lines. Taken together, our findings elucidate important aspects of the mechanism of action of the agents bevacizumab, erlotinib and DPDT, highlighting the promising activities of these drugs which, employed as single or in association, might contribute for the antitumor therapy.

Sumário

Lista de abreviaturas e siglas

5-FU: 5-Fluorouracil.

AS101*: Amonium trichloro (dioxoethylene-O,O') tellurate*

ATM: Cinase ataxia telangiectasia mutada (*ataxia telangiectasia mutated kinase*).

ATR: Cinase ataxia telangiectasia e RAD3 relacionada (*ATM and RAD3-related kinase*).

BSA: Albumina sérica bovina (*Bovine Serum Albumin*).

CDK: Cinase dependente de ciclina (*cyclin-dependent kinase*).

CHK1: Cinase checkpoint 1 (*checkpoint kinase 1*).

CHK2: Cinase checkpoint 2 (*checkpoint kinase 2*).

DAPI: 4'-6-Diamidino-2-fenilindol (*4',6-diamidino-2-phenylindole*)

DDR: Resposta a danos ao DNA (DNA Damage Response).

DNA: Ácido desoxirribonucléico (*deoxyribonucleic acid*).

DNA-Pk: Proteína cinase dependente de DNA (*DNA-dependent protein kinase*).

DOX: Doxorrubicina.

DSB: Quebra de cadeia dupla de DNA (double strand break)

DTDF: Ditelureto de difenila

EGFR: Receptor do fator de crescimento epidermal (*epidermal growth factor receptor*).

FDA: *Food and Drug Administration*.

FdUMP: Monofosfato de 5-fluordeoxiuridina.

GABA: Ácido γ-aminobutírico.

GPx: Glutationa peroxidase.

GSH: Glutationa.

GST: Glutationa-S-transferase.

HER2/ErbB2: Receptor do fator de crescimento epidermal humano 2

INCA: Instituto Nacional de Câncer.

MGMT: O-6-metilguanina metiltransferase.

MMS: Metil metano sulfonato

MTT: *3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide*.

OMS: Organização Mundial da Saúde.

PARP: Poli (adenosina difosfato ADP-ribose) polimerase.

PBS: Tampão fosfato salina.

PI3K: Fosfatidilinositol-3-quinase.

RTK: Receptores tirosina quinases

SOD: Superóxido dismutase.

TOP: Topoisomerases.

TR: Tiorredoxina redutase.

UV: Radiação ultravioleta.

VEGF: Fator de crescimento vascular endotelial (*vascular endothelial growth factor*).

VEGF-R: Receptor de fator de crescimento do endotélio vascular (vascular endothelial growth factor receptors).

Lista de Figuras

1. Introdução Geral

1.1 Epidemiologia do câncer

O câncer é uma doença resultante de sucessivas alterações genéticas capazes de conferir diversas características que viabilizam a sobrevivência e proliferação de células durante o processo de tumorigênese (Hanahan & Winberg, 2011). As características observadas nas células tumorais incluem: (i) a capacidade de manter a sinalização proliferativa; (ii) a evasão de sinais supressores de crescimento; (iii) a evasão da destruição imune; (iv) o potencial replicativo ilimitado; (v) a promoção de inflamação; (vi) a ativação da invasão e metástase; (vii) a indução da angiogênese; (viii) a instabilidade genômica e mutações; (ix) a resistência à morte celular e (x) a desregulação do metabolismo energético (Fig. 1).

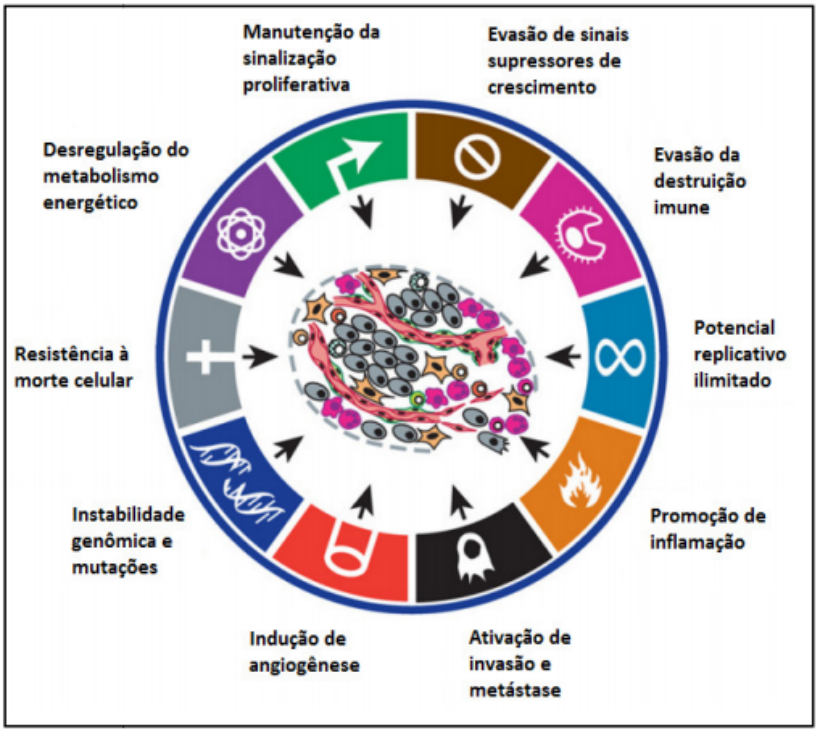

Figura 1. Caracterização das células tumorais. Figura adaptada de Hanahan & Winberg (2011).

De acordo com os dados mais recentes da Organização Mundial da Saúde (OMS), o câncer é a principal causa de morte nos países economicamente desenvolvidos, sendo considerada a segunda causa principal de óbitos nos países em desenvolvimento (Jemal *et al*., 2011). A Agência Internacional para Pesquisa sobre o Câncer (IARC/OMS) estimou em 2011 que ocorressem 12,4 milhões de casos novos e 7,6 milhões de óbitos por câncer no mundo. Destes, os mais incidentes foram o câncer de pulmão (1,52 milhões de casos novos), mama (1,29 milhões) e cólon e reto (1,15 milhões). Devido ao mau prognóstico, o câncer de pulmão foi considerado a principal causa de morte (1,31 milhões), seguido pelo câncer de estômago (780 mil óbitos) e pelo câncer de fígado (699 mil óbitos). Para América do Sul, Central e Caribe, estimou-se cerca de um milhão de casos novos de câncer e 589 mil óbitos. Em homens, o mais comum foi o câncer de próstata, seguido por pulmão, estômago, cólon e reto. Nas mulheres, o mais frequente foi o câncer de mama, seguido do colo do útero, cólon e reto, estômago e pulmão (IARC/OMS, 2011). Para o ano de 2030, a OMS estima que o câncer seja a causa de mais de 11 milhões de óbitos no mundo (OMS, 2012).

No Brasil, o câncer é considerado a segunda maior causa de mortes por doenças (Fig. 2), estimando-se, em 2016, números de novos casos bem maiores do que os encontrados em 2014 (576.580 mil novos casos e 122.600 mortes), demonstrando estatisticamente que a cada ano há um aumento de novos casos de câncer e mortes consecutivamente (INCA, 2016).

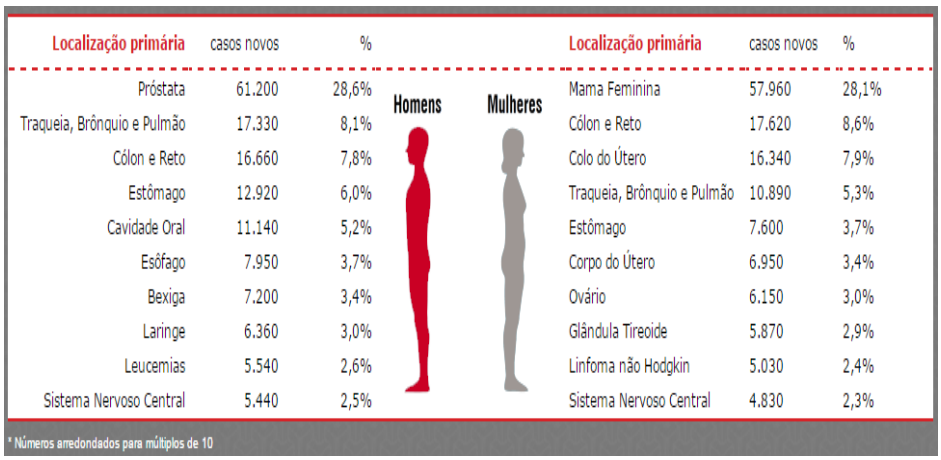

Figura 2. Distribuição proporcional dos dez tipos de câncer mais incidentes, estimados para 2016 por sexo, exceto pele não melanoma (INCA, 2016).

1.2 Processo de morte celular

Tratando mais especificamente da questão das mutações que decorrem em potenciais falhas nos mecanismos de morte celular programada e que podem determinar o aparecimento de um câncer, faz-se necessário entender que fisiologicamente o equilíbrio entre a sobrevivência e a morte das células está sob o estrito controle genético. Quando uma célula normal é exposta à lesão bioquímica, biológica ou física; ou estiver privada de substâncias necessárias à sua sobrevivência, ela ativa uma série de genes de resposta ao estresse. A partir daí, em alguns casos, esta célula pode eventualmente se recuperar, mas geralmente o que ocorre é a sua morte através de um mecanismo programado (Finlay *et al*., 2017).

Neste mecanismo de controle, tem sido considerado que a apoptose deve desempenhar um papel de protagonista. No entanto, se sabe que se o evento acomete um grande número de células, então isto sugere necrose, que é geralmente acompanhada de resposta inflamatória (Finlay *et al*., 2017). A necrose é um tipo de morte celular que ocorre quando a célula é exposta a um estado de estresse

extremo. Neste caso, a membrana plasmática é destruída, o conteúdo citoplasmático extravasa para o meio intercelular e em princípio, o núcleo permanece íntegro (Di Giacomo *et al*., 2017; Finlay *et al*., 2017). Apesar de a necrose ser tradicionalmente tratada como um destino acidental de morte para as células, alguns estudos recentes têm demonstrado que este mecanismo pode representar um destino autodeterminado da célula, que pode ser induzido via vários estímulos, tais como estresse, ativação de canais iônicos ou lesão no DNA. Além disso, nos casos onde as vias de apoptose estão deficientes ou ausentes, como observado em alguns casos na carcinogênese, a necrose parece por vezes possibilitar uma forma alternativa de morte celular (Junying & Kroemer *et al*., 2010, Galluzzi *et al*. 2016).

Se por um lado, as falhas nos mecanismos de morte celular podem ser a causa para um câncer, por outro, a ativação destes mecanismos é a explicação para o efeito antitumoral de muitos dos agentes utilizados para o tratamento desta doença (Galluzzi *et al*. 2016). A apoptose tem papel central na patogênese de várias doenças humanas quando os mecanismos de controle apoptótico estão suprimidos, super ou sub expressos ou alterados por mutação (Deka & Singh, 2017). Em princípio, a apoptose é considerada um processo fisiológico de morte celular pelo qual uma única célula pode ser eliminada do tecido vivo. Uma vez que o processo é mediado por proteínas específicas codificadas no genoma do hospedeiro, a apoptose é considerada um processo de morte celular programada (Galluzzi *et al*. 2016).

O processo apoptótico pode ser iniciado por duas vias, intrínseca ou extrínseca (Fig 3). Na via extrínseca (ou de receptores de morte celular), moléculas sinalizadoras de morte extracelulares, entre as quais se destacam FasL (ligante de

Fas), TRAIL (ligante indutor de apoptose relacionado ao TNF) e TNF (fator de necrose tumoral) se ligam a receptores de morte celular da superfície celular, desencadeando a clivagem e ativação da proteína caspase-8 que, por sua vez, induz a clivagem e ativação de caspases efetoras do processo apoptótico (Deka & Singh, 2017).

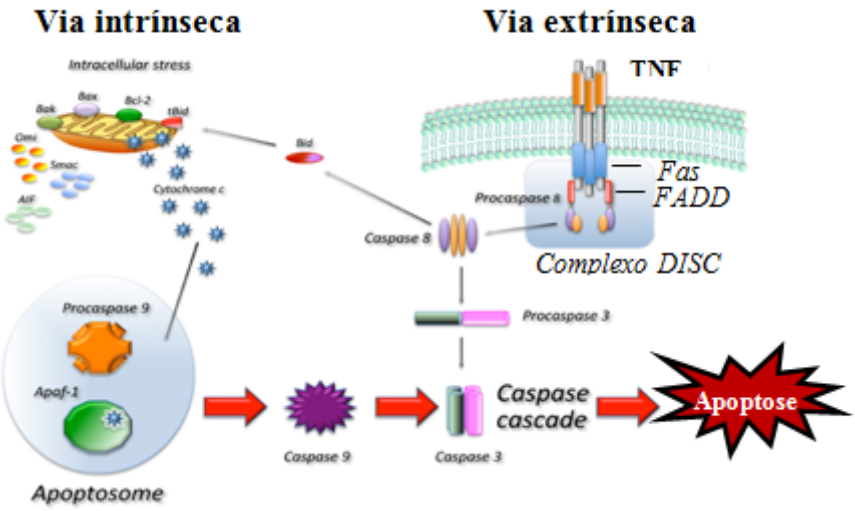

Figura 3 – Via intrínseca e extrínseca de apoptose. Adaptado de Favarolo *et al*., (2012).

A via intrínseca (ou mitocondrial) de apoptose, ativada quando a célula sinaliza algum dano ao DNA, estresse do retículo endoplasmático ou estresse oxidativo, é modulada por uma família de proteínas, a Família Bcl-2, cujos membros apresentam em sua estrutura um ou múltiplos domínios homólogos de Bcl-2 (domínio BH3) (Favarolo *et al*., (2012).

1.3 Receptores tirosina quinases (RTK)

A comunicação celular é mediada por alguns mecanismos, como a secreção de moléculas específicas, proteínas sinalizadoras ou até mesmo pela comunicação célula-célula. Todos esses processos são realizados com o objetivo de controlar

ações isoladas de um grupo de células, ou até mesmo de todo o órgão (Wee & Wang, 2017). Os receptores tirosina quinases (RTKs) são moléculas presentes na superfície das células e correspondem a uma família de proteínas altamente conservadas no processo evolutivo, descritas como chave regulatória de processos celulares como proliferação, migração e ciclo celular (para revisão ver Morin-Bem & Hirsh, 2017). Estes receptores possuem uma estrutura básica, onde se destaca um domínio N-terminal na região extramembrana, com alta afinidade a ligantes específicos; uma simples hélice de ancoragem transmembranica e, por final, uma região intracelular que possuem resíduos tirosina quinases na porção C-terminal da proteína (Wee & Wang, 2017).

Ancorados na membrana citoplasmática, todos os RTK se encontram na forma de monômeros, ou seja, individualizados. Entretanto, existe uma exceção, no qual o receptor de insulina já se encontra no estado dimerizado e inativo. A dimerização destes receptores acontece por meio da estimulação de ligantes específicos, capazes de alterar o estado conformacional da proteína receptora, além de autofosforilar os resíduos intracelulares. Estes resíduos ativam uma cascata de sinalização de proteínas quinases intracelulares responsáveis por importantes processos biológicos, entre eles, a proliferação celular (Morin-Bem & Hirsh, 2017; Wee & Wang, 2017).

1.4 Família ErbB

A família ErbB de proteínas tirosina quinases é composta por quatro membros que incluem o EGFR (Epidermal growth factor receptor), ErbB2/Neu/Her, ErbB3/Her3 e ErbB4/Her4 (Henson *et al*., 2017). A região extracelular dos receptores desta família apresenta quatro domínios, sendo que os domínios I e III

possuem aproximadamente 160 aminoácidos e os domínios II e IV são regiões ricas em cisteínas, constituídas por aproximadamente 150 aminoácidos. A dimerização de receptores ErbB é inteiramente mediada pelo ligante e provoca simultaneamente a ligação do receptor em dois locais (domínio I e domínio III) dentro da mesma molécula (Henson *et al*., 2017). Este fato promove mudanças conformacionais no receptor que libera a região do domínio II, anteriormente bloqueada, promovendo a dimerização de dois receptores na membrana citoplasmática (para revisão ver Henson *et al*., 2017).

A ativação dos receptores da família ErbB é modulada pela produção, secreção e ligação de proteínas ligantes naturais, que induzem a ativação de quinases intrínsecas. Esta ligação mediada por ligantes tais como: anfiregulinas, betacelulina, fator de crescimento epidermal (EGF), epiregulina, neuroregulina, EGF ligado à heparina, fator de crescimento transformante alfa e epigenina. Este mecanismo de fosforilação pode recrutar proteínas adaptadoras específicas levando a modulação de vias intracelulares (Miyamoto *et al*., 2017). Os quatro receptores membros desta família podem formar 10 possíveis combinações de dímeros, sendo 4 homodímeros e 6 heterodímeros. Cada combinação formada de receptores tem afinidade específica nos sinalizadores efetivos de vias intracelulares. De acordo com um estudo realizado com linhagens celulares, transfectadas com diferentes combinações heterodímicas dos receptores da família ErbB, existem algumas combinações que favorecem o aumento da proliferação celular, como no caso das combinações entre ErbB1/ErbB2, ErbB2/ErbB e ErbB1/ErbB3 (para revisão, ver Henson *et al*., 2017; Morin-Ben & Hirsh, 2017; Miyamoto *et al*., 2017).

Devido à diversidade de combinações e a íntima associação com regulações de vias intracelulares, o fenótipo celular pode ser influenciado pela diferença de

expressão destes receptores, como acontece nas células neoplásicas de mama na qual encontramos uma expressão alterada de ErbB2, que possui configurações estruturalmente favoráveis que expõem suas regiões de dimerização. O gene ErBB2, neste caso, assume o papel de um oncogene, quando ocorrem alterações de expressão causadas por amplificação (Miyamoto *et al.,* 2017).

A heterodimerização ErbB1/ErbB2, foi alvo de vários estudos na última década, que comprovaram o papel cooperativo destes receptores na tumorigênese. Estes estudos demonstraram um mecanismo de desligamento dos receptores após estimulação e dimerização, seguida da endocitose do receptor EGFR por vesículas mediadas por clatrina. A taxa de endocitose dos outros membros da família ErbB é baixa, em contrapartida, as taxas de reciclagem e nova ancoragem citoplasmática são maiores quando comparadas ao EGFR. Sendo assim, estes estudos observaram uma redução das taxas de endocitose e degradação e aumento da reciclagem de ErbB1 na associação ErbB1/ErbB2 (Henson *et al*., 2017).

1.4.1 Ativação do receptor ErbB1 (EGFR)

O gene EGFR está localizado no cromossomo 7 e codifica uma proteína madura que possui 170 kDa, formada por 1186 aminoácidos. O domínio intracelular do EGFR contém um resíduo de serina (Ser-1142), um resíduo de treonina (Thr-654) e um total de sete resíduos de tirosina (Try-845, 992, 1045, 1068, 1086, 1148, 1173). Estes resíduos de tirosina podem ser fosforilados de acordo com o estímulo indutor. O domínio intracelular do EGFR pode ativar várias vias intracelulares incluindo PI3K, MAPK, STAT e AKT (Fig. 4) (para revisão ver Appert-Collin *et al*., 2015; Henson *et al*., 2017). De forma detalhada, o EGFR ativa PI3KCA com o auxílio de uma proteína adaptadora chamada GRB2, que recruta outro adaptador chave conhecido como

GAB1, sendo que ambas as proteínas são capazes de ativar a cascata de sinalização mediada pela ativação de PI3KCA. Diferentemente, o receptor ErbB2 pode ativar a via MAPK, com auxílio da proteína adaptadora GRB2, SHC1, DOK-R e CRK (Cheng *et al*., 2014). Além disso, a fosforilação de EGFR pode ativar um grupo de proteínas da família Src que atuam como proteínas oncogênicas, podendo desencadear um aumento da proliferação celular através da ativação da via das MAPK (Appert-Collin *et al*., 2015).

A via intracelular STAT é constituída por proteínas que atuam como fatores de transcrição de genes alvos e codificam proteínas envolvidas em eventos de proliferação, diferenciação e sobrevivência como as ciclinas D1-D3 e a proteína p27. Os eventos descritos acima sofrem a influência da fosforilação de EGFR. O papel desempenhado pela ativação da via JAK mediada pela fosforilação de EGFR está vinculada a um maior recrutamento e uma ativação preferencial do braço de sinalização intracelular das proteínas da família STAT. Este fato foi comprovado experimentalmente in vitro, quando se inibiu a via JAK e notou-se a redução parcial da ativação de STAT em linhagens tumorais de mama (Cheng *et al*., 2014; Appert-Collin *et al*., 2015; Henson *et al*., 2017).

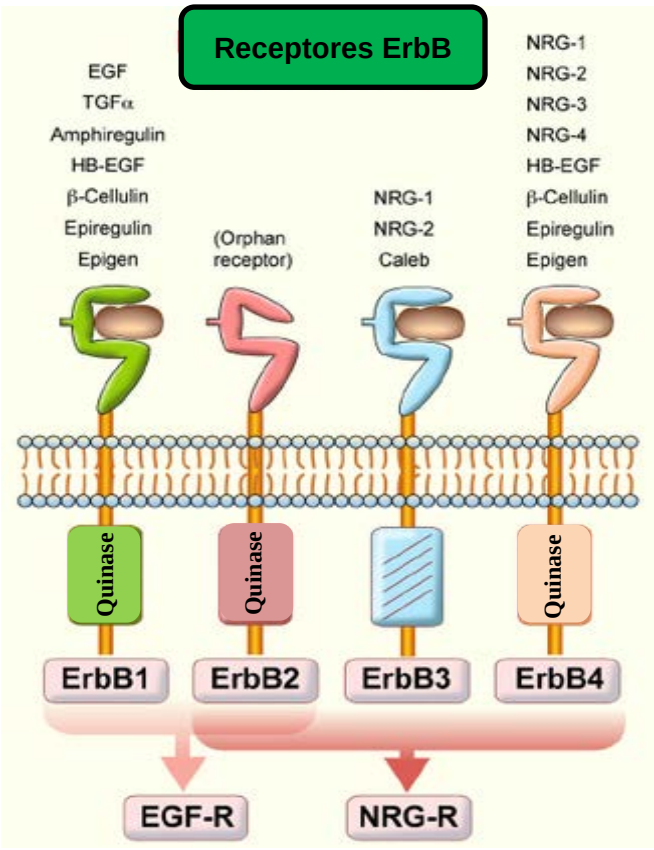

Figura 4. Ativação dos receptores da família ErbB. Adaptado de Guma *et al*., 2009.

1.4.2 O papel do EGFR no desenvolvimento tumoral

Devido á sua importância em processos regulatórios da proliferação celular, o equilíbrio da expressão e/ou ativação do EGFR deve ser mantido para garantir níveis controlados de proliferação celular. Este controle é mediado por uma variedade de mecanismos, incluindo o número de cópias do próprio gene, polimorfismos, *splicing* alternativo, disponibilidade dos ligantes e até mesmo dos outros membros da família ErbB41. De forma geral o EGFR tem expressão alterada em vários tipos tumorais, destacando o câncer de pulmão de células não pequenas (40-80%); câncer de esôfago (60-70 %); glioblastomas (40-60%) e carcinomas pancreáticos (40-90%). A desregulação do EGFR pode ser ocasionada pelos seguintes mecanismos: fatores parácrinos/autócrinos, amplificação do gene, mutações genéticas e translocação

nuclear do EGFR. Todas estas alterações levam à superexpressão ou ativação constitutiva das vias de sinalização mediadas pelo EGFR (Appert-Collin *et al*., 2015; Henson *et al*., 2017).

1.4.2.1 Fatores parácrinos e autócrinos

A transformação neoplásica de um tecido pode ser mediada por mecanismos autócrinos e/ou parácrinos dos ligantes específicos de EGFR, proporcionando o aumento da proliferação celular, sobrevivência, angiogênese, invasão e metástase (Mésage *et al*., 2016). Em pacientes portadores de câncer de cabeça e pescoço, a expressão de EGFR detectada por imunohistoquímica, pode variar de 43-100% dos casos. Esta discrepância entre as taxas de expressão pode ser resultado da variedade de anticorpos, fixadores, tempo de armazenamento da amostra e, por final, variações da técnica. Curiosamente, uma recente meta-análise avaliou o papel prognóstico da expressão de EGFR e encontrou uma correlação com a redução da sobrevida global destes pacientes (Dhomen *et al*., 2012; Batson *et al*., 2017). A superexpressão de EGFR, juntamente com a expressão endócrina dos seus respectivos ligantes, promove múltiplas vantagens que contribuem para a proliferação celular, sobrevivência, angiogênese, invasão e metástase. Uma década atrás, foi comprovado que a co-expressão de EGFR com seus ligantes no microambiente tumoral poderia exercer um importante papel na carcinogênese e progressão celular (Batson *et al*., 2017; Henson *et a.,l* 2017).

A família de genes TNF foi associada a um aumento da proliferação em linhagens de CCECP55, além disso, pacientes com aumento da expressão de TNF

apresentaram uma sobrevida global de 12,98 meses, em comparação a 21,85 meses dos pacientes com a expressão norma (Wang *et al*., 2016).

A superexpressão do ligante anfirregulina (AREG) está associada a um risco aumentado de desenvolver câncer de mama, pulmão, colorretal, ovário e próstata. Nos pacientes portadores de CCECP, a expressão de AREG foi detectada em 4% (12/279) dos pacientes e associada a uma sobrevida global média de 28,29 meses em comparação com 21,75 meses para os pacientes com expressão normal (para revisão ver Lian *et al*., 2016). O fator de crescimento epidérmico ligado à heparina (HBEGF) é um EGF produzido principalmente pelos macrófagos e monócitos. Alguns estudos têm associado a expressão de HBEGF com o desenvolvimento de tumores sólidos com fenótipos agressivos e mecanismos metastáticos em carcinomas de mama. O HBEGF é regulado positivamente em tumores de mama, ovário, gástricos, melanoma e pâncreas (para revisão ver Lian *et al*., 2016).

1.5 Estresse oxidativo: carcinogênese

As espécies reativas de oxigênio (ERO) são produtos normais do metabolismo celular e sua presença nas células pode ser benéfica ou não dependendo da concentração em que estão presentes. Todos os organismos vivos aeróbios utilizam o oxigênio como aceptor final de elétrons na cadeia transportadora de elétrons (Halliwell & Gutteridge, 2007, Gutteridge & Halliwell 2010, Halliwell 2012). As ERO podem ser formadas também por outros mecanismos como: produzidas pelas xantinas oxidases, NAD(P)H oxidases, citocromo P-450-oxidases, pela auto-oxidação de catecolaminas e lipoxigenases (Madamanchi *et al.* 2005; Barrera 2012).

As concentrações de ERO podem variar de acordo com o tipo celular. A sua presença em níveis medianos é importante para processos fisiológicos vitais em diferentes áreas, incluindo sinalização intracelular e regulação redox, assim como defesa contra agentes infecciosos (para revisão ver Carocho & Ferreira 2012). ERO podem atuar como segundos mensageiros na sinalização celular, como a ativação da guanilato ciclase com a formação do segundo mensageiro da guanosina monofosfato cíclica, e indução da divisão celular (Valko *et al*., 2007), a necrose e a apoptose (Carocho & Ferreira 2012; Halliwell 2012). Uma vez que foi observado que a produção de ERO leva à mudanças na transcrição de genes, via regulação redox e fosforilação de fatores de transcrição (Madamanchi *et al.,* 2005; Gutteridge & Halliwell 2010; Prochazkova *et al.* 2011). A regulação de proteínas por oxidação e redução, como ativação e inativação de fosfatases também é fundamental na sinalização celular (Prochazkova *et al.,* 2011; Halliwell 2012).

Quando o acúmulo destas moléculas ultrapassa a capacidade de defesa antioxidante das células, ocorre estresse oxidativo (Ma 2010; van Loon *et al*., 2010), podendo esta condição de estresse ser resultado de: (a) um aumento excessivo na produção das ERO; (b) uma diminuição da capacidade de defesa celular antioxidante; (c) ou ainda por ambos (Halliwell 2007).

Acredita-se que a origem de muitas situações patológicas está relacionada a um estresse oxidativo, sendo que as ERO podem desempenhar um papel importante no desenvolvimento do dano tecidual pelo ataque a biomoléculas como carboidratos, lipídios, proteínas e os ácidos nucléicos (Halliwell & Gutteridge, 2010, Halliwell 2012). Estes são alguns dos motivos para o crescente interesse sobre ERO, desde o início da década de oitenta. Os níveis elevados de ERO no organismo parecem ser um dos maiores responsáveis pelo processo de envelhecimento (Halliwell & Gutteridge,

2010). O ataque oxidativo sobre o DNA pode estar intimamente relacionado às mutações carcinogênicas (Halliwell & Gutteridge, 2010).

Por outro lado, no caso das células cancerosas, alguns estudos têm demonstrado que elas apresentam defesas antioxidantes alteradas e altos níveis basais de ERRO (Trachootham *et al*, 2006). Mais recentemente, este fato tem sido considerado uma vulnerabilidade a ser explorada no tratamento do câncer pelo uso de agentes moduladores do estado redox das células. Estes agentes atacam mais seletivamente as células tumorais. As células normais mantém sua homeostase redox com baixos níveis basais de ERO porque elas têm controle estrito do balanço entre geração e eliminação de ERO. Como consequência, as células normais podem tolerar certo nível de ERO exógenos, algo que as células cancerosas fazem com muito menos eficiência (Trachootham *et al*, 2006; Verrax *et al*., 2011). Neste sentido, pelo menos duas abordagens vêm sendo testadas: 1) o uso de agentes exógenos geradores de ERO e 2) o uso de compostos que inibem o sistema antioxidante (Verrax *et al*., 2011).

1.6 Tratamento do câncer

Em princípio, as três abordagens principais possíveis para o tratamento de câncer são: excisão cirúrgica, radioterapia e quimioterapia. Elas podem ser aplicadas isoladamente e/ou em associação. Estima-se que cerca de 1/3 dos casos possam ser resolvidos por medidas localizadas, feitas por cirurgia e/ou radioterapia. Contudo, a maioria dos casos caracteriza-se pelo desenvolvimento precoce de micrometástases, o que evidencia a necessidade de uma abordagem terapêutica sistêmica, que é realizada pela quimioterapia (Aragon-Ching, 2017).

1.6.1 Terapias antitumorais

A partir do ano 1960 a radioterapia começou a ser utilizada clinicamente para o tratamento de tumores locais. Entretanto, como a cirurgia, a radioterapia não era eficaz contra tumores metastáticos (Chabner & Roberts Jr, 2005). Assim, a busca por tratamentos que atingissem todos os órgãos do corpo, como a quimioterapia, tornou-se o foco dos esforços na cura do câncer.

A quimioterapia baseia-se no emprego de medicamentos antineoplásicos como agentes únicos (monoterapia) ou em associação com outros compostos (multiterapia) (Mésage *et al*., 2016). Em geral, a combinação de medicamentos demonstra ser mais eficiente do que a utilização de um único composto no tratamento de tumores metastáticos e em pacientes com alto risco de relapso após a intervenção cirúrgica (Chabner & Roberts Jr, 2005; para revisão ver Wild & Yamada., 2017) Além da quimioterapia, existem outros tratamentos utilizados contra o câncer, como a hormônio-terapia (estrógenos, anti-estrógenos, anti-andrógenos) e imuno-terapia (antígenos tumor-específicos, estimulantes do sistema imunológico, anticorpos monoclonais) (ACS, 2010). Diferentemente da quimioterapia tradicional, estas terapias enfocam algumas funções das células neoplásicas, o que aumenta a especificidade de ação no tumor e diminui os efeitos tóxicos ao paciente. No entanto, a hormônio-terapia e a imuno-terapia ainda são empregadas como adjuvantes da quimioterapia (ACS, 2010).

De maneira interessante, Bailly (2009) cita que a “guerra contra o câncer” foi iniciada nos anos 1950, ao mesmo tempo em que começou a corrida ao espaço. Enquanto Yuri Gagarin esteve pela primeira vez em órbita, alguns medicamentos antitumorais importantes como a doxorrubicina e mitomicina C foram descobertos

(Bailly, 2009). Nos 50 anos seguidos, várias moléculas foram descobertas, principalmente agentes citotóxicos, dentre os quais um grande número de compostos naturais que ainda são utilizadas na quimioterapia convencional (Fig. 5 Bailly, 2009).

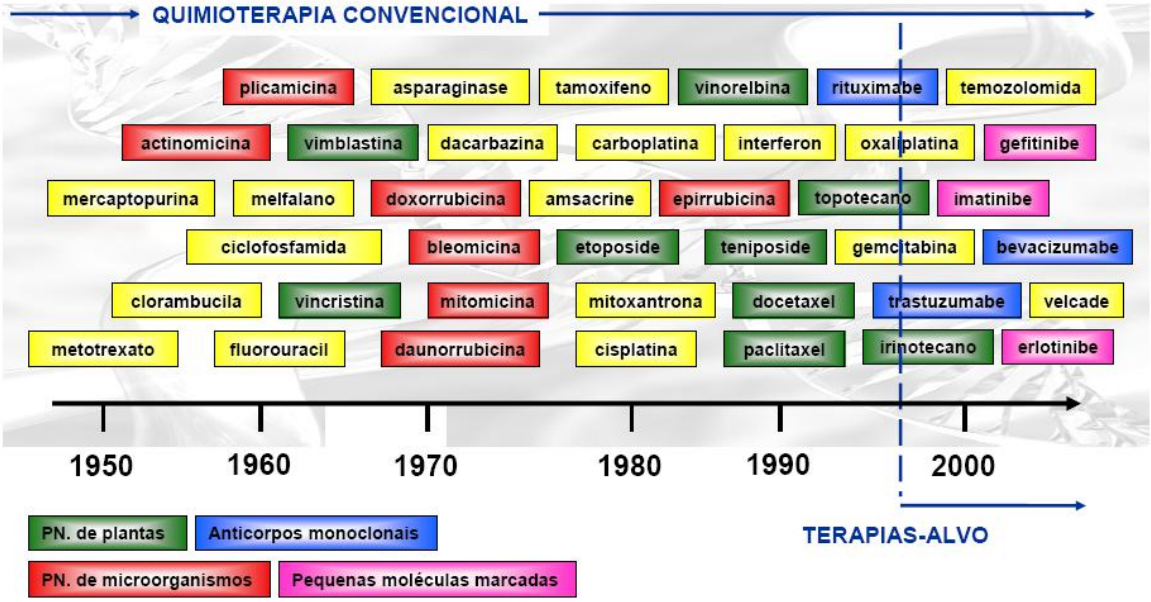

Figura 5. O desenvolvimento dos medicamentos utilizados no tratamento do câncer no período de 1950 - 2000 (adaptado de Baily, 2009). PN: produtos naturais.

Atualmente, os medicamentos utilizados no tratamento contra o câncer podem ser classificados em vários grupos e, considerando o mecanismo de ação, os medicamentos quimioterápicos são divididos principalmente em três grandes classes: inibidores mitóticos, agentes genotóxicos e compostos utilizados como terapia-alvo.

1.6.2 Inibidores Mitóticos

Os inibidores mitóticos são medicamentos que agem durante a mitose, interferindo principalmente nas funções dos microtúbulos, os quais participam da composição do citoesqueleto e estão envolvidos em vários processos celulares, dentre eles a mitose (Nogales, 2001, Penna *et al*., 2017). Na mitose, os microtúbulos formam o fuso mitótico, sendo responsáveis pela localização e separação dos

cromossomos nos pólos da célula (para revisão, ver Penna *et al*., 2017). Atualmente, vários agentes antineoplásicos interferem na atividade dos microtúbulos, levando, consequentemente, à catástrofe mitótica e morte celular. Como exemplos, os taxanos (paclitaxel, docetaxel) e epotilonas (ixabepilona) estabilizam os microtúbulos, enquanto os alcalóides da vinca (vimblastina, vincristina e vinorelbina) agem desestabilizando os microtúbulos (par arevisão, ver Penna *et al.,* 2017). A patupilona e eribulina são alguns exemplos de inibidores mitóticos que estão em fases I e II de testes clínicos para o tratamento do câncer de ovário, respectivamente (Bruning & Mylonas, 2010).

1.**6.3 Agentes genotóxicos**

A descoberta do DNA como molécula alvo na terapia anti-câncer aconteceu no início do século XX, principalmente durante a I e II Guerra Mundial (Hurley, 2002). Durante a II Guerra, em 1946, descobriu-se que os sintomas linfotóxicos apresentados pelos soldados expostos ao gás mostarda eram devidos à atividade genotóxica alquilante da mostarda sulfúrica presente no gás (Chabner & Roberts Jr, 2005; para revisão ver Penna *et al*., 2017). A ação das mostardas nitrogenadas em tumores que possuem uma alta taxa de divisão celular obtiveram efeitos citotóxicos eficazes contra leucemias e linfomas (para revisão ver Penna *et al.,* 2017). Estes resultados, assim como a descoberta da dupla hélice do DNA por Watson e Crick em 1953, encorajaram as pesquisas sobre as interações de pequenas moléculas com os ácidos nucléicos (Lenglet & Cordonnier, 2010). No período entre 1990 e 2006, aproximadamente 18 % das novas moléculas aprovadas pelo FDA para o tratamento do câncer são genotóxicas (Reichert & Wenger, 2008).

Os agentes genotóxicos exercem seus efeitos citotóxicos por interagirem com os ácidos nucléicos, interferindo nas suas funções e levando a vários tipos de lesões. Estes agentes podem causar danos ao DNA de maneira direta, ao se ligarem diretamente na molécula, ou indireta, ao agirem nas enzimas envolvidas na maquinaria nuclear (Granados-Principal *et al.*, 2010). Além destas atividades, alguns fármacos geram radicais livres, que podem promover oxidações das bases do DNA. As lesões geradas no DNA, dependendo do tipo e do número, podem levar à parada do ciclo celular e, caso não sejam passíveis de reparo, podem resultar em morte celular (Helleday *et al.*, 2008, Larsen & Escargueil, 2009, Granados-Principal *et al.*, 2010).

1.6.3.1 Agentes alquilantes

Os agentes alquilantes possuem a capacidade de se ligar covalentemente ao DNA, preferencialmente ao grupamento amino N7 de guaninas (Lenglet & Cordonnier, 2010). Estes compostos podem ser monofuncionais, formando adutos em um único sítio no DNA, como por exemplo, a temozolamida e trabectedina, ou bifuncionais, que se ligam em dois sítios no DNA, como a ciclofosfamida, clorambucila, melfalano bussulfano, mitomicina C e análogos da platina (Mlaadnov *et al.*, 2007, Larsen & Escargueil, 2009). Os agentes bifuncionais também podem levar à formação de pontes covalentes com o DNA, os chamados *crosslinks*. Estas pontes podem ser feitas entre bases da mesma fita (pontes intracadeia) ou fitas opostas do DNA (pontes intercadeia) e ainda estas ligações podem ocorrer entre o DNA e proteínas (Helleday *et al.*, 2008). Como estas pontes bloqueiam a separação das fitas do DNA e impedem o acesso de várias enzimas ao DNA, este tipo de lesão pode levar ao bloqueio de processos fundamentais como a replicação, transcrição e reparação do DNA, levando

consequentemente à morte celular (Hlavin *et al.*, 2010; Bordin *et al.,* 2013). Os agentes alquilantes continuam sendo amplamente utilizados no tratamento do câncer e constituem a base de várias multiterapias, especialmente aquelas empregadas no tratamento de doenças linfoproliferativas (Bordin *et al*., 2013).

1.6.3.2 Inibidores de topoisomerases

As enzimas topoisomerases (TOP) possuem múltiplas funções e desempenham papéis fundamentais nos processos de replicação, transcrição, recombinação, reparação do DNA e organização da cromatina. Particularmente, as topoisomerases relaxam as tensões torsionais da dupla hélice do DNA quando as fitas são separadas durante a passagem das forquilhas da replicação e transcrição (Salerno *et al.*, 2010). Em humanos, a família das topoisomerases é composta por seis enzimas, sendo que as enzimas TOP I e TOP II são os alvos principais de vários medicamentos antitumorais utilizados no tratamento de tumores de mama, pulmão, próstata e sarcomas (para revisão, ver Damiani *et al*., 2017). Por exemplo, os medicamentos antineoplásicos irinotecano, topotecano e belotecano inibem a enzima TOP I enquanto que doxorrubicina, daunorrubicina, etoposídeo e teniposídeo são inibidores da TOP II (para revisão, ver Damiani *et al*., 2017).

Estes compostos agem estabilizando a ligação covalente formada entre o DNA e a topoisomerase, induzindo a imobilização do complexo, ou inibindo a ligação desta enzima ao DNA (Larsen & Escargueil, 2009, Saffi *et al.*, 2010, Salemo *et al.*, 2010). Atualmente, vários compostos que agem nas topoisomerases estão sendo avaliados em ensaios clínicos, como por exemplo, o gimatecano, lurtotecano e exatecano (Pammier *et al.*, 2010; Damiani *et al*., 2017).

1.9.3.3 Antimetabólitos

Os agentes antimetabólitos são capazes de inibir enzimas nucleares necessárias para a síntese de nucleotídeos, e também podem ser incorporados no DNA e RNA, alterando suas estruturas (Larsen & Escargueil, 2009). Os antimetabólitos possuem estrutura similar aos nucleotídeos, moléculas precursoras de nucleotídeos, ou co-fatores necessários para a síntese de nucleotídeos. Assim, os antimetabólitos atuam inibindo o metabolismo dos nucleotídeos, levando à diminuição dos estoques intracelulares destes (Helleday *et al.*, 2008). Os agentes metotrexato e 5-fluorouracil (5-FU) foram introduzidos na terapia antitumoral na década de 1950 e são amplamente utilizados até o momento como agentes únicos ou em associação com outros compostos. O metotrexato, empregado no tratamento de vários tumores como carcinomas de mama, cabeça e pescoço, leucemia e linfomas, é análogo estrutural do ácido fólico. Este medicamento age inibindo a enzima diidrofolato redutase, que é essencial para a síntese de timidina, componente fundamental para a estrutura do DNA (para revisão ver Mazaud, 2017). Por outro lado, o 5-FU, como revisado em dois trabalhos do nosso grupo (Matuo *et al.*, 2009 e 2010), é um medicamento utilizado no tratamento de vários tipos de câncer, principalmente nos tumores de cólon e reto metastáticos, e que apresenta diferentes mecanismos de ação. Por ser análogo estrutural da uracila, o 5-FU é extensivamente incorporado como bases falsas na molécula de RNA, tanto no núcleo quanto no citoplasma, interferindo assim no processamento e função do RNA (Matuo *et al.*, 2009). Além disso, o principal metabólito do 5-FU, o monofosfato de 5-fluordeoxiuridina (FdUMP), tem a capacidade de inibir a enzima timidilato sintase, a qual é fundamental para a biossíntese de pirimidinas. Outros exemplos de compostos antimetabólitos utilizados

na terapia antitumoral incluem a gemcitabina, capecitabina e pemetrexedo (Mini *et al.*, 2006, Silvestris *et al.*, 2010, Galetta *et al.*, 2010).

1.6.3.4 Antibióticos

Os antibióticos antitumorais atuam nas células de diferentes maneiras, como a intercalação no DNA ou geração de danos oxidativos. A bleomicina e a doxorrubicina são exemplos de antibióticos que podem oxidar diretamente a molécula do DNA ou reagir com outros compostos celulares, como os lipídios, levando à formação de espécies reativas de oxigênio ou de nitrogênio (Saffi *et al.*, 2009). Estes radicais livres podem, por sua vez, modificar as bases ou induzir quebras nas pontes de hidrogênio do DNA, levando à formação de sítios abásicos (Hurley, 2002, Granados-Principal *et al.*, 2010). A doxorrubicina além de inibir a ação da topoisomerase, como citado anteriomente, também pode se intercalar entre as bases do DNA, causando alterações na dupla hélice. A dactinomicina, daunorrubicina e epirrubicina são outros exemplos de antibióticos antitumorais intercalantes (Helledey *et al.*, 2008, Saffi *et al.*, 2009, Lenglet & Cordonnier, 2010; para revisão ver Damiani *et* al., 2017).

1.6.4 Terapias-alvo

No início dos anos 90 houve uma explosão na descoberta de terapias-alvo, o que impulsionou a transformação da pesquisa e desenvolvimento de drogas antitumorais, inicialmente financiadas com baixos investimentos por agências governamentais e universidades, em um negócio industrial de bilhões de dólares (Chabner & Roberts Jr, 2005). No período de 1990 a 2006, 920 moléculas

antineoplásicas candidatas começaram a ser avaliadas em ensaios clínicos e dentre as aprovadas pelo FDA (Food and Drug Administration, USA), a maioria foram moléculas sintéticas inibidoras de quiinases (Reichert & Wenger, 2008). Este cenário reflete o avanço das pesquisas que têm como foco principal o desenvolvimento de medicamentos que atuam em diferentes alvos nas células tumorais, as terapias-alvo. Dentre os principais alvos, os fatores de crescimento, moléculas sinalizadoras, enzimas dos sistemas de reparação do DNA e proteínas envolvidas no ciclo celular, apoptose e angiogênese são os mais estudados até o momento (Larsen *et al*., 2011).

O imanitibe foi o primeiro composto antitumoral importante utilizado com terapia-alvo. Ele é um inibidor potente da enzima tirosina quinase BCR-ABL que é um produto da translocação cromossômica envolvida na patogênese da leucemia mielóide crônica (Chabner & Roberts Jr, 2005). Outros inibidores de quinases também foram aprovados pelo FDA para o tratamento do câncer, como o gefitinibe, empregado no câncer de pulmão de células não-pequenas, o sorafenibe, usado no carcinoma de células renais e carcinoma hepatocelular, entre outros (Reichert & Wenger, 2008, Bailly, 2009, Larsen *et al.*, 2011).

O receptor do fator de crescimento epidermal (EGFR) também é alvo de alguns compostos, como o gefitinibe, e o anticorpo monoclonal cetuximabe, empregado no tratamento do câncer de cólon e reto e, mais recentemente, o lapatinibe, aprovado para o tratamento do câncer de mama (Liu *et al*., 2017).

Em 2004 a FDA aprovou o primeiro agente biológico antiangiogênico, o bevacizumabe, que foi aprovado para o tratamento dos tumores de mama, cólon e reto e câncer renal. O bevacizumabe é um anticorpo monoclonal humanizado IgG1 dirigido contra o VEGFA, um membro da família do VEGF e principal mediador da cascata angiogênica. (Reichert & Wenger, 2008; Larsen *et al.*, 2011; Liu *et al*., 2017).

O Bevacizumab está associado a uma baixa incidência de efeitos colaterais graves e não potencializa a toxicidade dos quimioterápicos. O uso de Bevacizumab no tratamento do mCCR tem sido motivo de intenso debate, dado que a maioria dos estudos, quando analisados individualmente, não conseguiram chegar a resultados sobrepostos, deixando também dúvidas em relação ao custo-benefício. Uma recente meta-análise, com o objetivo de avaliar o impacto do Bevacizumab no tratamento de primeira linha do mCCR, demonstrou um aumento da sobrevivência livre de progressão quando em associação com 5-FU/LV ou com esquemas baseados em irinotecan, enquanto os benefícios da combinação com esquemas baseados em oxaliplatina se mostraram menos significativos (para revisão ver Roviello *et al*., 2017).

1.6.4.1 EGFR como terapia alvo: Inibidores de proteínas tirosina quinases

Devido ao grande relevo dos RTK no desenvolvimento tumoral, nas últimas décadas, a comunidade científica e farmacêutica desenvolveu várias estratégias terapêuticas para inibir estas moléculas. Os inibidores das proteínas tirosina quinases podem ser classificados em dois grandes grupos, de acordo com os mecanismos farmacológicos que eles desempenham em contato com o alvo para o qual foram desenvolvidos. A abordagem que utiliza anticorpos monoclonais contra EGFR (MoAb) classifica um grupo destas terapias e outro grupo é representado por pequenas moléculas inibidoras de receptores tirosina quinase (TKI). Estes por sua vez, penetram na membrana celular, inibindo a fosforilação de resíduos específicos no domínio tirosina quinase de cada receptor (Larsen *et al.*, 2011; Liu *et al*., 2017).

O EGFR foi o primeiro recetor de fatores de crescimento a ser alvo de terapia dirigida em contexto de doença neoplásica. Trata-se de um recetor do tipo

tirosina-quinase da família HER, cuja ativação pelo seu principal ligando, o EGF, resulta na sua dimerização e autofosforilação, ativando inúmeras vias metabólicas, como as vias RASRAF-MEK-ERK e PI3K-Akt-mTOR, resultando num aumento da proliferação, crescimento e sobrevivência das células tumorais. A expressão do EGFR está aumentada em 60-75% dos cancer coloretais (CCR) e esta sobre-expressão resulta na sua ativação independente de ligando, constituindo um marcador de mau prognóstico (Larsen *et al*., 2009).

Atualmente são três os anticorpos monoclonais dirigidos contra o EGFR aprovados pela FDA para o tratamento do mCCR. O Cetuximab, um anticorpo quimérico IgG1, e o Panitumumab, um anticorpo totalmente humanizado IgG2 e o erlotinib. Estes estão indicados, quer em monoterapia quer em combinação com QT, no tratamento do mCCR em doentes K-RAS não mutados (*National Comprehensive Cancer Network*. NCCN, 2013)

As mutações do oncogene K-RAS, que estão presentes em aproximadamente 40% dos CCR, resultam na ativação constitutiva, e independente do EGFR, da cascata RAS-RAF-ERK (Brand & Wheeler, 2012). Vários estudos demonstraram que apenas tumores K-RAS não mutados respondem significativamente à terapia com anti-EGFR, alcançando um aumento da sobrevivência livre de progressão, pelo que a pesquisa de mutações do gene K-RAS foi aprovada como indicador preditivo negativo de resposta a estes agentes. Assim, está preconizada a pesquisa da mutação K-RAS em doentes com mCCR, estando aterapêutica anti-EGFR aprovada apenas em doentes *K-RAS* não mutados. Além do seu papel como único biomarcador de resposta aos anti-EGFR, há vidência crescente de que as mutações *K-RAS* estão associadas a doença de pior prognóstico (Normanno *et al*., 2009; Brand & Wheeler, 2012).

1.7 Resistência à quimioterapia

A resistência aos quimioterápicos pode ser tanto intrínseca como adquirida. No primeiro caso, é proposto que fatores que medeiam a resistência já estejam presentes entre as células que compõem o tumor ou que tornaria a terapia antitumoral ineficaz. Já na resistência adquirida é proposto que durante o tratamento do tumor, inicialmente sensível a droga, possam ocorrer mutações em algumas células tumorais, que ativariam vias de sinalização compensatórias, possibilitando que essas células não respondam ao tratamento, o que conferiria a elas vantagens proliferativas em relação a massa tumoral total (Holohan *et al*., 2013).

Estima-se que a resistência à quimioterapia é a causa de fracasso terapêutico em 90% dos pacientes com câncer metastático (Longley, Johnston, 2005). Dessa forma, se fosse possível superar a resistência a drogas o impacto na sobrevida de pacientes seria imensa.

De fato, desde os primórdios da quimioterapia contra o câncer, muita atenção tem sido dada para tentar identificar quais mecanismos de resistência são responsáveis pelo fracasso terapêutico. Dessa forma, verificou-se que vários são os fatores que afetam a sensibilidade celular a uma determinada droga. Esses fatores incluem mecanismos que limitam a quantidade de droga que entra na célula (o influxo da droga). Por outro lado, a resistência ao tratamento pode ser resultado do aumento do efluxo da droga para fora da célula; inativação de processos envolvidos na ativação da droga; alterações que modificam os

alvos celulares da droga; indução da otimização do processamento do dano causado pela droga (Valent *et al*., 2012).

Ademais, os tumores são altamente adaptáveis e a ativação de vias de sinalização pró-sobrevivência e/ou a inativação da via indutora de morte está também intrinsecamente relacionado a quimiorresistência (Debatin, Krammer, 2004). Outro fator que tem sido cada vez mais aceito como fundamental para resistência a quimioterápicos é a presença de células tronco cancerosas (CSC, do Inglês *Cancer Stem Cells*) na massa tumoral, uma vez que tem sido mostrado que CSC são intrinsecamente altamente resistentes a quimioterapia (Singh, Settleman, 2010; Valent *et al*., 2012).

Além disso, é preciso que se tenha sempre em mente que normalmente os tumores possuem um grau elevado de heterogeneidade celular e molecular, o que justificaria que resistência a drogas poderiam ocorrer devido a pressão seletiva induzida pelo tratamento em que um pequeno grupo de células resistentes estaria presente no tumor original (Swanton, 2012).

1.8 Telúrio

O telúrio é um elemento raro, que tem sido considerado como um elemento traço não essencial. No entanto, um corpo humano típico possui 0,5 g de Te, excedendo os níveis de todos os outros elementos em humanos, exceto ferro, zinco e rubídio (Nogueira *et al*., 2004). Seu papel biológico se houver, não foi claramente estabelecido até o presente momento. A investigação de atividades terapêuticas de compostos de telúrio é bastante limitada na literatura, apesar da abundância relativa de telúrio no corpo humano. No entanto, as atividades antioxidantes, miméticas,

imunomoduladoras e antitumorais dos diferentes compostos de telúrio nas células malignas e não malignas são extremamente promissoras, embora muito complexas (Nogueira *et al*., 2004). Não surpreendente, um maior interesse nesse elemento alimentou a busca de novos compostos de telúrio com propriedades farmacológicas (para revisão ver Nogueira *et al*., 2004; Cunha *et al*., 2009).

Os compostos de telúrio podem existir na forma inorgânica e orgânica. Os teluritos e teluratos de metal alcalino podem ser utilizados em microbiologia como antibióticos e antifúgicos, os organotelurídeos e os diorganoditelurídeos possuem uma potente atividade antioxidante e os compostos inorgânicos e orgânicos são potentes inibidores da caspase e catepsina. Muitas substâncias à base de telúrio possuem potencial redox, apresentando o telúrio nos seus estados de oxidação de 6, 5, 4, 2 ou −2 (para revisão ver Chasteen *et al*., 2009).

Os compostos de telúrio que exercem as atividades biológicas mais pronunciadas são AS101 [tricloro de amônio (dioxoetileno-O, O) telurato] e SAS [octa-O-bis- (R, R) -tartarato ditelurano]. AS101, um pequeno composto de telúrio, atualmente em ensaios clínicos de fase II em pacientes com câncer, é um potente imunomodulador (tanto *in vitro* como *in vivo*). As propriedades imunomoduladoras do AS101 foram consideradas cruciais para as atividades clínicas, demonstrando os efeitos protetores do AS101 em modelos de ratos infectados com parasitas e vírus, em doenças auto-imunes, em camundongos sépticos e em doenças renais (para revisão ver Sredni, 2012; Halpert & SrednIl., 2014).

1.8.1 Ditelureto de Difenila (DTDF)

O DTDF é um composto orgânico de telúrio, sólido, e altamente hidrofóbico. É um importante intermediário em reações de síntese orgânica, em especial de drogas contendo telúrio (Muniz Alvarez *et al.* 2005). Esse composto tem sido estudado devido às suas propriedades toxicológicas e interessantes atividades farmacológicas, com fins ao desenvolvimento de novos fármacos organotelurados. (Cunha *et al*., 2009; Hassan *et al*., 2009a). Em concentrações mais elevadas, o DTDF apresenta efeitos tóxicos, como a capacidade de oxidar grupamentos tiólicos em proteínas, afetando uma série de proteínas importantes como as enzimas δ-ALA-D e Na^+/K^+ATPase (Borges *et al*., 2005; Souza *et al*., 2010).

Resultados do nosso grupo mostraram que o DTDF apresenta efeito genotóxico e mutagênico em diferentes modelos biológicos (Degrandi *et al.* 2010). Nesse estudo, o DTDF induziu alteração no quadro de leitura em *Salmonella typhimurium* e em linhagem selvagem haplóide da levedura *Saccharomyces cerevisiae*. Os mutantes de *S. cerevisiae* deficientes na reparação por excisão de bases (BER) e na reparação recombinacional (HR) mostraram elevada sensibilidade ao DTDF. Ainda neste trabalho, foi demonstrado que o DTDF foi citotoxico a partir da concentração de 1 μmol após 2 horas de exposição em células V79. Consistentemente, o tratamento das células por 2 horas com concentrações citotóxicas do DTDF aumentou os níveis de peroxidação lipídica e diminuíram os níveis de GSH/GSSH em levedura e em células V79, indicando que DTDF pode levar ao aumento da peroxidação lipídica e da oxidação da glutationa intracelular, caracterizando um estado de estresse oxidatívo. Além disso, o DTDF induziu danos oxidativos ao DNA, determinados pelo ensaio cometa modificado empregando as

endonucleases formamidopirimidina DNA-glicosilase (Fpg) e endonuclease III (EndoIII) com e sem ativação metabólica. Nas concentrações mais elevadas, o DTDF induziu a formação de quebras simples e duplas de DNA em células V79, como evidenciado pelo ensaio cometa, nas versões alcalina e neutra, na presença e ausência de ativação metabólica. O tratamento com o DTDF também induziu aumento na frequência de micronúcleos em células V79, demonstrando potencial mutagênico dessa molécula em altas concentrações. Entretanto, quando realizado o pré-tratamento com N-acetilcisteina, que restaura o GSH ao nível normal, houve redução dos efeitos oxidativos, genotóxicos e mutagênicos do DTDF em levedura e em células V79 (Degrandi *et al.* 2010).

No estudo de Jorge e colaboradores (2015) foi demonstrado que o DTDF pode induzir apoptose e parada no ciclo celular em fase S. No ensaio de relaxamento de DNA plasmidial, o DTDF foi capaz de inibir atividade de enzima topoisomerase I (TopoI). Em complementação a esses resultados, estes autores também verificaram que linhagens de leveduras deficientes em TopoI mostraram-se resistentes aos tratamentos com DTDF quando comparadas as linhagens selvagens. Portanto, o conjunto destas observações evidencia uma possível ação inibitória do DTDF para a enzima TopoI.

Utilizando modelo *in vitro* de linhagens de câncer de colorectal HT-29 e Caco-2, o DTDF apresentou efeitos antiproliferativo. Nesse estudo, foi possível observar um decréscimo significativo da viabilidade celular para ambas as linhagens em uma faixa de concentração de 62,5 até 1000 µM. Além disso, foi observado aumento da atividade de caspases 3/7 e 9 após exposição ao DTDF (500-1000 µM) em células HT-29, decréscimo de GSH/GSSH e apoptose, confirmado através de coloração fluorescente (Vij e Hardej, 2012).

Apesar dos efeitos tóxicos do DTDF mencionados no parágrafo anterior, em concentrações menores esse composto organotelurado reduziu o nível de peroxidação lipídica em cérebro, rim e fígado de ratos induzida por vários oxidantes (Rossato *et al.* 2002; Hassan *et al.* 2009). Além disso, o DTDF apresentou efeito neuroprotetor, reduzindo a neurotoxicidade e estresse oxidativo induzido por 4-aminopiridina em camundongos adultos (Brito *et al.* 2009).

1.9 Pesquisa de novos fármacos antitumorais

O desenvolvimento de fármacos para o tratamento de câncer é mais difícil que o desenvolvimento de fármacos para outras finalidades, uma vez que fármacos antitumorais são os agentes mais tóxicos intencionalmente administrados a humanos. O desenvolvimento torna-se ainda mais complexo, porque o primeiro uso desses compostos em humanos é realizado em pacientes não responsivos a outros tratamentos com antitumorais convencionais, os quais já estão bastante debilitados em função da doença e efeitos colaterais. Logo, a dose inicial selecionada para o primeiro uso em humanos deve, além de ser segura, oferecer alta probabilidade de eficácia na fase pré-clínica do desenvolvimento desses fármacos (Newhouse *et al*., 2005).

A maioria dos quimioterápicos usados na terapêutica foi selecionada por sua capacidade de controlar a proliferação celular, entretanto, as estratégias para a descoberta de novos fármacos têm mudado ao longo dos anos.

A terapia combinada consiste na administração simultânea de um fármaco de quimioterapia convencional (ou às vezes, um protocolo de radioterapia), juntamente com um ou mais bioativos naturais (geralmente de origem vegetal ou fúngica) ou

sintéticos de pequeno peso molecular (Redondo-Blanco *et al*., 2017). Como regra geral, o projeto de combinações envolvendo um medicamento tradicional de quimioterapia (ou protocolo de radioterapia) mais um ou mais compostos bioativos poderiam ser uma abordagem promissora para potencialmente obter melhorias na remissão em parte ou completa de tumores (Redondo-Blanco *et al*., 2017). Ao mesmo tempo isso poderia minimizar os efeitos colaterais associados com este tratamento medicamentoso ou radioterapia, tais como neutropenia, diarréia, cardiotoxicidade, nefrotoxicidade, hepatotoxicidade, etc. (Fig. 6). A maioria dos efeitos sinérgicos dessas combinações foram relatados *in vitro* e usando modelos de tumor animal e são devido à bioatividade antioxidante, indução da apoptose (via intrínseca ou extrínseca) e / ou parada do ciclo celular (em qualquer ponto de verificação) (revisado por Redondo-Blanco *et al*., 2017).

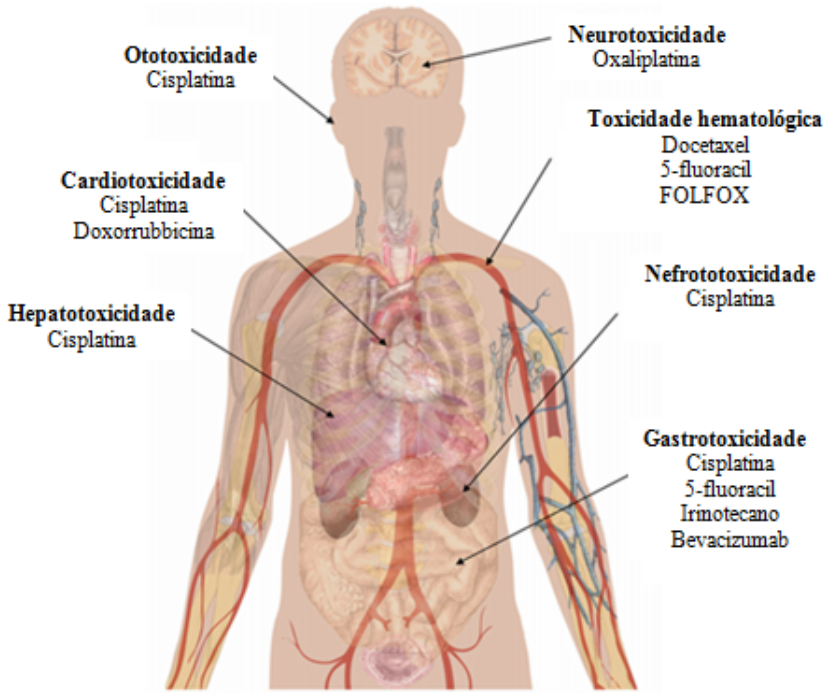

Figura 6. Efeitos colaterais dos quimioterápicos. Adaptado de Redondo-Blanco(2017).

2. OBJETIVOS

2.1 Objetivo Geral:

Elucidar os mecanismos de ação da combinação dos quimioterápicos bevacizumab e erlotinib em modelo de câncer colorectal e o potencial antigenotóxico, antimutagênico e antitumoral do ditelureto de difenila, visando a aplicação destes agentes na terapia antitumoral.

2.2 Objetivos específicos

- Determinar o potencial antitumoral da combinação de bevacizumab e erlotinib em diferentes modelos de câncer colorretal, com presença do *KRAS* selvagem ou mutado;

- Avaliar a sinalização celular (*dowstream)* da combinação de bevacizumab e erlotinib em diferentes modelos de câncer colorretal, em relação do estado mutacional do *KRAS*;

- Verificar a atividade antigenotóxica e antimutagênica do ditelureto de difenila (DTDF) em fibroblastos de pulmão de hamster chinês – células V79;

- Estabelecer o potencial citotóxico do DTDF em diferentes linhagens tumorais e seu efeito protetor na indução de danos oxidativos pelo quimioterápico doxorrubicin.

Capítulo I

Antitumor activity of joint VEGF- and EGFR-targeting in *RAS* mutant colorectal cancer (CRC) models

A ser submetido a revista *Clinical Cancer Research*.

Os resultados apresentados nesse capítulo foram obtidos durante o estágio de doutorado-sandwich pelo Projeto CAPES/COFECUB n° 583/07 no Laboratório de *Biologie et Thérapeutiques du Cancer* e no *Centre de Recherche Saint-Antoine* INSERM – UPMC (Paris, França) coordenado pela Dr Annette k. Larsen na França e pelo Dr João A.P. Henriques no Laboratório de Reparação de DNA de eucariotos da UFRGS (Porto Alegre, Brasil).

Este capítulo consiste em um manuscrito de dados a ser submetido ao periódico *Clinical Cancer Research,* onde foram avaliadas as combinações de um anticorpo direcionado ao VEGF e um inibidor de EGFR de molécula pequena possuem atividade superior à inibição de VEGF sozinha, *in vivo* como *in vitro*, independentemente da sensibilidade ao bevacizumab e do estado mutacional do *KRAS*, indicando que pequenas moléculas e anticorpos-alvo visando os mesmos caminhos moleculares podem ser ativos em diferentes populações de pacientes e combinado com distintos agentes anticancer.

Antitumor activity of joint VEGF- and EGFR-targeting in *RAS* mutant colorectal cancer (CRC) models

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Keywords: Colorectal cancer, combined VEGF/EGFR inhibition, *KRAS* mutation, bevacizumab-resistance

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Background: There is extensive cross-talk between VEGF- and EGFR-pathway signaling in colorectal cancer (CRC). However, combinations of VEGF- and EGFR-targeted antibodies (mAbs) show disappointing activity, in particular for patients with mutant *RAS*. Previous results show that tyrosine kinase inhibitors (TKIs) can be active in CRC models resistant to mAbs. This promoted us to examine whether the activity of bevacizumab can be increased by combination with erlotinib.

Methods: The antitumor activity of bevacizumab, erlotinib and their combination was determined in CRC models with different *RAS* status and bevacizumab sensitivity. EGFR/VEGF pathway activation was characterized by immunohistochemistry, Western blot and ELISA assays. The influence of cetuximab and erlotinib on EGF-mediated migration and the EGFR-EGF ligand feed-back loop was established in CRC cell lines with different *RAS* status.

Results: The addition of erlotinib increased bevacizumab activity in all models independent of *RAS* status. Bevacizumab exposure was accompanied by marked EGFR activation in tumor cells as well as in tumor-associated endothelial cells and resulted in strong accumulation of intracellular EGFR, which could be attenuated by erlotinib. In cellular models, erlotinib was able to attenuate EGF-mediated functions in all cell lines independent of *RAS* status while cetuximab only showed activity in *RAS* wt cells.

Conclusions: The results presented here provided the rational for the GERCOR DREAM phase III clinical trial and elucidate the molecular framework to explain the clinical activity of the combination. Differential

activity of mAbs and TKIs targeting the same signaling pathway is likely applicable for other tumor types.

Introduction

The epidermal growth factor receptor (EGFR) and the vascular endothelial growth factor (VEGF) pathways are validated targets for treatment of patients with metastatic colorectal cancer (mCRC). There is extensive cross-talk between these two signaling pathways (1) and combinations of VEGF(R) and EGFR-directed compounds have consistently shown at least additive activity in preclinical models (2). However, the results of clinical trials were disappointing since the addition of EGFR-targeted monoclonal antibodies (mAbs) to bevacizumab plus chemotherapy was no better, in terms of overall survival, than bevacizumab plus chemotherapy alone, even for patients with wild-type *KRAS* tumors, and was detrimental, in terms of progression-free survival, for most patients with *KRAS* mutant tumors (3,4).

To determine if these disappointing results were pathway- or agent-related, we compared the antitumor activity of two mAbs with two small molecule tyrosine kinase inhibitors (TKIs) in the same CRC xenograft models (5). The results showed that the combination of the two antibodies was no more active than either agent alone while the combination of the TKIs showed synergistic antitumor activity. Unexpectedly, the TKI combination was also active in CRC models with mutant *RAS* status (5) indicating that mutant *RAS* may not be a limiting factor for EGFR-directed TKIs like it is for the mAbs (6-8). These findings provided the rational for the current study which shows that erlotinib enhances the *in vivo* activity of bevacizumab independent of *RAS* status. We further demonstrate a

differential activity of cetuximab and erlotinib on EGFR-mediated functions in CRC cells revealing fundamental differences between mAbs and TKIs targeting the same pathway.

Materials and Methods

Drugs and antibodies

Bevacizumab (Avastin) was provided by Roche, cetuximab was from Merck while erlotinib (Tarceva) was purchased from LC Laboratories (Woburn, MA). Antibodies are detailed in Supplementary Materials (available online).

Tumor cells

CRC cells were maintained in cell culture as described previously (9). Characterization of the cell lines is detailed in Supplementary Materials (available online).

In vivo studies

The antitumor effects of bevacizumab, erlotinib and their combination were evaluated in athymic mice (female NMRI-Foxn1n, 6 weeks old) from Taconic (Skensved, Danmark) bearing SW48, HT-29 or SW620 xenografts. Two to five million cells were injected into the right flank, and the treatments were started when the tumors were palpable. Animals were weighed daily and the tumor size was determined three times per week (10). Tumor volumes (mm^3) were calculated according to formula: [(length x $width^2$)/2]. Boxplot analysis was carried out using the graphpad prism 6.04 software (Graphpad, La Jolla, CA). Treated/Control (T/C) values were calculated as follows: average tumor volume of treated animals/average

tumor volume of control animals x 100. Animals were treated according to institutional guidelines and the protocol was approved by the local ethics committee for animal experimentation.

Immunohistochemistry

Biomarker analysis was carried out with tumors collected after 4 weeks of treatment. IHC staining is detailed in Supplementary Materials (available online).

ELISA assay and Western blot of xenograft samples

Tumor tissues were collected from untreated, frozen tumors (three tumors per treatment) and protein extracts were prepared in RIPA buffer according to the manufacturer's instructions. Amphiregulin levels were determined by ELISA as detailed in the Supplementary Materials (available online).

For Western blot, equal amounts of proteins (100 µg/lane) were loaded into SDS-PAGE gels, transferred onto nitrocellulose membranes and blotted with antibodies directed against phosphorylated and total EGFR as detailed in Supplementary Materials (available online). Protein expression was quantified by densitometric analysis of the immunoblots using Image Lab software (Biorad) after normalization with β-actin.

ELISA assays of conditioned media.

Cells were seeded and allowed to attach for 24 hrs followed by 72 hrs incubation in 5% FCS in the presence of cetuximab (1 µg/mL) or erlotinib (3 µM) as described (11). The levels of secreted TGF-alpha and amphiregulin were determined as detailed in Supplementary Materials (available online).

Tumor cell migration

Cell migration was assessed by the trans-well assay (Boyden chamber, Dutscher, Issy-les-moulineaux, France) according to the manufacturer's instructions. Briefly, 50 000 to 100 000 cells in serum-free media with or without erlotinib or cetuximab were plated in the upper chamber on membranes with a pore size of 8 µm while the lower chamber contained culture media with EGF (20 ng/ml) as chemoattractant. After 6 hr, remaining cells were removed from the top-side of the inserts whereas migrating cells on the bottom of the inserts were stained with Diff-Quik (ThermoFischer Scientific, Waltham, MA) and all migrating cells were counted. Results are expressed as means ± standard error of the means (SEM) and represent triplicate samples from at least two independent experiments.

Statistical analysis

ANOVA was performed to determine the significance of observed differences between groups using the tool pack from Excel (Microsoft). Post hoc comparisons were made using Student's paired *t*-test using GraphPad Prism (GraphPad Software). Differences between two groups are presented as the mean ± SEM or mean ± SD as noted in the figure legends. All tests were two-sided and p values <0.05 were considered statistically significant.

Results

Erlotinib increases the antitumor activity of bevacizumab

Three different CRC xenograft models were used including SW48 (*KRAS* wt, bevacizumab sensitive), HT-29 (*KRAS* wt, bevacizumab resistant) and SW620 (*KRAS* mutant and bevacizumab sensitive) (10). The

antitumor activities of bevacizumab (1 mg/kg) and erlotinib were comparable for SW48 and SW620 xenografts with T/C values (average tumor volume of treated animals/average tumor volume of control animals x 100) of 55 and 54 for bevacizumab and 65 and 74 for erlotinib, respectively (Figure 1). Combined treatment with bevacizumab and erlotinib increased the antitumor activity to T/C values of 33 and 39, respectively, which is significantly different from bevacizumab alone ($p < 0.01$). To confirm the activity of the bevacizumab + erlotinib combination toward the *KRAS* mutant SW620 tumors the experiment was repeated with a standard dose of bevacizumab (5 mg/kg). Under these conditions, the T/C value was 27 for bevacizumab alone and 16 for the combination ($p < 0.001$) (data not shown).

Bevacizumab exposure (5 mg/kg) was accompanied by modest tumor growth inhibition for HT-29 xenografts (T/C of 68) whereas erlotinib treatment was associated with unexpected strong antitumor activity (T/C of 36). The combination of the two drugs gave a T/C value of 26, which is significantly ($p < 0.001$) more than what was observed for bevacizumab alone (Figure 1), whereas the difference between erlotinib and erlotinib + bevacizumab did not reach significance ($p = 0.07$).

Angiogenic signaling

Bevacizumab has preferential activity for human VEGF. To estimate the relative contribution of human tumor-derived and murine stromal-derived VEGF within the tumor microenvironment, tissue extracts were prepared from untreated SW48, HT-29 and SW620 tumors and the concentration of murine and human VEGF was determined by species-specific ELISA analysis (data not shown). The results show that human

VEGF represents at least 95% of the total VEGF in agreement with previous findings (10, 12).

The microvascular density of SW48, HT-29 and SW620 xenografts was compared by immunohistochemistry with a CD31-directed antibody followed by quantitative image analysis. The results (Figure 2, upper panel left) indicate that bevacizumab reduced the microvascular density by ~ 65% in the bevacizumab-sensitive SW48 and SW620 xenografts compared to only ~ 30% in the bevacizumab-resistant HT-29 xenografts. In comparison, erlotinib treatment was accompanied by ~ 50% reduction of the vascular density in all 3 xenograft models. Interestingly, the addition of erlotinib to bevacizumab strongly diminished the microvascular density of HT-29 xenografts (from 30% to 64% of the vehicle control) suggesting that EGFR-signaling contributes to the bevacizumab resistance of these tumors. The combination of bevacizumab + erlotinib was also significantly more effective than bevacizumab alone for the SW48 and SW620 tumor models, although the effect was less pronounced than for HT-29.

Bevacizumab exposure was accompanied by ~ 40% increased expression of tissue-associated VEGF that diminished significantly ($p < 0.001$) when erlotinib was added to bevacizumab (Figure 2, upper panel right). Most CRC cells and tumors express functional VEGFR1/Flt-1 that promotes cellular survival under environmental stress (13). Bevacizumab exposure was accompanied by increased levels of active phospho-VEGFR1 for all xenograft models which was attenuated ($p < 0.001$) by the addition of erlotinib. This was most marked for the HT-29 xenografts where the levels of phospho-VEGFR1 were 171% in the presence of bevacizumab alone and 71% in the presence of bevacizumab + erlotinib, compared to untreated vehicle controls. The differences in VEGFR1

phosphorylation were not linked to altered protein levels, as determined by ELISA of total VEGFR1 in tumor extracts (Supplemental figure S1). In bevacizumab-treated tumors, activated pVEGFR1 was mostly localized inside the cells as indicated by a prominent cytoplasmic signal (Figure 2, indicated with a white arrow).

In addition to VEGFR1, CRC xenografts may express low levels of VEGFR2/Flk-1. Bevacizumab exposure was accompanied by up to 2-fold increase in the levels of phospho-VEGFR2 that was significantly attenuated in the presence of erlotinib, with the exception of the SW620 model, where the modest decrease did not reach significance (Figure 2). It is noticeable that the levels of VEGFR1 and VEGFR2 protein are very different, with about 20-fold less VEGFR2, compared with VEGFR1, in SW48 and SW620 tumors and almost 80-fold less VEGFR2, compared to VEGFR1, in HT-29 xenografts (Supplemental figure S1). These differences may explain the current controversy regarding the expression of VEGFR2 by CRC cells (13). Taken together, our findings show that bevacizumab activates autocrine VEGF-signaling in all three xenograft models which is attenuated in the presence of erlotinib.

EGFR signaling

Next, the levels of total and phosphorylated EGFR were determined (Figure 3). The results show that erlotinib exposure was accompanied by decreased levels of the active, autophosphorylated form of EGFR which was most pronounced for SW48 (66% inhibition, compared to the vehicle control) followed by SW620 (50% inhibition) and HT-29 xenografts (35% inhibition). Unexpectedly, bevacizumab treatment was accompanied by strong EGFR activation ranging from ~ 140% for the bevacizumab-

sensitive SW48 and SW620 xenografts to more than 200% for the bevacizumab-resistant HT-29 xenografts. The addition of erlotinib to bevacizumab was accompanied by a highly significant ($p < 0.001$) decrease in phospho-EGFR levels in all models shown by quantitative immunohistochemistry as well as by Western blot analysis. In comparison, bevacizumab had no detectable influence on total EGFR levels whereas erlotinib exposure, alone or in combination, was accompanied by a modest increase of total EGFR that never reached significance.

As observed for pVEGFR1, bevacizumab treatment was accompanied by a prominent cytoplasmic signal of pEGFR (indicated with a white arrow).

EGFR signaling in endothelial cells

Tumor-associated endothelial cells (TECs) frequently express functional EGFR (14,15). To characterize the expression of active EGFR on the TECs, a semi-quantitative approach was developed as illustrated (Figure 4, upper panel, left). Tumors were double-labeled for phospho-EGFR and CD31 and the degree of colocalization was determined as detailed in the figure legend. The results show that bevacizumab treatment was accompanied by a strong increase in TEC-associated phospho-EGFR while phospho-EGFR was attenuated following treatment with erlotinib. Importantly, the addition of erlotinib to bevacizumab was associated with a highly significant ($p < 0.001$) decrease in TEC-associated phospho-EGFR, compared to bevacizumab alone, for all three xenograft models.

The EGFR ligands amphiregulin (AREG) and transforming growth factor-alpha (TGF-alpha) are known to form positive feed-back loops with activated EGFR (16-18). Amphiregulin is of particular interest, since it is expressed at high levels in CRC and has potential predictive value for the

response to EGFR-targeted agents (19,20). Tumor extracts were prepared and the ligand expression was determined by ELISA analysis. A modest increase in amphiregulin expression was observed in the bevacizumab-treated tumors while erlotinib treatment resulted in a ~ 50% decrease. Importantly, amphiregulin expression was significantly decreased in all bevacizumab + erlotinib groups, compared to bevacizumab alone. For TGF-alpha, the levels were too close to the detection limit to give reproducible results.

It has been reported that amphiregulin promotes endothelial tube formation *in vitro* suggesting a direct role for amphiregulin in tumor angiogenesis (21). To determine if tumor-derived amphiregulin co-localize with the TECs, tumors were double-labeled for human amphiregulin and CD31. The results (Figure 4, lower panel) show prominent co-localization of amphiregulin and the TECs (indicated in yellow) compared to the fainter red labeling of amphiregulin in the tumor cells, coherent with a close *in vivo* association between the TECs and tumor-derived amphiregulin.

HIF induction

Bevacizumab-induced modifications of receptor tyrosine kinase activity and localization may be associated with tumor hypoxia due to vascular pruning. The results show that bevacizumab treatment was accompanied by a clear increase in the expression of HIF2alpha, and to a lesser degree, HIF1alpha (Figure 5). In comparison, the expression of both HIFs was decreased when erlotinib was added to bevacizumab, in agreement with the notion that HIF alpha proteins are under dual regulation by hypoxia and tyrosine kinase signaling (22).

EGFR-signaling in *RAS* mutant cells

EGFR activation is accompanied by a positive feed-back loop with some of its ligands including TGF-alpha and amphiregulin (16-18). Intriguingly, a recent study reports that EGFR-directed antibodies are unable to downregulate amphiregulin and TGF-alpha in *RAS* mutant CRC cells, which could, at least in part, explain the lack of activity of the mAbs toward such cells (11). The activity of erlotinib and cetuximab toward different *RAS* mutant CRC cell lines was compared (Figure 6). The results show that cetuximab exposure increased TGF-alpha expression up to 4-fold in all cellular models, but had no significant influence on amphiregulin. Importantly, erlotinib decreased both TGF-alpha and amphiregulin secretion, with highly significant ($p < 0.001$) differences between cetuximab and erlotinib in all cases (Figure 6 upper panels).

EGFR activation is also associated with tumor cell migration. Four CRC cell lines with good migratory capacity were selected including LIM1215 (*KRAS* wt), HCT-116, LS174T and SW480 (all *KRAS* mutant). EGF strongly stimulated the migration of all cell lines which could be attenuated by erlotinib (Figure 6 middle and lower panels). In clear contrast, cetuximab was only able to attenuate migration of the *KRAS* wt LIM1215 cells, without any detectable influence on the migration of the *KRAS* mutant cells. Importantly, the viability at the end of the incubation period was comparable for all groups (Supplemental figure S2).

Discussion

We here report that erlotinib increases the activity of bevacizumab in CRC models independent of *RAS* status. These findings provided the rational for the DREAM Phase III clinical trial which showed that administration of bevacizumab plus erlotinib as maintenance therapy

resulted in three months increase in overall survival compared to bevacizumab alone (23). In addition, these results provide a rational for clinical evaluation of EGFR-directed TKIs in combination with VEGF-blockers like bevacizumab and aflibercept.

Usually, EGFR inhibition is believed to principally influence tumor cells while VEGF-targeting is acting on endothelial cells. The results presented here provide evidence for a more complex model. Pruning of the tumor-associated microvasculature was accompanied by tumor hypoxia, HIF induction and activation of tumor-associated VEGF signaling as well as EGFR activation. Hypoxia also interfered with receptor recycling as evidenced by a prominent cytoplasmic signal for both pVEGFR1 and pEGFR in agreement with an influence of the oxygen-sensing pathway on endocytosis (24). Bevacizumab exposure was also accompanied by EGFR signaling activation and accumulation of tumor-derived amphiregulin in the TECs. These results suggest that both tumor cells and TECs are targeted by EGFR blockage.

Our findings underline the discrepancy between EGFR-targeting alone compared to EGFR-targeting in combination with VEGF-directed agents. The mAbs show clinical activity in CRC while the TKIs have been less active and/or more toxic (25,26). However, addition of erlotinib to bevacizumab increased the antitumor activity, which was not the case for the combination of EGFR- and VEGF-targeted mAbs (3-5). Furthermore, the activity of EGFR-targeted mAbs requires wt *RAS* which was not the case for the TKIs. Intriguingly, we found that the impact of *RAS* status could be reproduced in cellular models since erlotinib consistently inhibited EGFR-mediated cellular functions independent of *RAS* status while cetuximab was only active in *RAS* wt cells.

It should be noted that the mAbs and the TKIs owe their activity to different mechanisms. Binding of the mAbs to EGFR is followed by endocytosis and lysosomal degradation, thereby decreasing receptor density on the cell surface (27). In the case of the TKIs, it is the catalytic activity of EGFR that is inhibited without an immediate effect on EGFR protein levels. Importantly, the activity of the TKIs is not restricted to surface-associated EGFR but includes additional pools of EGFR, like the active EGFR contained in endosomes following receptor internalization. Intriguingly, recent results suggest that the interaction between EGFR and Ras proteins ceases within minutes after EGFR activation, with EGFR accumulating in the endosomes while Ras remain associated with the plasma membrane, thereby providing a physical separation between the two molecules and rapid signal downregulation (28).

Traditionally, EGFR signaling has been associated with canonical cellular signaling pathways like Ras/MAPK, PI3K/Akt, Phospholipase C/PKC, STAT and Src pathways (Figure 7). However, several novel EGFR signaling pathways have been described recently including the binding of EGFR to and phosphorylation of the tyrosine kinase Lyn, which then phosphorylate MCM7, a licensing factor critical for DNA synthesis (29), beclin1 (BCN-1), a crucial mediator of autophagy (30) and argonaute 2 (AGO2) a modulator of microRNA processing (31). Importantly, the phosphorylation of AGO2 and Beclin1 is mediated by catalytically active EGFR present on intracellular vesicles after internalization, but before lysosomal degradation (30, 31), thereby representing a subfraction of EGFR that can be inhibited by EGFR-directed small molecules, but not by EGFR-directed mAbs.

In conclusion, our study provides strong evidence that mAbs and TKIs target different elements of the EGFR signaling pathway, both as single agents and in combination with VEGF-targeted therapies. We further suggest that differential activity of mAbs and TKIs targeting the same signaling pathway is likely applicable for other tumor types.

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References

1. Larsen AK, Ouaret D, El Ouadrani K, et al. Targeting EGFR and VEGF(R) pathway cross-talk in tumor survival and angiogenesis. *Pharmacol Ther*. 2011;131(1):80-90.

2. Tabernero J. The role of VEGF and EGFR inhibition: implications for combining anti-VEGF and anti-EGFR agents. *Mol Cancer Res*. 2007;5(3):203-220.

3. Tol J, Koopman M, Cats A, et al. Chemotherapy, bevacizumab, and cetuximab in metastatic colorectal cancer. *N Engl J Med*. 2009;360(6):563-572.

4. Hecht JR, Mitchell E, Chidiac T, et al. A randomized phase IIIB trial of chemotherapy, bevacizumab, and panitumumab compared with chemotherapy and bevacizumab alone for metastatic colorectal cancer. *J Clin Oncol*. 2009;27(5):672-680.

5. Ouaret D, Poindessous V, El Ouadrani K, et al. EGFR- and VEGF (R)-targeted small molecules show synergistic activity in colorectal cancer models refractory to combinations of monoclonal antibodies. *Clin Cancer Res*. 2011;17(20):6522-6530.

6. Lièvre A, Bachet JB, Boige V, et al. KRAS mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. J Clin Oncol. 2008;26(3):374-379.

7. Douillard JY, Oliner KS, Siena S, et al. Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. N Engl J Med. 2013;369(11):1023-1034.

8. Atreya CE, Corcoran RB, Kopetz S. Expanded RAS: refining the patient population. *J Clin Oncol*. 2015;33(7):682-685.

9. Ouaret D, Larsen AK. Protein kinase C β inhibition by enzastaurin leads to mitotic missegregation and preferential cytotoxicity toward colorectal cancer cells with chromosomal instability (CIN). *Cell Cycle*. 2014;13(17):2697-2706.

10. Mésange P, Poindessous V, Sabbah M, et al. Intrinsic bevacizumab resistance is associated with prolonged activation of autocrine VEGF signaling and hypoxia tolerance in colorectal cancer cells and can be overcome by nintedanib, a small molecule angiokinase inhibitor. *Oncotarget*. 2014;5(13):4709-4721.

11. Hobor S, Van Emburgh BO, Crowley E, et al. TGFα and amphiregulin paracrine network promotes resistance to EGFR blockade in colorectal cancer cells. *Clin Cancer R*es. 2014;20(24):6429-6438.

12. Chiron M, Bagley RG, Pollard J, et al. Differential antitumor activity of aflibercept and bevacizumab in patient-derived xenograft models of colorectal cancer. *Mol Cancer Ther*. 2014;13:1636-1644.

13. Larsen AK, de Gramont A, Bouygues A, et al. Functions and clinical implications of autocrine VEGF signaling in colorectal cancer. *Curr Colorectal Cancer Rep*. 2013;9(3):270-277.

14. Yokoi K, Thaker PH, Yazici S, et al. Dual inhibition of epidermal growth factor receptor and vascular endothelial growth factor receptor phosphorylation by AEE788 reduces growth and metastasis of human colon carcinoma in an orthotopic nude mouse model. *Cancer Res*. 2005;65(9):3716-3725.

15. Amin DN, Hida K, Bielenberg DR, Klagsbrun M. Tumor endothelial cells express epidermal growth factor receptor (EGFR) but not ErbB3 and are responsive to EGF and to EGFR kinase inhibitors. *Cancer Res*. 2006;66(4):2173-2180.

16. Coffey RJ Jr, Derynck R, Wilcox JN, et al. Production and auto-induction of transforming growth factor-alpha in human keratinocytes. *Nature*. 1987;328(6133):817-820.

17. Gunaratnam L, Morley M, Franovic A, et al. Hypoxia inducible factor activates the transforming growth factor-alpha/epidermal growth factor receptor growth stimulatory pathway in VHL(-/-) renal cell carcinoma cells. *J Biol Chem*. 2003;278(45):44966-44974.

18. Panupinthu N, Yu S, Zhang D, et al. Self-reinforcing loop of amphiregulin and Y-box binding protein-1 contributes to poor outcomes in ovarian cancer. *Oncogene*. 2014;33(22):2846-2856.

19. Khambata-Ford S, Garrett CR, Meropol NJ, et al. Expression of epiregulin and amphiregulin and K-ras mutation status predict disease control in metastatic colorectal cancer patients treated with cetuximab. *J Clin Oncol*. 2007;25:3230-3237.

20. Salazar R, Capellà G, Tabernero J. Paracrine network: another step in the complexity of resistance to EGFR blockade? *Clin Cancer Res*. 2014;20(24):6227-6229.

21. Bordoli MR, Stiehl DP, Borsig L, et al. Prolyl-4-hydroxylase PHD2- and hypoxia-inducible factor 2-dependent regulation of amphiregulin contributes to breast tumorigenesis. *Oncogene*. 2011;30:548-560.

22. Nilsson MB, Zage PE, Zeng L, et al. Multiple receptor tyrosine kinases regulate HIF-1alpha and HIF-2alpha in normoxia and hypoxia in neuroblastoma: implications for antiangiogenic mechanisms of multikinase inhibitors. *Oncogene*. 2010 29(20):2938-2949.

23. Tournigand C, Chibaudel B, Samson B, et al. Bevacizumab with or without erlotinib as maintenance therapy in patients with metastatic colorectal cancer (GERCOR DREAM; OPTIMOX3): a randomised, open-label, phase 3 trial. *Lancet Oncol*. 2015;16(15):1493-1505.

24. Wang Y, Roche O, Yan MS, et al. Regulation of endocytosis via the oxygen-sensing pathway. *Nat Med*. 2009;15(3):319-324.

25. Santoro A, Comandone A, Rimassa L, et al. A phase II randomized multicenter trial of gefitinib plus FOLFIRI and FOLFIRI alone in patients with metastatic colorectal cancer. *Ann Oncol*. 2008;19(11):1888-1893.

26. Meyerhardt JA, Stuart K, Fuchs CS, et al. Phase II study of FOLFOX, bevacizumab and erlotinib as first-line therapy for patients with metastatic colorectal cancer. *Ann Oncol*. 2007;18(7):1185-1189.

27. Spangler JB, Neil JR, Abramovitch S, et al. Combination antibody treatment down-regulates epidermal growth factor receptor by inhibiting endosomal recycling. *Proc Natl Acad Sci USA*. 2010;107(30):13252-13257.

28. Pinilla-Macua I, Watkins SC, Sorkin A. Endocytosis separates EGF receptors from endogenous fluorescently labeled HRas and diminishes receptor signaling to MAP kinases in endosomes. *Proc Natl Acad Sci USA*. 2016;113(8):2122-2127.

29. Huang TH, Huo L, Wang YN, et al. Epidermal growth factor receptor potentiates MCM7-mediated DNA replication through tyrosine phosphorylation of Lyn kinase in human cancers. *Cancer Cell*. 2013;23(6):796-810.

30. Wei Y, Zou Z, Becker N, et al. EGFR-mediated Beclin 1 phosphorylation in autophagy suppression, tumor progression, and tumor chemoresistance. *Cell*. 2013;154(6):1269-1284.

31. Shen J, Xia W, Khotskaya YB, et al. EGFR modulates microRNA maturation in response to hypoxia through phosphorylation of AGO2. *Nature*. 2013;497(7449):383-387.

Figure and Table legends

Figure 1. Influence of bevacizumab, erlotinib and their combination on tumor growth of CRC xenografts. *Left*, nude mice with SW48, HT-29 or SW620 CRC xenografts were dosed with vehicle (striped squares), bevacizumab (1 mg/kg *i.p.* every 3 days for SW48 and SW620 or 5 mg/kg *i.p.* every 3 days for HT-29) (black circles), erlotinib (75 mg/kg *p.o.* once daily) (white circles) or bevacizumab and erlotinib together (light grey circles). The curves represent the average tumor growth of at least 7 animals per group. Right, box and whisker plots of tumor volumes in mice with SW48, HT-29 or SW620 CRC xenografts after 4 weeks treatment with vehicle, bevacizumab (beva), erlotinib (erlo) or their combination (beva + erlo). Lines, medians; boxes, 25^{th} to 75^{th} percentile interquartile ranges; whiskers, the highest and lowest value for a given treatment. The brackets indicate the difference between the bevacizumab groups and the corresponding bevacizumab + erlotinib group P values were calculated using Student's paired *t*-test. ** $p < 0.01$; *** $p < 0.001$.

Figure 2. Influence of bevacizumab, erlotinib and their combination on tumor angiogenesis. Animals with SW48 (grey columns), HT-29 (hatched columns) or SW620 (black columns) human CRC xenografts were treated

with bevacizumab (beva), erlotinib (erlo) or their combination (beva + erlo) for 4 weeks as described in the legend to Figure 1, followed by immunohistochemistry and quantitative image analysis. The photos illustrate the typical staining patterns for tumors derived from animals treated with bevacizumab (B) or with bevacizumab + erlotinib (B+E). For the microvascular density, CD31-positive endothelial blood vessels are outlined in white while the bar diagram indicate the CD31-positive area, as % of total, and represent the averages of at least 6 fields/tumor for at least 3 different tumors. For the quantitative analysis of VEGF, phospho-VEGFR1 and phospho-VEGFR2, the data represent the average fluorescence intensities of treated tumors compared to the treatment intensity of control tumors and are the averages of 6 fields/tumor for at least 3 different tumors. Bars indicate the mean ± SEM. The brackets indicate the difference between the bevacizumab groups and the corresponding bevacizumab + erlotinib groups. P values were calculated using Student's paired *t*-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Bottom panels, cellular distribution of phospho-VEGFR1 (pVEGFR1) after treatment with bevacizumab, erlotinib and their combination in HT-29 tumors after 4 weeks treatment as described in the legend to Figure 1. The presence of phospho-VEGFR1 was determined by immunohistochemistry and is indicated in red, whereas the nuclei appear in blue. The white arrow indicates the prominent intracellular accumulation of phosphorylated VEGFR1.

Figure 3. Influence of bevacizumab, erlotinib and their combination on EGFR. Animals with SW48 (grey columns), HT-29 (hatched columns) or SW620 (black columns) human CRC xenografts were treated with

bevacizumab (beva), erlotinib (erlo) or their combination (beva + erlo) for 4 weeks as described in the legend to Figure 1. Top panels, the expression of phospho-EGFR and total EGFR was determined by immunohistochemistry followed by quantitative image analysis. The photos illustrate the typical staining patterns for tumors derived from animals treated with bevacizumab (B) or with bevacizumab + erlotinib (B+E). The bars indicate the mean ± SEM and represent the average fluorescence intensities of treated tumors compared to the treatment intensity of control tumors and are the averages of 6 fields/tumor for at least 3 different tumors. The brackets indicate the difference between the bevacizumab groups and the corresponding bevacizumab + erlotinib groups. P-values were calculated using Student's paired *t*-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Middle panel, Western blot analysis of pEGFR and EGFR expression in the tumors described above. The columns indicate the pEGFR signal (% of control) after normalization with beta-actin that was used as loading control. Data represent typical results from 3 different experiments. Bottom panels, cellular distribution of phosphorylated EGFR (pEGFR) after treatment with bevacizumab, erlotinib and their combination in HT-29 tumors after 4 weeks treatment as described in the legend to Figure 1. The presence of phospho-EGFR was determined by immunohistochemistry and is indicated in red, whereas the nuclei appear in blue. The white arrow indicates the prominent intracellular accumulation of phosphorylated EGFR.

Figure 4. EGFR-signaling in tumor-associated endothelial cells (TECs).

Top, to identify phosphorylated EGFR on the tumor-associated endothelial cells, double labeling was carried out for phospho-EGFR and CD31 and the degree of colocalization was determined by semi-quantitative analysis as illustrated (top, left). Only blood vessels where the entire rim could be assessed were included in the analysis, with at least 12 blood vessels per group. For each blood vessel, no colocalization was characterized by a uniform green color and given a value of 0, some colocalization was characterized by a mixture of yellow and green cells and given a value of 1 and strong colocalization was characterized by predominance of yellow cells and given a value of 2 thereby giving an estimate of the average levels of co-localization for each group. The results of the semi-quantitative analysis are indicated in the bottom (right). P values were calculated using Student's paired *t*-test. The brackets indicate the difference between the bevacizumab groups and the corresponding bevacizumab + erlotinib groups. P-values were calculated using Student's paired *t*-test. $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Bottom, to identify human, tumor-derived amphiregulin on the tumor-associated endothelial cells, double labeling was carried out for CD31 (in green) and human amphiregulin (AREG in red) to visualize the degree of colocalization (Merge, in yellow). The brackets indicate the difference between the bevacizumab groups and the corresponding bevacizumab + erlotinib groups. P-values were calculated using Student's paired *t*-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 5. Influence of bevacizumab, erlotinib and their combination on the expression of hypoxia-induced factors (HIFs). Animals with SW48 (grey columns), HT-29 (hatched columns) or SW620 (black columns) human CRC xenografts were treated with bevacizumab, erlotinib or their

combination for 4 weeks as described in the legend to Figure 1. The expression of HIF-1alpha and HIF-2alpha was determined by immunohistochemistry followed by quantitative image analysis. The photos illustrate the typical staining patterns for tumors derived from animals treated with bevacizumab (B) or with bevacizumab + erlotinib (B+E). The bars indicate the mean ± SEM and represent the average fluorescence intensities of treated tumors compared to the treatment intensity of control tumors and are the averages of 6 fields/tumor for at least 3 different tumors. The brackets indicate the difference between the bevacizumab groups and the corresponding bevacizumab + erlotinib groups. P-values were calculated using Student's paired *t*-test. $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 6. Influence of erlotinib and cetuximab on EGFR signaling in CRC cells. Upper panels, secretion of TGF-alpha (left) or amphiregulin (right) in HCT-116, DLD-1, SW620 and SW480 cells after 72 hrs incubation in the absence (hatched columns) or presence of erlotinib (3 µM, grey columns) or cetuximab (1 µg/mL, black columns). The values represent the averages of 3 independent experiments, each done in duplicate. Middle and lower panels, migration of LIM1215, HCT-116, LS174T and SW480 cells as determined by the trans-well assay (Boyden chamber) in the absence (white columns) or presence of EGF in the lower chamber and in the absence (hatched columns) or presence of erlotinib (grey columns) or cetuximab (black columns) in the upper chamber. The values represent the averages of 3 independent experiments, each done in duplicate. The brackets indicate the differences between erlotinib and cetuximab-treated cells. P values were calculated using Student's paired *t*-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

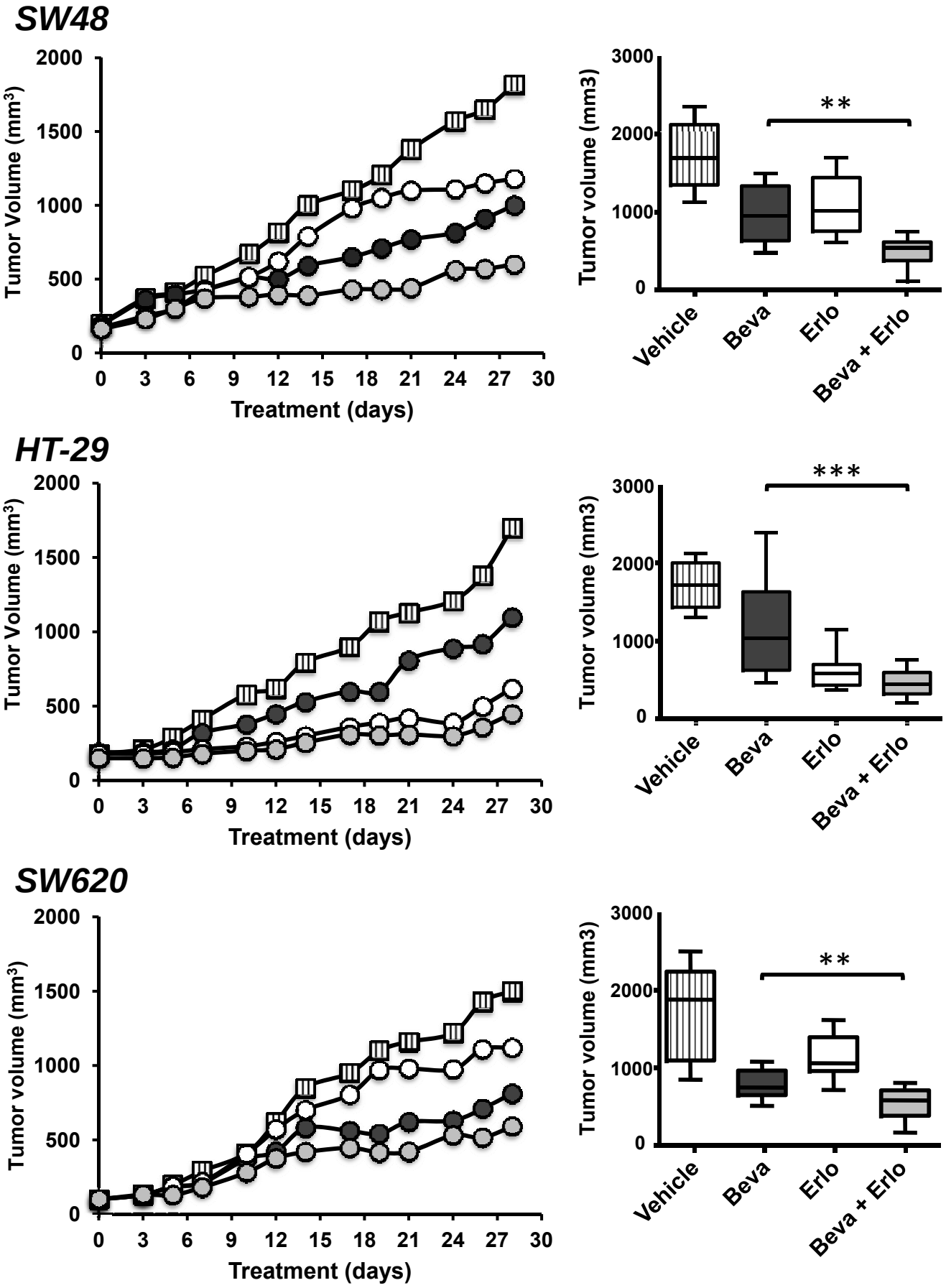
Figure 7. Outline of classical and novel EGFR signaling pathways. EGFR mediates a wide variety of cellular functions including both classical mechanisms and newly identified pathways that plays a role in the regulation of DNA synthesis (Lyn/MCM7), autophagy (BCN-1) and miRNA processing (AGO2).

Supplementary figures

Figure S1. Expression of total VEGFR1 and VEGFR2. Animals with SW48 (grey columns), HT-29 (hatched columns) or SW620 (black columns) xenografts were treated with bevacizumab, erlotinib or their combination for 4 weeks as described in the legend to Figure 1. The expression of total VEGFR1 and VEGFR2 was determined by ELISA analysis of tumor extracts (3 tumors per treatment). The values represent the average of 3 independent experiments, each done in duplicate. The brackets indicate the difference between the bevacizumab groups and the corresponding bevacizumab + erlotinib groups. P-values were calculated using Student's paired *t*-test. * $p < 0.05$.

Figure S2. The influence of 72 hrs exposure to vehicle, erlotinib (Erlo, 3 μM) or cetuximab (Cetux, 1 μg/mL) on the viability of the indicated CRC cell lines as measured by the TUNEL assay. Apoptosis is expressed as the % area of TUNEL-positive cells compared to the total area of viable cells, and is the average of 6 fields/slides (each field representing approximately 500 cells), all done in duplicate.

Figure 1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1500
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Treatment (days)
Tumor volume (mm3)
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Vehicle
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Erlo
Beva + Erlo
HT-29
Tumor Volume (mm³)
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Treatment (days)
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Vehicle
Beva
Erlo
Beva + Erlo
SW620
Tumor volume (mm³)
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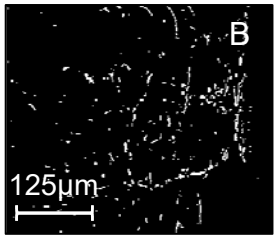
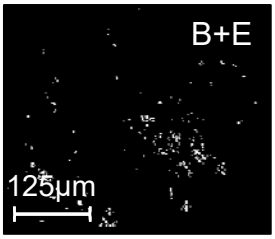
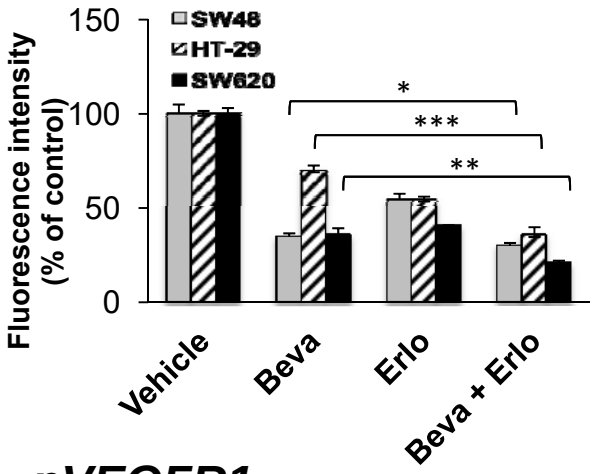
Vehicle
Beva
Erlo
Beva + Erlo

Figure 2

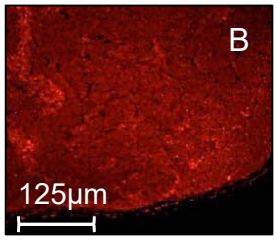
CD31

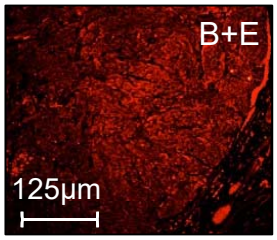


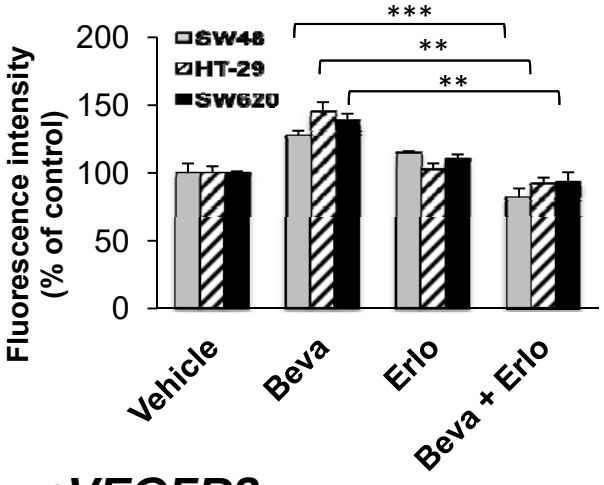




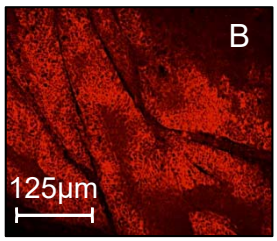
VEGF

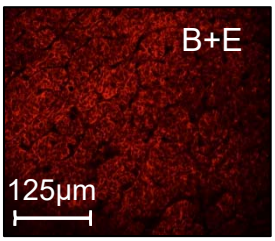


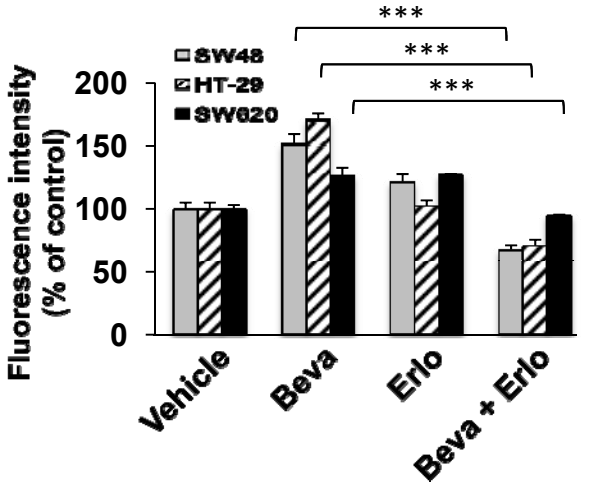




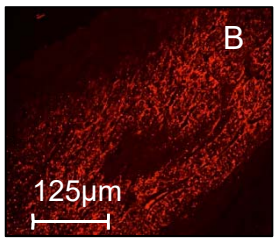
pVEGFR1

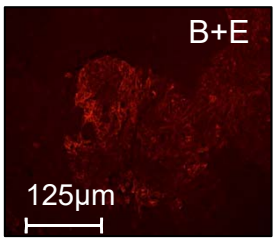


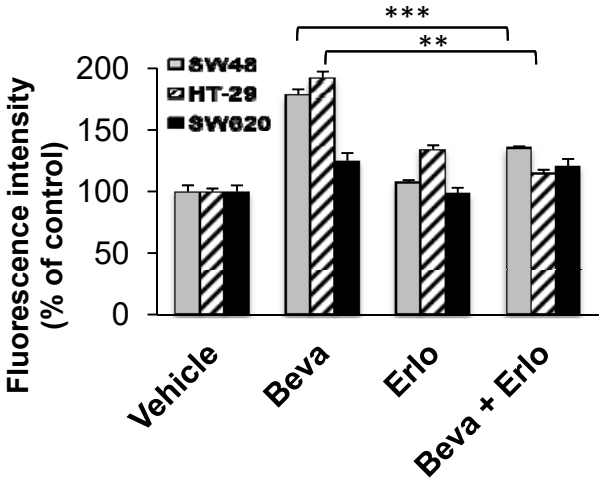




pVEGFR2







pVEGFR1

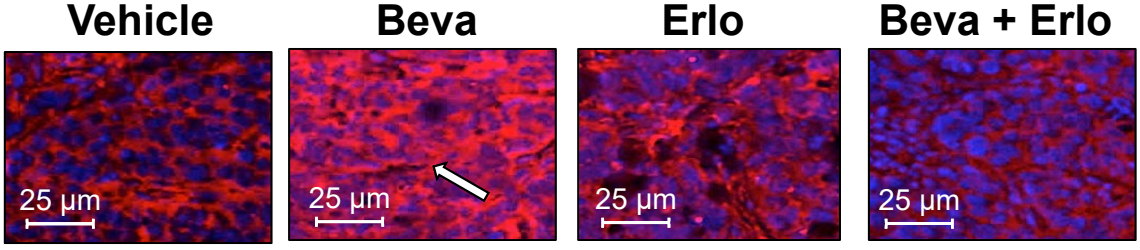


Figure 3

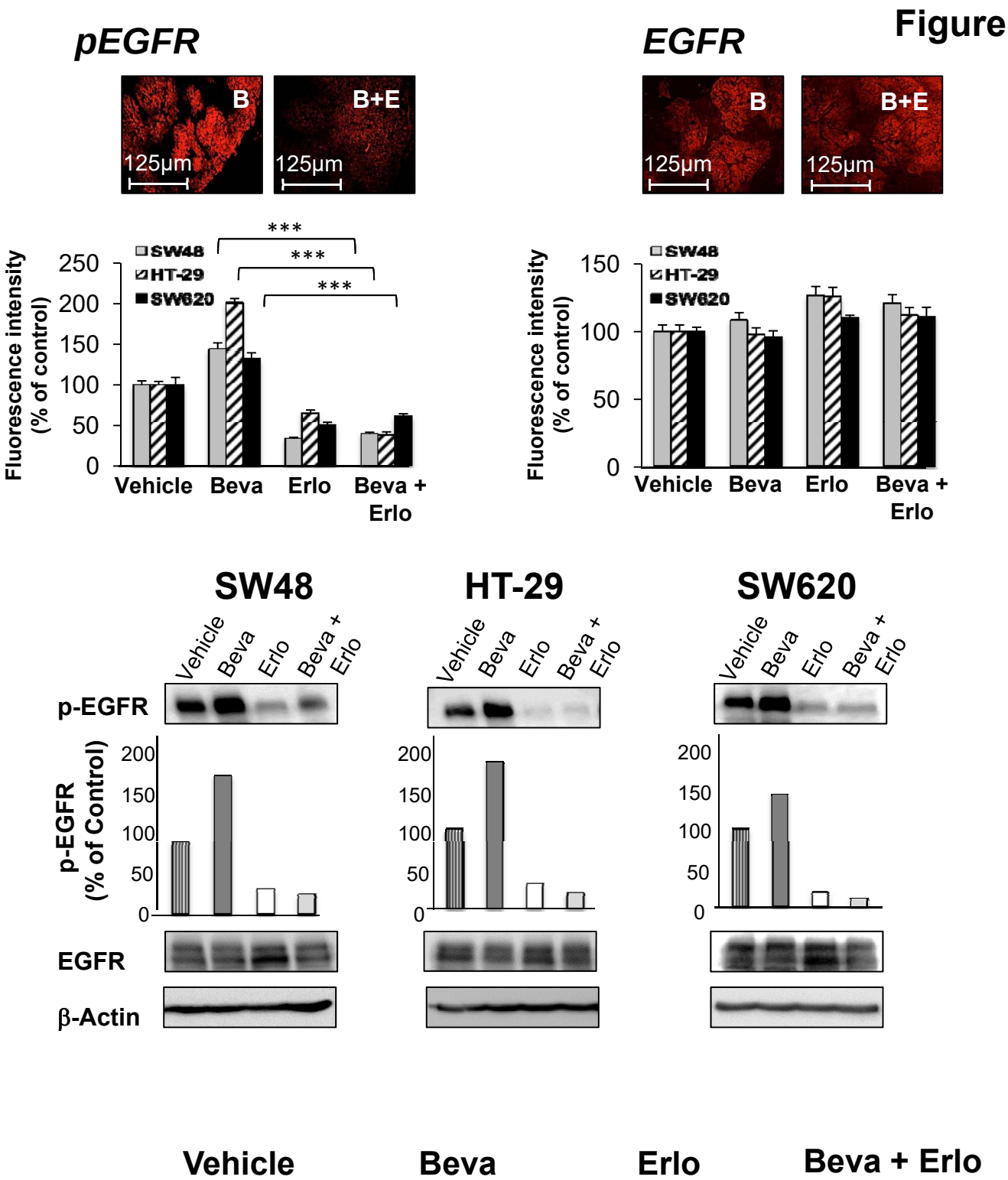
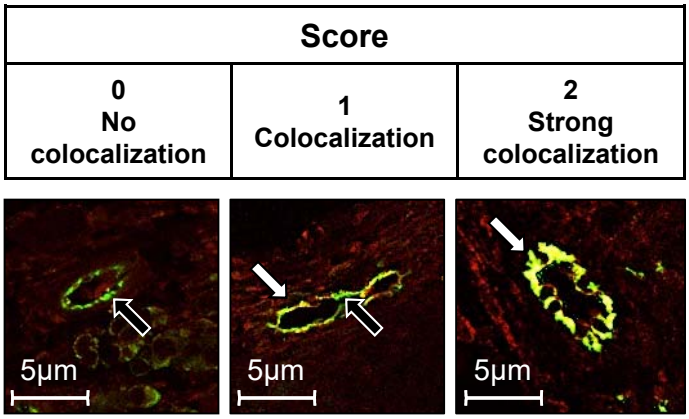
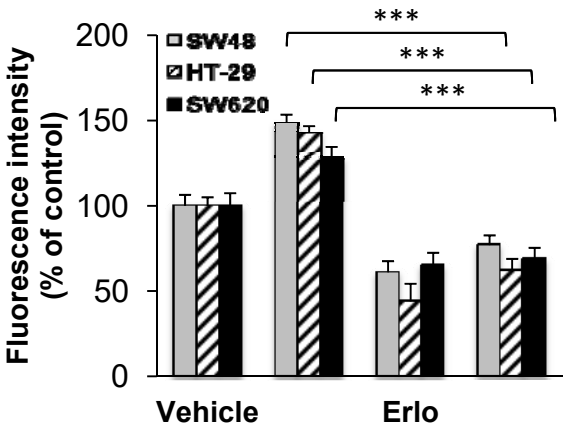
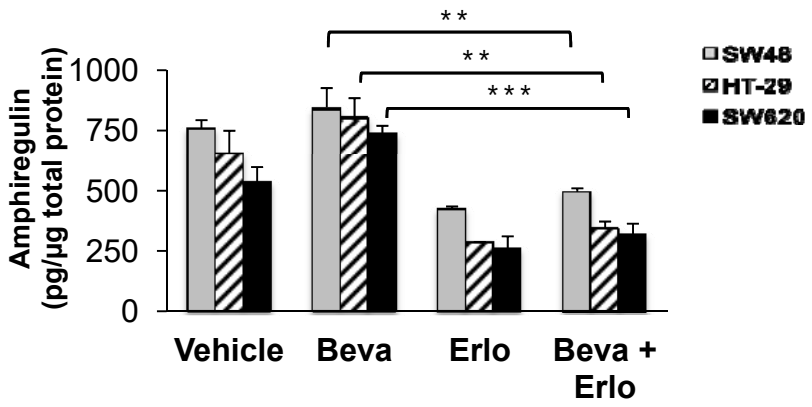

pEGFR
B
B+E
125µm
125µm
EGFR
B
B+E
125µm
125µm
Fluorescence intensity (% of control)
SW48
HT-29
SW620
***
***
***
250
200
150
100
50
0
Vehicle
Beva
Erlo
Beva + Erlo
Fluorescence intensity (% of control)
SW48
HT-29
SW620
150
100
50
0
Vehicle
Beva
Erlo
Beva + Erlo
SW48
HT-29
SW620
Vehicle
Beva
Erlo
Beva + Erlo
p-EGFR
p-EGFR (% of Control)
200
150
100
50
0
EGFR
β-Actin
pEGFR
Vehicle
Beva
Erlo
Beva + Erlo
25 µm
25 µm
25 µm
25 µm

Figure 4


Score
0
No
colocalization
1
Colocalization
2
Strong
colocalization
5µm
5µm
5µm


Fluorescence intensity
(% of control)
200
150
100
50
0
SW48
HT-29
SW620
***
***
***
Vehicle
Erlo


Amphiregulin
(pg/µg total protein)
1000
750
500
250
0
**
**
***
SW48
HT-29
SW620
Vehicle
Beva
Erlo
Beva +
Erlo

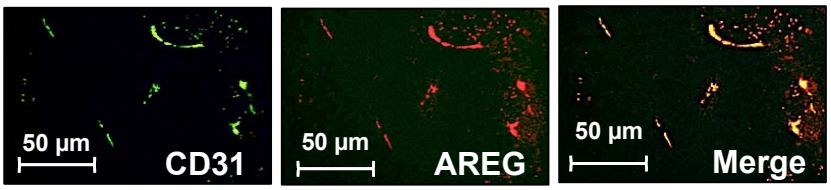

50 µm
CD31
50 µm
AREG
50 µm
Merge

Figure 5

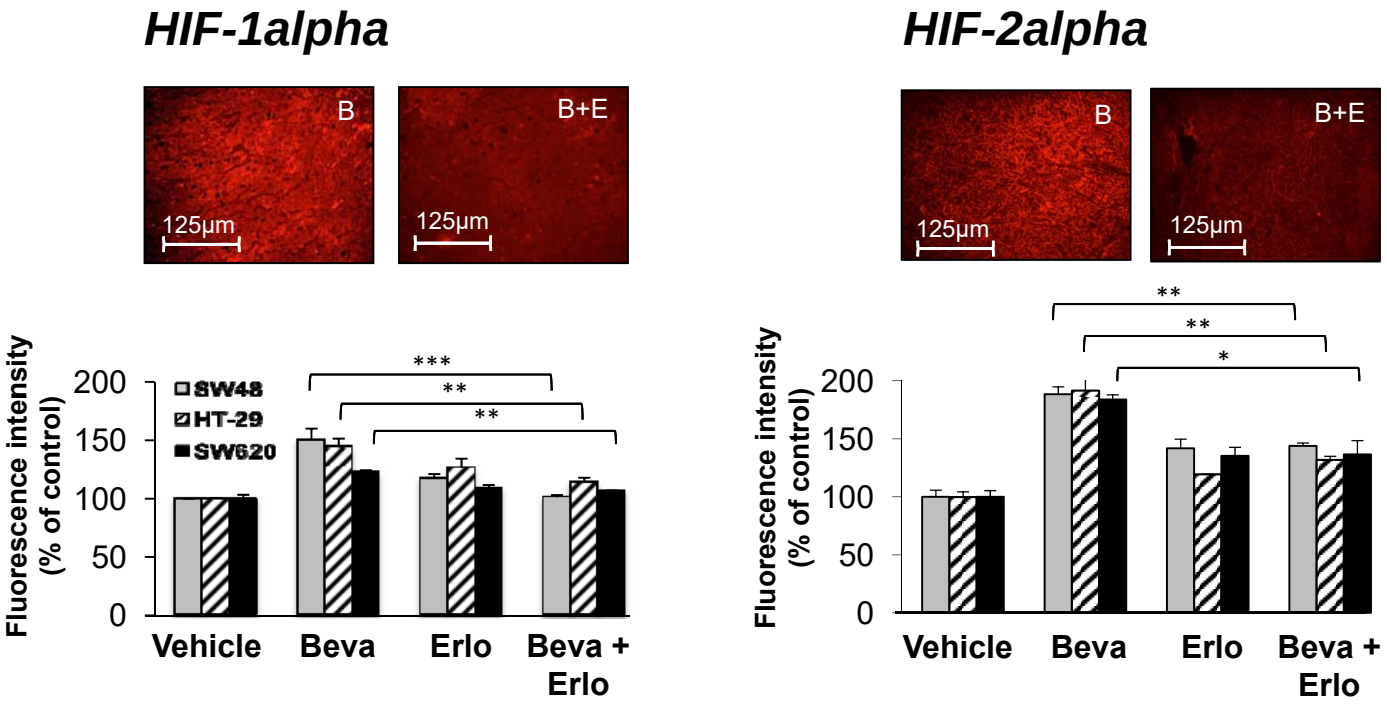
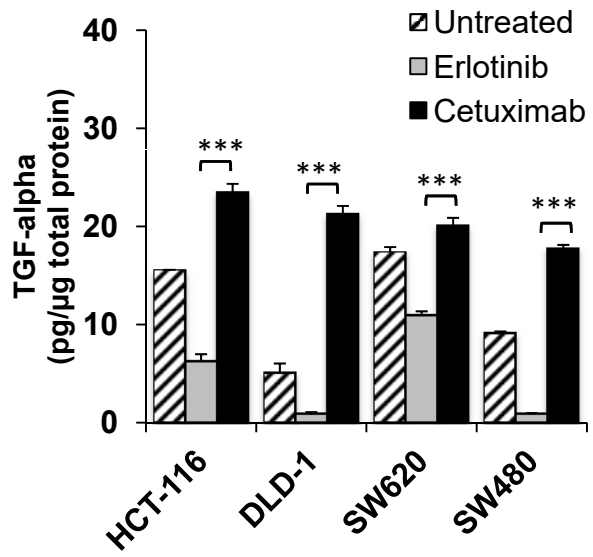
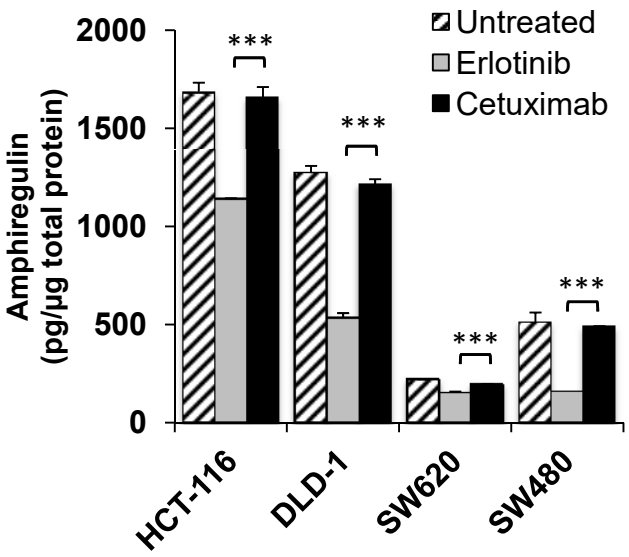
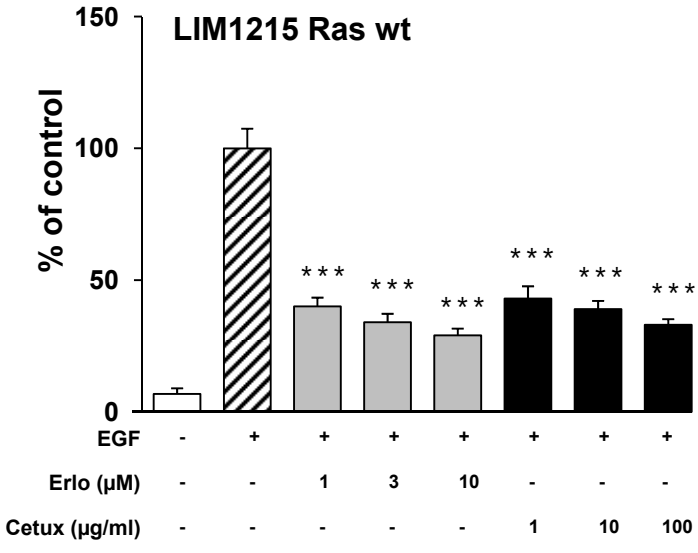
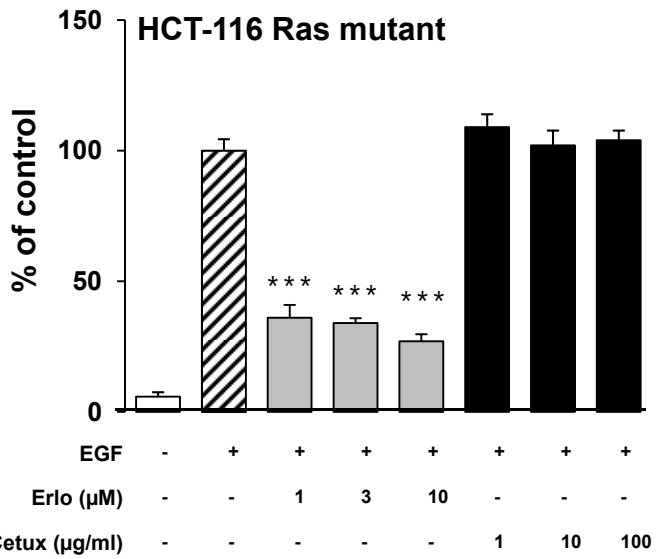

HIF-1alpha
B
B+E
125µm
125µm
Fluorescence intensity (% of control)
200
150
100
50
0
SW48
HT-29
SW620
***
**
**
Vehicle
Beva
Erlo
Beva + Erlo
HIF-2alpha
B
B+E
125µm
125µm
Fluorescence intensity (% of control)
200
150
100
50
0
**
**
*
Vehicle
Beva
Erlo
Beva + Erlo

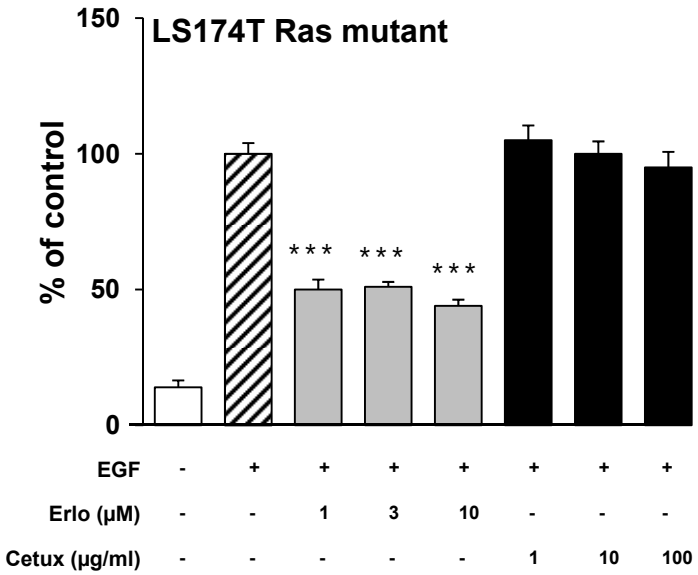
Figure 6


Untreated
Erlotinib
Cetuximab
TGF-alpha (pg/µg total protein)
40
30
20
10
0
***
HCT-116
DLD-1
SW620
SW480


Untreated
Erlotinib
Cetuximab
Amphiregulin (pg/µg total protein)
2000
1500
1000
500
0
***
HCT-116
DLD-1
SW620
SW480


LIM1215 Ras wt
% of control
150
100
50
0
***
EGF - + + + + + + +
Erlo (µM) - - 1 3 10 - - -
Cetux (µg/ml) - - - - - 1 10 100


HCT-116 Ras mutant
% of control
150
100
50
0
***
EGF - + + + + + + +
Erlo (µM) - - 1 3 10 - - -
Cetux (µg/ml) - - - - - 1 10 100


LS174T Ras mutant
% of control
150
100
50
0
***
EGF - + + + + + + +
Erlo (µM) - - 1 3 10 - - -
Cetux (µg/ml) - - - - - 1 10 100

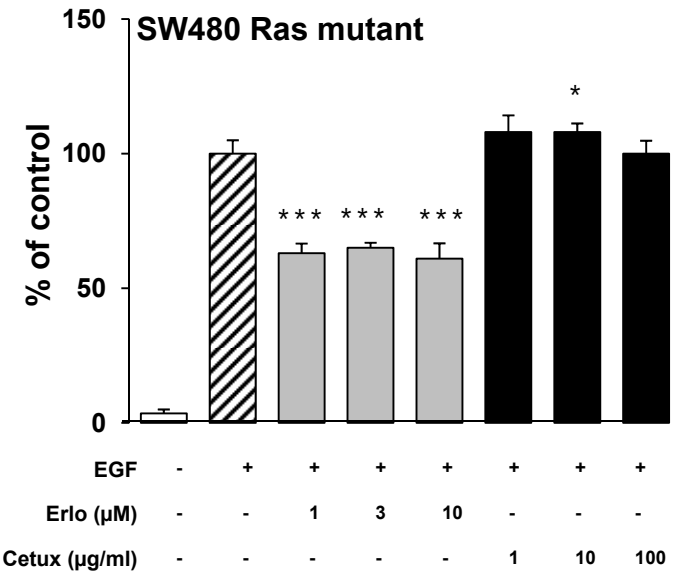
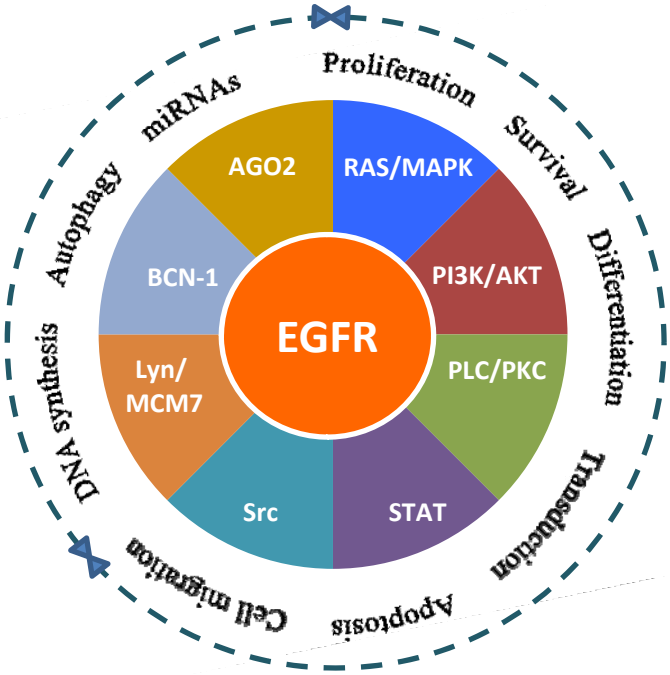

SW480 Ras mutant
% of control
150
100
50
0
***
*
EGF - + + + + + + +
Erlo (µM) - - 1 3 10 - - -
Cetux (µg/ml) - - - - - 1 10 100

Figure 7

Novel Pathways



Classical Mechanisms

Figure S1

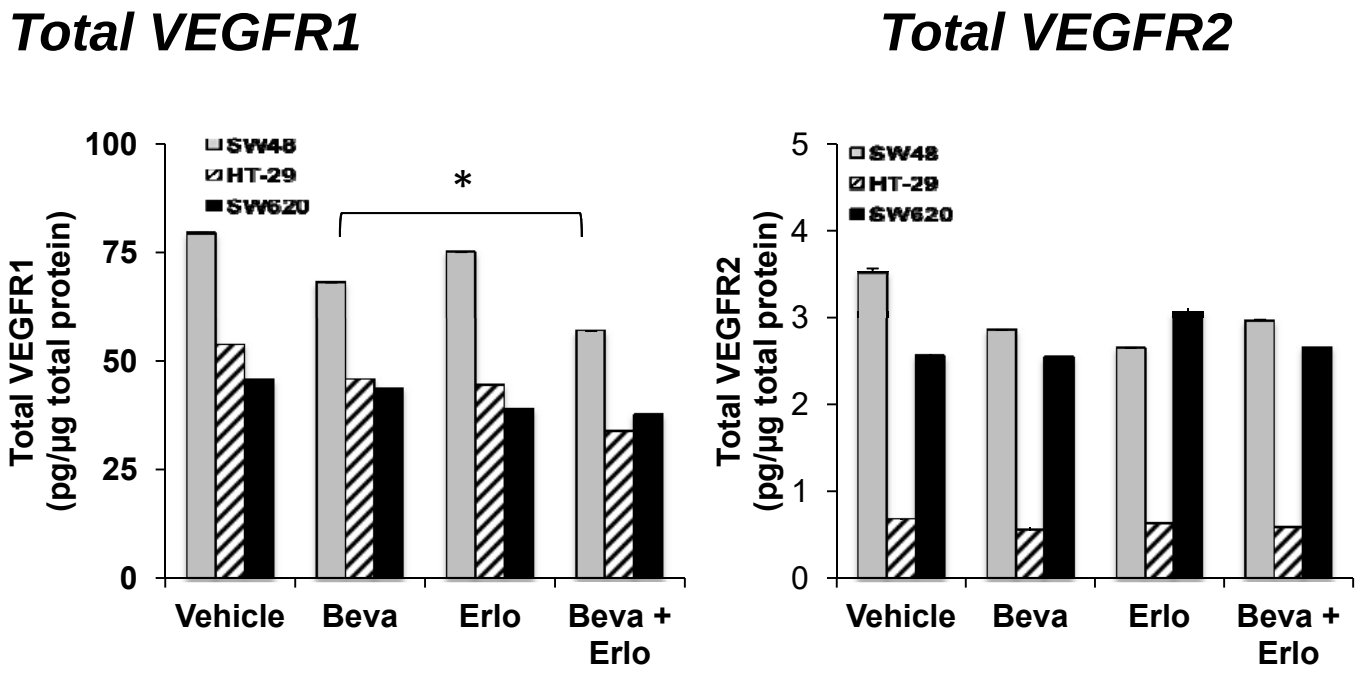
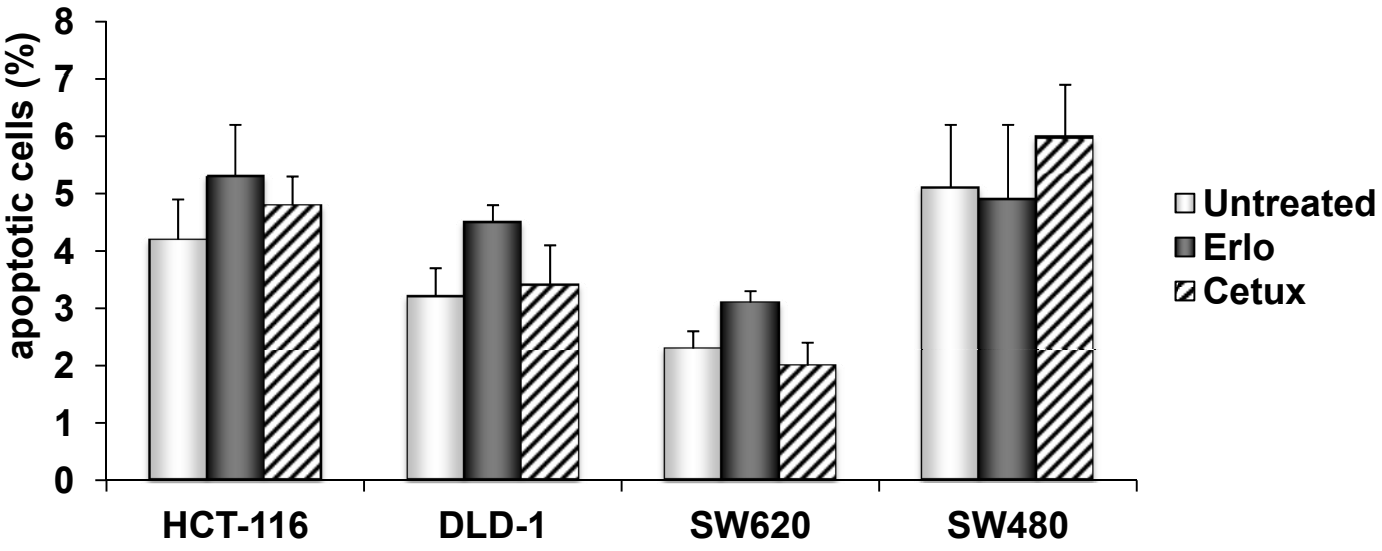

Total VEGFR1
Total VEGFR2
SW48
HT-29
SW620
Total VEGFR1 (pg/µg total protein)
Total VEGFR2 (pg/µg total protein)
100
75
50
25
0
5
4
3
2
1
0
*
Vehicle
Beva
Erlo
Beva + Erlo

Figure S2


apoptotic cells (%)
0
1
2
3
4
5
6
7
8
HCT-116
DLD-1
SW620
SW480
Untreated
Erlo
Cetux

Capítulo II

Antigenotoxic and antimutagenic effects of diphenyl ditelluride against several known mutagens in Chinese hamster lung fibroblasts

Publicado em *Mutagenesis*, 2015, 30, 799–809

Nesse capítulo, mostramos que o pre-tratamento em baixas concentrações com o ditelureto de difenila (DTDF) reduz a citotoxicidade, genotoxicidade e mutagenicidade dos mutágenos peróxido de hidrogênio (H_2O_2), metil metanosulfonato (MMS), *tert*-butil hidroperóxido (*t*-BOOH) ou radiação ultravioleta – C (UVC) em fibroblastos de pulmão de Hamster chinês (células V79). Além disso, com o uso de endonucleases específicas (FPG e Endo III) evidenciamos que o pre-tratamento com o DTDF reduz a quantidade de bases oxidadas do DNA e pelo ensaio de DCFH-DA, reduziu a formação de espécies reactivas de oxigênio (ROS). Esses resultados podem estar associados a modulação das defesas antioxidantes.

Mutagenesis, 2015, 30, 799–809
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Original Manuscript
Advance Access publication 22 May 2015

OXFORD

Original Manuscript

Antigenotoxic and antimutagenic effects of diphenyl ditelluride against several known mutagens in Chinese hamster lung fibroblasts

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Abstract

The present study evaluates antigenotoxic and antimutagenic properties of diphenyl ditelluride (DPDT) against several known mutagens in Chinese hamster lung fibroblasts (V79 cells). DPDT was not cytotoxic and genotoxic at concentrations ranging from 0.01 to 0.1 μM. The pre-treatment for 2 h with this organotellurium compound at non-cytotoxic dose range (0.01, 0.05 and 0.1 μM) increased cell survival after challenge with hydrogen peroxide (H_2O_2), *t*-butyl hydroperoxide (*t*-BOOH), methylmethanesulphonate (MMS) or ultraviolet (UV)C radiation. In addition, the pre-treatment with DPDT decreased the DNA damage and Formamidopyrimidine DNA-glycosylase (Fpg)- and Endonuclease III (Endo III) sensitive sites induction by the studied genotoxic agents, as verified by comet assay and modified comet assay, respectively. The pre-treatment also reduced micronucleus frequency, revealing the protector effect of DPDT against MMS and UVC-induced mutagenesis. Our results demonstrate that DPDT-treated cells at concentration range of 0.01–0.1 μM do not change thiobarbituric acid reactive species (TBARS) levels and ROS generation. Moreover, DPDT pre-treatment at this concentration range decreases the ROS induction by H_2O_2 and *t*-BOOH treatment indicating antioxidant potential. On the other hand, concentrations higher than 0.1 μM increase TBARS formation and inhibited superoxide dismutase (SOD) activity, suggesting pro-oxidative effect of this compound at high concentrations. Our results suggest that DPDT presents antigenotoxic and antimutagenic properties at concentration range of 0.01–0.1 μM. The protection effect could be attributed to antioxidant capacity of DPDT at this concentration range in V79 cells.

Introduction

In the fields of chemistry and biochemistry, it has been shown that organotellurium (OT) compounds could induce a variety of toxic effects that depend on the dose employed. (1, 2) On the other hand, these compounds have been pointed out as promising and useful alternatives for pharmaceutical industry. (2, 3) OT compounds that inhibit thioredoxin reductase (TrxR) could be useful as antitumor agents with effects including induction of oxidative stress, cell cycle arrest and apoptosis. (4) Consequently, it is essential to increase the

knowledge about pharmacological properties of OT compounds, particularly in view of their toxic potential or antioxidant and antigenotoxic activities. (5)

OT compounds can exhibit potent antioxidant activity mediated by their glutathione peroxidase (GPx) mimetic properties (6, 7) and/or by their ability to scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS) in mammalian models. (8) Moreover, OT compounds revealed anti-inflammatory, immunomudulatory, anticancer, hepato- and neuroprotective properties. (9–11)

The diphenyl ditelluride (DPDT) is a solid, simple and stable OT compound. It is an important and versatile intermediate in organic synthesis. The DPDT in high doses is extremely toxic in rodents, causing neurotoxic effects in mice after acute or prolonged exposure and neurodegenerations in rats. (12–14) Also, previous studies of our laboratory showed cytotoxic, genotoxic and mutagenic properties of DPDT in mammalian V79 cells and in strains of yeast *Saccharomyces cerevisiae* both proficient and deficient in several DNA repair pathways. (3) Moreover, DPDT has been shown to inhibit TrxR and GPx in adult mice. (15)

On the other hand, Rossato (7) reported an antioxidant action of DPDT by reducing the level of lipid peroxidation induced by some oxidants in rat brain. Indeed, Brito *et al.* (16) showed that DPDT afforded protection in adult mice against 4-aminopyridine-induced neurotoxicity and oxidative stress. Moreover, it has been reported that DPDT at low concentrations (1–4 µM) significantly increased Na^+/K^+-ATPase activity in rat brain, suggesting that DPDT could be an antioxidant agent. (13)

The aim of this study was to establish the concentration range of DPDT with possible protective, antigenotoxic and antimutagenic effects in V79 cells. For this purpose we evaluated cell survival and DNA damage induction in comet assay and cytokinesis block micronucleus test. Oxidative stress parameters and activity of antioxidant enzymes following low concentration DPDT treatment are also determined.

Materials and methods

Chemicals

DPDT (CAS registry number 32294-60-3) was provided by Dr Antônio Braga, Federal University of Santa Catarina, Brazil. The analysis of ^{1}HNMR and ^{13}CNMR spectra showed that DPDT analytical and spectroscopic data fully agreed with their assigned structure. The chemical purity of DPDT (99.9%) was determined by gas chromatography/high-performance liquid chromatography. Dulbecco's modified Eagle Medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA, l-glutamine and antibiotics were purchased from Gibco BRL (Grand Island, NY, USA); cytochalasin-B (Cyt-B), methylmethanesulfonate (MMS), hydrogen peroxide (H_2O_2), thiobarbituric acid (TBA), trichloroacetic acid (TCA) and hydrolysed 1,1,3,3-tetramethoxypropane (TMP) were purchased from Sigma (St. Louis, MO, USA). Low-melting point agarose and normal agarose were obtained from Invitrogen (Carlsbad, CA, USA). Formamidopyrimidine DNA-glycosylase (Fpg) and Endonuclease III (Endo III) were obtained from New England BioLabs (Beverly, MA, USA). Giemsa stain was from Merck (Darmstadt, Germany) and 2′,7′-dichlorfluorescein-diacetate (DCFH-DA) from Invitrogen (PoortGebouw, The Netherlands). All other reagents were of analytical grade. The tissue culture flasks (bottles and dishes) were supplied by TPP (Trasadingen, Switzerland).

Cell culture and treatments

V79 cells were grown as monolayers under standard conditions in DMEM supplemented with 10% heat-inactived FBS, 0.2 mg/ml l-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were maintained in tissue culture flasks at 37°C in a humidified atmosphere containing 5% carbon dioxide, and were harvested by treatment with 0.15% trypsin-0.08% EDTA in phosphate-buffered saline (PBS) solution. Cells were seeded in complete medium and grown to 80–90% confluence prior to the treatment with the test substance. DPDT was dissolved in dimethyl sulfoxide (DMSO) and added to FBS-free medium to reach the different desired concentrations. The final DMSO concentration in the medium never exceeded 0.2%, and the control group was exposed to an equivalent concentration of solvent. Cells were treated with DPDT for 2 h, at concentrations from 0.01 to 1 µM, in DMEM culture without serum, then washed with PBS at pH 7.4 and submitted to the mutagen treatment.

For oxidative challenge, cells were exposed to 150 µM H_2O_2 or to 200 µM *t*-butyl hydroperoxide (*t*-BOOH) for 2 h in the dark at 37°C. For the MMS exposure, cells were incubated with 40 µM MMS for 2 h in the dark at 37°C. For the ultraviolet (UV)C exposure, medium was removed after incubation, and the monolayer was rinsed with PBS at pH 7.4. Each dish received PBS at pH 7.4, and cells were exposed to a dose of UV light (254 nm) at a rate of 0.3 J/m²/s at room temperature. UVC dosimetry was performed in air with Blak-Ray Ultraviolet Meter (Upland, CA, USA) and the irradiation dose was 5 J/m². Prior to exposure, lids of the dishes were removed, and the covering solution aspirated from the dishes.

Cytotoxicity evaluation using a lactate dehydrogenase leakage assay

V79 cells (1×10^4 cells/well) in logarithmic growth phase were seeded in 96-well plates. After treatment, the fraction of total lactate dehydrogenase (LDH) activity in the supernatant was taken as an indicator of membrane leakage or cell lysis. CytoTox assay kit (Promega, Madison, WI, USA) was used for enzymatic assessment of LDH release, following the manufacturer's instructions. This method involves assessing the rate of conversion of NADH (1.5 mmol/l) to NAD^+ in the presence of l-(+)-lactic acid (50 mmol/l) in culture supernatants (*S*) and in the remaining cells (*C*) after lysis with serum-free medium containing 1% Triton X-100. The percentage of LDH leakage was calculated as follows: % LDH leakage = $S \div (S + C) \times 100$, where *S* corresponds to the LDH activity measured in the supernatants and *C* to the LDH activity measured in the remaining cells of each sample after lysis.

Colony-forming ability (clonal survival)

Exponentially growing V79 cells were treated according to the experimental protocol of Mirabelli *et al.* (17) After treatment, cells were trypsinised, and 200 cells per 60-mm dish were seeded in triplicate to determine colony-forming ability. After 5–7 days incubation, colonies were fixed with ethanol, stained with crystal violet (0.1%), counted, and their survival was calculated as a percentage relative to the control treatment.

Comet assay

The alkaline comet assay was performed as described (3) with minor modifications. V79 cells were pre-treated at low concentrations of DPDT and exposed to mutagens. After treatment, cells were washed with ice-cold PBS, trypsinised and resuspended in complete medium. Then, 15 µl of cell suspension were mixed with low-melting point

agarose, and immediately spread onto a glass microscope slide pre-coated with a layer of 1.5% normal melting point agarose. Agarose was allowed to set at 4°C for 5 min. Slides were incubated in ice-cold lysis solution at 4°C for at least 1 h to remove cell proteins, leaving DNA as 'nucleoids'. The alkaline comet assay was performed at pH 13. In the modified comet assay, slides were removed from the lysis solution, washed three times in enzyme buffer and incubated with 60 µl of Fpg (45 min 37°C) or Endo III (30 min 37°C) solutions. After lysis, slides were placed on a horizontal electrophoresis unit, covered with fresh buffer for 15 min at 4°C to allow DNA unwinding and the expression of alkali-labile sites. Electrophoresis was performed for 20 min at 25 V and 300 mA (0.90 V/cm). Slides were then neutralised (4 mM Tris, pH 7.5), washed in bi-distilled water and stained using a silver nitrate. One hundred cells (50 cells from each of the two replicate slides) were selected, and analysed for each concentration of the test substance. Cells were visually scored according to tail length into five classes: (i) class 0: undamaged, without tail; (ii) class 1: with a tail shorter than the diameter of the head (nucleus); (iii) class 2: with a tail length 1–2 × the diameter of the head; (iv) class 3: with a tail longer than 2 × the diameter of the head and (v) class 4: comets with no heads. A value damage index (DI) was assigned to each sample. DI ranged from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4). The formation of Fpg- and Endo III sensitive sites was calculated as a difference between the score obtained after incubation with the respective enzyme and with the enzyme buffer only.

Micronucleus test

The micronucleus assay in binucleated cells was performed according to Thomas and Fenech, (18) with minor modifications. V79 cells were incubated with various DPDT concentrations for 2 h in FBS-free medium. Cultures were then washed twice with medium, and Cyt-B was added to a final concentration of 3 µg/ml. As positive controls, the classical mutagenic agents MMS and UVC were used. Cultures were harvested 21 h after Cyt-B addition. Cells were separated from the bottle by trypsinisation, and the cell suspension was centrifuged at 1000 × g for 5 min. Cells were resuspended in 75 mM KCl, and maintained at 4°C for 3 min (mild hypotonic treatment). They were then centrifuged, and a methanol/acetic acid (3:1) solution was slowly added. This fixation step was repeated twice, and the cells were finally resuspended in a small volume of methanol/acetic acid, dropped on clean slides, and stained with 3% Giemsa (pH 6.8) for 5 min. Slides were mounted, and codified prior to microscopic analysis. Micronuclei were counted in 2000 binucleated cells (BNC) with well-preserved cytoplasm. Nuclear division index (NDI) was also calculated employing the formula, NDI = [M1 + 2(M2) + 3(M3) + 4(M4)]/N

Where M1–M4 represents the number of cells with one to four nuclei and *N* is the total number of cells scored (excluding necrotic and apoptotic cells).

Determination of ROS levels

The levels of intracellular ROS were determined by DCFH-DA. DCFH-DA enters the cells and predominantly reacts with highly oxidising ROS to produce the fluorophore dichlorofluorescein (DCF). (19) Briefly, 5×10^4 cells/well were plated in 24-well culture plates, grown overnight, pre-treated with DPDT for 2 h and submitted to mutagen treatment. For oxidative challenge, cells were exposed to 500 µM H_2O_2 or 400 µM *t*-BOOH for 2 h in the dark at 37°C. After that, the cells were washed twice with PBS and incubated with 10 µM DCFH-DA in PBS at 37°C for 30 min, protected from light. After incubation, cells were washed with PBS, harvested with trypsin/EDTA and for quantification of ROS, the fluorescence intensity was measured using a Guava easyCyte Plus Flow Cytometry (Guava Technologies, Hayward, CA, USA). Ten thousand cells were measured for each experimental condition. Relative ROS production was expressed as the change in fluorescence of the test samples compared with that of the appropriate controls (100%).

Superoxide dismutase

Superoxide dismutase (SOD) activity was spectrophotometrically determined according to the method described by Bannister and Calabrese. (20) The V79 cells (1×10^6 cells/well) were plated in 6-well culture plates, grown overnight and treated with DPDT for 2 h, washed three times with phosphate buffer, scraped and centrifuged at 3000 × *g* for 10 min, and the supernatant was collected. The SOD was measuring by the inhibition of self-catalytic adrenochrome formation rate at 480 nm in a reaction medium containing 1 mmol/l adrenaline (pH 2.0) and 50 mmol/l glycine (pH 10.2). This reaction was performed at 30°C for 3 min and was expressed as U SOD/mg protein; protein was measured by Pierce™ BCA protein assay kit (Thermo, Rockford, IL, USA).

Catalase

The catalase (CAT) activity was determined according to the method described by Aebi (21) in cell extracts obtained as follows. V79 cells (1×10^6 cells/well) were plated in 6-well culture plates, grown overnight and treated with DPDT for 2 h. Cells were washed three times with 50 mM phosphate buffer (pH 7.0). The resulting suspension was centrifuged at 3000 × *g* for 10 min, and the supernatant was incubated with phosphate buffer containing H_2O_2 3 mM. CAT activity was determined by measuring the decrease in absorbance (H_2O_2 degradation) at 240 nm for 3 min and expressed as U CAT/mg protein. One unit of catalase activity was defined as 1 µM of H_2O_2 consumed/min; protein was measured by Pierce™ BCA protein assay kit (Thermo).

Measurement of lipid peroxidation

The extent of DPDT-induced lipid peroxidation was determined by the formation of thiobarbituric acid reactive species (TBARS) during an acid-heating reaction. The assays were performed according to Salgo and Pryor (22), with minor modifications. V79 cells (1×10^7 cells) were seeded in 25 cm^2 flask. After the treatments, the cells were washed with cold PBS, scraped and homogenised in ice-cold PBS. Samples containing 400 µl of the cell lysates were combined with 600 µl of 15% TCA and 0.67% TBA. The mixture was heated at 100°C for 25 min. After cooling to room temperature, the samples were centrifuged at 4000 r.p.m. for 10 min, the supernatant fraction was isolated and the absorbance was measured at 532 nm. Hydrolysed TMP was used as a standard and results are expressed as malondialdehyde equivalents.

Statistical analysis

The data were obtained from at least three independent experiments in duplicate, and the results are expressed as mean ± SD. Data were analysed by one-way analysis of variance (ANOVA), followed by test of Tukey with $P < 0.05$ considered as statistically significant.

Results

DPDT range finder study

In order to choose DPDT concentrations for the study, LDH leakage assay and clonal survival were employed. DPDT treatment at concentration range of 0.01–0.5 µM was not cytotoxic, while a concentration of 1 µM DPDT significantly increased LDH leakage (to 19% of

total, $P < 0.001$) in relation to the control (8% of total, Figure 1A) and decreased clonal survival to 15% (Figure 2A). In addition, the treatment with DPDT at non-cytotoxic concentrations (0.01–0.5 µM) was unable to induce DNA strand breaks as shown by the alkaline comet assay (Figures 3A and 4A). Therefore, we decided to use concentration range of 0.01–1.0 µM DPDT in all subsequent experiments.

Protective effect of DPDT in V79 cells

The ability of non-toxic concentrations of DPDT to reduce the H_2O_2 toxicity was measured in the LDH leakage assay and by clonal survival. Figure 1B shows that the cytotoxicity of 150 µM H_2O_2 treatment (74% of total LDH) was significantly suppressed by DPDT pre-treatment at concentrations 0.01 (58% of total, $P < 0.05$), 0.05 (51% of total, $P < 0.01$) and 0.1 µM (39% of total, $P < 0.001$). As can be seen in Figure 2B, the exposure at 150 µM H_2O_2 decreased the clonal survival to 47% ($P < 0.001$) when compared to the negative control. However, when cells were pre-treated with DPDT prior exposure to H_2O_2, the clonal survival was significantly higher compared to cells only exposed to mutagens at DPDT concentrations of 0.01 (70%, $P < 0.05$), 0.05 (84%, $P < 0.001$) and 0.1 µM (80%, $P < 0.001$), showing protective effect against toxicity generated by H_2O_2.

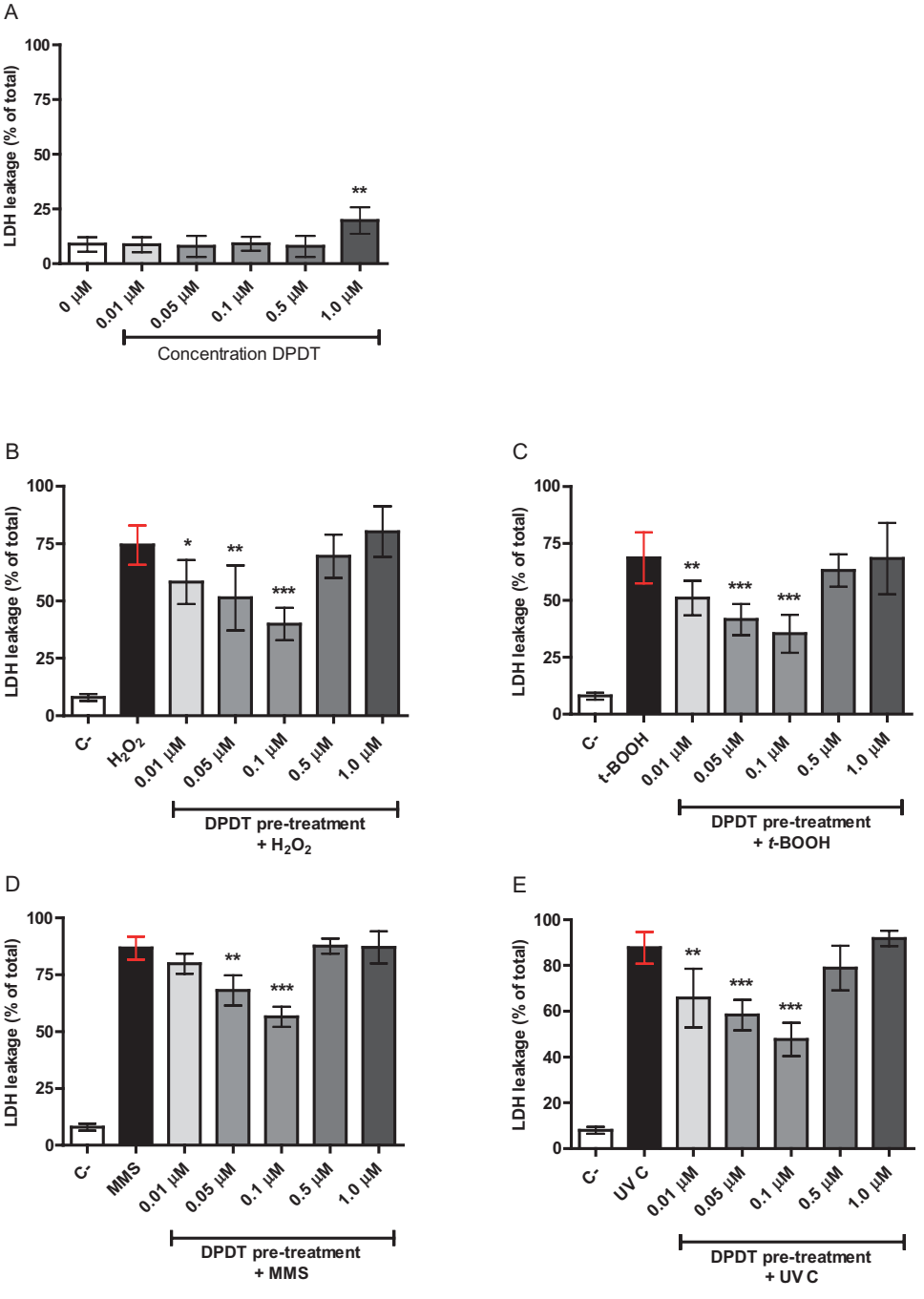

Figure 1. Effects of DPDT on extracellular LDH leakage in V79 cells. (**A**) DPDT treatment was compared with the negative control. (**B–E**) DPDT pre-treated cells were compared to cells only exposed to mutagens. Results are expressed as mean ± SD ($n = 6$). Data significantly different in relation to the control group: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$/one-way ANOVA Tukey's multiple comparison test.

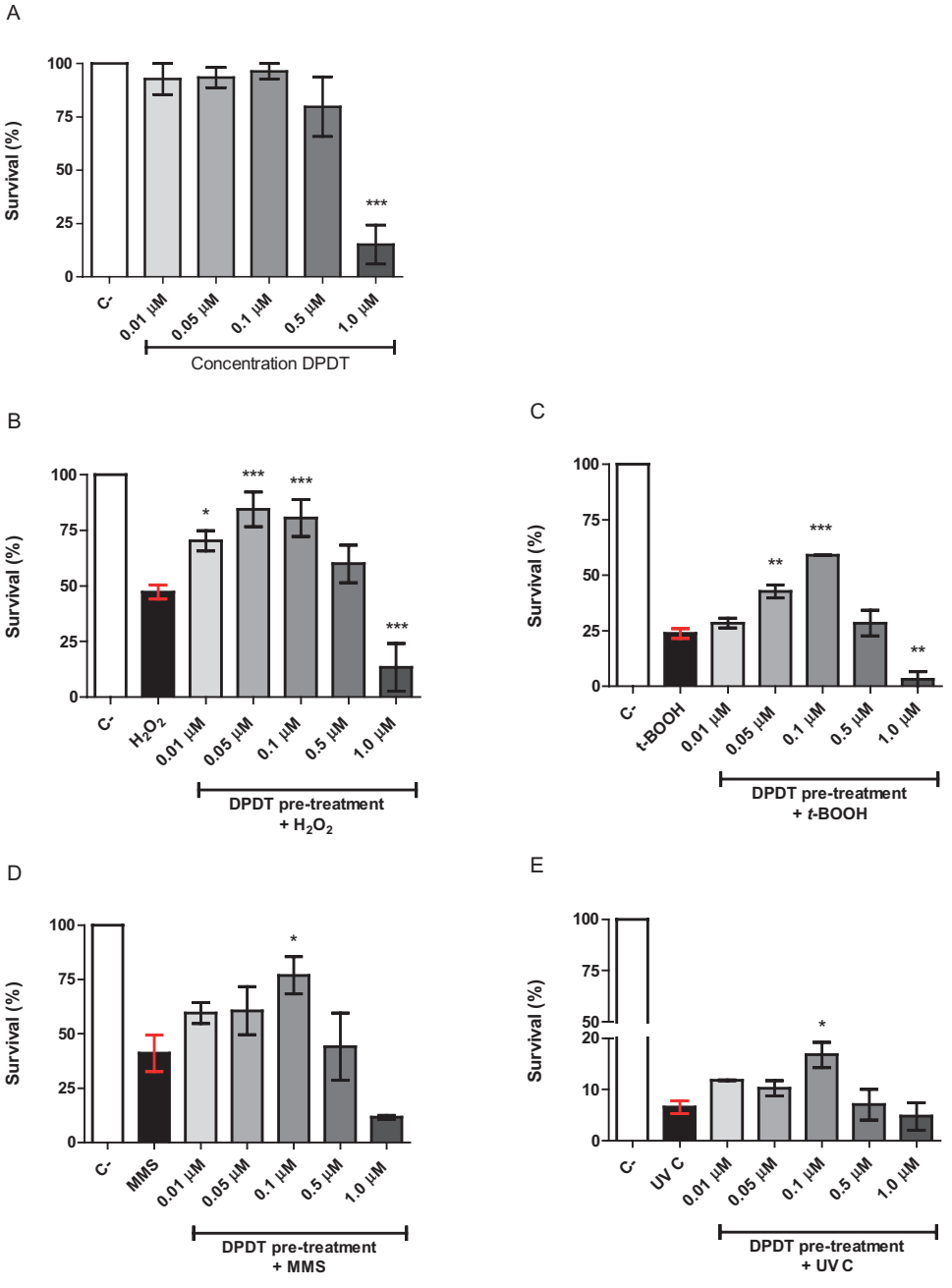

Figure 2. Clonogenic survival in V79 cells. (**A**) DPDT treatment was compared to the negative control. (**B–E**) DPDT pre-treated cells were compared to cells only exposed to mutagens. Results are expressed as mean ± SD, $n = 3$. Data significantly different in relation to the control group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$/ one-way ANOVA Tukey's multiple comparison test.

The DPDT pre-treatment also decreased the leakage of LDH in V79 cells after 200 µM *t*-BOOH oxidative challenge (68% of total, Figure 1C), to 50% at concentration of 0.001 µM ($P < 0.05$), to 41% at 0.05 µM ($P < 0.001$) and to 35% at 0.1 µM ($P < 0.001$), indicating an increase of cell viability in a concentration-dependent manner. Figure 2C shows that the pre-treatment at concentrations of 0.05 (42% survival, $P < 0.05$) and 0.1 µM (59% survival, $P < 0.001$), DPDT prevented the cell death and increased the clonal survival about two times in comparison with cells exposed to 200 µM *t*-BOOH alone (23% survival, $P < 0.001$). As can be seen in Figure 1D, the pre-treatment with DPDT at concentrations 0.05 (68% of total LDH leakage, $P < 0.05$) and 0.1 µM (56% of total LDH leakage, $P < 0.001$) decreased the cytotoxicity induced by MMS (86% of total). Figure 2D shows that MMS decreased the clonal survival to 41% when compared to the negative control; however, the pre-treatment with DPDT at concentration of 0.1 µM (76% survival, $P < 0.05$) decreased the toxicity generated by 40 µM MMS. It can be seen in Figure 1E that the exposure to 5 J/m^2 UVC was highly cytotoxic, as measured in LDH leakage assay (87% of total LDH leakage, $P < 0.001$), when compared to the negative control (8% of total); however, pre-treatment with DPDT at doses 0.01 (65% of total LDH, $P < 0.05$), 0.05 (58% of total LDH, $P < 0.001$) and 0.1 µM (47% of total LDH, $P < 0.001$) decreased cytotoxicity induced by UVC (Figure 1E). The exposure

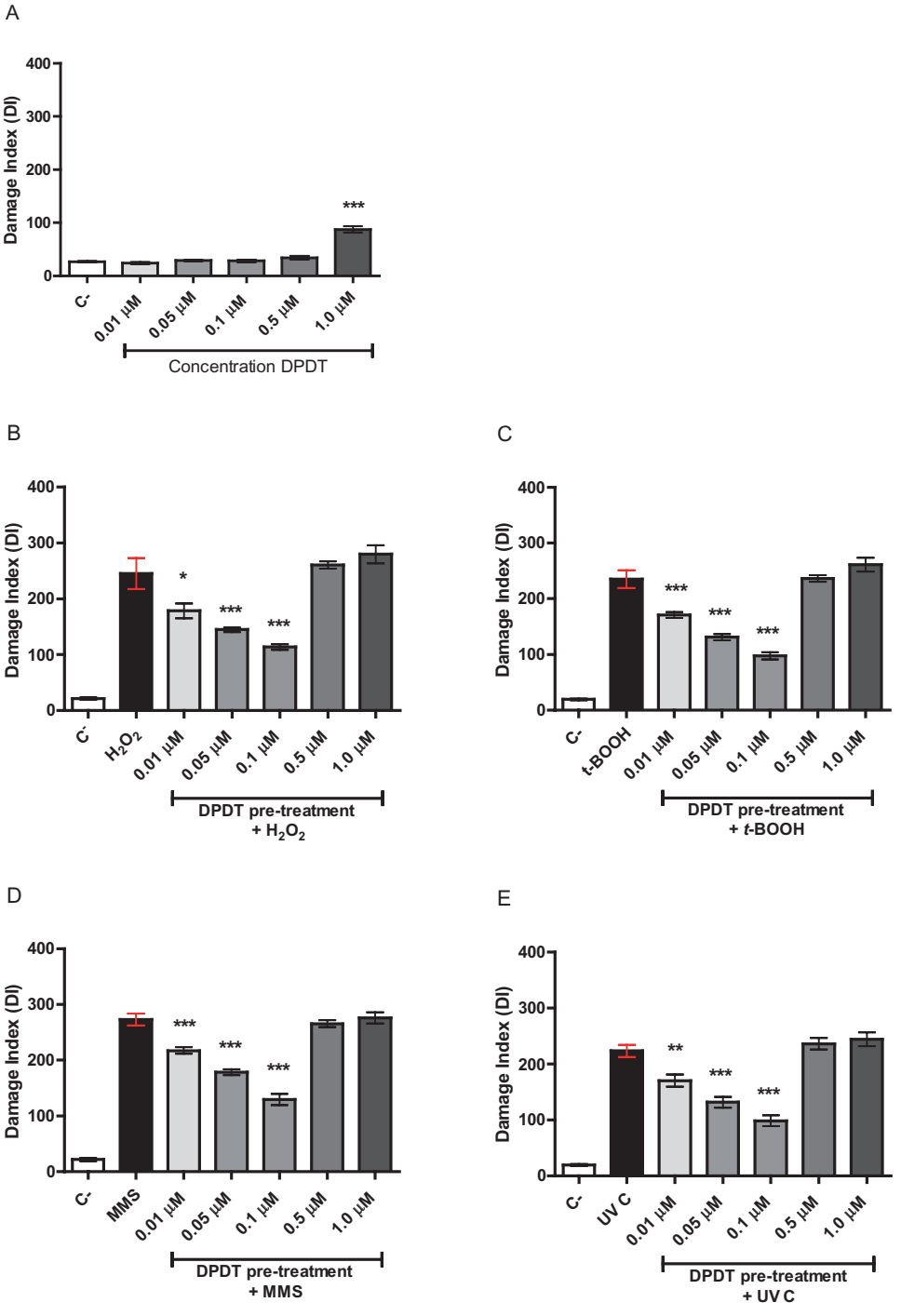

Figure 3. Antigenotoxic evaluation in V79 cells by comet assay. (**A**) DPDT treatment was compared to the negative control. (**B–E**) DPDT pre-treated cells were compared to cells only exposed to mutagens. Solvent was used as a negative control. Data are expressed as means ± SD, $n = 6$. Data significantly different in relation to the control group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$/one-way ANOVA Tukey's multiple comparison test.

of cells to UVC leads to significant decrease in clonal survival (7% survival, $P < 0.001$); however, pre-treatment with DPDT at concentration 0.1 µM (16% survival, $P < 0.05$) increases clonal survival (Figure 2E).

Antigenotoxic effects of DPDT

In order to verify possible antigenotoxic effect, we tested DPDT action against the oxidative agents H_2O_2 or *t*-BOOH (Figure 3B and C, respectively). When the cells were pre-treated with DPDT at concentrations of 0.01, 0.05 and 0.1 µM prior exposure to the genotoxic agents, the DNA strand breaks and Fpg- and Endo III sensitive sites were significantly reduced, as verified in traditional ($P < 0.05$) (Figure 3B and C) and modified Comet assay ($P < 0.05$) (Figure 4B and C). DPDT pre-treatment afforded best protection against the MMS and UVC genotoxicity at concentration of 0.1 µM ($P < 0.001$), reducing DI ($P < 0.001$) (Figure 3D and E) and Fpg- and Endo III sensitive sites ($P < 0.05$) (Figure 4D and E) induced by MMS or UVC, respectively.

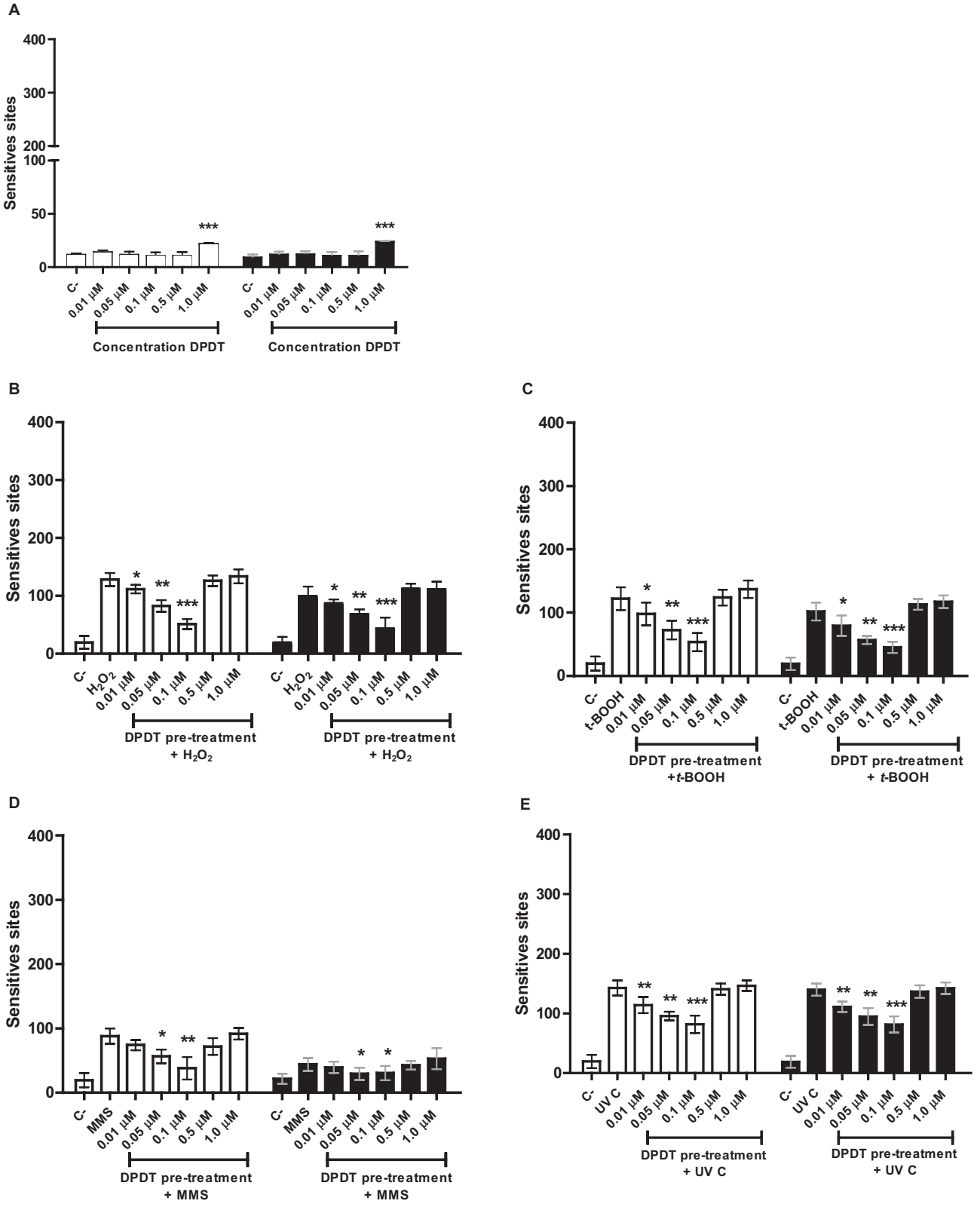

Figure 4. DPDT pre-treatments were compared to cells only exposed to mutagen as evaluated by a modified comet assay using Fpg (clear bars) and Endo III (black bars) enzymes. The Fpg- and Endo III sensitive sites were calculated as a difference between the score obtained after incubation with the respective enzyme and with the enzyme buffer only. (**A**) DPDT treatment was compared with the negative control. (**B–E**) DPDT pre-treated cells were compared to cells only exposed to mutagens. Solvent was used as a negative control. Data are expressed as means ± SD, $n = 5$. Data significantly different in relation to the control group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$/one-way ANOVA Tukey's multiple comparison test.

Antimutagenic properties of DPDT

As can be seen in Figure 5A, the DPDT did not induce micronuclei at concentration range of 0.01 to 0.1 µM. The concentrations 0.5 (12.6 ± 3.5 MN, $P < 0.001$) and 1 µM (18.6 ± 1.5 MN, $P < 0.001$) of DPDT increased the micronucleus frequency three and four times, respectively, when compared to the negative control (4.3 ± 0.5 MN) (Figure 5A). Therefore, for the evaluation of the antimutagenic effects, concentrations of 0.01, 0.05 and 0.1 µM DPDT were used. Figure 5B and C shows that MMS (13.5 ± 0.6 MN, $P < 0.001$) and UVC (18.1 ± 2.1 MN, $P < 0.001$) induced micronucleus formation and decrease cell proliferation (59%, $P < 0.001$ and 58%, $P < 0.05$, respectively) (Figure 5D and E). However, the pre-treatment with DPDT decreased micronucleus frequency for MMS and UVC at concentrations of 0.01 (4.0 ± 0.8 MN, $P < 0.01$ and 6.1 ± 1.0 MN, $P < 0.001$), 0.05 (4.0 ± 0.7 MN, $P < 0.01$ and 5.3 ± 0.7 MN, $P < 0.001$) and

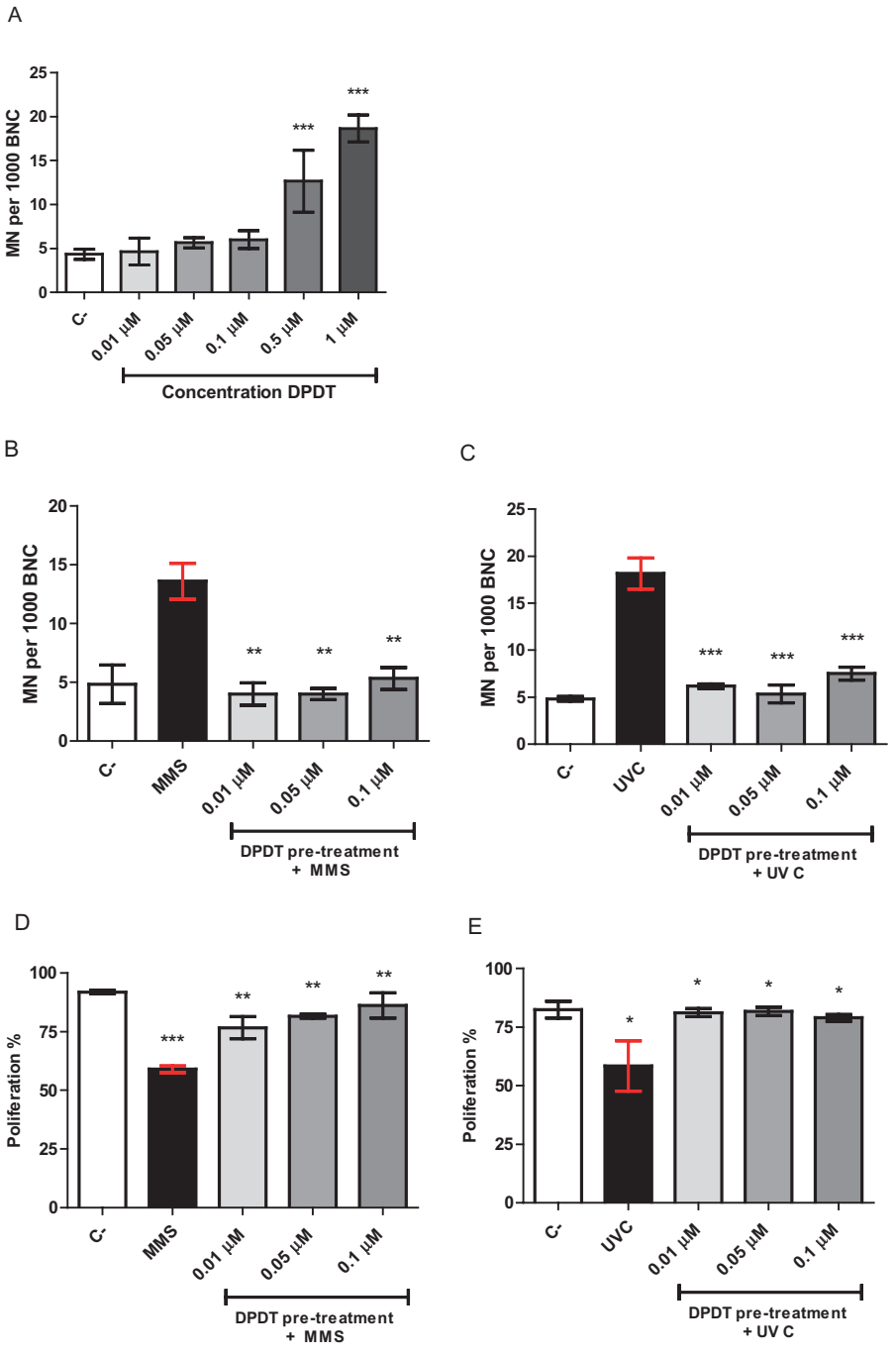

Figure 5. Micronucleus induction in V79 cells by 2 h DPDT treatment (**A**). DPDT pre-treated cells were compared to cells only exposed to MMS (40 µM) (**B**), or UV C 5 J/m² (**C**). Effect of the treatment with non-mutagenic DPDT concentrations on the cell proliferation measured by NDI. (**D**) Treatment with MMS (40 µM) following DPDT pre-treatment at the indicated concentrations. (**E**) Treatment with UV C 5 J/m² following DPDT pre-treatment at the indicated concentrations. Results are expressed as mean ± SD, $n = 3$. Data significantly different in relation to the control group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$/one-way ANOVA Tukey's multiple comparison test. The groups with pre-treatment were compared to the treatment with mutagen alone. Positive control was compared to the solvent control.

0.1 µM (5.3 ± 1.5 MN, $P < 0.01$ and 7.5 ± 1.5 MN, $P < 0.001$) (Figure 5B and C, respectively) and increased the cell proliferation in all tested concentrations ($P < 0.05$ and $P < 0.01$), assessed by NDI (Figure 6A and B). Thus, DPDT afforded protection against both mutagenic agents (MMS and UVC), suggesting antimutagenic effects.

Determination of intracellular ROS accumulation

As can be seen in Figure 6A, 2 h incubation with 0.01, 0.05 and 0.1 µM DPDT did not increase ROS generation. Moreover, pre-treatment at these concentrations decreased the ROS induction by H_2O_2, from193% to about 150% ($P < 0.05$), and by *t*-BOOH, from 228% to about 170% ($P < 0.01$) (Figure 6B and C, respectively). However, pre-treatment with 1 µM DPDT (230% of total) potentiates ROS generation by H_2O_2 alone (193% of total) (Figure 6B).

Oxidative stress biomarkers

We also investigate the enzymatic antioxidant defenses by measuring SOD and CAT activity as well as TBARS concentration following DPDT treatment. Table 1 shows that DPDT at concentrations above 0.1 µM reduced SOD activity, whereas CAT activity remained unaltered. The concentration of TBARS significantly increased after exposure at 0.5 ($P < 0.001$) and 1 µM ($P < 0.001$) DPDT indicating oxidative stress induction (Table 1).

Discussion

OT compounds can have contrasting behaviours depending on the concentration used. The equilibrium between toxicological and therapeutic effects of a compound is a very important parameter when evaluating its usefulness as a pharmacological drug. Several authors have described that OT compounds are effective protector agents, even more active than their organoselenium (OS) and sulfur analogues. (16, 23) Accordingly, in low concentrations DPDT reduces lipid peroxidation induced by quinolinic acid and sodium nitroprusside in rat brain. (7) Also, it has been reported that DPDT concentrations below 5.0 µM are not cytotoxic and genotoxic in human leucocytes. (24) In this study, we demonstrate for the first time that the DPDT pre-treatment at non-toxic concentrations, ranging from 0.01 to 0.1 µM, presents potent antigenotoxic and antimutagenic effects against several known mutagens in V79 cells. The concentration of 0.1 µM was the most effective against the H_2O_2 and *t*-BOOH oxidative agents, as well as against UVC-radiation (that can damage DNA directly or via production of free radicals), reducing the observed genotoxic and mutagenic effect over 50% in relation to the respective mutagen alone. In conformity, DPDT at concentration range from 0.01 to 0.1 µM was able to reduce intracellular ROS accumulation generated by H_2O_2 and *t*-BOOH (Figure 6B and C) suggesting antioxidant properties.

Moreover, pre-treatment with DPDT resulted in survival improvement, antigenotoxic and antimutagenic effects following

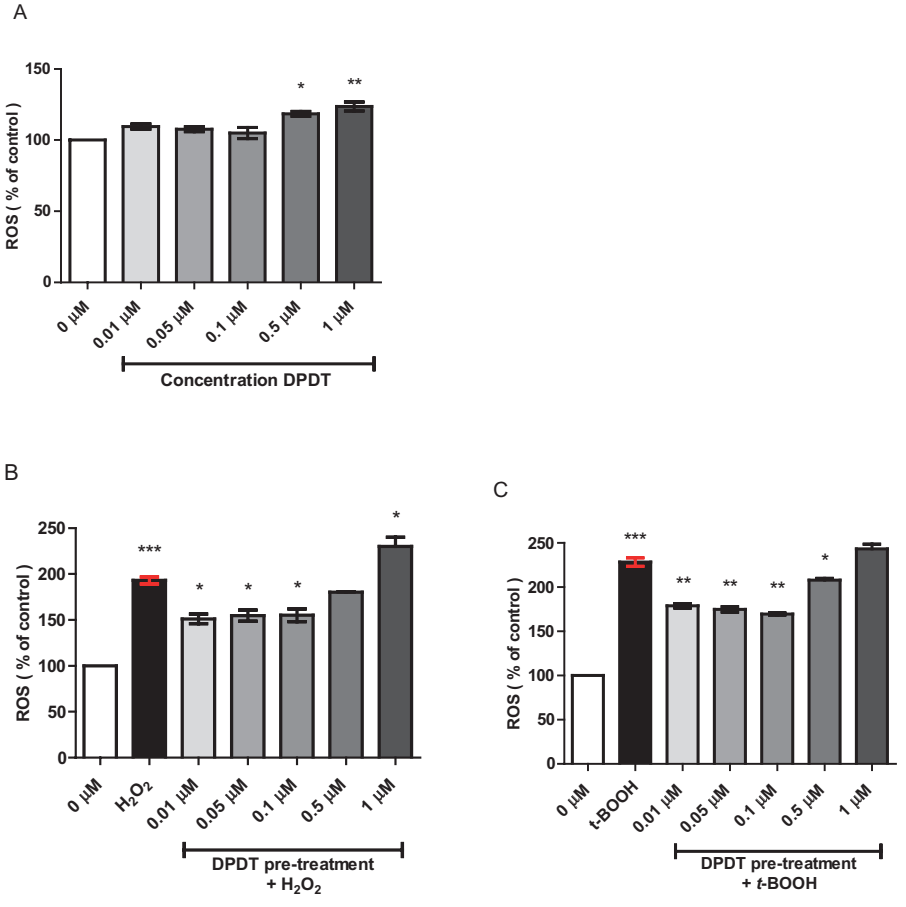

Figure 6. ROS generation in V79 cells by 2 h DPDT treatment (**A**). Cells treated with H_2O_2 (**B**) and *t*-BOOH (C) following DPDT pre-treatment. DPDT pre-treated cells were compared to cells only exposed to the oxidative agents H_2O_2 (500 µM) or *t*-BOOH (400 µM). Results are expressed as mean ± SD, $n = 3$. Data significantly different in relation to the control group $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$/one-way ANOVA Tukey's multiple comparison test.

Table 1. Effect of DPDT treatment on oxidative stress biomarkers in V79 cells[a]

Concentration (μmol)	SOD activity (U SOD/mg protein)	CAT activity (U CAT/mg protein)	TBARS (MDA equivalents nmol/mg protein)
0	185.48 ± 18.77	3.51 ± 0.84	1.2 ± 0.04
0.01	184.17 ± 32.65	3.71 ± 0.77	1.45 ± 0.27
0.05	163.99 ± 14.78	3.57 ± 1.49	0.7 ± 0.04
0.1	117.58 ± 28.66*	3.86 ± 1.41	0.65 ± 0.21
0.5	109.44 ± 24.89**	2.44 ± 0.83	3.6 ± 0.04***
1	115.01 ± 23.08*	2.01 ± 1.31	4.9 ± 0.22***

MDA, malondialdehyde.

[a]Data expressed as mean ± SD of three independent experiments performed in duplicate. Significantly different in relation to the control group, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$/one-way ANOVA Tukey's multiple comparison test.

MMS treatment. MMS is used as an experimental model compound for several decades to elucidate the toxicity mechanisms of alkylating agents relative to mutagenesis. (25, 26) It is a direct-acting alkylating agent whose action is mediated by base tautomerisation. (27) Numerous reports describe rapid glutathione (GSH) depletion by alkylating agents in mammalian cells leading to generation of oxidative stress. (28, 29) GSH is an intracellular peptide responsible for the maintenance of thiol status, peroxide metabolism, detoxification and antioxidant defense. (30) It has been postulated that a decrease of GSH may compromise cellular antioxidant defenses, leading to the accumulation of ROS generated as products of the normal cellular metabolism or external agents. The treatment with oxidative stress inducing agents such as UVC, H_2O_2 and *t*-BOOH, or activators of Phase II detoxifying enzymes such as MMS, can induce GSH synthesis. (30, 31) Detoxification of xenobiotics or their metabolites is one of the major functions of GSH. These compounds are electrophiles and form conjugates with GSH either spontaneously or in reactions catalysed by the enzyme Glutathione *S*-transferase. Moreover, GSH plays a crucial role in the metabolism, inducing adaptive responses to different metals and metalloids. (32)

Interestingly, the co-treatment with DPDT presented lower reduction in the genotoxic damage induced by H_2O_2, *t*-BOOH and MMS, and the post-treatment had no significant effect (data not shown). These data suggest that the protection effect of DPDT could be result of cellular antioxidant defense modulation rather than direct antioxidant action. DPDT is an electrophilic compound, which can suffer thiol nucleophilic attack in the tellurium atom. (15, 33) In this sense, it could interact with GSH and thiol group containing proteins. Such interaction of DPDT with a thiol containing redox sensitive blood enzyme δ-aminolevulinic acid dehydratase (δ-ALA-D) leading to the enzyme inhibition was observed in mice. (15) Consistently, previous works of our group showed that DPDT at concentrations above 50 and 0.5 μM in yeast and V79 cells, respectively, induced depletion of GSH resulting in decreased reduced/oxidized glutathione ratio. (3) On the other hand, Comparsi *et al.* (15) showed that the thiol content in mice brain increased as an adaptive response of cerebral tissue to the redox disruption caused by the compound. In this manner, one may suppose that DPDT, depleting GSH due to oxidation and/or as possible substrate of GSH conjugation, could modulate cellular antioxidant defense inducing GSH synthesis. Such induction could explain the antigenotoxic and antimutagenic effect of DPDT pre-treatment observed in our study. In support of this, diphenyl diselenide (the OS structural analog of DPDT) is detoxified by conjugation with GSH in rat liver fractions (34) and induced depletion of GSH in V79 cells. (26) Diphenyl diselenide also showed antigenotoxic activities against H_2O_2-, MMS- and UVC- induced DNA damage in V79 cells, which is observed at higher concentration range (1.62–12.5 μM) in relation to the DPDT. (26)

Furthermore, DPDT treatment at low concentrations did not induce changes in the SOD and CAT enzyme activities (Table 1). In contrast, concentrations above 0.1 μM increase lipid peroxidation and inhibit SOD activity in V79 cells (Table 1), indicating an oxidative stress induction. In accordance, Comparsi *et al.* (15) reported that 10 μmol/kg DPDT decreased SOD and CAT activity in rat brain. Previous studies reported that inhibition of SOD activity could be a result from the enzyme inactivation by oxidant agents. (35, 36) In addition, synthetic Se- and Te-containing organic redox modulators were shown to generate high levels of ROS at concentrations of 100 μM. (37) Tellurium can generate compounds that are structurally related to their selenium analogues and consequently can exhibit some similar chemical properties. In this sense, a series of OS compounds, as well as the structural analog diphenyl diselenide, also exhibit dual nature displaying both antimutagenic and mutagenic effects. (26, 38, 39) Multiple mechanisms of the protection against genotoxicity and oxidative stress induction by these compounds were suggested, including the influence on the activity of antioxidant enzymes (such as SOD, CAT and GPx) and on the level of GSH, which is capable of scavenging free radicals directly and may also play a role in protection against DNA damage. (38, 39)

In summary, the present study shows that pre-treatment with DPDT at concentration range of 0.01 to 0.1 μM confers protection against H_2O_2, *t*-BOOH, MMS- and UVC-induced cytotoxicity and genotoxicity. The chemoprotective effect is, most probably, due to the antioxidant action of the compound in low concentrations. Thus, investigation of the balance between the cytotoxic and antimutagenic potential of DPDT and its structural derivatives may help to develop novel and more efficient chemotherapy regimens.

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Conflict of interest statement: None declared.

References

1. Carvalho, C. A., Gemelli, T., Guerra, R. B., Oliboni, L., Salvador, M., Dani, C., Araújo, A. S., Mascarenhas, M. and Funchal, C. (2009) Effect of in vitro exposure of human serum to 3-butyl-1-phenyl-2-(phenyltelluro)oct-en-1-one on oxidative stress. *Mol. Cell. Biochem.*, 332, 127–134.
2. Nogueira, C. W., Zeni, G. and Rocha, J. B. (2004) Organoselenium and organotellurium compounds: toxicology and pharmacology. *Chem. Rev.*, 104, 6255–6285.

3. Degrandi, T. H., de Oliveira, I. M., d'Almeida, G. S., Garcia, C. R., Villela, I. V., Guecheva, T. N., Rosa, R. M. and Henriques, J. A. (2010) Evaluation of the cytotoxicity, genotoxicity and mutagenicity of diphenyl ditelluride in several biological models. *Mutagenesis*, 25, 257–269.
4. Urig, S. and Becker, K. (2006) On the potential of thioredoxin reductase inhibitors for cancer therapy. *Semin. Cancer Biol*., 16, 452–465.
5. Avila, D. S., Benedetto, A., Au, C., Manarin, F., Erikson, K., Soares, F. A., Rocha, J. B. and Aschner, M. (2012) Organotellurium and organoselenium compounds attenuate Mn-induced toxicity in Caenorhabditis elegans by preventing oxidative stress. *Free Radic. Biol. Med*., 52, 1903–1910.
6. Ren, X., Xue, Y., Zhang, K., Liu, J., Luo, G., Zheng, J., Mu, Y. and Shen, J. (2001) A novel dicyclodextrinyl ditelluride compound with antioxidant activity. *FEBS Lett*., 507, 377–380.
7. Rossato, J. I., Ketzer, L. A., Centurião, F. B., Silva, S. J., Lüdtke, D. S., Zeni, G., Braga, A. L., Rubin, M. A. and Rocha, J. B. (2002) Antioxidant properties of new chalcogenides against lipid peroxidation in rat brain. *Neurochem. Res*., 27, 297–303.
8. Briviba, K., Tamler, R., Klotz, L. O., Engman, L., Cotgreave, I. A. and Sies, H. (1998) Protection by organotellurium compounds against peroxynitrite-mediated oxidation and nitration reactions. *Biochem. Pharmacol*., 55, 817–823.
9. Engman, L., Kandra, T., Gallegos, A., Williams, R. and Powis, G. (2000) Water-soluble organotellurium compounds inhibit thioredoxin reductase and the growth of human cancer cells. *Anticancer. Drug Des*., 15, 323–330.
10. Avila, D. S., Colle, D., Gubert, P. *et al*. (2010) A possible neuroprotective action of a vinylic telluride against Mn-induced neurotoxicity. *Toxicol. Sci*., 115, 194–201.
11. Sredni, B. (2012) Immunomodulating tellurium compounds as anti-cancer agents. *Semin. Cancer Biol*., 22, 60–69.
12. Nogueira, C. W., Rotta, L. N., Perry, M. L., Souza, D. O. and da Rocha, J. B. (2001) Diphenyl diselenide and diphenyl ditelluride affect the rat glutamatergic system in vitro and in vivo. *Brain Res*., 906, 157–163.
13. Borges, V. C., Rocha, J. B. and Nogueira, C. W. (2005) Effect of diphenyl diselenide, diphenyl ditelluride and ebselen on cerebral Na(+), K(+)-ATPase activity in rats. *Toxicology*, 215, 191–197.
14. Heimfarth, L., Loureiro, S. O., Dutra, M. F., Andrade, C., Pettenuzzo, L., Guma, F. T., Gonçalves, C. A., da Rocha, J. B. and Pessoa-Pureur, R. (2012) In vivo treatment with diphenyl ditelluride induces neurodegeneration in striatum of young rats: implications of MAPK and Akt pathways. *Toxicol. Appl. Pharmacol*., 264, 143–152.
15. Comparsi, B., Meinerz, D. F., Franco, J. L. *et al*. (2012) Diphenyl ditelluride targets brain selenoproteins in vivo: inhibition of cerebral thioredoxin reductase and glutathione peroxidase in mice after acute exposure. *Mol. Cell. Biochem*., 370, 173–182.
16. Brito, V. B., Rocha, J. B., Folmer, V. and Erthal, F. (2009) Diphenyl diselenide and diphenyl ditelluride increase the latency for 4-aminopyridine-induced chemical seizure and prevent death in mice. *Acta Biochim. Pol*., 56, 125–134.
17. Mirabelli, C. K., Sung, C. M., McCabe, F. L., Faucette, L. F., Crooke, S. T. and Johnson, R. K. (1988) A murine model to evaluate the ability of in vitro clonogenic assays to predict the response to tumors in vivo. *Cancer Res*., 48, 5447–5454.
18. Thomas, P. and Fenech, M. (2011) Cytokinesis-block micronucleus cytome assay in lymphocytes. *Methods Mol. Biol*., 682, 217–234.
19. Wang, H. and Joseph, J. A. (1999) Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radic. Biol. Med*., 27, 612–616.
20. Bannister, J. V. and Calabrese, L. (1987) Assays for superoxide dismutase. *Methods Biochem. Anal*., 32, 279–312.
21. Aebi, H. (1984) Catalase in vitro. *Methods Enzymol*., 105, 121–126.
22. Salgo, M. G. and Pryor, W. A. (1996) Trolox inhibits peroxynitrite-mediated oxidative stress and apoptosis in rat thymocytes. *Arch. Biochem. Biophys*., 333, 482–488.
23. Pessoa-Pureur, R., Heimfarth, L. and Rocha, J. B. (2014) Signaling mechanisms and disrupted cytoskeleton in the diphenyl ditelluride neurotoxicity. *Oxid. Med. Cell. Longev*., 2014, 458601.
24. Caeran Bueno, D., Meinerz, D. F., Allebrandt, J., Waczuk, E. P., dos Santos, D. B., Mariano, D. O. and Rocha, J. B. (2013) Cytotoxicity and genotoxicity evaluation of organochalcogens in human leucocytes: a comparative study between ebselen, diphenyl diselenide, and diphenyl ditelluride. *Biomed Res. Int*., 2013, 537279.
25. Liu, H., Colavitti, R., Rovira, I. I. and Finkel, T. (2005) Redox-dependent transcriptional regulation. *Circ. Res*., 97, 967–974.
26. Rosa, R. M., Moura, D. J., Romano E Silva, A. C., Saffi, J. and Pêgas Henriques, J. A. (2007) Antioxidant activity of diphenyl diselenide prevents the genotoxicity of several mutagens in Chinese hamster V79 cells. *Mutat. Res*., 631, 44–54.
27. Zhang, J. G., Nicholls-Grzemski, F. A., Tirmenstein, M. A. and Fariss, M. W. (2001) Vitamin E succinate protects hepatocytes against the toxic effect of reactive oxygen species generated at mitochondrial complexes I and III by alkylating agents. *Chem. Biol. Interact*., 138, 267–284.
28. Liu, H., Lightfoot, R. and Stevens, J. L. (1996) Activation of heat shock factor by alkylating agents is triggered by glutathione depletion and oxidation of protein thiols. *J. Biol. Chem*., 271, 4805–4812.
29. van de Water, B., Zoeteweij, J. P. and Nagelkerke, J. F. (1996) Alkylation-induced oxidative cell injury of renal proximal tubular cells: involvement of glutathione redox-cycle inhibition. *Arch. Biochem. Biophys*., 327, 71–80.
30. Lu, S. C. (2009) Regulation of glutathione synthesis. *Mol. Aspects Med*., 30, 42–59.
31. Lu, S. C. (1999) Regulation of hepatic glutathione synthesis: current concepts and controversies. *FASEB J*., 13, 1169–1183.
32. Srikanth, K., Pereira, E., Duarte, A. C. and Ahmad, I. (2013) Glutathione and its dependent enzymes' modulatory responses to toxic metals and metalloids in fish–a review. *Environ. Sci. Pollut. Res. Int*., 20, 2133–2149.
33. Rigobello, M. P., Folda, A., Citta, A. *et al*. (2011) Interaction of selenite and tellurite with thiol-dependent redox enzymes: Kinetics and mitochondrial implications. *Free Radic. Biol. Med*., 50, 1620–1629.
34. Prigol, M., Nogueira, C. W., Zeni, G., Bronze, M. R. and Constantino, L. (2012) In vitro metabolism of diphenyl diselenide in rat liver fractions. Conjugation with GSH and binding to thiol groups. *Chem. Biol. Interact*., 200, 65–72.
35. Anbarasi, K., Vani, G., Balakrishna, K. and Devi, C. S. (2006) Effect of bacoside A on brain antioxidant status in cigarette smoke exposed rats. *Life Sci*., 78, 1378–1384.
36. Pinton, S., da Rocha, J. T., Zeni, G. and Nogueira, C. W. (2010) Organoselenium improves memory decline in mice: involvement of acetylcholinesterase activity. *Neurosci. Lett*., 472, 56–60.
37. Mániková, D., Letavayová, L. M., Vlasáková, D. *et al*. (2014) Intracellular diagnostics: hunting for the mode of action of redox-modulating selenium compounds in selected model systems. *Molecules*, 19, 12258–12279.
38. Słoczyńska, K., Powroźnik, B., Pękala, E. and Waszkielewicz, A. M. (2014) Antimutagenic compounds and their possible mechanisms of action. *J. Appl. Genet*., 55, 273–285.
39. Santos, R. A. and Takahashi, C. S. (2008) Anticlastogenic and antigenotoxic effects of selenomethionine on doxorubicin-induced damage in vitro in human lymphocytes. *Food Chem. Toxicol*., 46, 671–677.

Capítulo III

Redox-modulating and antiproliferative properties of diphenyl ditelluride

A ser submetido a revista *Molecules.*

Neste capítulo esta apresentada uma mini-revisão sobre os efeitos antioxidantes e antiproliferativos do DTDF que será submetida para publicação no periódico *Molecules.* Essa revisão aborda em um de seus sub-capítulos os mecanismos e efeitos quimioprotetores do DTDF em baixas concentrações contra a genotoxicidade induzida por diferentes mutágenos em diferentes modelos de estudo. Em outro sub-capítulo são abordados os efeitos antiproliferiativos do DTDF, como por exemplo: (i) o efeito inibidor da enzima TrxR; (ii) o efeito inibidor da enzima TOP I; (iii) potencial citotóxico; (iv) parada no ciclo celular; (v) indução de apoptose; (vi) aumento das espécie reativas de oxigênio; (vii) possível interação com AKT1; (viii) alteração da sinalização celular mediada por cálcio.

Redox-modulating and antiproliferative properties of diphenyl ditelluride

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Abstract

Tellurium is a rare element which has been regarded as a toxic, non-essential trace element and its biological role is not clearly established to date. Besides of that, the biological effects of elemental tellurium and some of its inorganic and organic derivatives have been studied, leading to a set of interesting and promising applications. As an example, the diphenyl ditelluride (DPDT) showed antioxidant, antigenotoxic, antimutagenic and anticancer properties. The antioxidant and pro-oxidant properties of DPDT are complex and depend on experimental conditions, which may explain contradictory literature reports of their nature. Also, the DPDT may exert its effects through different pathways, including distinct ones to those responsible for the resistance phenotypes: transcription factors, membrane receptors, adhesion and structural molecules, cell cycle regulatory components, and apoptosis pathways.

1. Introduction

The discovery of tellurium (Te) in 1782 is credited to Muller in work with Hungarian gold mines. The discovery was not so much a determination of a new element as an exclusion of other alternatives, the last being proof that the element, which Muller had isolated, was not antimony. Somewhat surprisingly, this isolation came 35 years before the lighter, sister metalloid selenium's discovery by Berzelius in 1817. Sulfur had been known since ancient times and oxygen was isolated in 1774 (for comprehensive review, see Chasteen *et al*., 2009).

Te is an element sharing the same group of sulfur and selenium in the periodic table; that is, it is the heaviest of the stable chalcogens (group 16) and is classified as a metalloid. In contrast to oxygen, sulfur, and selenium, Te has no essential physiological role in cell biology (for comprehensive review, see Larner, 1995). A number of studies have shown that trace amounts of Te are present in body fluids such as blood and urine (Chasteen *et al*., 2009). Moreover, Te has been shown to be present as tellurocysteine and telluromethionine in several proteins in bacteria, yeast and fungi. However, until now, no telluro proteins have been identified in animal cells (Bienert *et al*., 2008). In a recent and broad ranging overview of the biological activities of Te compounds it was pointed out that Te may face the same prejudice as selenium once did and that natural biological functions for Te may be revealed in time. Also salient was the observation that tellurium– carbon bonds are more labile than their selenium counterparts and so bond cleavage occurs much more readily, a possible explanation of why tellurium-containing amino acids have yet to be observed naturally (Petragnani *et al.,* 2005).

Currently, inorganic Te is used in the vulcanization of rubber, in metal oxidizing solutions used to blacken or tarnish metals and in the nanoparticulate semiconductor industry. Moreover, the use of organic Te compounds will increase due to their importance as catalysts in inorganic and organic synthesis, as stabilizers for polymers, as components of insecticides and phase-change optical magnetic disks and as compounds used in the photography industry (Zeni *et al*., 2006; Comasseto *et al*., 2008; Princival *et al*., 2010). In addition, Te has also been used in the composition of quantum dots in thermoelectric materials, in digital versatile disk-random access memory (DVD-RAM), and in DVD-recordable disks (DVD-RW) (Hardman *et al*., 2006; Comasseto *et al*., 2008; Princival *et al*., 2010; Ferrarini *et al*., 2012).

The presence of Te in different types of electronic materials and nanomaterials is an important health issue. These materials usually contain a variety of toxic elements and there is a paucity of research about the environmental and occupational toxicity of those materials (Dopp et al., 2004; Lovvric et al., 2005; Klaine et al., 2008; Ogra, 2009). Risks from occupational and environmental human exposure to this element may be implied due to this increased use. The main focus on the biological effects of this element has been its toxicity since Te is a non-essential and harmful metalloid. The toxicity of elemental Te and its ionic forms have also been scarcely explored in the literature (Babula *et al*., 2008; Gagné *et al.,* 2008). After its release in the environment, Te can be biomethylated to more volatile intermediates and, consequently, can be mobilized from soil or from aquatic bodies to the atmosphere (Taylor, 1996; Dopp *et al*., 2004). In short, the presence of tellurium in the environment is expected to increase in the next years or decades.

Synthetic organotellurium (OT) compounds have found limited use in the past, but they have become a promising and advantageous alternative for numerous applications, as seen in the increase of reports on OT chemistry appearing in the literature (Nogueira *et al.,* 2004;Friedman *et al*., 2009). In the last few decades, evidence has been accumulating that OT molecules are promising pharmacological agents. Several reports have been published showing immunomodulatory, antioxidant, antiproliferative and anti-inflammatory properties of OT compounds (Sailer *et al*., 2003; Nogueira *et al.,* 2004;Friedman *et al*., 2009).

Here in this review, we will give emphasis to the OT compound diphenyl ditelluride (DPDT) (Figure 1), which is used as an intermediate in organic synthesis and has been described to possess very contrasting and interesting biological activities, such as antioxidant (Trindade *et al*., 2015), cytotoxic (Degrandi *et al*., 2010) and antiproliferative properties (Sailer *et al*., 2003).

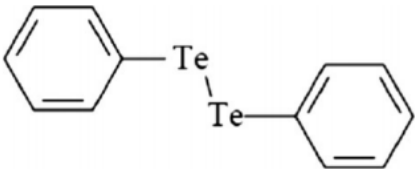

Figure 1.Chemical structure of diphenyl ditelluride.

2. Antioxidant and chemopreventive effects

Antioxidant effect is based on the ability of certain molecules to retard or inhibit oxidative damage. The role of antioxidants is to block oxidative reactions induced by highly reactive oxidant molecules that damage other molecules by capturing electrons and modifying the chemical structure (Rossato *et al*., 2002). These damaging molecules are the so-called free radicals or reactive oxygen species (ROS). The antioxidant

properties of substances such as OT compounds can protect the membrane and other components of the cellular structure (Briviba *et al.,* 1998; Rossato et al., 2002; Ávila *et al*., 2008). OT compounds are readily oxidized from the divalent to the tetravalent state. This property makes them attractive as scavengers of reactive oxidizing agents such as hydrogen peroxide, hypochlorite, and peroxyl radicals, and as inhibitors of lipid peroxidation in chemical and biological systems (reviewed in Nogueira et al., 2004).

Oxidative stress is associated with the pathogenesis of several diseases, such as cardiovascular diseases, neurodegenerative diseases, autoimmune disorders, diabetes, and cancer (reviewed in Raza *et al*., 2017). It is well established that one of the mechanisms for cancer development is oxidative stress (Raza *et al*., 2017). Using mammalian models, researchers have studied the molecular basis of ROS generation, its cellular effects and the efficacy of various antioxidants in mitigating ROS-induced cellular damage (Briviba *et al.,* 1998; Rossato *et al*., 2002; Ávila *et al*., 2008). The potential for OT compounds to offer efficient treatment for disease models associated with oxidative stress has been of interest to several research groups (Engman et al., 2003; Nogueira et al., 2004; Ba et al., 26) The ROS scavenging activity and glutathione peroxidase mimetic property of the organochalcogens likely accounts for their efficacy in attenuating oxidative stress both in *in vitro* and *in vivo* rodent models (Briviba *et al*., 1998; Sarma *et al*., 2008; Sausen *et al*., 2010).

In vitro studies comparing the antioxidant properties of organochalcogenide compounds (diphenyldiselenide $(PhSe)_2$, diphenylditelluride $(PhTe)_2$, diphenyl disulfide $(PhS)_2$, p-Cl-diphenyldiselenide $(pCl\text{-}PhSe)_2$, bis-[S-4-isopropyl 2-phenyl oxazoline] diselenide $(AA\text{-}Se)_2$, bis-[S-4-isopropyl 2-phenyl oxazoline] ditelluride $(AA\text{-}Te)_2$ and bis-[S-4-isopropyl 2-phenyl oxazoline] disulfide $(AA\text{-}S)_2$) demonstrated that their protective

efficacy against lipid peroxidation reaction were mediated by free radical-scavenging activities (Table 1) (Rossato *et al*., 2002; Ávila *et al.,* 2012; Sredni, 2012). In fact, DPDT (1.63 μM) inhibited lipid peroxidation (50%) in rat brain homogenates induced by quinolinic acid (QA) and sodium nitroprusside (SNP), with higher potency than selenides and with potency similar to that of ebselen (a classical antioxidant) (32). Indeed, Brito *et al.* (2009) showed that DPDT afforded protection in adult mice against 4-aminopyridine-induced neurotoxicity and oxidative stress. Moreover, it has been reported that DPDT at low concentrations (1 – 4 μM) significantly increased Na^+/K^+-ATPase activity in rat brain, suggesting that DPDT could be an antioxidant agent (Table 1) (Borges *et al*., 2005).

In this scenario, Trindade *et al*. (2015) showed that the pre-treatment for 2h with DPDT at non-cytotoxic dose range (0.01, 0.05 and 0.1 μM) in Chinese hamster fibroblast cells (V79), increased cell survival after challenge with hydrogen peroxide (H_2O_2), *t*-butyl hydroperoxide (*t*-BOOH), methyl methanesulphonate (MMS) or ultraviolet (UV)C radiation. In this way, the pre-treatment with DPDT decreased the DNA damage and formamidopyrimidine DNA-glycosylase (Fpg, specific for oxidized purines) and Endonuclease III (Endo III, recognizes mainly oxidized pyrimidines) sensitive sites and decreases the ROS induction by the studied genotoxic agents. All these observations demonstrate clearly the protector effect of DPDT. Thus, the chemopreventive effect could be attributed to antioxidant capacity of DPDT at this concentration range (0.01, 0.05 and 0.1 μM) in V79 cells (Table 1).

Some chemotherapeutic approaches have proposed the use of antioxidants to minimize cytotoxicity and the damage induced in normal tissues by antitumor agents

that produce free radicals (reviewed in 61). The doxorubicin (DOX) is one of the commonly used chemotherapeutic agents in the treatment of hematological malignancies (Damiani *et al*., 2016). DOX-induced cardiotoxicity is believed to be related to the generation of ROS by at least two mechanisms: enzymatic reduction of the quinone with subsequent redox cycling and/or formation of an iron-anthracycline complex capable of intramolecular reduction and redox cycling (reviewed in Damiani *et al*., 2016).

In view of antioxidant effect of DPDT, we evaluate the effect of low DPDT concentrations on DOX-induced toxicity and genotoxicity in Chinese hamster fibroblasts V79, as well as in human fibroblasts proficient (MRC5) and deficient (XPD) in Nucleotide Excision Repair (NER). For this purpose, the cell lines MRC5 and V79 were treated with DOX in the presence or absence of DPDT pre-treatment. Measurement of cell viability was performed using MTT assay. As can be seen on the Figure 2, the pre-treatment with DPDT (10 nM and 50nM) in V79, MRC5 and XPD cell lines, increased cell survival after challenge with DOX (0.6 μg/mL).

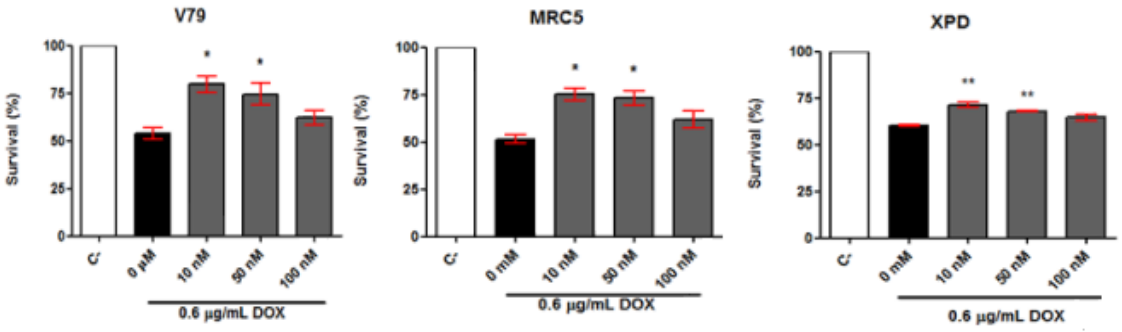

Figure 2. Protective effect of the pre-treatment with diphenyl ditelluride for 2h in serum-free medium on doxorubicin citotoxicity in Chinese hamster fibroblasts (V79), as well as in human fibroblasts proficient (MRC5) and deficient (XPD) in NER cell lines evaluated by MTT assay 72 hours after pre-treatment. Data are reported as means ± SD of 3 independent experiments. *Significantly different at $p<0.05$, **$p<0.01$, compared to cells treated with doxorubicin (ANOVA one-way followed by Tukey test).

The DNA damage induced by DOX was studied in the comet assay and modified comet assay including incubation with the enzymes Fpg and Endo III. DOX at concentration of 0.6 µg/mL induced genotoxicity, increase in the Fpg- and Endo III sensitive sites (Figure 3) and elevated intracellular ROS levels after 3h treatment (Figure 4). The effect of a range of DPDT concentrations (10 nM, 50 nM and 100 nM) on DOX induced cytotoxicity and genotoxicity at same conditions was evaluated. All concentrations tested of DPDT decreased DOX-induced genotoxicity (Figure 3) and ROS formation in mammalian cells (Figure 4). Our results showed that low DPDT concentrations exhibit chemopreventive effect on DOX-induced DNA damage without decreasing its cytotoxicity in mammalian cells. This finding suggests that DPDT can be useful for preventing the DOX-induced genotoxic damage in normal tissues.

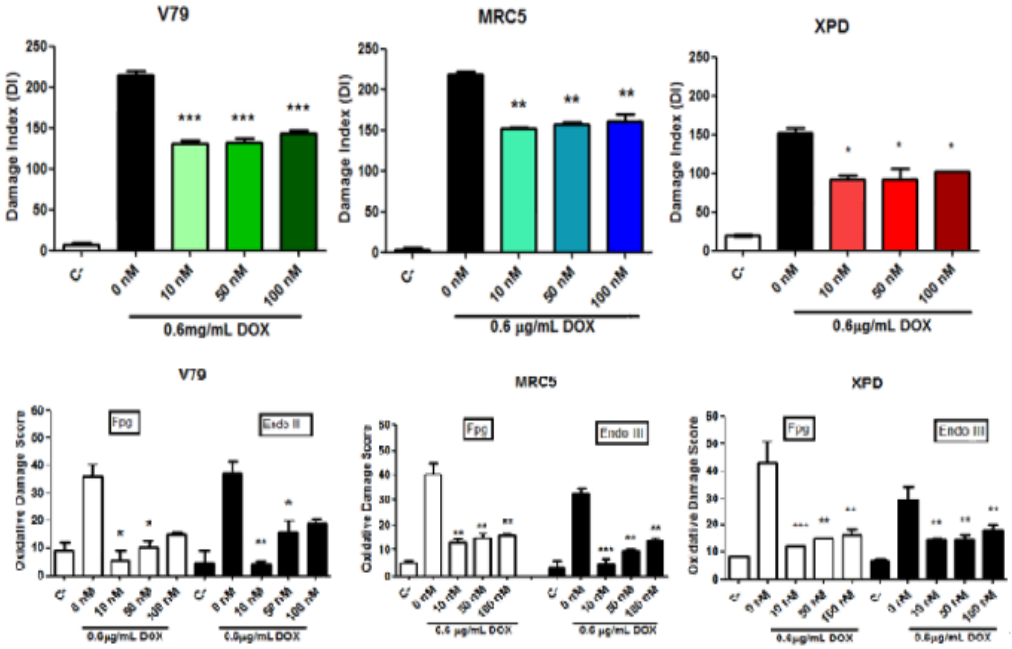

Figure 3. Antigenotoxic effect of the pre-treatment with diphenyl ditelluride for 2h in serum-free medium on the genotoxicity of doxorubicin in Chinese hamster fibroblasts (V79), as well as in human fibroblasts (MRC5) and deficient (XPD) in NER cell lines evaluated by comet assay and modified comet assay. Data are reported as means ± SD of 3 independent experiments. *Significantly different at $p<0.05$, **$p<0.01$,***$p<0.001$, compared to cells treated with doxorubicin (ANOVA one-way followed by Tukey test). Damage index is an arbitrary score calculated from cells in different damage classes, which are classified by visual score considering the DNA migration length and the amount of DNA in the tail. The oxidative damage score represents the difference in the damage score between cells incubated with the Fpg and EndoIII enzymes and with the incubation buffer only.

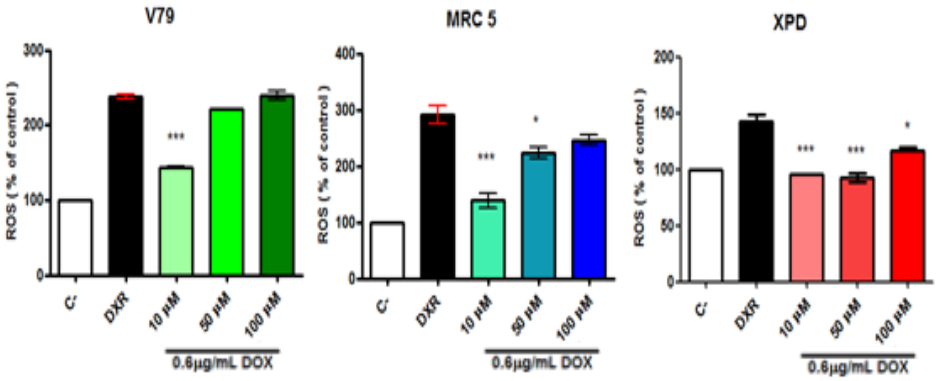

Figure 4. Effect of DPDT on DOX-induced ROS generation. ROS induction was evaluated in cells pre-treated with diphenyl ditelluride for 2h in serum-free medium and treated with doxorubicin for 3h – V79 Chinese hamster fibroblasts; MRC5 and XPD - human fibroblasts proficient and deficient in NER, respectively, evaluated by DCFH-DA assay. Data are reported as means ± SD of 3 independent experiments. *Significantly different at $p<0.05$, **$p<0.01$,***$p<0.001$, compared to cells treated with doxorubicin only (ANOVA one-way followed by Tukey test).

Compounds modulating the cellular antioxidant defense could render loss of effectiveness during chemotherapy. Recently, some mechanisms related to DPDT antioxidant properties have been proposed to explain the chemoprotective effects of this organotellurium compound (Trindade *et al.,* 2015). DPDT is an electrophilic compound, which can suffer thiol nucleophilic attack in the tellurium atom (Rigobello *et al*., 2011; Comparsi *et al*., 2012). In this sense, it could interact with GSH and thiol group containing proteins. Such interaction of DPDT with a thiol containing redox sensitive blood enzyme δ-aminolevulinic acid dehydratase (δ-ALA-D) leading to the enzyme inhibition was observed in mice (Comparsi *et al*., 2012). Consistently, previous works of our group showed that DPDT at concentrations above 50 µM in yeast and 0.5 µM in V79 cells, induced depletion of GSH resulting in decreased reduced/oxidized glutathione ratio (Degrandi *et al*., 2010). On the other hand, Comparsi *et al*. (Comparsi *et al*., 2012) showed that the thiol content in mice brain increased as an adaptive response of cerebral tissue to the redox disruption caused by DPDT. In this manner,

one may suppose that DPDT, depleting GSH due to oxidation and/or as possible substrate of GSH conjugation, could modulate cellular antioxidant defense inducing GSH synthesis (Degrandi *et al*., 2010). Such induction could explain the antigenotoxic and antimutagenic effect of DPDT pre-treatment observed in our study. In support of this, diphenyl diselenide (compound analog of DPDT) is detoxified by conjugation with GSH in rat liver fractions (Prigol *et al*., 2012) and induced depletion of GSH in V79 cells (Rosa *et al.,* 2007). Diphenyl diselenide also showed antigenotoxic activities against H_2O_2-, MMS- and UVC- induced DNA damage in V79 cells, which is observed at higher concentration range (1.62 – 12.5 µM) in relation to the DPDT (Rosa *et al.,* 2007).

The evaluation of antimutagenic potential is vital for compounds that display antioxidant activity. The search for synthetic antimutagens is an important trend in the area of antimutagenicity research (Gordaliza, 2007). The compounds may act through multiple antioxidant mechanisms, including the influence on the activity of SOD and catalase (CAT), the level of GSH, and the removal of ROS. Accordingly, the DPDT significantly decreased the mutagenicity induced by two mutagens, namely MMS and UVC, possibly restoring the GSH content, thus revealing its antioxidant and protector effects (Trindade *et al.,* 2015). It was found that the antimutagenic potential of variety of compounds could be attributed to their antioxidant activity (Table 1) (Trindade *et al.,* 2015). Based on current knowledge, antioxidant activity is a desirable property, since it can provide antimutagenic effects of a compound.

Table 1. The antioxidant and chemopreventive effects of diphenyl ditelluride.

Model	DPDT	Effects	Agent	Ref.
Rat brain	1.63 μM	Inhibition of thiobarbituric reactive species (TBARS) formation by 50%	Quinolic acid (QA) and sodium nitroprusside (SNP)	(Rossato *et al.,* 2002)
Rat brain	150 μmol/kg	Neuroprotective activity	4-aminopyridine	(Brito *et al*., 2009)
Rat brain	1 - 4 μM	Increased Na^+/K^+-ATPase	-	(Borges *et al*., 2005)
V79 cell line	0.01 - 0.1 μM	Reduced cytotoxicity, DNA damage-induction , micronucleus- and ROS formation	Hydrogen peroxide (H_2O_2), t-butyl hydroperoxide (*t*-BOOH), methyl methanesulphonate (MMS) and UV-C.	(Trindade *et al.,* 2015)
V79, MRC5, XPD cell lines	0.01 - 0.1 μM	Reduced DNA damage and ROS formation	Doxorubicin (DOX)	Fig. 2-4

3. Diphenyl ditelluride mechanism of antiproliferative action in non-cancer and cancer cells

The mechanisms behind the mediated cell death are diverse, and it is widely recognized that the effectiveness of tellurium compounds as cancer agents is dependent on the chemical form and dose, as well as on redox state and experimental model (Sredni, 2012; Sekhon, 2013; Halpert & Sredni, 2014; Jorge *et al*., 2015). There is emerging evidence that cell death by tellurium compounds is associated with ROS formation, cell growth arrest, induction of programmed cell death and immunomodulatory effects (Sredni, 2012). Moreover, tellurium compounds may induce cell death by distinct pathways, either caspase dependent and independent, depending

on chemical form and system studied (Degrandi et al., 2010; Halpert & Sredni, 2014; Jorge et al., 2015). Mechanisms of actions of DPDT and other tellurium compounds are discussed below.

3.1 Stress response and cellular targets

Due to increasing evidence suggesting the vulnerability of cancer cells to oxidative stress, the idea of targeting the antioxidant capacity of tumor cells has risen as promising therapeutic strategy and has evolved as the rational design of new anticancer agents (Hardman, 2006). In general, healthy cells are characterized by a low steady-state level of ROS and in some way constant levels of reducing equivalents, while cancer cells are endowed with increased levels of ROS and reducing equivalents due to accelerated glycolysis (the Warburg effect) and pentose phosphate cycle (Gordaliza, 2007; Ferrarini *et al*., 2012; Redondo-Blanco *et al*., 2017). In addition, cancer cells develop an increased and maximized antioxidant capacity, as a compensatory mechanism to evade ROS-induced cell death that makes them extra vulnerable to an additional ROS induction (Raza *et al*., 2017). It is widely recognized that the balance between ROS and reducing equivalents in cells and tissues determines their redox state, and that it is detrimental to uphold the redox balance within the cell (Ferrarini *et al*., 2012; Redondo-Blanco *et al*., 2017). The overall cellular redox state is tightly regulated by systems that modulate the cellular redox status, counteracting ROS formation, and/or reversing the formation of disulfides. These systems are either dependent on the glutathione systems or on the thioredoxin (Trx) system (Comparsi *et al*., 2012).

Trx and thioredoxinreductase (TrxR) comprise a thioredoxin system in the cells. It functions in thiol-disulfide exchange reactions essential to control of the intracellular

redox environment, cellular growth, defense against oxidative stress and control of apoptosis, thus having multiple roles in mammalian cells including implications in cancer (for comprehensive review, see Zhang *et al*., 2017). The potential switch from an antioxidant to a pro-oxidant TrxR species is the aim of many TrxR inhibitors *(in vitro* and *in vivo*). As many tumors expressing elevated levels of TrxR are resistant to chemotherapy, TrxR-targeting may contribute to prevent resistance mechanisms. There is evidence that the expression of TrxR correlates with apoptotic resistance in various cancer cell types (for comprehensive review, see Zhang *et al*., 2017).Inhibition of TrxR and its related redox reactions may thus contribute to a successful single, combinatory or adjuvant cancer therapy.

Among cancer cell redox modulators, tellurium compounds have gained substantial attention due to their promising chemotherapeutic potential (reviewed by 64). A great number of effective natural and synthetic TrxR inhibitors are now available possessing antitumor potential ranging from induction of oxidative stress to cell cycle arrest and apoptosis (for comprehensive review, see Urig & Becker, 2006; Zhang *et al*., 2017). Cyclodextrin-derived diorganyl tellurides were identified as novel inhibitors of thioredoxin reductase with tumor growth inhibition capacities in the submicromolar range (Urig & Becker, 2006). Also, inhibition of the cerebral TrxR activity was shown in mice after acute exposure to 10 and 50 µmol/kg DPDT (Table 2) (Comprasi *et al.,* 2012).

Table 2. Diphenyl ditelluride mechanism of action in non-cancer and cancer cells.

Model	DPDT	Results	Ref.
V79 cell line	*0.5 – 1* μM	Reduced superoxide dismutase (SOD) activity, increased TBARS and ROS formation.	(Trindade *et al*., 2015)
V79 cell line	0.5 - 50 μM	Increased TBARS, reduced GSH:GSSH ratio	(Degrandi *et al.,* 2010)
Mouse brain	*10 – 50 μmol/Kg*	Reduced SOD, catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and thioredoxin reductase (TrxR) activity	(Comparsi *et al*., 2012)
V79 cell line	1 – 50 μM	Cytotoxic and genotoxic effects	(Degrandi *et al.,* 2010)
Caco-2 cell line	62.5 – 1000 μM	Antiproliferative effect	(Vij *et al.,* 2012)
HL-60 cell line	1 μM	Antiproliferative effect, apoptosis induction and accumulation of S phase cells.	(Trindade *et al*., 2015)
HL-60, HCT-8, SF-295, MDAMB-43 and CMSPH cell lines	0.03 – 2.16 μg/mL	Antiproliferative effect (IC50)	Table 2
C6, U87 and U251 cell lines	0.28 – 2.8 mM	Antiproliferative effect, increase of G2/M cells (C6 cell line) and sub-G1 phase cells (C6, U87 and U251 cell line).	Fig. 5-6
HT-29 and CCD-18Co cell lines	500 - 1000 μM	Apoptosis induction, increase in caspases 3/7 and caspase 9 activity	(Vij *et al*., 2012)
V79 cell line	1 – 10 μM	Increased caspase 3/7 activity, apoptosis, necrosis, inhibition of Human Topo I activity	(Jorge *et al*., 2015)
C6 cell line (systems biology)	-	Interaction with AKT1 protein kinase	Fig. 7
Astrocytes and neurons	0.1 – 0.5 μM	Hyperphosphorylation of GFAP, vimentin mediated by N-methyl-d-aspartate (NMDA) and calcium channels. Activation of MAPKs, Erk and p38MAPK	(Heimfarth *et al*., 2016)

As mentioned above, tellurium compounds have the ability to generate ROS, mainly through cycling with GSH or the Trx systems and oxygen to produce superoxide

and hydrogen peroxide, and thereby generating oxidative stress and a ROS promoting cellular stress response (Urig & Becker, 2006). As a consequence of the increased ROS formation, as well as by direct interaction and binding, DPDT is also known to cause genotoxicity and an altered DNA damage response (Table 2) (Degrandi *et al.,* 2010; Jorge *et al*., 2015).

Previous results from our research group demonstrated increase in TBARS production in V79 cells at concentrations starting at 0.5 μM DPDT. Indeed, it was shown that 2h treatments within a concentration range of 0.5 – 50 μM significantly reduced the GSH:GSSG ratio in V79 cells (Degrandi *et al.,* 2010). Comparsi *et al* (Comparsi *et al*., 2012) showed that DPDT increase TBARS formation and reduce the activity of the antioxidant enzymes CAT, SOD, GR, GPx, and TrxR, suggesting a possible involvement of oxidative stress in brain injury caused by DPDT. Recently, it has been shown by Trindade *et al.* (2015) that treatment with DPDT reduced activity of enzyme SOD in V79 cells.

3.2 Cytotoxic and antiproliferative effects

Degrandi *et al.* (2010) evaluated the toxic and mutagenic properties of DPDT in bacteria, yeast and cultured mammalian cells. The cytotoxic threshold of DPDT was different in each biological model. In *Salmonella* TA100, the DPDT was cytotoxic at concentrations higher than 20 μM, whereas in *Saccharomyces cerevisiae*, such effects were observed starting at a concentration of 100 μM. It is important to note that the cytotoxicity effects of DPDT treatment (1 μM – 50 μM) in V79 reported in this study was

confirmed recently by Jorge *et al.* (2015) in the same system at concentrations of 1 μM – 10 μM (Table 2).

In another study, significant decrease in cell viability was observed in HT-29 and heterogeneous human epithelial colorectal adenocarcinoma cells (Caco-2) treated at the concentration range of 62.5 - 1000 μM DPDT in MTT and luminescence assays (Table 2) (Vij *et al*., 2012). In order to evaluate the cytotoxic effects in different tumor cell lines by MTT assay, we treated the acute promyelocytic leukemia (HL-60), human ileocecal adenocarcinoma (HCT-8), human glioblastoma (SF-295) and melanoma (MDAMB-435) cell lines for 72 h at concentration range of 0,019-10 μg/mL DPDT. As can be seen in Table 3, the IC_{50} concentration of DPDT was quite low for HL-60 (IC_{50} - 0.03 μg/mL), HCT-8 (IC_{50} - 0.25 μg/mL) and SF-295 (IC_{50} - 0.28 μg/mL) cell lines. The IC_{50} in a cancer MDAMB-435 cell line (2.16 μg/mL) was higher than in a normal human peripheral blood mononuclear (CMSPH) cells (0.4 μg/mL). It is important to note that the toxic effect of DPDT in HL60 cells (IC_{50} – 0.03 μg/mL) was observed at a similar concentration range of the toxicity induced by the antitumor agent DOX (IC_{50} – 0.02 μg/mL). This concentration is more than 14× lower in comparison with the concentration toxic for the normal CMSPH cells (0.4 μg/mL). The citotoxicity of DPDT cannot be attributed to unspecific damage to cell membranes, because its hemolytic potential in erythrocytes was observed at much higher concentration of 244.25 μg/mL (Table 3).

Table 3. Cytotoxic effects of diphenyl ditelluride compared to doxorubicin on different cell lines.

Compound (µg/mL)	Cell line					
	HL-60	HCT-8	SF-295	MDAMB-435	CMSPH	Erythrocyte
DPDT (IC50)	0,03	0,25	0,28	2,16	0,40	> 100 µg/mL (244,25)
DOX (IC50)	0,02	0,01	0,24	0,48	0,97	Nd*

* Not determined.

Based on the cytotoxicity results on Table 3, we evaluated the antiproliferative effects of DPDT in human glioblastoma U87 and U251 cell lines, and in rat glial tumour cell line (C6) by clonogenic assay. The cells were treated for 72 h at concentration of 0.028mM, 0.28mM and 2.8mM DPDT and the concentration of 0.28mM showed decrease of the clonogenic capacity of about 40 - 50% for the all cell lines tested (Figure 5).

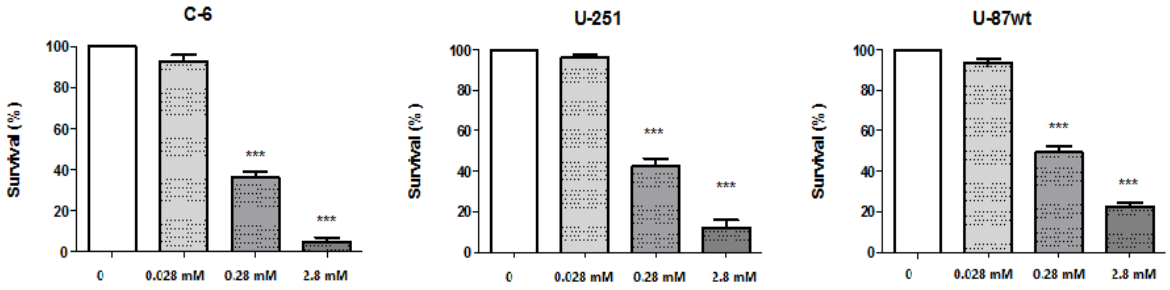

Figure 5. The cells were treated for 72 hr with DPDT and the clonogenic capacity was evaluated in human glioblastoma (U87) and (U251), and in rat glial tumour (C6) cell lines. Data are reported as means ± SD of 3 independent experiments. *Significantly different at $p<0.05$, **$p<0.01$,***$p<0.001$, compared to untreated control cells (ANOVA one-way followed by Tukey test).

3.3 Cell cycle arrest and cell death

Sailer *et al.* (2003) were the first to evaluate the effects of DPDT on the progression of the cell cycle, and showed an increase of S phase in human

promyelocytic (HL-60) cells after DPDT exposure (1 μM) (Table 2). In another study, Jorge *et al.* (2015) showed that the treatment with DPDT (5 μM) in V79 cells increased the proportion of cells in S phase. Furthermore, another study of our group (Juchem et al., in preparation) showed that the cell cycle arrest in G2/M phase was more pronounced in the colon cancer HCT116 cell line than in normal fibroblast MRC5 cells after 24, 48 and 72 h of DPDT exposure. Moreover, for all exposure times, a concentration of 1 μM DPDT did not affect the percentage of cells in any phase of the cell cycle (Table 2) (68). In order to evaluate the effects of DPDT (0.028 – 2.8 mM) on cell cycle in C6, U251 and U87 cell lines, we performed flow citometry analysis. As can be seen on Figure 6, after 24 h of treatment with DPDT (2.8 mM), the sub-G1 fraction of cells increased in C6 and U87 cell lines. Moreover, after 48 hr DPDT (2.8 mM) treatment, the increase of sub-G1 cells was detected in all cell lines tested. It is important to note that the 72 h of DPDT (0.28 mM) treatment induced also an increase in the percentage of cells in G2 / M phase in C6 cell line (Figure 6).

The study conducted by Vij and Hardej (2012) showed an increase in caspases 3/7 and 9 activity in HT-29 and human colon (CCD-18Co) cells treated with DPDT (500-1000 μM) (Table 2). In another study, Jorge *et al.* (2015) showed apoptosis and/or necrosis induction and an increasing in caspase 3/7 activity in all treatment concentrations (1-10μM) in V79 cells. In contrast, the study conducted by Roy and Hardej (2011) for DPDT treatment in rat hippocampal astrocytes did not demonstrate apoptosis induction in the cells but only the induction of necrosis. These discrepancies may be due to variation of glutathione content in the different cell types (Table 2) (Degrandi *et al*., 2010). Considering the ability of Te to bind to sulfhydryl groups,

differences in the GSH levels might be at least partially responsible for the choice of either apoptosis or necrosis induction (Degrandi *et al*., 2010; Jorge *et al*., 2015). In this sense, the organochalcogens can inhibit the activity of mitochondrial complexes I and II by interaction of these compounds with essential cysteinyl residues of mitochondrial complexes. Thus, the organochalcogens should be considered as putative candidates for apoptotic cell death induction via mitochondrial dysfunction caused by thiol oxidation (Puntel *et al*., 2013).

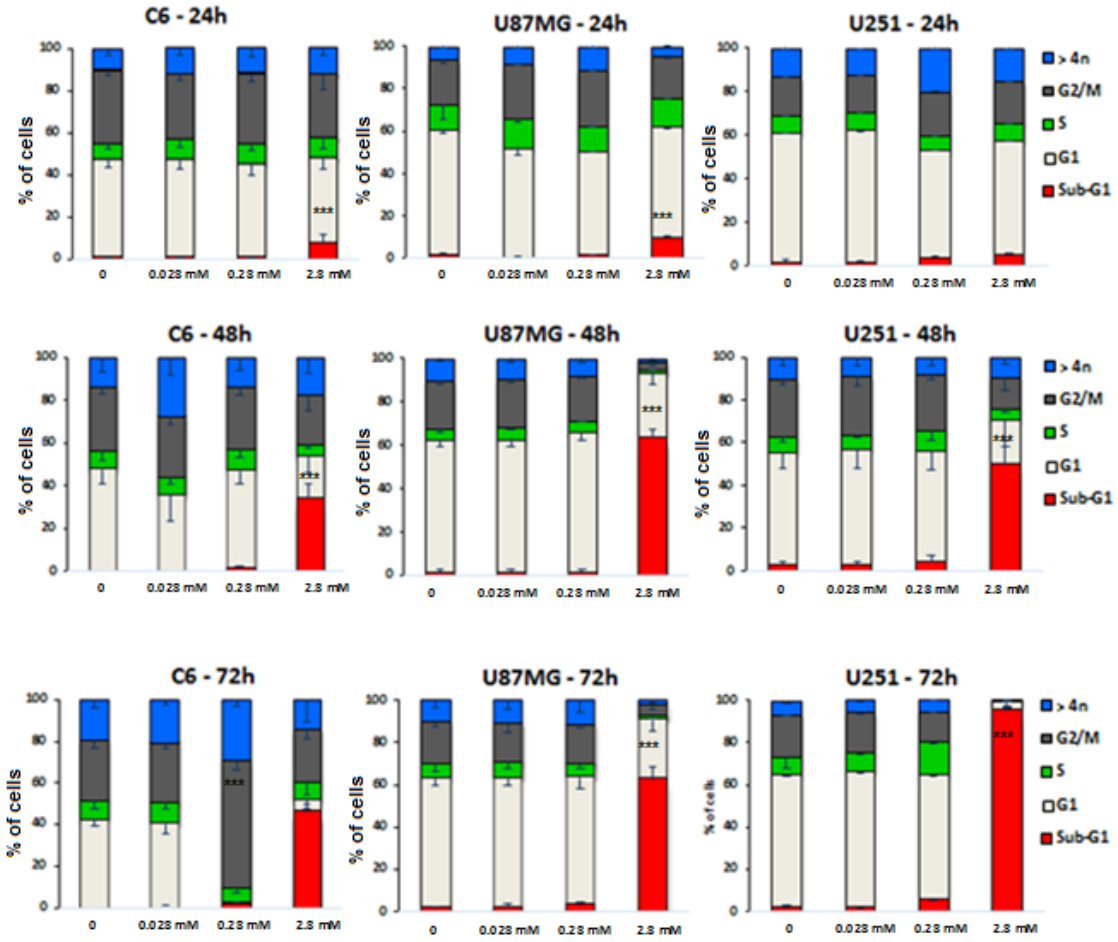

Figure 6. Effect of the diphenyl ditelluride (DPDT) treatment on the cell cycle distribution in U87, U251 and C6 cells after DPDT treatment for 24, 48 and 72hr at concentrations of 0.028, 0.28 and 2.8 mM. Values are the relative numbers of cells in the sub-G1, G1, S1, G2/M phase and >4n cells of four independent experiments performed in triplicate. The error bars indicate the standard error means of 3 independent experiments. *Significantly different at $p<0.05$, **$p<0.01$,***$p<0.001$, compared to untreated control cells (ANOVA one-way followed by Tukey test).

Chemical compounds with planar topologies are often capable of intercalation between the bases in DNA (Snyder & Arnore, 2002). Intercalating agent-induced genotoxicity manifests itself primarily as frameshift mutation in bacterial and yeast systems and as clastogenicity in mammalian systems (Gordaliza, 2007). So, the results reported by Degrandi *et al.* (2010) showing frameshift mutation induction by DPDT in *Salmonella typhimurium* and *S. cerevisiae* and an increase in double strand breaks in V79 cells, suggested intercalation activity and/or interaction with DNA topoisomerase enzymes (Table 2). In this sense, studying the response of *S. cerevisiae* mutants defective in topoisomerase enzymes to treatment with DPDT, Jorge *et al* (2015) showed pronounced tolerance in *top1Δ* cell line. The same study also reported DPDT-induced inhibition of human Topo I activity *in vitro*. These results suggest that DPDT could interact with the Top1p enzyme, when present in the cell, inducing DNA lesion responsible for the induced cell death.

The intertwining of DNA strands and helices, produced during essential cellular processes of replication, recombination, transcription, and chromosome segregation, must be resolved in order to maintain cell viability and genome stability. DNA topoisomerases provide an important solution for resolving such topological problems (Pommier *et al*., 2016). However, they do so through the formation of a covalent enzyme– DNA reaction intermediate, which is a potentially toxic lesion itself. Indeed, targeting of topoisomerase–DNA complexes has been widely exploited in the identification and development of antibacterial and anticancer chemotherapics (Pommier *et al*., 2016; Tse-Dinh, 2016). The majority of these agents are termed “poisons” to indicate a mechanism of covalent enzyme–DNA complex stabilization, versus “inhibitor,” which would signify the lack of DNA binding or cleavage by the

enzyme. A critical aspect of these differences in the mechanism of drug action is that in an isogenic cell system, increased expression of the enzyme would increase the cytotoxic activity of a “poison” through increased production of drug-stabilized topoisomerase–DNA adducts. In contrast, elevated levels of enzyme would confer resistance to “inhibitors.” In addition, many of the topoisomerase-targeting agents act as interfacial inhibitors (poisons) (Pommier *et al*., 2016; Cuya *et al*., 2017). Interfacial inhibitors are different from competitive (orthosteric) inhibitors and non-competitive (allosteric) inhibitors since they interact at the interface between two or more molecules. The collision of DNA replication forks with the ternary complexes, or the positively supercoiled DNA domains induced by these complexes, produces the irreversible DNA lesions and double-strand breaks that ultimately lead to cell death (reviewed in Cuya *et al*., 2017).

3.4 Systems biology and signaling

Computational biology tackles cancer in two distinct ways. The first involves analysis of massive quantities of genomic, proteomic, microarray, cell and tissue imaging data produced by experiments, as well as clinical data relating to the tumors, the patients and the results of clinical trials. The second, complementary way through which computations contribute to cancer research is by revealing the mechanism through which particular genetic or acquired aberration works. To understand mechanisms, and design or computationally screen drugs (Gordaliza, 2007).

A contrast analysis was applied and differentially expressed genes (DEG) after simulation of DPDT treatment were selected using a Rank Product. The technique is based on calculating Rank Products (RP) from replicate experiments. We use three

samples (C6 rat glioma cell line, untreated, under similar *culture conditions with our experimental model*) of GEO database (GSE1139 accession number). For each sample, the average of the signal between the same probes was calculated and applied to the normalized microarray data using Limma package in R/Bioconductor. The parameters used to run the RP were: permutation = 1000 and p-value ≤ 0.01.

As can be seen on Figure 8, there are predicted interaction between DPDT and AKT1. AKT1 is a serine/threonine-protein kinase also known as AKT kinase, involved in the regulation of various signaling downstream pathways that regulate cell metabolism, cell proliferation, survival, growth, and angiogenesis. It is also a member of the most frequently activated proliferation and survival pathway in cancer. AKT recognizes and phosphorylates the consensus sequence RXRXX(S/T) of the target proteins when surrounded by hydrophobic residues. The activation of AKT1 is driven by membrane localization, which is in turn initiated by the binding of the pleckstrin homology (PH) domain to phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3) or phosphatidyl inositol- 3,4-bisphosphate (PtdIns(3,4)P2), followed by phosphorylation of the regulatory

amino acids serine 473 (Ser 473) and threonine 308 (Thr 308) (Kumar *et al.,* 2013).

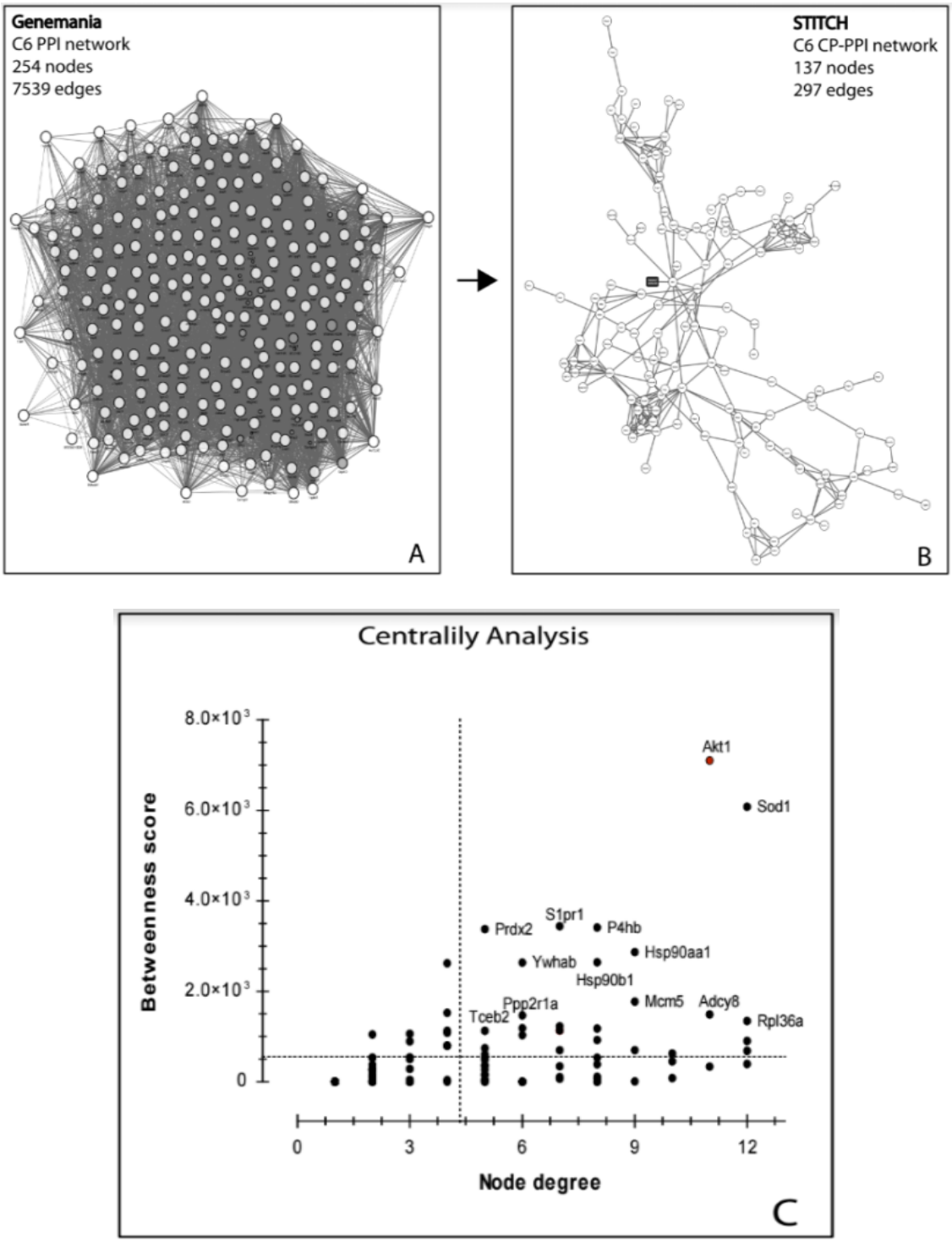

Figure 8. A - C6 PPI network obtained from GeneMANIA software and cell line microarrays data analysis. B - C6 CP-PPI network showing interactions between diphenyl ditelluride and specific targets. C- Network centralities analysis to define H-B nodes from CP-PPI network. Diphenyl ditelluride´s H-B targets are detailed in red.

Heimfarth *et al* (2016) showed DPDT-induced hyperphosphorylation of glial fibrillary acidic protein (GFAP), vimentin and neurofilament subunits (NFL, NFM and NFH) from primary astrocytes and neurons, respectively. These mechanisms were mediated by N-methyl-d-aspartate (NMDA) receptors, L-type voltage-dependent calcium channels (L-VDCCs), as well as metabotropic glutamate receptors upstream of phospholipase C (PLC). Upregulated Ca(2+) influx activated protein kinase A (PKA) and protein kinase C (PKC) in astrocytes causing hyperphosphorylation of GFAP and vimentin. Hyperphosphorylated (IF) together with RhoA-activated stress fiber formation, disrupted the cytoskeleton leading to altered cell morphology. In neurons, the high intracellular Ca(2+) levels activated the MAPKs, Erk and p38MAPK, beyond PKA and PKC, provoking hyperphosphorylation of NFM, NFH and NFL (Table 2).

4. Concluding remarks

Investigation of the antioxidant and anticancer properties of DPDT, against ROS generated damage in the treatment of diseases, is an active and promising area of research. DPDT are potent anti-proliferative and antioxidant agent at low and moderate concentrations (Fig. 9). The exact mechanism by which the antioxidant and anti-tumor effects are achieved remains unclear, although some mechanisms have been proposed, and is distinct depending on the system studied.

This compound may be useful in the development of rational combination therapies that can be predicted to have synergistic or additive effects with conventional quimioterapics. In addition, the role of DPDT in the prevention of ROS-mediated diseases warrants additional studies to better understand and elucidate a mechanism of the antioxidant and pro-oxidant activities.

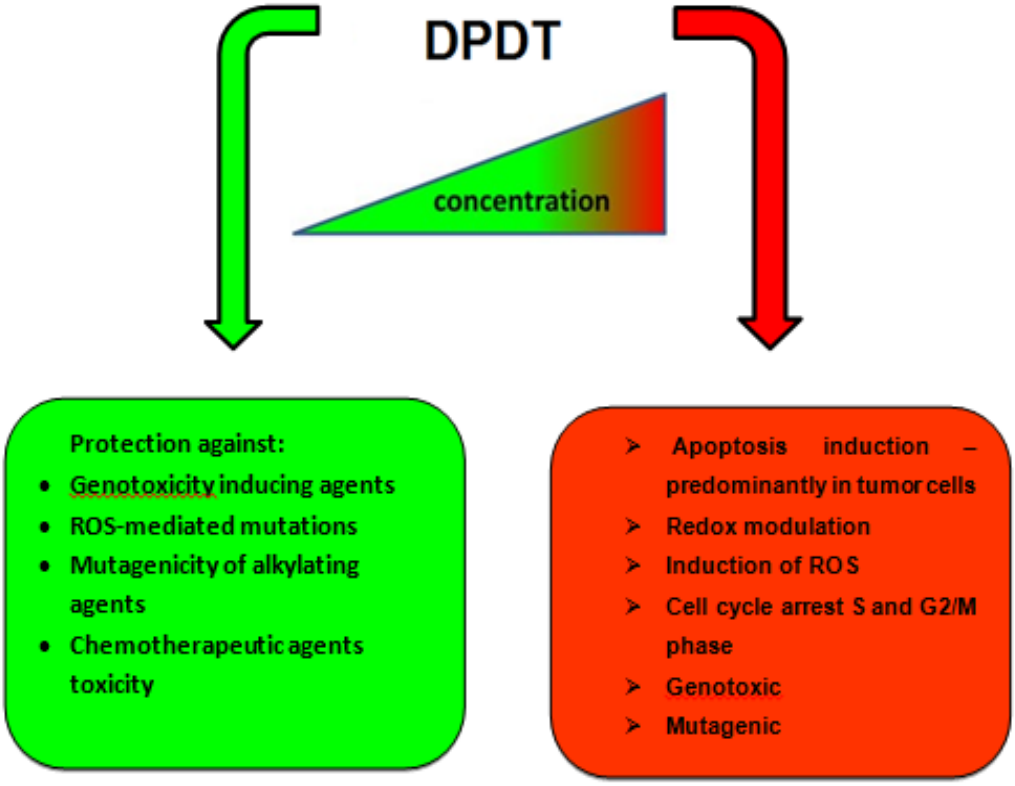

FIG. 9. Biological effects of Diphenyl ditelluride. Low concentrations of DPDT showed protective effects that could be attributed to its antioxidant capacity. DPDT at moderate concentrations showed selective cytotoxic effect inducing apoptosis preferentially in tumor cells. The observed cytotoxic effect can be explained by increased ROS formation and redox modulation. High concentrations of DPDT induced toxicity and mutagenicity.

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References

Avila DS, Benedetto A, Au C, et al. Organotellurium and organoselenium compounds attenuate Mn-induced toxicity in Caenorhabditis elegans by preventing oxidative stress. Free Radical Biology and Medicine. 2012;52(9):1903–1910.

Ávila DS, Gubert P, Palma A, et al. An organotellurium compound with antioxidant activity against excitotoxic agents without neurotoxic effects in brain of rats. Brain Research Bulletin. 2008;76(1-2):114–123.

Ba LA, Doring M, Jamier V, Jacob C. Tellurium: an element with great biological potency and potential. Organic & biomolecular chemistry. 8:4203–4216.

Babula P, Adam V, Opatrilova R, Zehnalek J, Havel L, Kizek R. Uncommon heavy metals, metalloids and their plant toxicity: a review. Environmental Chemistry Letters. 2008;6(4):189–213.

Bienert GP, Schüssler MD, Jahn TP. Metalloids: essential, beneficial or toxic? Major intrinsic proteins sort it out, Trends Biochem. Sci. , 2008, vol. 33 (pg. 20-26).

Borges, V. C., Rocha, J. B. and Nogueira, C. W. Effect of diphe-nyl diselenide, diphenyl ditelluride and ebselen on cerebral Na(+), K(+)-ATPase activity in rats. Toxicology, 2005, 215, 191–197.

Brito, V. B., Rocha, J. B., Folmer, V. and Erthal, F. Diphenyl disele-nide and diphenyl ditelluride increase the latency for 4-aminopyridine-induced chemical seizure and prevent death in mice. Acta Biochim. Pol.,2009, 56, 125–134.

Briviba K, Tamler R, Klotz LO, Engman L, Cotgreave IA, Sies H. Protection by organotellurium compounds against peroxynitrite-mediated oxidation and nitration reactions. Biochem Pharmacol. 1998;55:817–823.

Chabner BA, Roberts TG Jr. Timeline: Chemotherapy and the war on cancer. Nat Rev Cancer. 2005 Jan;5(1):65-72.

Chasteen, T. G., Fuentes, D. E., Tantaleán, J. C. and Vásquez, C. C. (2009), Tellurite: history, oxidative stress, and molecular mechanisms of resistance. FEMS Microbiology Reviews,2009:33: 820–832. doi:10.1111/j.1574-6976.2009.00177.x.

Comasseto JV, Dos Santos AA. Organotellurides as precursors of reactive organometallics. Phosphorus, Sulfur and Silicon and the Related Elements. 2008;183(4):939–947.

Comparsi, B., Meinerz, D. F., Franco, J. L. et al. (2012) Diphenyl ditellu-ride targets brain selenoproteins in vivo: inhibition of cerebral thioredoxin reductase and glutathione peroxidase in mice after acute exposure. Mol. Cell. Biochem., 370, 173–182.

Cunha RL; Gouvea IE; Juliano L. A glimpse on biological activities of tellurium compounds. An Acad Bras Cienc. 2009 Sep;81(3):393-407.

Cuya SM, Bjornsti MA, van Waardenburg RCAM. DNA topoisomerase-targeting chemotherapeutics: what's new? Cancer Chemother Pharmacol. 2017 Jul;80(1):1-14

Damiani RM, Moura DJ, Viau CM, et al. Pathways of cardiac toxicity: comparison between chemotherapeutic drugs doxorubicin and mitoxantrone. Arch Toxicol. 2016 Sep;90(9):2063-76.

Degrandi,T. H., de Oliveira, I. M., d'Almeida, G. S., Garcia, C. R.,Villela, I.V., Guecheva,T. N., Rosa, R. M. and Henriques, J.A. (2010) Evaluation of the cytotoxicity, genotoxicity and mutagenicity of diphenyl ditelluride in several biological models. Mutagenesis, 25, 257–269.

Dhawan A, Kayani MA, Parry JM, Parry E, Anderson D. Aneugenic and clastogenic effects of doxorubicin in human lymphocytes. Mutagenesis. 2003 Nov;18(6):487-90.

Dopp E, Hartmann LM, Florea A-M, Rettenmeier AW, Hirner AV. Environmental distribution, analysis, and toxicity of organometal(loid) compounds. Critical Reviews in Toxicology. 2004;34(3):301–333.

Douillard JY, Rong A, Sidhu R. RAS mutations in colorectal cancer. N Engl J Med. 2013 Nov 28;369(22):2159-60.

Edward R. T. Tiekink. Therapeutic potential of selenium and tellurium compounds: Opportunities yet unrealised. Dalton Trans., 2012, 41, 6390

Engman L, Al-Maharik N, McNaughton M, Birmingham A, Powis G. Thioredoxin reductase and cancer cell growth inhibition by organotellurium compounds that could be selectively incorporated into tumor cells. Bioorg Med Chem. 2003;11:5091–5100.

Ferrarini RS, Dos Santos AA, Comasseto JV. Tellurium in organic synthesis: a general approach to buteno- and butanolides. Tetrahedron. 2012;68(51):10601–10610.

Friedman M, Bayer I, Letko I, Duvdevani R, Zavaro-Levy O, Ron B, Albeck M, Sredni B. Topical treatment for human papillomavirus associated genital warts in humans with the novel tellurium immunomodulator AS101: assessment of its safety and efficacy, Br. J. Dermatol. , 2009, vol. 160 (pg. 403-408).

Gagné F, Auclair J, Turcotte P, et al. Ecotoxicity of CdTe quantum dots to freshwater mussels: impacts on immune system, oxidative stress and genotoxicity. Aquatic Toxicology. 2008;86(3):333–340.

Gordaliza M. Natural products as leads to anticancer drugs. Clin Transl Oncol (2007) 9:767-776.

Halpert G & Sredni B. The effect of the novel tellurium compound AS101 on autoimmune diseases. Autoimmun Rev. 2014 Dec;13(12):1230-5.

Hardman R. A toxicologic review of quantum dots: toxicity depends on physicochemical and environmental factors. Environmental Health Perspectives. 2006;114(2):165–172.

Heimfarth L, da Silva Ferreira F, Pierozan P, Loureiro SO, Mingori MR, Moreira JC, da Rocha JB, Pessoa-Pureur R. Calcium signaling mechanisms disrupt the cytoskeleton of primary astrocytes and neurons exposed to diphenylditelluride. Biochim Biophys Acta. 2016 Nov;1860(11 Pt A):2510-20.

Jorge, P. M., de Oliveira, I. M., Filippi Chiela, E. C., Viau, C. M., Saffi, J., Horn, F., Rosa, R. M., Guecheva, T. N. and Pêgas Henriques, J. A. Diphenyl Ditelluride-Induced Cell Cycle Arrest and Apoptosis: A Relation with Topoisomerase I Inhibition. Basic Clin Pharmacol Toxicol, 2015:116: 273–280.

Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011 Mar-Apr;61(2):69-90.

Klaine SJ, Alvarez PJJ, Batley GE, et al. Nanomaterials in the environment: behavior, fate, bioavailability, and effects. Environmental Toxicology and Chemistry. 2008;27(9):1825–1851.

Kumar A, Rajendran V, Sethumadhavan R, Purohit R. AKT kinase pathway: a leading target in cancer research. ScientificWorldJournal. 2013 Nov 13;2013:756134.

Larner AJ. Biological effects of tellurium: a review. Trace Elements and Electrocytes. 1995;12(1):26–31.

Lian C, Ruan L, Shang D, Wu Y, Lu Y., et al. Heparin-Binding Epidermal Growth Factor-Like Growth Factor as a Potent Target for Breast Cancer Therapy. Cancer Biother Radiopharm. 31(3):85-90. 2016.

Lovrić J, Cho SJ, Winnik FM, Maysinger D. Unmodified cadmium telluride quantum dots induce reactive oxygen species formation leading to multiple organelle damage and cell death. Chemistry and Biology. 2005;12(11):1227–1234.

Lugokenski TH, Mu¨ller LG, Taube PS, Rocha JBT, Pereira ME Inhibitory effect of ebselen on lactate dehydrogenase activity from mammals: a comparative study with diphenyl diselenide and diphenyl ditelluride. Drug Chem Toxicol. 2011: 34:66–76.

Maciel EN, Bolzan RC, Braga AL, Rocha JBT. Diphenyl diselenide and diphenyl ditelluride affect delta-aminolevulinate dehydratase from liver, kidney and brain of mice. J Biochem Mol Toxicol. 2000:14:310–319.

Mazaud C, Fardet L. Relative Risk of and Determinants for Adverse Events of Methotrexate Prescribed at a Low Dose: A Systematic Review and Meta-Analysis of Randomized, Placebo-Controlled Trials. Br J Dermatol. 2017 Feb 9. doi: 10.1111/bjd.15377.

Moretto MB, Boff B, Franco J, Posser T, Roessler TM, Souza DO, Nogueira CW, Wofchuk S, Rocha JB. Ca(2+) influx in rat brain: effect of diorganylchalcogenides compounds, Toxicol. Sci. , 2007, vol. 99 (pg. 566-571).

Nogueira CW, Borges VC, Zeni G, Rocha JB. Organochalcogens effects on delta-aminolevulinate dehydratase activity from human erythrocytic cells in vitro, Toxicology , 2003, vol. 191 (pg. 169-178).

Nogueira CW, Zen G, Rocha JB. Organoselenium and organotellurium compounds: toxicology and pharmacology, Chem. Rev. , 2004, vol. 104 (pg. 6255-6285).

Ogra Y. Toxicometallomics for research on the toxicology of exotic metalloids based on speciation studies. Analytical Sciences. 2009;25(10):1189–1195.

Petragnani N, Stefani HA. Advances in organic tellurium chemistry. Tetrahedron. 2005;61(7):1613–1679.

Pommier Y, Sun Y, Huang SN, Nitiss JL. Roles of eukaryotic topoisomerases in transcription, replication and genomic stability. Nat Rev Mol Cell Biol. 2016 17(11):703–721.

Prigol, M., Nogueira, C. W., Zeni, G., Bronze, M. R. and Constantino, L. In vitro metabolism of diphenyl diselenide in rat liver fractions. Conjugation with GSH and binding to thiol groups. Chem. Biol. Interact.2012: 200, 65–72.

Princival JL, Dos Santos AA, Comasseto JV. Reactive organometallics from organotellurides: application in organic synthesis. Journal of the Brazilian Chemical Society. 2010;21(11):2042–2054.

Puntel RL, Roos DH, Seeger RL, Rocha JB. Mitochondrial electron transfer chain complexes inhibition by different organochalcogens. Toxicol In Vitro 2013;27:59-70.

Raza MH, Siraj S, Arshad A, Waheed U4, Aldakheel F, Alduraywish S, Arshad M7. ROS-modulated therapeutic approaches in cancer treatment. J Cancer Res Clin Oncol. 2017 Jun 24.

Redondo-Blanco S, Fernández J, Gutiérrez-del-Río I, Villar CJ and Lombó F New Insights toward Colorectal Cancer Chemotherapy Using Natural Bioactive Compounds. Front. Pharmacol.2017 : 8:109.doi: 10.3389/fphar.2017.00109.

Rigobello, M. P., Folda, A., Citta, A. *et al*. Interaction of selenite and tellurite with thiol-dependent redox enzymes: Kinetics and mitochon-drial implications. *Free Radic. Biol. Med*.2011:50, 1620–1629.

Rosa, R. M., Moura, D. J., Romano E Silva,A. C., Saffi, J. and Pêgas Hen-riques, J. A. Antioxidant activity of diphenyl diselenide prevents the genotoxicity of several mutagens in Chinese hamster V79 cells. *Mutat. Res*.2007:631, 44–54.

Rossato JI, Ketzer LA, Centurião FB, et al. Antioxidant properties of new chalcogenides against lipid peroxidation in rat brain. Neurochemical Research. 2002;27(4):297–303.

Roviello G, Zanotti L, Cappelletti MR, Gobbi A, et al. Are EGFR tyrosine kinase inhibitors effective in elderly patients with EGFR-mutated non-small cell lung cancer? Clin Exp Med. 2017 Apr 8. doi: 10.1007/s10238-017-0460-7.

Roy S, Hardej D. Tellurium tetrachloride and diphenyl ditelluride cause cytotoxicity in rat hippocampal astrocytes. Food Chem Toxicol 2011; 49:2564-74.

Sailer BL, Liles N, Dickerson S, Chasteen TG. Cytometric determination of novel organotellurium compound toxicity in a promyelocytic (HL-60) cell line, Arch. Toxicol. , 2003, vol. 77 (pg. 30-36).

Sarma BK, Mugesh G. Antioxidant activity of the anti-inflammatory compound ebselen: a reversible cyclization pathway via selenenic and seleninic acid intermediates. Chemistry. 2008;14:10603–10614.

Sausen de Freitas A, de Souza Prestes A, Wagner C, Haigert Sudati J, Alves D, Oliveira Porciuncula L, Kade IJ, Teixeira Rocha JB. Reduction of diphenyl diselenide and analogs by mammalian thioredoxin reductase is independent of their gluthathione peroxidase-like activity: a possible novel pathway for their antioxidant activity. Molecules. 2010;15:7699–7714.

Sekhon BS. Metalloid compounds as drugs. Res Pharm Sci. 2013 Jul;8(3):145-58.

Snyder RD, Arnone MR. Putative identification of functional interactions between DNA intercalating agents and topoisomerase II using the V79 in vitro micronucleus assay. Mutat Res 2002;503:21-35.

Sredni B. Immunomodulating tellurium compounds as anti-cancer agents. Semin Cancer Biol. 2012 Feb;22(1):60-9.

Stangherlin EC, Ardais AP, Rocha JBT, Nogueira CW. Exposure to diphenyl ditelluride, via maternal milk, causes oxidative stress in cerebral cortex, hippocampus and striatum of young rats. Archives of Toxicology. 2009;83(5):485–491.

Taylor A. Biochemistry of tellurium. Biological Trace Element Research. 1996;55(3):231–239.

Trindade C, Juchem AL, de Albuquerque NR, et al. Antigenotoxic and antimutagenic effects of diphenyl ditelluride against several known mutagens in Chinese hamster lung fibroblasts. Mutagenesis. 2015 Nov;30(6):799-809.

Tse-Dinh YC. Targeting bacterial topoisomerases: how to counter mechanisms of resistance. Future Med Chem. 2016:8(10):1085–1100. doi:10.4155/fmc-2016-0042.

Urig S, Becker K. On the potential of thioredoxin reductase inhibitors for cancer therapy. Semin Cancer Biol. 2006 Dec;16(6):452-65.

Vij, P., eD. Hardej, 2012 Evaluation of tellurium toxicity in transformed and non-transformed human colon cells. Environ Toxicol Pharmacol 34: 768-782.

Wang JC. Cellular roles of DNA topoisomerases: a molecular perspective. Nat Rev Mol Cell Biol. 2002: 3(6):430–440. doi:10.1038/nrm831.

Zeni G, Lüdtke DS, Panatieri RB, Braga AL. Vinylic tellurides: from preparation to their applicability in organic synthesis. Chemical Reviews. 2006;106(3):1032–1076.

Zhang J, Li X, Han X, Liu R, Fang J. Targeting the Thioredoxin System for Cancer Therapy. Trends Pharmacol Sci. 2017 Jun 22. pii: S0165-6147(17)30119-0.

3. Discussão geral

Na última década, os progressos nas áreas da biologia celular e molecular proporcionaram a caracterização de vários processos celulares importantes para a patogênese do câncer, como por exemplo, as sinalizações envolvidas no crescimento, proliferação, angiogênese e metástase (Hanahan & Weinberg, 2011). Por outro lado, o DNA, que há mais de 50 anos é o alvo principal da maioria dos agentes antitumorais, continua desempenhando um papel importante nas novas terapias contra o câncer (D'Incalci & Galmarini, 2010; Underhiill *et al.*, 2011).

Os progressos recentes no sequenciamento genômico e caraterização molecular levaram ao conhecimento das vias intracelulares que comandam a proliferação, divisão e apoptose celulares, o que levou à investigação de terapêuticas dirigidas a alvos específicos (Redondo *et al.,* 2017). A descoberta recente de agentes dirigidos levou à necessidade de classificação molecular do tumor, a qual pode permitir uma melhor estimativa de prognóstico e identificação mais correta dos doentes mais responsivos a uma dada terapêutica. O tratamento personalizado e dirigido parece ser o futuro, tendo em conta a heterogeneidade desta neoplasia, que constitui um espectro de várias doenças na qual interagem fatores de cariz anatómico, histológico, cromossómico e molecular (Greystoke & Mullamitha, 2012). No presente trabalho, buscamos—investigar os mecanismos de ação da combinação dos quimioterápicos bevacizumab e erlotinib em modelos de câncer colorectal e o potencial antigenotóxico, antimutagênico e antitumoral do ditelureto de difenila, visando a aplicação destes agentes na terapia antitumoral. O bevacizumab é um anticorpo monoclonal humanizado IgG1 dirigido contra o VEGFA, um membro da

família do VEGF e principal mediador da cascata angiogênica e o erlotinib, o qual é um inibidor oral da tirosina quinase/EGFR relacionado ao domínio intra celular do EGFR. Acredita-se que os compostos direcionados a EGFR exercem principalmente sua atividade nas células tumorais, enquanto as terapias direcionadas ao VEGF funcionam principalmente nas células endoteliais de tumores colorretais (TECs) (Huang *et al*., 2013; Wei *et al*., 2013). No entanto, os resultados apresentados no Capítulo I não mostram distinção clara entre os dois compartimentos celulares, pelo menos não dentro do contexto do CRC. As células CRC exibem receptores VEGF funcionais e fornecem a maioria do VEGF no microambiente tumoral, dando assim origem à sinalização autocrina e paracrina do VEGF (Larsen *et al*., 2013; Mésage *et al.,* 2013; Salazar et al., 2014). Por outro lado, os TEC expressam EGFR funcionais, bem como anfiregulina derivada de tumor, promovendo assim a função e sobrevivência de TEC (Panupinthu *et al*., 2014). O conjunto dessas observações pode explicar a falta de concordância entre a sensibilidade *in vitro* e *in vivo* de nossos modelos tumorais ao erlotinib, uma vez que o erlotinib não apenas interage com as células tumorais e os TEC, mas também é capaz de perturbar a sinalização celular entre os dois compartimentos celulares através da sua influência na expressão do ligando.

A inibição da sinalização de VEGF visa eliminar a vascularização tumoral de tumores sólidos, resultando em hipóxia e apoptose induzida pela hipóxia (Shen *et al.,* 2013). Portanto, a sobrevivência das células tumorais depende do grau de supressão vascular, bem como sobre a capacidade das células tumorais para resistir ao estresse hipóxico (Mésage *et al*., 2014). É importante ressaltar que o tratamento com bevacizumab provocou níveis aumentados de VEGFR1, VEGFR2 e EGFR fosforilados

ativos (Capítulo I, Figs. 2 e 3), em todos os três modelos de tumor, que poderiam ser atenuados pela adição de erlotinib, resultando em diminuição da sinalização de sobrevivência em células tumorais e TEC, aumentando assim a atividade antitumoral. Este é o primeiro trabalho que relata a ativação do EGFR pelo bevacizumab.

Outra grande descoberta deste trabalho foi que a combinação de bevacizumab e erlotinib também foi ativa em modelos xenográficos de CRC com *KRAS* mutante. O *KRAS* é mutado em até 45% dos pacientes com CRC (Cancer Genome Atlas Network, 2012) e é um fator preditivo negativo reconhecido para os anticorpos dirigidos a EGFR (Khambata-Ford *et al*., 2007; Lelievre *et al*., 2008; Douliard *et al*., 2013; Atreyia *et al*., 2015).

A resistência adquirida ao cetuximab foi associada a mutações *KRAS* em tumores que inicialmente eram *KRAS* wt (Misale *et al*., 2012), acompanhada de secreção aumentada de ligantes EGFR (Troiani *et al*., 2013;), que aumentam, em vez de diminuir, na presença de cetuximab (Hobor *et al*., 2014). Consequentemente, sugerimos que a secreção aumentada de ligantes de EGFR e, em particular, o TGF-alfa, pode, pelo menos em parte, explicar a resistência de tumores mutantes *KRAS* ao cetuximab (Hobor *et al*., 2014). O papel crucial proposto para os ligantes de EGFR é totalmente coerente com os achados apresentados neste trabalho, uma vez que o erlotinib, mas não o cetuximab, conseguiu minimizar a secreção de ligantes de EGFR em todas as células mutantes *KRAS* examinadas.

Um mecanismo adicional associado à resistência ao cetuximab é a regulação positiva do HER2, que foi relatado em células CRC, bem como para modelos xenográficos derivados do paciente (Rajput *et al*., 2007; Bertolli *et al*., 2011). Curiosamente, o HER2 fosforilado total e ativo foi significativamente aumentado nos

três modelos de xenográficos quando erlotinib foi administrado sozinho, mas não quando o erlotinib foi combinado com bevacizumab (Capítulo I, Figs. 2 e S2). Estes resultados indicam que a ativação de HER2 pode ser um mecanismo de escape fundamental para células CRC em face da inibição de EGFR, o que pode ser prevenido ou, pelo menos, reduzida se o inibidor de EGFR for combinado com bloqueio de VEGF.

Em conclusão, no Capítulo I mostramos que as combinações de bevacizumab e erlotinib foram significativamente mais ativas que o bevacizumab sozinho em diferentes modelos de CRC, independentemente do estado do *KRAS* e sensibilidade ao bevacizumab. Em um contexto mais geral, nossos dados fornecem um quadro mecanístico para novas combinações de drogas para o tratamento do mCRC e apresentam evidências clinicamente relevantes (Fig. 6, Capítulo I).

Em contraste ao bevacizumab e erlotinib, o ditelureto de difenila (DTDF) é um composto sintético organotelurado e atualmente apresenta-se em fase de estudos *in vitro* para diferentes aplicações farmacológicas (Degrandi et, al. 2010; Trindade et al., 2015).

Um dos primeiros desafios na busca de novos efeitos biológicos para moléculas com potencial farmacológico é delinear o intervalo de dose tóxica e a distância, em termos de concentração, para a faixa com possibilidade de encontrarem-se atividades farmacológicas interessantes. Nas condições experimentais utilizadas, concentrações acima de 1.0 µmol de DTDF são citotóxicas e genotóxicas, enquanto que a zona de segurança encontra-se abaixo de 0.5 µmol (Capítulo II, Fig. 1).

Como pode ser observado no Capítulo II, após o pré-tratamento com o DTDF, pode-se perceber uma redução importante dos efeitos do H_2O_2, *t*-BOOH, MMS e UVC

em células V79, em termos de citotoxicidade, genotoxicidade e mutagenecidade. Notavelmente, a concentração de 0,1 μmol do DTDF foi mais efetiva, reduzindo em mais de 50% os danos no DNA e aumentando a viabilidade celular na mesma proporção em comparação com os mutágenos oxidantes (H_2O_2, *t*-BOOH). Reforçando esses resultados, o pré-tratemento com o DTDF diminui os danos oxidativos pela redução no número de purinas e pirimidinas oxidadas, determinado pelo ensaio cometa modificado e a geração de ROS pelo ensaio de DCFH-DA (Capitulo II, Fig. 6).

Comparando o efeito protetor do DTDF na concentração de 0,1 μmol com o disseleneto de difenila (12,5 μmol) no mesmo modelo de estudo e sob as mesmas condições (Rosa *et al.* 2007b), foi confirmado que compostos organotelurados podem apresentar efeito protetor tão significante quanto organoselenados. No caso do DTDF, a concentração protetora é 125 vezes menor que a do disseleneto de difenila, o que torna esse composto um bom candidato para futuros estudos com aplicação terapêutica.

Com o objetivo de aumentar o conhecimento a respeito do efeito protetor do DTDF, foram investigados a formação de espécies reativas de oxigênio e peroxidação lipídica em células V79. Como pode ser observado na Fig. 6A e Tabela 1, respectivamente (Capítulo II), o tratamento com o DTDF nas duas maiores concentrações testadas, foi detectado aumento nos níveis de ROS. Além disso, este tratamento aumentou significativamente a intensidade de ROS em todas as concetrações testadas (Fig. 6, Capítulo II). Reforçando esses resultados, quando foi determinado o nível de peroxidação lipídica em células V79 tratadas com o DTDF, verificou-se um aumento nos níveis de TBARS em concentrações maiores que 0,1 μmol (Capítulo II, Tabela 1), sugerindo que ROS tem um papel importante na

citotoxicidade do DTDF em células V79. Para entender melhor estes resultados, foram avaliadas as defesas antioxidantes enzimáticas. O tratamento com o DTDF em todas as concentrações aumentou a atividade da enzima SOD, porém, esse composto organotelurado inibiu a atividade de CAT em células V79 (Tabela 1, Capítulo II).

Os resultados obtidos neste trabalho sugerem que o efeito protetor do DTDF é devido a ação pró-oxidante. O aumento da atividade de SOD, acompanhado por inibição da atividade de CAT gera desequilíbrio em defesas antioxidantes da célula. Por outro lado, o aumento da atividade de SOD em nosso estudo poderia ser resultado de uma resposta adaptativa ao efeito pró-oxidante do DTDF. Esse organotelurado é um composto eletrofílico, que pode sofrer ataque nucleofílico tiol no átomo de telúrio, conferindo propriedade pró-oxidante (Rigobello *et al.* 2011; Comparsi *et al.* 2012).

Vários estudos têm demonstrado que ERO podem induzir a ativação de numerosas proteínas de sinalização, incluindo as do Receptor Tirosina Kinase, MAPKs e fatores de transcrição (Boonstra & Post, 2011). Ávila e colaboradores (2012) mostraram que o composto organotelurado dietil-2-fenil-2-telurofenil vinilfosfonato causa a translocação de DAF-16 do citosol para o núcleo em *C*aenorhabditis *elegans.* Esse achado indica que compostos organotelurados podem modular essa via, induzindo a produção de defesas antioxidantes enzimáticas, como SOD e glutationa peroxidase. Neste cenário, é possível que a resistência ao estresse oxidativo induzido pelo pré-tratamento com o DTDF em nossos estudos poderia depender da ativação de fatores de transcrição.

Considerando que vários tipos de câncer, como os tumores de mama, cólon e reto e leucemias, apresentam desequilíbrio redox com acúmulo de danos oxidativos e altos níveis de ERO (Kryston *et al.*, 2011). Neste sentido, tendo em

vista o efeito antioxidante do DTDF em baixas concentrações, foi verificado o efeito protetor deste composto em relação a danos induzidos por DOX, visando uma possível aplicação clínica como adjuvante. O pré-tratamento com este composto organotelurado diminui os níveis de ROS gerados pela DOX em células MRC5, V79 e XPD (Fig. 4, capítulo III). Além disso, o pré-tratamento com o DTDF reduziu a toxicidade gerada pela DOX (Fig.2, Capítulo III), diminuindo os danos ao DNA e o número de bases oxidadas (Fig.2, Capítulo III). A redução da geração de ROS, após tratamento com DOX pode ser explicada por aumento nas defesas antioxidantes em uma resposta adaptativa a ação pró-oxidante do DTDF. Por outro lado, em concentrações mais altas as propriedades pro-oxidantes do DTDF poderiam ser empregadas na terapia antitumoral, sensibilizando as células tumorais por aumentar ROS intracelular e induzindo as células tumorais a morte celular.

Para avaliar o potencial antitumoral do DTDF, foi realizado estudo em colaboração com a Dra. Claudia Ó Pessoa, pesquisadora responsável do Laboratório de Oncologia Experimental da Universidade do Ceará. Como pode ser observado no Capítulo II (Tabela 3), o DTDF apresentou elevada citotoxicidade (IC_{50} < 2 μg/mL) contra as linhagens celulares de câncer humano utilizadas, HL-60 (leucemia), HCT-8 (cólon), SF-295 (glioblastoma) e MDA-MB435 (melanoma). Além disso, é importante ressaltar que quando comparamos a IC_{50} do DTDF em HL-60 com a IC_{50} em leucócitos, observa-se uma seletividade para células neoplásicas de 14 vezes (Tabela 3, Capítulo III). Também foi avaliado o potencial do DTDF em causar lesões na membrana plasmática da célula, seja pela formação de poros ou pela ruptura total em células mononucleares de sangue periférico humano

(CMSPH). O resultado em eritrócitos de camundongos é extremamente importante porque sugere que o mecanismo de morte celular não envolve um dano direto a membrana plasmática, e sim uma via de sinalização mais específica (Tabela 3, Capítulo III).

Como pode ser observado no Capítulo III (Fig. 6), o DTDF (0.28 mM) induziu parada no ciclo celular em fase G2/M em células de glioma de camundongos (C6) e na concentração de 2.8 mM, o DTDF aumentou a população de células em fase sub-G1 em células de glioma humano (U87 e U251). Esses resultados mostram que a citotoxicidade do DTDF, além dos seus efeitos genotóxicos observados por Degrandi e colaboradores (2010), está consequentemente relacionada à parada no ciclo celular. Visto que o bloqueio na replicação causado pelo DTDF são induzidos por lesões no DNA, deve-se considerar que esse fenômeno geralmente vem acompanhado de ativação de proteínas de *checkpoints* que podem levar a parada no ciclo celular (Kang et al., 2017). De maneira geral, a parada no ciclo celular é um efeito dos compostos genotóxicos utilizados como agentes antineoplásicos, assim como evidenciado nesse estudo. As respostas ao dano no DNA causadas por esses agentes podem ser diversas, como modificações no genoma que ocorrem em algumas células, por exemplo, em células de câncer propensas a instabilidade genômica (Swift & Golsteyn, 2014).

Em outro estudo conduzido por Vij e Hardej (2016), os quais utilizaram o DTDF como agente antiproliferativo em células HT-29, se observou uma diminuição significativa na viabilidade celular pelo ensaio de MTT numa faixa de concentrações de 62,5 a 1000 µM. Ainda que essa faixa de concentrações seja distinta das utilizadas no nosso estudo, é interessante observar que nesse mesmo trabalho foi

determinada a ativação de caspase 3/7 e caspase 9 induzida pela exposição ao DTDF, confirmando a ação antiproliferativa desse composto por apoptose. Além disso, foi observado um decréscimo na taxa de GSH/GSSG o que sugere que o telúrio reage com grupos tiol, particularmente GSH. Neste contexto, Comparsi e colaboradores (2012) mostraram que o DTDF inibe a atividade da enzima TrxR. Ainda em células HT-29, outro estudo mostrou alterações em expressão de genes e proteínas antioxidantes (SOD, CAT, GPx), além de um aumento significativo de radical hidroxila à exposição ao DTDF numa faixa de concentrações de 500 a 1000 µM, o que fortalece a hipótese de que a ação antiproliferativa e genotóxica do DTDF seja consequência do estresse oxidativo (Vij & Hardej, 2016).

Recentemente, Heinfarth e colaboradores (2016) mostraram que o DTDF induz hiperfosforilação em neuronios e astrócitos. Esses mecanismos foram mediados por receptores de N-metil-d-aspartato (NMDA), canais de cálcio dependentes da tensão do tipo L (L-VDCCs), bem como receptores metabotrópicos de glutamato a montante da fosfolipase C (PLC). Aumentando o influxo de cálcio intracelular. Nos neurônios, os altos níveis intracelulares de Ca^{2+} ativaram MAPKs, Erk e p38MAPK, e como consequencia deve ter ocasionado desbalanço redox celular, aumentando ROS e induzindo a morte celular.

Os resultados apresentados nesse trabalho evidenciam claramente os efeitos antiproliferativos do DTDF em diferentes modelos de estudo. Os efeitos celuluares do DTDF parecem ser complexos e dependentes da concentração. Os resultados apresentados nos capítulos II e III sugerem que a citotoxicidade desse composto está diretamente relacionada ao seu potencial oxidativo, levando a decréscimo na razão GSH/GSSG, e inibição da atividade das enzimas CAT, SOD,

GR, GPx, e TrxR, diminuindo a capacidade anti-oxidante da célula e os níveis de ROS aumentam exponencialmente (Fig 9).

O DTDF também pode induzir hiperfosforilação em alvos, em procesos mediados por receptores de NMDA, canais de cálcio L-VDCCs, bem como receptores metabotrópicos de glutamato mGluR a montante da fosfolipase C (PLC), aumentando o influxo de cálcio intracelular (Heinfarth *et al.,* 2016). Nos neurônios, os altos níveis intracelulares de Ca^{2+} ativam as MAPKs, Erk e p38MAPK, ocasionado desbalanço redox celular, aumentando ROS e induzindo a morte celular (Heinfarth *et al*., 2016). A sobrecarga mitocondrial de Ca^{2+} é conhecida como um evento crítico na crise bioenergética associada à morte celular por necrose (um exemplo prototípico é a excitotoxicidade dos neurônios) e atua como um sinal crítico de sensibilização nas vias de apoptose intrínseca (Ivanova *et al*., 2017). De fato, a sobrecarga mitocondrial de Ca^{2+} resulta em alterações dramáticas nas funções mitocondriais, incluindo diminuição da produção de ATP e aumento da geração de espécies reativas de oxigênio (ROS) (Ivanova *et al*., 2017).

As alterações nos níveis de expressão e / ou a fosforilação do 1, 4, 5 fosfatidilinositol trifosfato (IP3) resultaram em maior ou menor susceptibilidade à morte celular. Vários oncogenes exercem seus efeitos regulando a expressão e a atividade de IP3. Exemplo clássico deste grupo é o oncogene AKT, que regula a apoptose controlando a liberação de Ca^{2+} do restículo endoplamático (RE) (Castaño et al., 2009). Como pode ser observado no capítulo III (Fig. 8), o DTDF interage com AKT, provavelmente reduzindo / inibindo a atividade e assim pode (i) levar a um descontrole em IP3, aumentando a sinalização intracelular de Ca^{2+}, ocasionando um desbalanço redox e levando a morte celular (ii) e / ou o DTDF

pode causar interferência no processo replicativo das células, podendo levar a parada no ciclo celular (Fig. 7). Como já revisado na literatura, agentes que atuam por esse mecanismo de ação seguem o mesmo padrão de efeitos biológico aos encontrados nesse estudo (Wu *et al*., 2010; Swift e Golsteyn, 2014). Assim, a investigação mais aprofundada de mecanismos moleculares se faz necessário em estudos futuros com o DTDF.

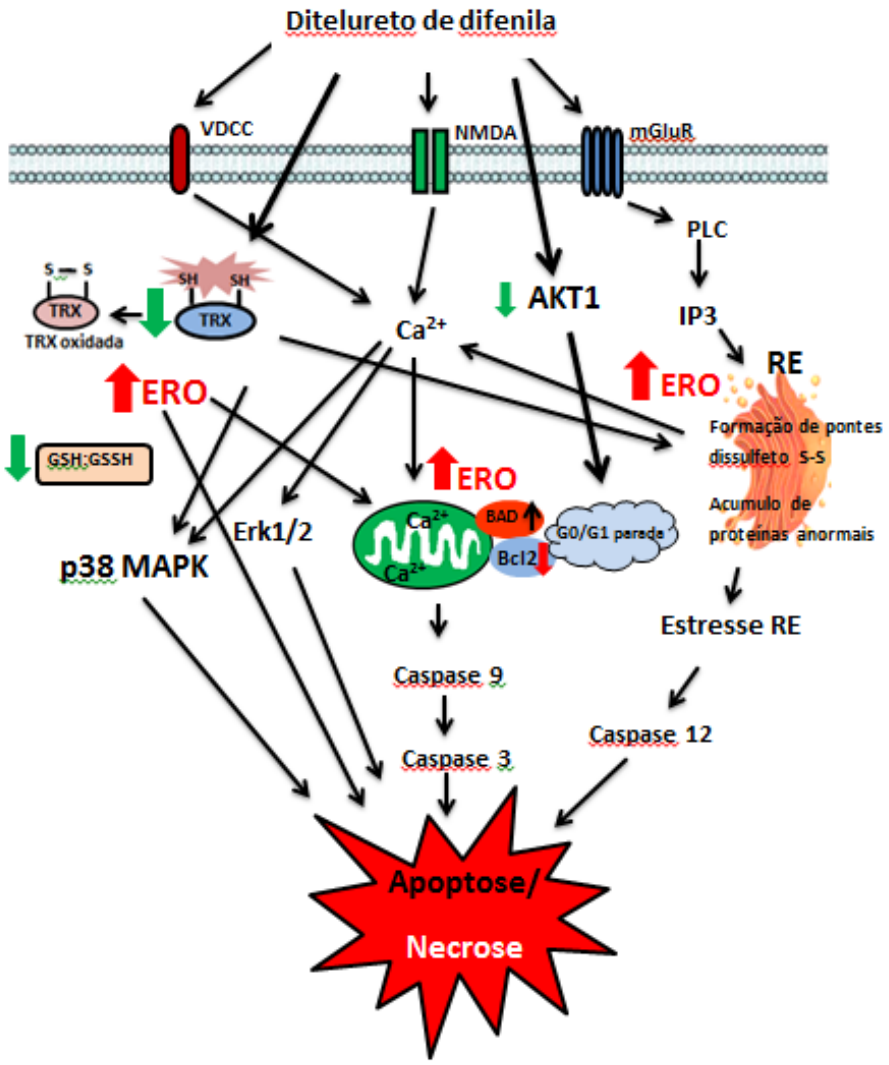

Figura 7. Modelo dos possíveis mecanismos de ação do ditelureto de difenila. Por ser um agente modulador do estado redox celular, pode interferir com o estado de diferentes proteínas redox-ativas, levando a um desbalanço redox e induzindo a morte celular.

4. Conclusões

4.1 Conclusão Geral

O conjunto de resultados desta tese permite concluir que as combinações de bevacizumab com erlonitib, uma molécula pequena inibidora de receptor tirosina quinase atuando no EGFR, são significativamente mais ativas do que o bevacizumab em modelos CRC independente da presença de *KRAS* mutado.

Além desses resultados, o composto DTDF apresentou efeito antigenotóxico e antimutagênico, possivelmente por atividade antioxidante e apresentou atividade antitumoral contra diferentes linhagens tumorais.

4.2 Conclusões Específicas

- A combinação dos agentes bevacizumab mais erlotinib foi mais efetiva que bevacizumab sozinho em modelos de câncer colorretal independente do estado mutacional do *KRAS*;
- Bevacizumab ativa a sinalização de sobrevivência mediada por EGFR nas células tumorais, bem como nas células endoteliais associadas ao tumor;
- Erlotinib reduz a secreção de anfiregulina e de TGF-alfa *in vitro* e *in vivo*, independente do estado mutacional do *KRAS*;
- O composto ditelureto de difenila (DTDF) em baixas concentrações (0,01 – 0,1 μM) confere proteção celular ao DNA, contra a ação genotóxica do peróxido de hidrogênio, *tert*-butil peróxido, metil metano sulfonato e radiação ultravioleta-C;
- O pré-tratamento com o DTDF em baixas concentrações (0,01 – 0,1 μM) reduziu a peroxidação lipídica e a formação de espécies reatiavas de oxigênio gerada pelos agentes oxidantes peróxido de hidrogênio e *tert*-butil peróxido;
- O DTDF apresenta potencial antitumoral em concentrações subgenotóxicas em diferentes linhagens celulares *in vitro.*

5. Perspectivas

Embora os resultados obtidos neste trabalho permitissem elucidar aspectos importantes no mecanismo de ação dos agentes testados, algumas questões permanecem controversas e devem ser exploradas para uma melhor compreensão das vias implicadas nas respostas celulares a estes agentes.

- Verificar em outros tumores a resposta celular ao tratamento combinado com bevacizumab e erlotinib;
- Analisar por western blotting marcadores celulares que corroborem com nossos resultados de imunohistoquímica;
- Avaliar o potencial antitumoral do DTDF em terapia combinada com diferentes quimioterápicos em células de glioblastoma multiforme;
- Determinar o potencial antitumoral do DTDF *in vivo*, em tumores xenográficos de glioblastoma multiforme;
- Avaliar a geração de espécies reativas de oxigênio (DCFH-DA) e analisar a morte celular por apoptose e necrose em células de glioblastoma multiforme por citometria de fluxo;
- Investigar o perfil redox das células tumorais e não tumorais expostas ao DTDF.

6. Referências bibliográficas

Abreu, F. C.; Ferraz, P. A. L.; Goulart, M. O. F. Some applications of electrochemistry in Biomedical Chemistry. Emphasis on the correlation of electrochemical and bioactive properties. J. Braz. Chem. Soc. v. 13, n. 1. p. 19-35, 2002.

Adhami, V.M. et al. Activation of pro death Bcl-2 family proteins and mitochondrial apoptosis pathway by sanguinarine in immortalized human HaCaT keratinocytes. Clin. Cancer Res. v.1, n.9, p. 3176-82, 2003.

Ahlquist, D. A., H. Zou, M. Domanico, D. W. Mahoney, T. C. Yab et al. Nextgeneration stool DNA test accurately detects colorectal cancer and large adenomas. Gastroenterology 142: 248-256; quiz e225-246, 2012.

Ahmed, D., P. W. Eide, I. A. Eilertsen, S. A. Danielsen, M. Eknaes et al. Epigenetic and genetic features of 24 colon cancer cell lines. Oncogenesis 2: e71, 2013.

Aithal, B.K. et al. Juglone, a naphthoquinone from walnut, exerts cytotoxic and genotoxic effects against cultured melanoma tumor cells. Cell Biol. Int. v. 33, 1039-49, 2009.

Ajith, T. A.; Janardhanan, K. K. Cytotoxic and antitumor activities of a polypore macrofungus, Hellinusrimosus (Berk) Pilat. J. Ethnopharmacol. v.84, p.157-162, 2003.

Albini, A.; Fagnoni, M. Photochemistry of N-oxides . In: Horspool, W.M.; Lenci, F. (ed.) CRC Handbook in Organic Photochemistry and Photobiology. 2.ed. Pisa (Italy): CRC Press, p.99-1-99-21, 2004.

Al-Harbi, S. et al. An antiapoptotic BCL-2 family expression index predicts the response of chronic lymphocytic leukemia toABT-737. Blood. v. 118, n.13, p. 3579-89, 2011.

Ali, R., Z. Mirza, G. M. Ashraf, M. A. Kamal, S. A. Ansari et al. New anticancer agents: recent developments in tumor therapy. Anticancer Res 32: 2999-3005, 2012.

Amaral, J.D. et al. The role of p53 in apoptosis.Discov Med. v.9, n.45, p.145-52, 2010.

Amin DN, Hida K, Bielenberg DR, Klagsbrun M. Tumor endothelial cells express epidermal growth factor receptor (EGFR) but not ErbB3 and are responsive to EGF and to EGFR kinase inhibitors. Cancer Res. 66:2173-80, 2006.

Appert-Collin, A., Hubert, P., Crémel, G., Bennasroune, A. Role of ErbB Receptors in Cancer Cell Migration and Invasion. Front Pharmacol 24;6:283. 2015.

Aragon-Ching, J.B and Trump, D.L. Targeted therapies in the treatment of urothelial cancers. Urol Oncol. 35(7):465-472. 2017.

Argiris, K., C. Panethymitaki e M. Tavassoli. Naturally occurring, tumor-specific, therapeutic proteins. Exp Biol Med (Maywood) 236: 524-536, 2011.

Arrowsmith, J. Trial watch: phase III and submission failures: 2007-2010. Nat Rev Drug Discov 10: 87, 2011.

Atreya CE., Corcoran, R.B., Kopetz, S. Expanded RAS: refining the patient population. J Clin Oncol. 1;33(7):682-5, 2015.

Austin, C. A., J. H. Sng, S. Patel e L. M. Fisher. Novel HeLa topoisomerase II is the II beta isoform: complete coding sequence and homology with other type II topoisomerases. Biochim Biophys Acta 1172: 283-291, 1993.

Avila DS, Benedetto A, Au C, et al. Organotellurium and organoselenium compounds attenuate Mn-induced toxicity in Caenorhabditis elegans by preventing oxidative stress. Free Radical Biology and Medicine. 52(9):1903–1910, 2012.

Ávila DS, Gubert P, Palma A, et al. An organotellurium compound with antioxidant activity against excitotoxic agents without neurotoxic effects in brain of rats. Brain Research Bulletin. 76(1-2):114–123, 2008.

Ayadi M, Bouygues A, Ouaret D, Ferrand N, Chouaib S, Thiery JP, Muchardt C, Sabbah M, Larsen AK. Chronic chemotherapeutic stress promotes evolution of stemness and WNT/beta-catenin signaling in colorectal cancer cells: implications for clinical use of WNT-signaling inhibitors. Oncotarget. May 11, 2015.

Ba LA, Doring M, Jamier V, Jacob C. Tellurium: an element with great biological potency and potential. Organic & biomolecular chemistry. 8:4203–4216, 2010.

Babula P, Adam V, Opatrilova R, Zehnalek J, Havel L, Kizek R. Uncommon heavy metals, metalloids and their plant toxicity: a review. Environmental Chemistry Letters. 6(4):189–213, 2008.

Bailly, C. Topoisomerase I poisons and suppressors as anticancer drugs. Curr Med Chem 7: 39-58, 2000.

Bakkenist, C. J., eM. B. Kastan. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature 421: 499-506, 2003.

Baldwin, E. L., eN. Osheroff. Etoposide, topoisomerase II and cancer. Curr Med Chem Anticancer Agents 5: 363-372, 2005.

Barnes, S., T. G. Peterson e L. Coward. Rationale for the use of genistein-containing soy matrices in chemoprevention trials for breast and prostate cancer. J Cell Biochem Suppl 22: 181-187, 1995.

Barrera, G. Oxidative stress and lipid peroxidation products in cancer progression and therapy. ISRN Oncol. 137289. 2012.

Barretina, J., G. Caponigro, N. Stransky, K. Venkatesan, A. A. Margolin et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. Nature 483: 603-607, 2012.

Bartek, J., eJ. Lukas, DNA damage checkpoints: from initiation to recovery or adaptation. Curr Opin Cell Biol 19: 238-245, 2007.

Batson, S., Mitchell, S.A., Windisch, R., et al., Tyrosine kinase inhibitor combination therapy in first-line treatment of non-small-cell lung cancer: systematic review and network meta-analysis. Onco Targets Ther. 10:2473-2482. 2017.

Bertotti A, Migliardi G, Galimi F, Sassi F, Torti D, Isella C, et al. A molecularly annotated platform of patient-derived xenografts ("xenopatients") identifies HER2 as an effective therapeutic target in cetuximab-resistant colorectal cancer. Cancer Discov. 1:508-23, 2011.

Bhonde, M. R., M. L. Hanski, M. Notter, B. F. Gillissen, P. T. Daniel et al. Equivalent effect of DNA damage-induced apoptotic cell death or long-term cell cycle arrest on colon carcinoma cell proliferation and tumour growth. Oncogene 25: 165-175 2006.

Bienert GP, Schüssler MD, Jahn TP. Metalloids: essential, beneficial or toxic? Major intrinsic proteins sort it out, Trends Biochem. Sci. vol. 33 pg. 20-26, 2008.

Bordin DL, Lima M, Lenz G, Saffi J, Meira LB, Mésange P, Soares DG, Larsen AK, Escargueil AE, Henriques JA. DNA alkylation damage and autophagy induction. Mutat Res. Oct-Dec;753(2):91-9, 2013.

Bordoli MR, Stiehl DP, Borsig L, Kristiansen G, Hausladen S, Schraml P, et al. Prolyl-4-hydroxylase PHD2- and hypoxia-inducible factor 2-dependent regulation of amphiregulin contributes to breast tumorigenesis. Oncogene. 30:548-60, 2011.

Borges, V. C., J. B. Rocha e C. W. Nogueira. Effect of diphenyl diselenide, diphenyl ditelluride and ebselen on cerebral Na(+), K(+)-ATPase activity in rats. Toxicology 215: 191-197, 2005.

Brand, T.M., Wheeler, D.L. KRAS mutant colorectal tumors: past and present. Small GTPases. 3(1):34-9. 2012.

Braun, M. S., eM. T. Seymour. Balancing the efficacy and toxicity of chemotherapy in colorectal cancer. Ther Adv Med Oncol 3: 43-52, 2011.

Brito, V. B., Rocha, J. B., Folmer, V. and Erthal, F. Diphenyl disele-nide and diphenyl ditelluride increase the latency for 4-aminopyridine-induced chemical seizure and prevent death in mice. Acta Biochim. Pol., 56, 125–134, 2009.

Briviba K, Tamler R, Klotz LO, Engman L, Cotgreave IA, Sies H. Protection by organotellurium compounds against peroxynitrite-mediated oxidation and nitration reactions. Biochem Pharmacol. 55:817–823, 1998.

Brozmanova, J., D. Manikova, V. Vlckova e M. Chovanec. Selenium: a double-edged sword for defense and offence in cancer. Arch Toxicol 84: 919-938, 2010.

Bruning, A and Mylonas, I. New emerging drugs targeting the genomic integrity and replication machinery ovarian cancer. Arch Gynecol Obstet. 283(5):1087-96. 2011.

Bucher, N., eC. D. Britten. G2 checkpoint abrogation and checkpoint kinase-1 targeting in the treatment of cancer. Br J Cancer 98: 523-528, 2008.

Burden, D. A., eN. Osheroff. Mechanism of action of eukaryotic topoisomerase II and drugs targeted to the enzyme. Biochim Biophys Acta 1400: 139-154, 1998.

Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. Nature. 487:330-7, 2012.

Carocho, M and Ferreira, I.C. A review on antioxidants, prooxidants and related controversy: natural and synthetic compounds, screening and analysis methodologies and future perspectives. Food Chem Toxicol. 51:15-25. 2013.

Castaño JP, Martínez-Fuentes AJ, Gutiérrez-Pascual E, Vaudry H, Tena-Sempere M, Malagón MM. Intracellular signaling pathways activated by kisspeptins through GPR54: do multiple signals underlie function diversity? Peptides. Jan;30(1):10-5, 2009.

Castedo, M., J. L. Perfettini, T. Roumier, K. Andreau, R. Medema et al. Cell death by mitotic catastrophe: a molecular definition. Oncogene 23: 2825-2837, 2004.

Chasteen, T. G., Fuentes, D. E., Tantaleán, J. C. and Vásquez, C. C. Tellurite: history, oxidative stress, and molecular mechanisms of resistance. FEMS Microbiology Reviews, 33: 820–832, 2009.

Chasteen, T.G., Fuentes, D.E., tantaleán, J.C., Vásquez, C.C. Tellurite: history, oxidative stress, and molecular mechanisms of resistance. FEMS Microbiol Rev. 33(4):820-32. 2009.

Chibaudel B, Tournigand C, Samson B, Scheithauer W, Mésange P, Lledo G, et al. Bevacizumab-erlotinib as maintenance therapy in metastatic colorectal cancer. Final results of the GERCOR DREAM study. Ann Oncol. 25(Supp4):4970, 2014.

Chiron M, Bagley RG, Pollard J, Mankoo PK, Henry C, Vincent L, et al. Differential antitumor activity of aflibercept and bevacizumab in patient-derived xenograft models of colorectal cancer. Mol Cancer Ther. 13:1636-44, 2014.

Comasseto JV, Dos Santos AA. Organotellurides as precursors of reactive organometallics. Phosphorus, Sulfur and Silicon and the Related Elements. 183(4):939–947, 2008.

Comparsi, B., Meinerz, D. F., Franco, J. L. et al. Diphenyl ditellu-ride targets brain selenoproteins in vivo: inhibition of cerebral thioredoxin reductase and glutathione peroxidase in mice after acute exposure. Mol. Cell. Biochem., 370, 173–182, 2012.

Corcoran RB, Rothenberg SM, Hata AN, Faber AC, Piris A, Nazarian RM, et al. TORC1 Suppression Predicts Responsiveness to RAF and MEK Inhibition in BRAF-Mutant Melanoma. Sci Transl Med. 5:196ra98, 2013.

Cunha RL; Gouvea IE; Juliano L. A glimpse on biological activities of tellurium compounds. An Acad Bras Cienc. Sep;81(3):393-407, 2009.

Cuya SM, Bjornsti MA, van Waardenburg RCAM. DNA topoisomerase-targeting chemotherapeutics: what's new? Cancer Chemother Pharmacol. Jul;80(1):1-14, 2017.

D'Incalci, M., Galmarini, C.M. A review of trabectedin (ET-743): a unique mechanism of action. Mol Cancer Ther. 9(8):2157-63. 2010.

Damiani RM, Moura DJ, Viau CM, et al. Pathways of cardiac toxicity: comparison between chemotherapeutic drugs doxorubicin and mitoxantrone. Arch Toxicol. Sep;90(9):2063-76, 2016.

Das B, Yeger H, Tsuchida R, Torkin R, Gee MF, Thorner PS, et al. A hypoxia-driven vascular endothelial growth factor/Flt1 autocrine loop interacts with hypoxia-inducible factor-1alpha through mitogen-activatedprotein kinase/extracellular signal-regulated kinase 1/2 pathway in neuroblastoma. Cancer Res. 65:7267-75, 2005.

Degrandi,T. H., de Oliveira, I. M., d'Almeida, G. S., Garcia, C. R.,Villela, I.V., Guecheva,T. N., Rosa, R. M. and Henriques, J.A. Evaluation of the cytotoxicity, genotoxicity and mutagenicity of diphenyl ditelluride in several biological models. Mutagenesis, 25, 257–269, 2010.

Deka, B and Singh, K.k. Multifaced Regulation of Gene Expression by the Apoptosis- and Splicing-Associated Protein Complex and Its Components. Int J Biol Sci. 13(5):545-560. 2017.

Dhawan A, Kayani MA, Parry JM, Parry E, Anderson D. Aneugenic and clastogenic effects of doxorubicin in human lymphocytes. Mutagenesis. 18(6):487-90, 2003.

Dhomen, N.S., Mariadason, J., Tebbutt, N., Scott, A.M. Therapeutic targeting of the epidermal growth factor receptor in human cancer. Crit Rev Oncog. 17(1):31-50. 2012.

Di Giacomo, S., Di Sotto, A., Mazzanti, G., Wink, M. Chemosensitizing properties of b-caryophyllene and b-caryophyllene oxide in combination with doxorubicin in human cancer cells. Anticancer Res. 37(3):1191-1196. 2017.

Dopp E, Hartmann LM, Florea A-M, Rettenmeier AW, Hirner AV. Environmental distribution, analysis, and toxicity of organometal(loid) compounds. Critical Reviews in Toxicology. 34(3):301–333, 2004.

Douillard JY, Oliner KS, Siena S, Tabernero J, Burkes R, Barugel M, et al. Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. N Engl J Med. 369:1023-34, 2013.

Edward R. T. Tiekink. Therapeutic potential of selenium and tellurium compounds: Opportunities yet unrealised. Dalton Trans. 41, 6390, 2012.

Elkabets M, Vora S, Juric D, Morse N, Mino-Kenudson M, Muranen T, et al. mTORC1 Inhibition Is Required for Sensitivity to PI3K p110α Inhibitors in PIK3CA-Mutant Breast Cancer. Sci Transl Med. 5: 196ra99, 2013.

Engelman JA, Jänne PA, Mermel C, Pearlberg J, Mukohara T, Fleet C, et al. ErbB-3 mediates phosphoinositide 3-kinase activity in gefitinib-sensitive non-small cell lung cancer cell lines. Proc Natl Acad Sci USA. 102:3788-93, 2005.

Engman L, Al-Maharik N, McNaughton M, Birmingham A, Powis G. Thioredoxin reductase and cancer cell growth inhibition by organotellurium compounds that could be selectively incorporated into tumor cells. Bioorg Med Chem. 11:5091–5100, 2003.

Favaloro, B., Allocati, N., Graziano, V., Di Llio, C., De Laurenzi, V. Role of apoptosis in disease. Aging. 4(5):330-49, 2012.

Ferrarini RS, Dos Santos AA, Comasseto JV. Tellurium in organic synthesis: a general approach to buteno- and butanolides. Tetrahedron. 68(51):10601–10610, 2012.

Finlay, D., Teriete, P., Vamos, M., Costoford, NDP., Vouri, K. Inducing death in tumor cells: roles of the inhibitor of apoptosis proteins. F1000Res. 6:587, 2017.

Friedman M, Bayer I, Letko I, Duvdevani R, Zavaro-Levy O, Ron B, Albeck M, Sredni B. Topical treatment for human papillomavirus associated genital warts in humans with the novel tellurium immunomodulator AS101: assessment of its safety and efficacy, Br. J. Dermatol. vol. 160 (pg. 403-408), 2009.

Gagné F, Auclair J, Turcotte P, et al. Ecotoxicity of CdTe quantum dots to freshwater mussels: impacts on immune system, oxidative stress and genotoxicity. Aquatic Toxicology. 86(3):333–340, 2008.

Galetta, D., Rossi, A., Pisconti, S., Millaku, A, Colucci, G. Maintenance or non-maintenance therapy in the treatment of advanced non-small cell lung câncer: that is the question. Cancer Treat Rev 36 (3):S30-3, 2010.

Galluzzi, L., Bravo-San Pedro, J.M., Kepp, O., Kroemer, G. Regulated cell death and adaptive stress responses. Cell Mol Life Sci. 73(11-12):2405-10, 2016.

Gordaliza M. Natural products as leads to anticancer drugs. Clin Transl Oncol 9:767-776, 2007.

Gossage L, Eisen T. Targeting multiple kinase pathways: a change in paradigm. Clin Cancer Res. 16:1973-8, 2010.

Granados-Principal, S., Quiles, J.L., Ramirez-Tortosa, C et al., New advances in molecular mechanisms and the prevention of adriamycin toxicity by antioxidant nutrients. Food Chem Toxicol. 48(6):1425-38, 2010.

Greystoke, A and Mullamitha, S.A. How many diseases are colorectal cancer. Gastroenterol res Pract. 2012:564741, 2012.

Gumá, M., Rius, J., Duong-Polk, KX, Haddad GG., et al. Genetic and pharmacological inhibition of JNK ameliorates hypoxia-induced retinopathy through interference with VEGF expression. Proc Natl Acad Sci. 106(21):8760-5, 2009.

Gunaratnam L, Morley M, Franovic A, de Paulsen N, Mekhail K, Parolin DA,et al. Hypoxia inducible factor activates the transforming growth factor-alpha/epidermal growth factor receptor growth stimulatory pathway in VHL(-/-) renal cell carcinoma cells. J Biol Chem. 278:44966-74, 2003.

Gutteridge, J.M and Halliwell, B. Antioxidants: Molecules, medicines, and myths. Biochem Biophys Res Commun. 19;393(4):561-4, 2010.

Halliwell B. Cell culture, oxidative stress, and antioxidants: avoiding pitfalls. Biomed J. 3(3):99-105, 2014.

Halliwell B. Free radicals and antioxidants: updating a personal view. Nutr Rev. 70(5):257-65, 2012.

Halpert G & Sredni B. The effect of the novel tellurium compound AS101 on autoimmune diseases. Autoimmun Rev. Dec;13(12):1230-5, 2014.

Halpert, G., Sredni, B. The effect of the novel tellurium compound AS101 on autoimmune diseases. Autoimmun Rev. 13(12):1230-5, 2014.

Hanahan, D., Weinberg, R.A. Hallmarks of cancer: the next generation. Cell 144(5):646-74, 2011.

Hardman R. A toxicologic review of quantum dots: toxicity depends on physicochemical and environmental factors. Environmental Health Perspectives. 114(2):165–172, 2006.

Hecht JR, Mitchell E, Chidiac T, Scroggin C, Hagenstad C, Spigel D, et al. A randomized phase IIIB trial of chemotherapy, bevacizumab, and panitumumab compared with chemotherapy and bevacizumab alone for metastatic colorectal cancer. J Clin Oncol. 27:672-80, 2009.

Heimfarth L, da Silva Ferreira F, Pierozan P, Loureiro SO, Mingori MR, Moreira JC, da Rocha JB, Pessoa-Pureur R. Calcium signaling mechanisms disrupt the cytoskeleton of primary astrocytes and neurons exposed to diphenylditelluride. Biochim Biophys Acta. Nov;1860(11 Pt A):2510-20, 2016.

Helleday, T., Peterman, E., Lundin, C., et al. DNA repair patways as targets for cancer therapy. Nature Rev 8:193-204, 2008.

Henson, E., Chen, Y, Gibson S. EGFR family member'sregulation of autophagy is at a crossroads of cell survival and death in cancer. Cancer (Basel). 24;9(4), 2017.

Hlavin Em, Smeaton MB, Noronha, AM et al. Cross-linck structure affects replication-independent DNA interstrand cross-linck repair in mammalian cells. Biochemistry. 49(18):3977-88, 2010.

Hobor S, Van Emburgh BO, Crowley E, Misale S, Di Nicolantonio F, Bardelli A. TGFα and amphiregulin paracrine network promotes resistance to EGFR blockade in colorectal cancer cells. Clin Cancer Res. 20:6429-38, 2014.

Hristina Ivanova, Martijn Kerkhofs, Rita M. La Rovere, and Geert Bultynck. Endoplasmic Reticulum–Mitochondrial $Ca2^{+}$ Fluxes Underlying Cancer Cell Survival. Front Oncol. 7: 70, 2017.

Huang TH, Huo L, Wang YN, Xia W, Wei Y, Chang SS, et al. Epidermal growth factor receptor potentiates MCM7-mediated DNA replication through tyrosine phosphorylation of Lyn kinase in human cancers. Cancer Cell. 23:796-810, 2013.

INCA. Estimativa 2012., Instituto Nacional de câncer. 2012. Incidência de câncer no Brasil/Instituto Nacional de câncer – Rio de Janeiro, 2012.

Ivanova H, Kerkhofs M, La Rovere, Bultynck G. Endoplasmic Reticulum-Mitochondrial Ca2+ Fluxes Underlying Cancer Cell Survival. Front Oncol. 3:7:70. 2017.

Jia LT, Zhang R, Shen L, Yang AG. Regulators of carcinogenesis: emerging roles beyond their primary functions. Cancer Lett. 357:75-82, 2015.

Jorge, P. M., de Oliveira, I. M., Filippi Chiela, E. C., Viau, C. M., Saffi, J., Horn, F., Rosa, R. M., Guecheva, T. N. and Pêgas Henriques, J. A. Diphenyl Ditelluride-Induced Cell Cycle Arrest and Apoptosis: A Relation with Topoisomerase I Inhibition. Basic Clin Pharmacol Toxicol, 116: 273–280, 2015.

Junying Yuan and Guido Kroemer. Alternative cell death mechanisms in development and beyond. Genes Dev. Dec 1; 24(23): 2592–2602, 2010.

Kang S, Kang MS, Ryu E, Myung K. Eukaryotic DNA replication: Orchestrated action of multi-subunit protein complexes. Mutat Res. S0027-5107(17)30056-8, 2017.

Khambata-Ford S, Garrett CR, Meropol NJ, Basik M, Harbison CT, Wu S, et al. Expression of epiregulin and amphiregulin and K-ras mutation status predict disease control in metastatic colorectal cancer patients treated with cetuximab. J Clin Oncol. 25:3230-7, 2007.

Kitagawa D, Yokota K, Gouda M, Narumi Y, Ohmoto H, Nishiwaki E, et al. Activity-based kinase profiling of approved tyrosine kinase inhibitors. Genes Cells. 18:110-22, 2013.

Klaine SJ, Alvarez PJJ, Batley GE, et al. Nanomaterials in the environment: behavior, fate, bioavailability, and effects. Environmental Toxicology and Chemistry. 27(9):1825–1851, 2008.

Krishnamachary B, Berg-Dixon S, Kelly B, Agani F, Feldser D, Ferreira G, Iyer N, LaRusch J, Pak B, Taghavi P, Semenza GL. Regulation of colon carcinoma cell invasion by hypoxia-inducible factor 1. Cancer Res. 63:1138-43, 2003.

Kryston, T.B., Georgiev, A.B., Pissis, P., Georgakilas, A.G. Role of oxidative stress and DNA damage in human carcinogenesis. Mutat Res: Fundam Mol Mech Mutagen. 2011.

Kumar A, Rajendran V, Sethumadhavan R, Purohit R. AKT kinase pathway: a leading target in cancer research. ScientificWorldJournal. Nov 13;2013:756134, 2013.

Kuwai T, Nakamura T, Sasaki T, Kim SJ, Fan D, Villares GJ, et al. Phosphorylated epidermal growth factor receptor on tumor-associated endothelial cells is a primary target for therapy with tyrosine kinase inhibitors. Neoplasia. 10:489-500, 2008.

Larner AJ. Biological effects of tellurium: a review. Trace Elements and Electrocytes. 12(1):26–31, 1995.

Larsen AK, de Gramont A, P.oindessous V, Bouygues A, Ayadi M, Mésange P. Functions and clinical implications of autocrine VEGF signaling in colorectal cancer. Curr Colorectal Cancer Rep. 9:270-7, 2013.

Larsen AK, Ouaret D, El Ouadrani K, Petitprez A. Targeting EGFR and VEGF(R) pathway cross-talk in tumor survival and angiogenesis. Pharmacol Ther. 131:80-90, 2011.

Larsen, A., Escargueil, A.E. Les ciblés liées à la réplication, à l'expression et à la stabilité du génome, em Raymond, E. Thérapie ciblée des cancers – Le concept de cicle em cancerologie, 2009.

Lenglet, G., David-Cordonnier, M.H. DNA-destabilizing agents as an alternative approach for targeting DNA : mechanisms of action and cellular consequenses. J Nucleic Acids, 2010.

Lièvre A, Bachet JB, Boige V, Cayre A, Le Corre D, Buc E, et al. KRAS mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. J Clin Oncol. 26:374-9, 2008.

Liu H, Wang J, Chen Y, Chen Y, Ma X, Bihl JC, Yang Y. NPC-EXs Alleviate Endothelial Oxidative Stress and Dysfunction through the miR-210 Downstream Nox2 and VEGFR2 Pathways. Oxid Med Cell Longev. 2017:9397631, 2017.

Longley DB, Johnston PG. Molecular mechanisms of drug resistance. J Pathol. Jan;205(2):275-92, 2005.

Lovrić J, Cho SJ, Winnik FM, Maysinger D. Unmodified cadmium telluride quantum dots induce reactive oxygen species formation leading to multiple organelle damage and cell death. Chemistry and Biology. 12(11):1227–1234, 2005.

Lugokenski TH, Muller LG, Taube PS, Rocha JBT, Pereira ME. Inhibitory effect of ebselen on lactate dehydrogenase activity from mammals: a comparative study with diphenyl diselenide and diphenyl ditelluride. Drug Chem Toxicol 34:66–76, 2011.

Maciel EN, Bolzan RC, Braga AL, Rocha JBT. Diphenyl diselenide and diphenyl ditelluride affect delta-aminolevulinate dehydratase from liver, kidney and brain of mice. J Biochem Mol Toxicol 14:310–319, 2000.

Madamanchi NR, Vendrov A, Runge MS. Oxidative stress and vascular disease. Arterioscler Thromb Vasc Biol. Jan;25(1):29-38, 2005.

Martínez-Reyes I, Chandel NS. Mitochondrial one-carbon metabolism maintains redox balance during hypoxia. Cancer Discov. 4:1371-3, 2014.

Matuo, R., Sousa, F.G., Escarguiel, A,E., Grivicich, I., et al., 5-Fluoracil and its active metabolite FdUMP cause DNA damage in human SW620 colon adenocarcinoma cell line. J Appl Toxicol 29(4):308-16, 2009.

Mésange P, Poindessous V, Sabbah M, Escargueil AE, de Gramont A, Larsen AK. Intrinsic bevacizumab resistance is associated with prolonged activation of autocrine VEGF signaling and hypoxia tolerance in colorectal cancer cells and can be overcome by nintedanib, a small molecule angiokinase inhibitor. Oncotarget. 5:4709-21, 2014.

Mini E, Nobili S, Caciagli B, Landini I, Mazzei T. Cellular pharmacology of gemcitabine. Ann Oncol. May;17 Suppl 5:v7-12, 2006.

Misale S, Yaeger R, Hobor S, Scala E, Janakiraman M, Liska D, et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature. 486:532-6, 2012.

Miyamoto Y, Suyama K, Baba H. Recent Advances in Targeting the EGFR Signaling Pathway for the Treatment of Metastatic Colorectal Cancer. Int J Mol Sci. Apr 2;18(4). pii: E752, 2017.

Moore MJ, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, et al. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. J Clin Oncol. 25:1960-6, 2007.

Moretto MB, Boff B, Franco J, Posser T, Roessler TM, Souza DO, Nogueira CW, Wofchuk S, Rocha JB. Ca(2+) influx in rat brain: effect of diorganylchalcogenides compounds, Toxicol. Sci., vol. 99 (pg. 566-571), 2007.

Morin-Ben Abdallah S, Hirsh V. Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors in Treatment of Metastatic Non-Small Cell Lung Cancer, with a Focus on Afatinib. Front Oncol. May 16;7:97, 2017.

Mukohara T, Engelman JA, Hanna NH, Yeap BY, Kobayashi S, Lindeman N, et al. Differential effects of gefitinib and cetuximab on non-small-cell lung cancers bearing epidermal growth factor receptor mutations. J Natl Cancer Inst. 97:1185-94, 2005.

Muñiz Alvarez JL, García Calzón JA, López Fonseca JM. Electrochemistry of diphenylditelluride at the hanging mercury drop electrode in a protic medium. J Colloid Interface Sci. Jul 15;287(2):592-6, 2005.

Newhouse, J.P. et al. Federal agency roles in cancer drug development from preclinical research to new drug approval. Washington: National Academic Press, 2005.

Nogales E, Grigorieff N. Molecular Machines: putting the pieces together. J Cell Biol. Jan 8;152(1):F1-10, 2001.

Nogueira CW, Borges VC, Zeni G, Rocha JB. Organochalcogens effects on delta-aminolevulinate dehydratase activity from human erythrocytic cells in vitro, Toxicology, vol. 191 (pg. 169-178), 2003.

Nogueira CW, Zen G, Rocha JB. Organoselenium and organotellurium compounds: toxicology and pharmacology, Chem. Rev. vol. 104 (pg. 6255-6285), 2004.

Normanno N, Tejpar S, Morgillo F, De Luca A, Van Cutsem E, Ciardiello F. Implications for KRAS status and EGFR-targeted therapies in metastatic CRC. Nat Rev Clin Oncol. Sep;6(9):519-27, 2009.

Ogra Y. Toxicometallomics for research on the toxicology of exotic metalloids based on speciation studies. Analytical Sciences. 25(10):1189–1195, 2009.

Ouaret D, Larsen AK. Protein kinase C β inhibition by enzastaurin leads to mitotic missegregation and preferential cytotoxicity toward colorectal cancer cells with chromosomal instability (CIN). Cell Cycle. 13:2697-706, 2014.

Panupinthu N, Yu S, Zhang D, Zhang F, Gagea M, Lu Y, et al. Self-reinforcing loop of amphiregulin and Y-box binding protein-1 contributes to poor outcomes in ovarian cancer. Oncogene. 33:2846-56, 2014.

Pencreach E, Guérin E, Nicolet C, Lelong-Rebel I, Voegeli AC, Oudet P, Larsen AK, Gaub MP, Guenot D. Marked activity of irinotecan and rapamycin combination toward colon cancer cells in vivo and in vitro is mediated through cooperative modulation of the mammalian target of rapamycin/hypoxia-inducible factor-1alpha axis. Clin Cancer Res. 15:1297-307, 2009.

Penna, L.S., Henriques, J.A.P., Bonatto, D. Anti-mitotic agentes: Are they emerging molecules for câncer treatment. Pharmacol Ther. 173:67-82. 2017.

Petragnani N, Stefani HA. Advances in organic tellurium chemistry. Tetrahedron. 61(7):1613–1679, 2005.

Poindessous V, Ouaret D, El Ouadrani K, Battistella A, Mégalophonos VF, Kamsu-Kom N, et al. EGFR- and VEGF(R)-targeted small molecules show synergistic activity in colorectal cancer models refractory to combinations of monoclonal antibodies. Clin Cancer Res. 17:6522-30, 2011.

Pommier Y, Sun Y, Huang SN, Nitiss JL. Roles of eukaryotic topoisomerases in transcription, replication and genomic stability. Nat Rev Mol Cell Biol 17(11):703–721, 2016.

Pommier, Y., Leo, E., Zhang, H.L., Marchand, C. DNA Topoisomerase and their poisoning by anticancer and antibacterial drugs. Chem Biol 28;17(5):421-33, 2010.

Prigol, M., Nogueira, C. W., Zeni, G., Bronze, M. R. and Constantino, L. In vitro metabolism of diphenyl diselenide in rat liver fractions. Conjugation with GSH and binding to thiol groups. Chem. Biol. Interact., 200, 65–72, 2012.

Princival JL, Dos Santos AA, Comasseto JV. Reactive organometallics from organotellurides: application in organic synthesis. Journal of the Brazilian Chemical Society. 21(11):2042–2054, 2010.

Procházková, D., Bousová, I., Wilhelmová, N. Antioxidant and prooxidant properties of flavonoids. Fitoterapia. 82(4):513-23. 2011.

Puntel RL, Roos DH, Seeger RL, Rocha JB. Mitochondrial electron transfer chain complexes inhibition by different organochalcogens. Toxicol In Vitro 27:59-70, 2013.

Rajput A, Koterba AP, Kreisberg JI, Foster JM, Willson JK, Brattain MG. A novel mechanism of resistance to epidermal growth factor receptor antagonism in vivo. Cancer Res. 67:665-73, 2007.

Raza MH, Siraj S, Arshad A, Waheed U4, Aldakheel F, Alduraywish S, Arshad M7. ROS-modulated therapeutic approaches in cancer treatment. J Cancer Res Clin Oncol. Jun 24, 2017.

Redondo-Blanco S, Fernández J, Gutiérrez-del-Río I, Villar CJ and Lombó F. New Insights toward Colorectal Cancer Chemotherapy Using Natural Bioactive Compounds. Front. Pharmacol. 8:109, 2017.

Reichert JM, Wenger JB. Development trends for new cancer therapeutics and vaccines. Drug Discov Today. Jan;13(1-2):30-7, 2008.

Rigobello, M. P., Folda, A., Citta, A. et al. Interaction of selenite and tellurite with thiol-dependent redox enzymes: Kinetics and mitochon-drial implications. Free Radic. Biol. Med., 50, 1620–1629, 2011.

Rosa, R. M., Moura, D. J., Romano E Silva,A. C., Saffi, J. and Pêgas Hen-riques, J. A. Antioxidant activity of diphenyl diselenide prevents the genotoxicity of several mutagens in Chinese hamster V79 cells. Mutat. Res., 631, 44–54, 2007.

Rossato JI, Ketzer LA, Centurião FB, Silva SJ, Lüdtke DS, Zeni G, Braga AL, Rubin MA, Rocha JB. Antioxidant properties of new chalcogenides against lipid peroxidation in rat brain. Neurochem Res. Apr;27(4):297-303, 2002.

Roy S, Hardej D. Tellurium tetrachloride and diphenyl ditelluride cause cytotoxicity in rat hippocampal astrocytes. Food Chem Toxicol 49:2564-74, 2011.

Saffi J, Agnoletto MH, Guecheva TN, Batista LF, Carvalho H, Henriques JA, Stary A, Menck CF, Sarasin A. Effect of the anti-neoplastic drug doxorubicin on XPD-mutated DNA repair-deficient human cells. DNA Repair (Amst). Jan 2;9(1):40-7, 2010.

Sailer BL, Liles N, Dickerson S, Chasteen TG. Cytometric determination of novel organotellurium compound toxicity in a promyelocytic (HL-60) cell line, Arch. Toxicol. vol. 77 (pg. 30-36), 2003.

Salazar R, Capellà G, Tabernero J. Paracrine network: another step in the complexity of resistance to EGFR blockade? Clin Cancer Res. 20:6227-9, 2014.

Salerno, S., Da Settimo, F., Taliani, S., et al., Recent advancesin the deveolpment of dual topoisomerase I and II inhibitors as anticâncer drugs. Curr Med Chem 17(35):4270-90, 2010.

Sarma BK, Mugesh G. Antioxidant activity of the anti-inflammatory compound ebselen: a reversible cyclization pathway via selenenic and seleninic acid intermediates. Chemistry. 14:10603–10614, 2008.

Sausen de Freitas A, de Souza Prestes A, Wagner C, Haigert Sudati J, Alves D, Oliveira Porciuncula L, Kade IJ, Teixeira Rocha JB. Reduction of diphenyl diselenide and analogs by mammalian thioredoxin reductase is independent of their gluthathione peroxidase-like activity: a possible novel pathway for their antioxidant activity. Molecules. 15:7699–7714, 2010.

Sekhon BS. Metalloid compounds as drugs. Res Pharm Sci. Jul;8(3):145-58, 2013.

Shen J, Xia W, Khotskaya YB, Huo L, Nakanishi K, Lim SO, et al. EGFR modulates microRNA maturation in response to hypoxia through phosphorylation of AGO2. Nature. 497:383-7, 2013.

Shepherd FA, Rodrigues Pereira J, Ciuleanu T, Tan EH, Hirsh V, Thongprasert S, et al. National Cancer Institute of Canada Clinical Trials Group. Erlotinib in previously treated non-small-cell lung cancer. N Engl J Med. 353:123-32, 2005.

Silvestris, N., Maiello, E., de Vita, F., et al., Update on capecitabine alone and in combination regimens in colorectal câncer patients. Cancer Treat Rev 36(S3): S46-S55, 2010.

Singh A, Settleman J. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. Oncogene. Aug 26;29(34):4741-51, 2010.

Snyder RD, Arnone MR. Putative identification of functional interactions between DNA intercalating agents and topoisomerase II using the V79 in vitro micronucleus assay. Mutat Res 503:21-35, 2002.

Soares DG, Escargueil AE, Poindessous V, Sarasin A, de Gramont A, Bonatto D, Henriques JA, Larsen AK. Replication and homologous recombination repair regulate DNA double-strand break formation by the antitumor alkylator ecteinascidin 743. Proc Natl Acad Sci U S A. Aug 7;104(32):13062-7, 2007.

Spangler JB, Neil JR, Abramovitch S, Yarden Y, White FM, Lauffenburger DA, Wittrup KD. Combination antibody treatment down-regulates epidermal growth factor receptor by inhibiting endosomal recycling. Proc Natl Acad Sci USA. 107:13252-7, 2010.

Sredni B. Immunomodulating tellurium compounds as anti-cancer agents. Semin Cancer Biol. Feb;22(1):60-9, 2012.

Stangherlin EC, Ardais AP, Rocha JBT, Nogueira CW. Exposure to diphenyl ditelluride, via maternal milk, causes oxidative stress in cerebral cortex, hippocampus and striatum of young rats. Archives of Toxicology. 83(5):485–491, 2009.

Swanton C. Intratumor heterogeneity: evolution through space and time. Cancer Res. Oct 1;72(19):4875-82, 2012.

Swift LH, Golsteyn RM. Genotoxic anti-cancer agents and their relationship to DNA damage, mitosis, and checkpoint adaptation in proliferating cancer cells. Int J Mol Sci. Feb 25;15(3):3403-31, 2014.

Tabernero J. The role of VEGF and EGFR inhibition: implications for combining anti-VEGF and anti-EGFR agents. Mol Cancer Res. 5:203-20, 2007.

Taylor A. Biochemistry of tellurium. Biological Trace Element Research. 55(3):231–239, 1996.

Tol J, Koopman M, Cats A, Rodenburg CJ, Creemers GJ, Schrama JG, et al. Chemotherapy, bevacizumab, and cetuximab in metastatic colorectal cancer. N Engl J Med. 360:563-72, 2009.

Trachootham D, Zhou Y, Zhang H, Demizu Y, et al., Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by beta-phenylethyl isothiocyanate. Cancer Cell. Sep;10(3):241-52, 2006.

Trindade C, Juchem AL, de Albuquerque NR, et al. Antigenotoxic and antimutagenic effects of diphenyl ditelluride against several known mutagens in Chinese hamster lung fibroblasts. Mutagenesis. Nov;30(6):799-809, 2015.

Troiani T, Martinelli E, Napolitano S, Vitagliano D, Ciuffreda LP, Costantino S, et al. Increased TGF-α as a mechanism of acquired resistance to the anti-EGFR inhibitor cetuximab through EGFR-MET interaction and activation of MET signaling in colon cancer cells. Clin Cancer Res. Dec 15;19(24):6751-65, 2013.

Tse-Dinh YC. Targeting bacterial topoisomerases: how to counter mechanisms of resistance. Future Med Chem 8(10):1085–1100, 2016.

Underhill, C., Toulmonde, M., Bonnefoi, H. A review of PARP inhibitors: from bench to bedside. Ann Oncol 22(2):268-79. 2011.

Urig S, Becker K. On the potential of thioredoxin reductase inhibitors for cancer therapy. Semin Cancer Biol. Dec;16(6):452-65, 2006.

Valent P, Gastl G, Geissler K, Greil R, Hantschel O, Lang A, Linkesch W, Lion T, Petzer AL, Pittermann E, Pleyer L, Thaler J, Wolf D. Nilotinib as frontline and second-line therapy in chronic myeloid leukemia: open questions. Crit Rev Oncol Hematol. Jun;82(3):370-7, 2012.

Verrax J, Beck R, Dejeans N, Glorieux C, et al., Redox-active quinones and ascorbate: an innovative cancer therapy that exploits the vulnerability of cancer cells to oxidative stress. Anticancer Agents Med Chem. Feb;11(2):213-21, 2011.

Vij, P., ED. Hardej. Evaluation of tellurium toxicity in transformed and non-transformed human colon cells. Environ Toxicol Pharmacol 34: 768-782, 2012.

Wang JC. Cellular roles of DNA topoisomerases: a molecular perspective. Nat Rev Mol Cell Biol 3(6):430–440, 2002.

Wang Y, Roche O, Yan MS, Finak G, Evans AJ, Metcalf JL, et al. Regulation of endocytosis via the oxygen-sensing pathway. Nat Med. 15:319-24, 2009.

Wee P, Wang Z. Epidermal Growth Factor Receptor Cell Proliferation Signaling Pathways. Cancers (Basel). May 17;9(5), 2017.

Wei Y, Zou Z, Becker N, Anderson M, Sumpter R, Xiao G, et al. EGFR-mediated Beclin 1 phosphorylation in autophagy suppression, tumor progression, and tumor chemoresistance. Cell. 154:1269-84, 2013.

Wild AT, Yamada Y. Treatment Options in Oligometastatic Disease: Stereotactic Body Radiation Therapy - Focus on Colorectal Cancer. Visc Med. Mar;33(1):54-61, 2017.

World Health Organization – WHO, disponível em: http://www-dep.iarc.fr/WHOdb/WHOdb.htm

Wu, N., X. W. Wu, K. Agama, Y. Pommier, J. Du et al. A novel DNA topoisomerase I inhibitor with different mechanism from camptothecin induces G2/M phase cell cycle arrest to K562 cells. Biochemistry 49: 10131-10136, 2010.

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Antimutagenic and antioxidant properties of the aqueous extracts of organic and conventional grapevine *Vitis labrusca* cv. Isabella leaves in V79 cells

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ABSTRACT

Grapes are one of the most commonly consumed fruit, in both fresh and processed forms; however, a significant amount is disposed of in the environment. Searching for a use of this waste, the antigenotoxic, antimutagenic, and antioxidant activities of aqueous extracts from organic and conventional *Vitis labrusca* leaves were determined using V79 cells as model. The antigenotoxic activity was analyzed by the alkaline comet assay using endonuclease III and formamidopyrimidine DNA glycosylase enzymes. The antimutagenic property was assessed through the micronucleus (MN) formation, and antioxidant activities were assessed using 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA) assay and 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical scavenging, as well as with superoxide dismutase (SOD) and catalase (CAT) activity assays. In addition, phenolic content and ascorbic acid levels of both extracts were determined. Data showed that both organic and conventional grapevine leaves extracts possessed antigenotoxic and antimutagenic properties. The extract of organic leaves significantly reduced intracellular reactive oxygen species (ROS) levels in V79 cells, and displayed greater ability for $DPPH^{\bullet}$ scavenging and higher SOD and CAT activities than extract from conventional leaves. Further, the extract from organic leaves contained higher phenolic and ascorbic acid concentrations. In summary, extracts from organic and conventional grape leaves induced important in vitro biological effects.

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Genomic instability may be induced by metabolic processes and exogenous factors such as diet, lifestyle, and environmental stress at several different cellular levels, ranging from DNA sequence changes to structural and numerical abnormalities at the chromosomal level. This instability plays a critical role in both cancer initiation and cancer progression. Evidence indicates that nutrient intake optimization plays a significant role in decreasing adverse impact on the genome. There is increasing evidence that chemoprophylactic agents may prevent cancer and other diseases. In recent years, an increasing number of biomarkers of genome integrity, including telomere length and mtDNA deletions, were utilized in establishing recommended daily intakes for nutrients (Ferguson et al., 2015; Neeha and Kinth, 2013). This strategy, referred to as chemoprevention, may be pursued by means of suitable pharmacological agents and/or by dietary factors (Ferguson et al., 2005). Plants and fruits are considered among the main sources of biologically active chemicals, and a wide number of new compounds that originated from natural resources were developed into commercial drugs (Beutler, 2009).

Grapes are one of the most commonly consumed fruits, in both fresh and processed forms. The best known and most cultivated vines in the world are *Vitis* genus, *V. labrusca* and *V. vinifera* species, whose fruits have been used for wine and juice production. Grapes are rich in phenolic compounds, including flavonoids such as catechin, epicatechin, quercetin, anthocyanins and procyanidins, and resveratrol (3,5,4'-trihydroxyl-stilbene) (Zhou and Raffoul, 2012). These phytochemicals

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possess antioxidant effects and free radical scavenging that protect the body against oxidative damage produced by reactive oxygen species (ROS) (Sato et al., 2001; Rockenbach et al., 2011; Keser et al., 2013; Santos et al, 2014). Therefore, polyphenols have been linked to reduction in the risk of pathological processes such as atherosclerosis, Alzheimer's and Parkinson's diseases, and several types of cancer (Firuzi et al., 2011; Hu, 2011).

Vitis vinifera leaves have been used to treat hypertension, diarrhea, hemorrhage, and varicosity (Oliboni et al., 2011). Some studies reported that grapevine leaves possess anti-inflammatory and analgesic properties (Kosar et al., 2007), as well as the ability to lower blood glucose levels in diabetics (Orhan et al., 2006). Few investigations assessed the biological effects of *V. labrusca* leaves. The ethanolic extracts of organic and conventional *V. labrusca* leaves (cv. Bordo) showed neuroprotective (Dani et al., 2010), hepatoprotective, renal protective, and cardioprotective effects (Oliboni et al., 2011). Pacifico et al. (2011) found antioxidant activity and antiproliferative properties of methanolic extract of *V. labrusca* cv. Isabella leaves in the human hepatoma cell line (HepG2) and human lung adenocarcinoma epithelial cell line (A549).

In this context, the aim of the present study was to examine and compare the antioxidant, antigenotoxic, and antimutagenic effects of aqueous extracts of organic and conventional cultivated *V. labrusca* cv. *Isabella* grapevine leaves in a lung fibroblast cell line derived from Chinese hamster lung fibroblast cells (V79 cells). The V79 cell line has high plating efficiency and short generation time, properties that make it useful for genotoxicity and mutagenicity testing (Krahn, 1983). In addition, the levels of vitamin C and polyphenols were determined in these aqueous extracts.

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin-ethylenediamine tetraacetic acid (EDTA), and antibiotics were purchased from Gibco BRL (Grand Island, NY). 2,2-Diphenyl-1-picrylhydrazyl (DPPH$^{\boxtimes}$), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide (MTT), hydrogen peroxide (H_2O_2), methyl methanesulfonate (MMS), and cytochalasin-B (cyt-B) were purchased from Sigma-Aldrich (St. Louis, MO). Low-melting-point agarose and normal agarose were obtained from Invitrogen (Carlsbad, CA). Formamidopyrimidine DNA glycosylase (FPG, 8,000 U/ml) and endonuclease (ENDO III, 10,000 U/ml) were purchased from New England BioLabs (Ipswich, MA). The high-performance liquid chromatography (HPLC) solvents were obtained from Mallinckrodt Baker, Inc. (Phillipsburg, NJ).

Plant Material and Preparation of Aqueous Extracts from Organic Grapevine leaf (OGL) and Conventional Grapevine Leaf (CGL)

Vitis labrusca was cultivated in the northeast region of the Serra Gaucha, Rio Grande do Sul, Brazil. Voucher specimens were identified and deposited in the herbarium of the University of Caxias do Sul, Rio Grande do Sul, Brazil (voucher HUCS 31066). The organic leaves received ECOCERT certification. The leaves were dried at 37ºC and sheltered from light. Extracts were obtained using 5 g leaves/100 ml distilled water under reflux (100°C) for 30 min. After cooling to 25°C, extracts were filtered and lyophilized (Edward freeze dryer) at 60°C and 10^{-1} bar. The extracts were stored and protected from humidity and light.

Determination of Total and Major Polyphenols

Total phenolic content of the grapevine leaf extracts were measured using the method of Singleton and Rossi (1965). Two-hundred-microliter extracts were assayed with 1000 µl Folin–Ciocalteau reagent and 800 µl sodium carbonate (7.5%, w/v). After 30 min, absorption was read at 765 nm and total phenolic content expressed as milligrams of gallic acid per milliliter.

Identification of the various compounds in organic grapevine leaf (OGL) and conventional grapevine leaf (CGL) samples of *V. labrusca* was performed by HPLC analysis utilizing an Agilent HP 1050 equipped with a MetaPhor column 150 × 4.6 mm, 5 µm ODS-3, and a diode array detector (DAD). Solvent A was water/glacial acetic acid (99:1 v/v), and solvent B was methanol. The solvents were delivered at a total flow rate of 1 ml /min. The gradient profile was as follows:

10% B isocratic for 5 min, 10% B to 30% B linearly in 5 min, then 30% B to 50% linearly in 10 min, 50% B to 70% linearly in 10 min, and finally 70% B to 90% linearly in 15 min and a return to 30% B linearly in 13 min. The injection volume was 20 µl, and chromatograms were read at 280, 258, and 355 nm. The ultraviolet (UV) spectra and retention times of each studied metabolite were used for identification purposes, comparing them with those of pure standards under equivalent conditions. The linearity of the standard curve was confirmed by plotting the peak areas (y, mAU*s) and their corresponding concentrations (x, mg/g). All solutions used in the HPLC analysis were filtered by a 0.45-µm polyamide membrane filter.

Ascorbic Acid Content

Ascorbic acid content of extracts was determined according to the method of Association of Official Analytical Chemists (Horwitz, 1990).

V79 Cell Culture and Treatments

The V79 cell line was obtained from the Cell Bank of Rio de Janeiro (BCRJ, Rio de Janeiro, RJ, Brazil). V79 cells were cultivated under standard conditions in DMEM supplemented with 10% heat-inactivated FBS, 100 IU/ml penicillin, and 100 IU/ml streptomycin. Cells were maintained in tissue-culture flasks at 37°C in a humidified atmosphere containing 5 % CO_2 and harvested by treatment with 0.15% trypsin–0.08% EDTA in phosphate-buffered saline solution (PBS). Cells (3×10^4) were seeded in 5 ml complete medium and grown to 60–70% confluence before treatment with extracts.

The lyophilized OGL and CGL extracts were added to FBS-free medium to achieve different selected concentrations and then added to cells that were incubated at 37°C for 3 h in a humidified atmosphere containing 5% CO_2. To evaluate the antioxidant and antigenotoxic potential of these extracts, cells were then washed with PBS. Oxidative challenge with H_2O_2 (150 µM) and genotoxic challenge with MMS (40 µM) were carried out for 30 min or 1 h, respectively, in the dark in FBS-free medium.

MTT Assay

The MTT assay was performed according to Denizot and Lang (1986). After treatment, cells were washed once with PBS before addition of serum-free medium containing yellow tetrazolium salt (MTT; 1 mg/ml). This process was followed by incubation for 3 h at 37°C. After incubation, supernatant was removed. The residual purple formazan product was solubilized in dimethyl sulfoxide (DMSO), stirred for 20 min, and then absorbance was read at 570 nm. The results were expressed as percent cell proliferation relative to control (cells without any test compounds).

Colony-Forming Ability (Clonal Survival)

Exponentially growing V79 cells were treated according to the experimental protocol of Rafehi et al. (2011). After treatment, cultures were trypsinized, and 200 cells were seeded for determination of their colony-forming ability. Cells were treated with OGL and CGL extracts (1 or 5 µg/ml) in FBS-free medium for 3 h at 37°C in a humidified atmosphere containing 5% CO_2, followed by exposure to 150 µM H_2O_2 for 30 min under similar conditions. Culture flasks were protected from direct light during treatment with OGL and CGL extracts and H_2O_2. After 5 d of incubation, the colonies were fixed with methanol and acetic acid (3:1), stained with crystal violet (0.1%), and counted. Survival was calculated as percent relative to survival of control cells.

Antigenotoxic Effects Evaluated by Alkaline Comet Assay

The alkaline comet assay was performed as described by Singh et al. (1988) with minor modifications of Trindade et al. (2015). After treatment, cells were washed with ice-cold PBS, trypsinized, and resuspended in a complete medium. Then, 20 µl cell suspension was dissolved in 0.75% low-melting-point agarose and spread onto a glass microscope slide that was precoated with a layer of 1.5% normal-melting-point agarose. Slides were incubated in ice-cold lysis solution at 4°C for at least 1 h to remove cell proteins, leaving DNA as "nucleoids." The alkaline comet assay was performed at pH 13. In the

modified comet assay, slides were removed from lysis solution, washed 3 times in enzyme buffer and incubated with 60 µl of FPG (45 min 37°C) or Endo III (30 min 37°C) solutions. After lysis, slides were placed on a horizontal electrophoresis unit, covered with fresh buffer for 15 min at 4°C to enable DNA unwinding and expression of alkali-labile sites. Electrophoresis was performed for 20 min at 25 V and 300 mA (0.90 V/cm). Slides were then neutralized (4 m*M* Tris, pH 7.5), washed in double-distilled water and stained using silver nitrate. One hundred cells (50 cells from each of the 2 replicate slides) were selected, and analyzed for each concentration of the test substance. Cells were visually scored according to tail length into five classes: (i) class 0: undamaged, without tail; (ii) class 1: with a tail shorter than diameter of the head (nucleus); (iii) class 2: with a tail length 1–2 × the diameter of the head; (iv) class 3: with a tail longer than 2 × diameter of the head; and (v) class 4: comets with no heads. A value damage index (DI) was assigned to each sample. DI ranged from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4). The formation of FPG- and Endo III-sensitive sites was calculated as a difference between the score obtained after incubation with the respective enzyme and with the enzyme buffer only.

Determination of Reactive Oxygen Species (ROS) Generation

The 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA) assay was used to estimate the intracellular generation of reactive oxygen species (ROS) (Bass et al., 1983). This assay is a reliable method for measuring intracellular ROS such as H_2O_2, hydroxyl radical ($OH^{\cdot}$), and hydroperoxides (ROOH). Intracellular peroxides oxidize DCFH-DA to a highly fluorescent compound (2',7'-dichlorofluorescein; DCF). After treatment with OGL and CGL extracts plus H_2O_2, V79 cells were washed and incubated with 10 µ*M* DCFH-DA for 30 min at 37°C in the dark. After incubation, cells were washed with PBS and analyzed within 30 min using FACSCalibur (Becton Dickinson, San Jose, CA). The DCFH oxidation was measured using 488 nm excitation and 530/30 nm band-pass emission filters. As a rule, 10,000 cells were counted in each experiment. Cell Quest software (Becton Dickinson) was used to calculate the mean fluorescence.

DPPH$^{\bullet}$ Scavenging, SOD- and CAT-Like Activities

The antioxidant activity of both extracts was measured by DPPH$^{\bullet}$ scavenging activity (Yamaguchi et al., 1998). The OGL and CGL extracts were added to a Tris-HCl buffer (100 m*M*, pH 7) and 250 µ*M* DPPH$^{\bullet}$ dissolved in ethanol. Samples were kept in the dark for 20 min, and absorbance was read at 517 nm (UV-1700 spectrophotometer, Shimadzu, Kyoto, Japan). The results are presented as IC_{50} (concentration of extract necessary to scavenge 50% of the DPPH$^{\bullet}$ radical). To evaluate the antioxidants' enzyme-like activities, extracts were prepared at a concentration of 1 µg/ml. The superoxide dismutase (SOD) activity was spectrophotometrically determined by measuring the inhibition of self-catalytic adrenochrome formation rate at 480 nm in a reaction medium containing 1 mmol/L adrenaline (pH 2) and 50 mmol/L glycine (pH 10.2). This reaction was performed at 30°C for 3 min. The results were expressed in units of SOD. The catalase (CAT) activity assay was performed according to the method described by Aebi (1984) by determining H_2O_2 decomposition rate at 240 nm. The results were expressed as micromoles H_2O_2 decomposed per minute. Ascorbic acid at 50 µg/ml was used as a positive control.

Cytokinesis Block Micronucleus (CBMN) Assay

The micronucleus (MN) assay was performed according to Thomas and Fenech (2011) with minor modifications. After treatment, cultures were washed twice with medium, and Cyt-B was added at a final concentration of 3 µg/ml. Cultures were harvested 21 h after Cyt-B addition. Cells were trypsinized and the cellular suspension was centrifuged at 800 × g for 5 min. Cells were then resuspended in 75 m*M* KCl solution and maintained at 4°C for 3 min (mild hypotonic treatment). Subsequently, cells were centrifuged and a methanol/acetic acid (3:1) solution was added. This fixation step was repeated twice. Cells were then resuspended in a small volume of methanol/acetic acid and placed dropwise onto clean slides.

The slides were stained with 3% Giemsa (pH 6.8) for 3–4 min. Micronuclei were counted in 2,000 binucleated cells (BNC) with well-preserved cytoplasm.

Statistical Analysis

All measurements were performed at least in triplicate and values reported as mean with standard deviation (SD). Data were subjected to Student's *t*-test or an analysis of variance (one-way, ANOVA), followed by Tukey's or Dunnett's test using the SPSS version 17.0 (SPSS, Chicago, IL) software package. In all comparisons, $p < .05$ was considered as indicating statistical significance.

Results

Total Contents of Phenolic Compounds, Ascorbic Acid, and Major Flavonoids in Aqueous Grapevine Leaf Extracts

Table 1 presents total phenolic content of aqueous grapevine leaf extracts. Polyphenolic compounds were 100% higher in OGL than CGL extract, whereas ascorbic acid levels in OGL were 40% greater than in OCL. HPLC-DAD analysis enabled identification in both extracts of quercetin 3-*O*-glucoside and rutin (querecitin 3-*O*-rhamnoglucopyranose). Similarly, higher concentrations of flavonoids were detected in OGL. Quercetin 3-*O*-glucoside concentration was greater in OGL extract (54.66 ± 0.01 mg/g) than in CGL (34.11 ± 0.05 mg/g). OGL extract contained 5.41 ± 0.05 mg/g rutin, while CGL extract possessed 3.27 ± 0.04 mg/g (Figure 1).

Table 1. Total polyphenol and ascorbic acid contents in grapevine leaf extracts.

Grape leaf extract	Polyphenol content (mg/g)	Ascorbic acid (μg/g)
Organic	56.05 ± 1.09*	9.7 ± 0.35*
Conventional	28.93 ± 0.88	6.5 ± 0.34

Note.
Data expressed as mean ± SD of three independent experiments. Significantly different in relation to the CGL extract, *$p < .05$/one-way ANOVA, Student's test.

Cytotoxic and Protective Effects of Grapevine Leaf Extracts in V79 Cells

None of the grapevine leaf extracts exerted a cytotoxic effect in V79 cells within the concentration range of 0.1–10 μg/ml (data not shown). The ability of OGL and CGL extracts to reduce H_2O_2-mediated toxicity was analyzed by MTT and clonal survival assays. The results of the MTT assay indicated that treatment with OGL and CGL extracts (at 0.5, 1, or 5 μg/mL) exerted a protective effect against toxicity induced by H_2O_2 (Figure 2). In addition, our findings demonstrated that exposure of cells to H_2O_2 resulted in significant decrease in clonal survival (50%) compared to negative control, whereas incubation with either OGL or CGL inhibited H_2O_2 induced toxicity (Figure 3).

Genotoxic and Antigenotoxic Effects of Grapevine Leaf Extracts by Comet Assay

Incubation with OGL and CGL extracts did not produce a significant amount of DNA strand breaks in V79 cells compared to the negative control (Table 2). Exposure of cells to H_2O_2 resulted in a significant increase in level of DNA damage, whereas treatment with OGL and CGL extracts at 1 or 5 μg/ml reduced DNA damage induced by H_2O_2. Employing a modified comet assay with ENDO III and FPG repair proteins, data demonstrated that OGL and CGL extracts did not induce oxidative damage (Table 2). The OGL and CGL extracts provided more effective protection against H_2O_2-mediated genotoxicity at 5 μg/ml, diminishing FPG- and Endo III-sensitive sites induced by the oxidative agent.

Antimutagenic Potential of Grapevine Leaf Extracts in Micronucleus (MN) Test

The OGL and CGL extracts used in this study were able to protect cells against H_2O_2-mediated genotoxicity. In parallel, the protective effects of the extracts against the mutagenic effect of H_2O_2 were assessed in the MN assay. As observed in Figure 4A, the number of BNC was similar in cultures treated with 5 μg/ml OGL extract alone, indicating no apparent toxicity in V79 cells. There was no marked change in MN frequency after treatment with both grapevine leaf

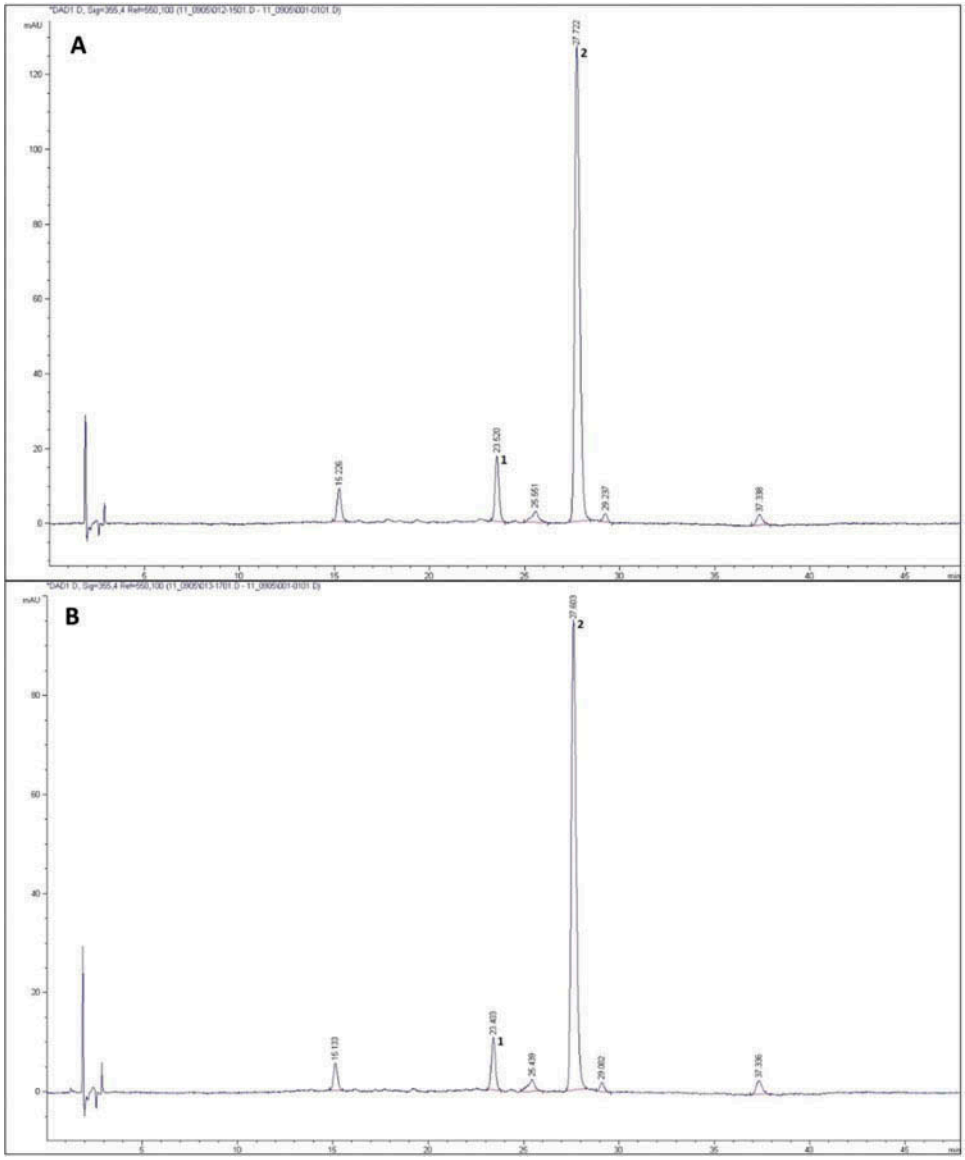

Figure 1. HPLC analysis of the water extracts of (A) organic and (B) conventional grapevine leaves with responses at 350 nm; 1, rutin; 2, quercetin 3-*O*-glucoside.

extracts in relation to MMS treatment (Figure 4B). Incubation with a 5-µg/ml concentration of OGL extract protected cells against MMS-induced MN formation, suggesting antimutagenic activity against a typical monofunctional alkylating agent.

Antioxidant Activities of Grapevine Leaf Extracts

The OGL extract presented a higher antioxidant activity (IC_{50}: 32.85 ± 0.01 mg%) than the CGL extract (83.8 ± 0.01 mg%) (Table 3). OGL extract showed the same scavenging activity as ascorbic acid. In addition, both extracts exhibited SOD and CAT activities that were ninefold and twofold higher, respectively, in OGL compared to CGL extract (Table 3).

Protective Action of OGL and CGL Extracts on ROS Generation

The results of the DCF assay indicated that treatment with OGL and CGL extracts (at 0.5, 1, or 5 µg/ml) decreased intracellular levels of ROS induced by H_2O_2. The relative DCF fluorescence intensities measured after exposure illustrated that at all three concentrations of OGL and CGL extracts reduced intracellular ROS in V79 cells, showing a protective effect against H_2O_2 (Figure 5).

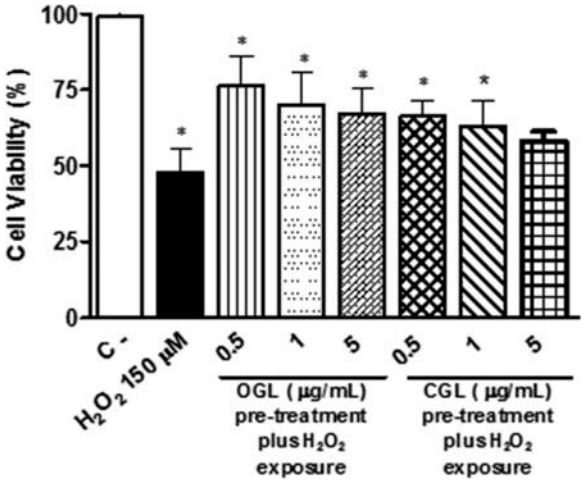

Figure 2. Viability of V79 cells treated for 3 h with organic grapevine leaf (OGL) and conventional grapevine leaf (CGL) extracts at the indicated concentrations in FBS-free medium and subsequently submitted to H_2O_2 (150 µ*M*) for 30 min in MTT assay. The results are expressed as the mean ± SD values, $n = 3$. One-way ANOVA, Dunnett's test. Positive control was compared to the solvent control. Asterisk indicates data are significantly different from positive control at $p < .05$.

Discussion

In this study, determination of polyphenol and ascorbic acid contents in the aqueous extract of grapevine leaves showed high levels of phenolic phytochemicals and vitamin C. Further, OGL extract contained higher levels of polyphenols and ascorbic acid than CGL (Table 1). These data

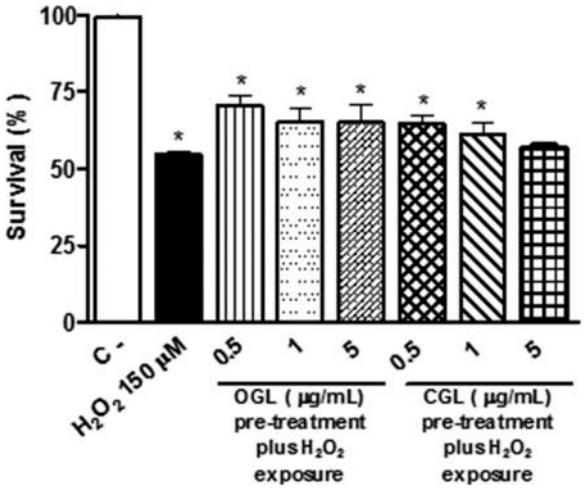

Figure 3. Clonogenic survival of V79 cells treated for 3 h with OGL and CGL at indicated concentrations in FBS-free medium and subsequently submitted to H_2O_2 (150 µ*M*) for 30 min. One-way ANOVA, Dunnett's test. Positive control was compared to the solvent control. Asterisk indicates data are significantly different from positive control at $p < .05$.

are in agreement with those reported for other *Vitis* species, whose phenols are mainly distributed in the leaf (Pacifico et al., 2011; Pastrana-Bonilla et al., 2003). The major phenolic compounds in the grapevine leaf extracts were quercetin 3-*O*-glucoside and rutin. The highest concentration of these components was found in the leaf extracts from organically managed grapevines. These observations might be attributed to (1) the effects of organic cultivation where pesticides are not used, (2) that plants are more exposed to the actions of phytopathogens, and (3) that this challenge stimulates plants to generate higher amounts of polyphenols as a self-defense mechanism (Dani et al., 2007; Soleas et al., 1997). However, in addition to the absence of fertilizers and/or fungicides, other factors, including different humidity and temperature conditions (Xia et al., 2010), as well as harvest season and age of the plants, are all known to play a key role in biosynthesis of phenolic compounds (Juroszek et al., 2009). Polyphenols and vitamins are important bioactive compounds present in *Vitis* leaves that were reported as primary contributors to protect biological systems in various ways (Dani et al., 2010; Edenharder et al., 2002; Pacifico et al., 2011). As phenolic compounds were noted to exert antioxidant effects (Xia et al. 2010), the involvement of these chemicals in the actions of OGL and CGL in providing protection against oxidant-mediated damage on V79 cells may be inferred. Polyphenols such as quercetin, rutin, and its glycosides were reported to be scavengers of the superoxide radical, H_2O_2, and methylglyoxal (Farrar et al., 2007; Xia et al., 2010; Zhou and Raffoul, 2012).

The present study demonstrated that treatment with OGL and OCL extract significantly increased cell viability as evidenced by MTT and clonal survival following H_2O_2-induced oxidative damage. This protective effect is in accordance with previous results described by Xia et al. (2010), who found that low concentrations of polyphenols provide significant protection to cells. Many bioflavonoids displayed antioxidant activity at low concentrations and pro-oxidant activity at high concentrations (Galati et al., 2002; Lambert and Elias, 2010). Dani et al. (2007) suggested that the antioxidant capacity of phenolics

Table 2. Evaluation of antigenotoxicity of organic grapevine leaf (OGL) and conventional grapevine leaf (CGL) extracts against H_2O_2-induced damage in V79 cells using a standard and modified comet assay.

Treatment	Damage index (DI)	Δ FPG (DI)	Δ ENDO III (DI)
NC	8.45 ± 0.89	7.15 ± 2.90	6.85 ± 0.99
H_2O_2 150 μ*M*	170.75 ± 7.01	54.88 ± 5.38	53.00 ± 5.18
OGL 1 μg/ml	7.85 ± 1.73	3.75 ± 0.63	2.25 ± 0.63
OGL 5 μg/ml	10.00 ± 0.76	3.25 ± 0.82	1.25 ± 0.63
CGL 1 μg/ml	10.44 ± 1.18	2.50 ± 0.38	2.63 ± 0.31
CGL 5 μg/ml	10.50 ± 0.38	3.00 ± 0.53	2.31 ± 0.62
Pretreatment OGL 1 μg/ml plus H_2O_2 150 μ*M*	99.13 ± 9.23*	18.00 ± 1.51*	22.39 ± 3.73*
Pretreatment OGL 5 μg/ml plus H_2O_2 150 μ*M*	79.50 ± 8.73*	14.40 ± 1.66*	20.33 ± 7.04*
Pretreatment CGL 1 μg/ml plus H_2O_2 150 μ*M*	139.38 ± 9.55*	43.18 ± 3.14 *	39.15 ± 4.52*
Pretreatment CGL 5 μg/ml plus H_2O_2 150 μ*M*	125.25 ± 13.31*	40.50 ± 5.35 *	34.13 ± 5.44*

Note. Data are presented as mean ± SD ($n = 4$). Treatments with extracts were compared to the negative control. Treatments with OGL or CGL extracts plus H_2O_2 150 μ*M* were compared to the cells only exposed to H_2O_2 150 μ*M*. Δ (delta) indicates the levels of FPG- and ENDO III-sensitive sites calculated as the score obtained after incubation with enzymes (FPG or ENDO III) minus the score obtained without enzyme (only with the enzyme buffer).
*Significantly different at $p < 0.05$ as tested by one-way ANOVA (Tukey test).

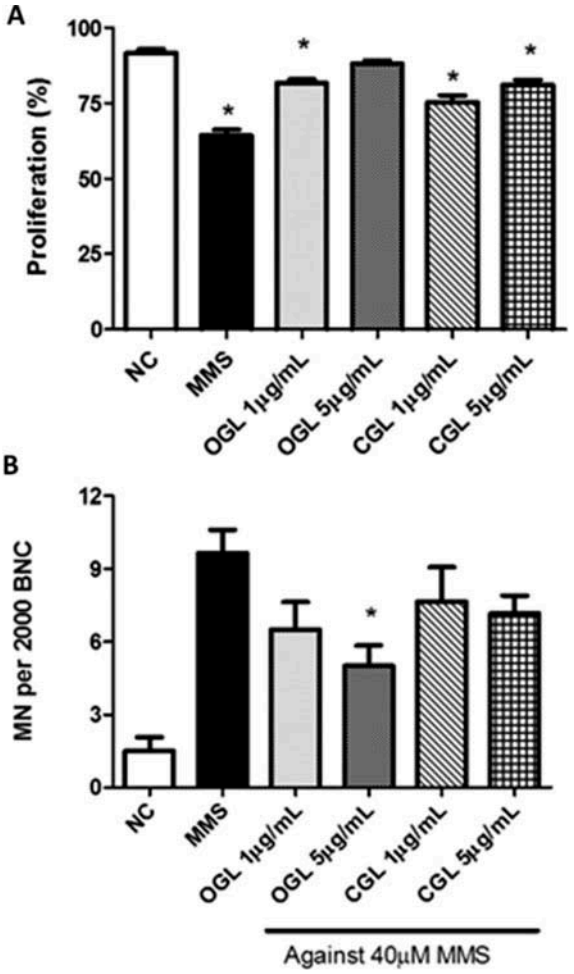

Figure 4. (A) Effect of OGL and CGL extracts treatment on cell proliferation, measured as percent BNC in MN assay. (B) Antimutagenic activity of V79 cells treated with OGL or CGL for 3 h in FBS-free medium and challenged with MMS (40 μ*M*) for 1 h. Results are expressed as the mean ± SD ($n = 4$). OGL or CGL was compared to the cells only exposed to a mutagen. MMS treatment was compared to the negative control. Asterisk indicates data are significantly different as tested by one-way ANOVA (Tukey test) at $p < .05$.

may have a concentration saturation limit, and above this limit, activity might not rise further as concentration is elevated. For some phenolic compounds, 50% and 25% (v/v) concentrations demonstrated equivalent antioxidant activities, with both being higher than that of the 10% (v/v) concentration. Several studies indicated that the phenolic compounds were the chemical structure (s) mainly responsible for the antioxidant activities of grape extracts (Dani et al., 2010; Keser et al., 2013; Kosar et al., 2007). However, the relationship between phenolic compounds and antioxidant capacity is not consistent among findings from different investigations, which noted that in addition to concentration, the antioxidant capacities of phenolic compounds were affected by other factors (Farrar et al., 2007; Ramos et al., 2013).

Our results from the alkaline comet assay showed that both OGL and CGL extracts prevented occurrence of DNA damage at 1 and 5 μg/ml concentrations. Reinforcing these results, treatment with both aqueous extracts led to decreased oxidative damage by reducing the number of FPG- and ENDO III-sensitive sites (Table 2). This effect may be attributed to reduction in formation of oxidized DNA bases. The OGL extract demonstrated a more effective protective effect to purine and pyrimidine base oxidation than did the CGL. In addition, using the DCF assay both OGL and CGL extracts were able to lower ROS produced by H_2O_2, confirming this scavenger effect. It is important to note that these results are consistent with previous studies

Table 3. In vitro antioxidant activity of grapevine extracts.

Sample	DPPH$^{\bullet}$ (IC50)[A]	SOD-like activity (U SOD)[B]	CAT-like activity (μmol decomposed H_2O_2/min)
Organic grape leaf extract	32.85 ± 0.001[a*]	5.98 ± 0.12[b]	30.00 ± 5.30[b]
Conventional grape leaf extract	83.80 ± 0.001[b]	0.66 ± 0.01[a]	13.13 ± 2.65[a]
Ascorbic acid	26.5 ± 0.001[a]	27.76 ± 0.33[c]	15.00 ± 2.65[a]

Note. Data represent mean ± SD values of three independent experiments.
[A]IC_{50} value (mg% of samples needed to scavenge 50% of DPPH[•]).
[B]U SOD-like value (U SOD).
[a,b,c]Different letters indicate a significant difference according to the analysis of variance and Tukey's post hoc test ($p \leq .05$).

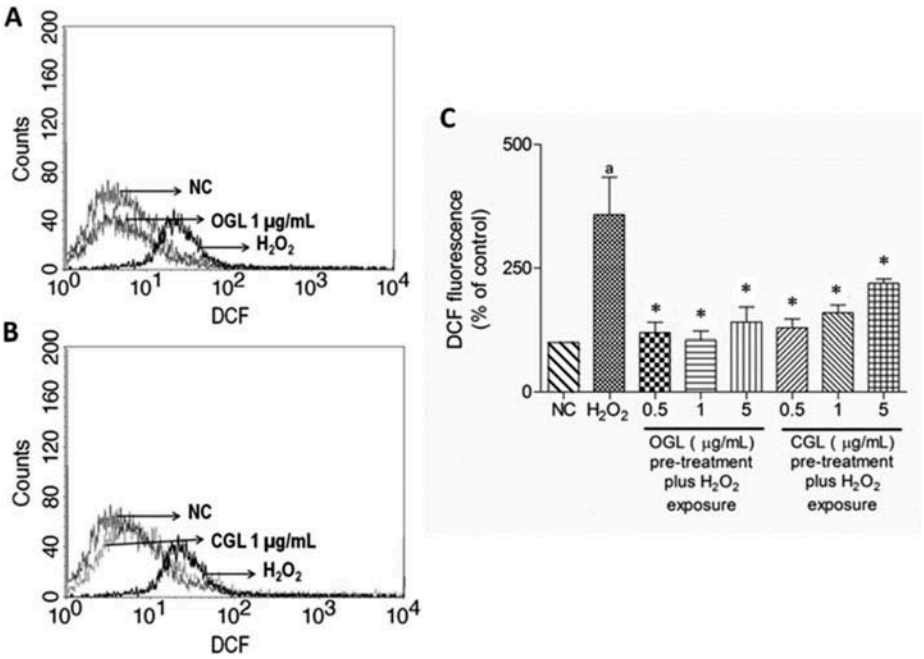

Figure 5. Effects of OGL and CGL extracts on the ROS production in V79 cells. (A) Histogram showing OGL results. (B) Histogram illustrating CGL results. (C) DCF fluorescence expressed as percent negative control values. Data represent mean and standard deviation of three independent experiments. ANOVA followed by Dunnett's test. Asterisk indicates data are significantly different from positive control at *$p < .05$.

demonstrating that polyphenols such as quercetin and rutin decreased the amount of DNA damage associated with oxidative stress (Araujo et al., 2011; García-Rodríguez et al., 2014; Min and Ebeler, 2009), and displayed strong radical-scavenging activities and antioxidant effects in several cell lines (Araujo et al., 2011; Cho et al., 2011; Min and Ebeler, 2009; Ramos et al., 2013; Santos et al., 2014; Watjen et al., 2005). It was suggested that quercetin and rutin may act as an antioxidant in cells by modulation of gene expression (Bouhlel et al., 2008; Granado-Serrano et al., 2010).

Celik and Arinc (2010) noted that quercetin at low concentrations (below 100 μ*M*) was able to prevent free-radical-mediated DNA damage produced by idarubicin and mitomycin C in the presence of P-450 reductase and cofactor NADPH using a cell-free agarose gel method. Khan et al. (2012a) found that treatment with rutin, one of the polyphenols presented in grapevine extracts, restored DNA fragmentation and 8-oxo-dG damage, as well as decreasing the p53 and CYP 2E1 expression induced by carbon tetrachloride (CCl_4) in rats. Further, the flavonoid rutin (1) decreased levels of ROS, nitric oxide (NO), glutathione disulfide (GSSG), and malondialdehyde (MDA), (2) lowered activity of inducible nitric oxide synthase (iNOS), (3) attenuated mitochondrial damage, (4) increased glutathione (GSH)/GSSG ratio, (5) enhanced activities of SOD, CAT, and glutathione peroxidase (GPx) (Khan et al., 2012a, 2012b; Wang

et al., 2012), and (6) modulated production of proinflammatory cytokines by decreasing tumor necrosis factor (TNF)-α and interleukin (IL)-1β generation (Wang et al., 2012).

This study showed the antimutagenic activity of *V. labrusca* cv. Isabella extracts against MMS, a commonly used alkylating mutagen. Incubation with OGL at a concentration of 5 μg/mL demonstrated potent antimutagenic activity against MMS-induced damage. The increase in cell proliferation (BNC) and decrease in MN frequency, when compared to MMS alone, indicate elevated repair activity. The protective effect of *V. labrusca* cv. Isabella might be attributed to its polyphenol content. Polyphenols are known to quench free radicals or modulate transcription factors with chemopreventive activity (such as Nrf2) by attaching to specific sequences of DNA, thus modulating expression of numerous antioxidant and anti-inflammatory genes and enzymes that inhibit effects of carcinogens (Niture et al., 2007). In addition, polyphenols enhance the activity of O^6-methylguanine-DNA methyltransferase (MGMT), which repairs alkylated DNA bases by removing the methyl group from O^6-methylguanine (Abarikwu et al., 2012).

In previous studies, antimutagenic activity against alkylating agents was positively associated with the presence of vitamin C, which competes with DNA as a target for alkylation, thereby reducing cellular alkylation by MMS (Franke et al., 2005; Melo-Cavalcante et al., 2011). In addition, Franke et al. (2005) noted that ascorbic acid takes part in the activation of genes involved in DNA repair, modulating the level of DNA damage in cells exposed to ROS in vivo and in vitro. It was suggested that vitamin C diminished endogenous oxidative damage, as evidenced by oxidized pyrimidines and strand breaks in lymphocyte DNA (Franke et al., 2005).

To verify the possible antioxidant mechanism of the protective effect of grapevine cv. Isabella, its $DPPH^{\bullet}$ scavenging ability was evaluated. The aqueous extracts of OGL demonstrated a stronger radical scavenging activity (IC_{50} 32.85 mg/ml) than for CGL extract (IC_{50} 83.8 mg/ml). In addition, extracts of OGL presented the highest SOD and CAT activities.

Our study demonstrated the antioxidant and antigenotoxic activity of grapevine cv. Isabella leaves extracts, more pronounced with organic cultivation, which suggested a possible use as a food supplement. Grapevine leaves are postulated to be a great potential source of polyphenols, vitamins, and possibly other compounds to be applied as functional food ingredients, particularly in relation to prevention of cardiovascular diseases (Scalbert et al., 2005). However, more studies are necessary to assess the possible changes in the antioxidant capacity of the ingredients encapsulated and stored during their commercial life on the market.

Conclusions

In summary, treatment with OGL or OCL significantly prevented genotoxic damage induced by H_2O_2 or MMS in V79 cells. Our data also suggest that the antigenotoxic and antimutagenic effects might be attributed to the synergistic action of complex mixture of polyphenols, vitamins and possibly other compounds in the extracts. These data may be of significant value for the food industry, dedicated to the formulation of dietary supplements containing *V. labrusca* cv. Isabella ingredients.

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Conflict of Interest

The authors declare that there is not any conflict of interest.

References

Abarikwu, S. O., Otuechere, C. A., Ekor, M., Monwuba, K., and Osobu, D. 2012. Rutin ameliorates cyclophosphamide-induced reproductive toxicity in male rats. *Toxicol. Int.* 19: 207–214.

Aebi, H. 1984. Catalase in vitro. *Methods Enzymol.* 105: 121–126.

Araujo, J. R., Goncalves, P., and Martel, F. 2011. Chemopreventive effect of dietary polyphenols in colorectal cancer cell lines. *Nutr. Res*. 31: 77–87.

Bass, D. A., Parce, J. W., Dechatelet, L. R., Szejda, P., Seeds, M. C., and Thomas, M. 1983. Flow cytometric studies of oxidative product formation by neutrophils: A graded response to membrane stimulation. *J. Immunol*. 130: 1910–1917.

Beutler, J. A. 2009. Natural products as a foundation for drug discovery. *Curr. Protocols Pharmacol*. 46: 9-11-11–19-11-21.

Bouhlel, I., Kilani, S., Skandrani, I., Ben Amar, R., Nefatti, A., Laporte, F., Hininger-Favier, I., Ghedira, K., and Chekir-Ghedira, L. 2008. *Acacia salicina* extracts protect against DNA damage and mutagenesis in bacteria and human lymphoblast cell K562 cultures. *Nutr. Res*. 28: 190–197.

Celik, H., and Arinc, E. 2010. Evaluation of the protective effects of quercetin, rutin, naringenin, resveratrol and trolox against idarubicin-induced DNA damage. *J. Pharm. Pharm. Sci*. 13: 231–241.

Cho, J. H., Park, S. Y., Lee, H. S., Whang, W. K., and Sohn, U. D. 2011. The protective effect of quercetin-3-*O*-beta-D-glucuronopyranoside on ethanol-induced damage in cultured feline esophageal epithelial cells. *Korean J. Physiol. Pharmacol*. 15: 319–326.

Dani, C., Oliboni, L. S., Agostini, F., Funchal, C., Serafini, L., Henriques, J. A., and Salvador, M. 2010. Phenolic content of grapevine leaves (*Vitis labrusca* var. Bordo) and its neuroprotective effect against peroxide damage. *Toxicol. In Vitro* 24: 148–153.

Dani, C., Oliboni, L. S., Vanderlinde, R., Bonatto, D., Salvador, M., and Henriques, J. A. 2007. Phenolic content and antioxidant activities of white and purple juices manufactured with organically- or conventionally-produced grapes. *Food Chem. Toxicol*. 45: 2574–2580.

Denizot, F., and Lang, R. 1986. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* 89: 271–277.

Edenharder, R., Sager, J. W., Glatt, H., Muckel, E., and Platt, K. L. 2002. Protection by beverages, fruits, vegetables, herbs, and flavonoids against genotoxicity of 2-acetylaminofluorene and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in metabolically competent V79 cells. *Mutat. Res*. 521: 57–72.

Farrar, J. L., Hartle, D. K., Hargrove, J. L., and Greenspan, P. 2007. Inhibition of protein glycation by skins and seeds of the muscadine grape. *Biofactors* 30: 193–200.

Ferguson, LR., Bronzetti, G., and de Flora, S. 2005. Mechanistic approaches to chemoprevention of mutation and cancer. *Mutat. Res*. 591: 3–7.

Ferguson, L. R., Chen, H., Collins, A.R., Connell, M., Damia, G., Dasgupta, S., Malhotra, M., Meeker, A. K., Amedei, A., Amin, A., Ashraf, S. S., Aquilano, K., Azmi, A. S., Bhakta, D., Bilsland, A., Boosani, C. S., Chen, S., Ciriolo, M. R., Fujii, H., Guha, G., Halicka, D., Helferich, W. G., Keith, W. N., Mohammed, S. I., Niccolai, E., Yang, X., Honoki, K., Parslow, V. R., Prakash, S., Rezazadeh, S., Shackelford, R. E., Sidransky, D., Tran, P. T., Yang, E. S., and Maxwell, C. A. 2015. Genomic instability in human cancer: Molecular insights and opportunities for therapeutic attack and prevention through diet and nutrition. *Semin. Cancer Biol*. 35(Suppl.): S5–S24.

Firuzi, O., Miri, R., Tavakkoli, M., and Saso, L. 2011. Antioxidant therapy: Current status and future prospects. *Curr. Med. Chem*. 18: 3871–3888.

Franke, S. I., Pra, D., Erdtmann, B., Henriques, J. A., and da Silva, J. 2005. Influence of orange juice over the genotoxicity induced by alkylating agents: An in vivo analysis. *Mutagenesis* 20: 279–283.

Galati, G., Sabzevari, O., Wilson, J. X., and O'Brien, P. J. 2002. Prooxidant activity and cellular effects of the phenoxyl radicals of dietary flavonoids and other polyphenolics. *Toxicology* 177: 91–104.

García-Rodríguez, M. del C., Nicolás-Méndez, T., Montaño-Rodríguez, A. R., and Altamirano-Lozano, M. A. 2014. Antigenotoxic effects of (–)-epigallocatechin-3-gallate (EGCG), quercetin, and rutin on chromium trioxide-induced micronuclei in the polychromatic erythrocytes of mouse peripheral blood. *J. Toxicol. Environ. Health A* 77: 324–336.

Granado-Serrano, A. B., Martin, M. A., Bravo, L., Goya, L., and Ramos, S. 2010. Quercetin modulates NF-kappa B and AP-1/JNK pathways to induce cell death in human hepatoma cells. *Nutr. Cancer* 62: 390–401.

Horwitz, W. 1990. *Official methods of analysis*, *AOAC*, 15th ed., Vol. II, pp. 1058–1059. Arlington, VA: Association of Official Analytical Chemists.

Hu, M. L. 2011. Dietary polyphenols as antioxidants and anticancer agents: More questions than answers. *Chang Gung Med. J*. 34: 449–460.

Juroszek, P., Lumpkin, H. M., Yang, R. Y., Ledesma, D. R., and Ma, C. H. 2009. Fruit quality and bioactive compounds with antioxidant activity of tomatoes grown on-farm: Comparison of organic and conventional management systems. *J. Agric. Food Chem*. 57: 1188–1194.

Keser, S., Celik, S., and Turkoglu, S. 2013. Total phenolic contents and free-radical scavenging activities of grape (*Vitis vinifera* L.) and grape products. *Int. J. Food Sci. Nutr*. 64: 210–216.

Khan, R. A., Khan, M. R., and Sahreen, S. 2012a. CCl4-induced hepatotoxicity: Protective effect of rutin on p53, CYP2E1 and the antioxidative status in rat. *BMC Complement. Altern. Med*. 12: 178.

Khan, R. A., Khan, M. R., and Sahreen, S. 2012b. Protective effects of rutin against potassium bromate induced nephrotoxicity in rats. *BMC Complement. Altern. Med*. 12: 204.

Kosar, M., Kupeli, E., Malyer, H., Uylaser, V., Turkben, C., and Baser, K. H. 2007. Effect of brining on biological activity of leaves of *Vitis vinifera* L. (Cv. Sultani Cekirdeksiz) from Turkey. *J. Agric. Food Chem*. 55: 4596–4603.

Krahn, D. F. 1983. Chinese hamster cell mutagenesis: A comparison of the CHO and V79 systems. *Ann. NY Acad. Sci*. 407: 231–238.

Lambert, J. D., and Elias, R. J. 2010. The antioxidant and pro-oxidant activities of green tea polyphenols: A role in cancer prevention. *Arch. Biochem. Biophys*. 501: 65–72.

Melo-Cavalcante, A. A., Dantas, S. M., Leite Ade, S., Matos, L. A., Sousa, J. M., Picada, J. N., and da Silva, J. 2011. In vivo antigenotoxic and anticlastogenic effects of fresh and processed cashew (*Anacardium occidentale*) apple juices. *J. Med. Food* 14: 792–798.

Min, K., and Ebeler, S. E. 2009. Quercetin inhibits hydrogen peroxide-induced DNA damage and enhances DNA repair in Caco-2 cells. *Food Chem. Toxicol*. 47: 2716–2722.

Mirabelli, C. K., Sung, C. M., McCabe, F. L., Faucette, L. F., Crooke, S. T., and Johnson, R. K. 1988. A murine model to evaluate the ability of *in vitro* clonogenic assays to predict the response to tumors in vivo. *Cancer Res*. 48: 5447–5454.

Neeha, V. S., and Kinth, P. 2013. Nutrigenomics research: A review. *J. Food Sci. Technol*. 50: 415–428.

Niture, S. K., Velu, C. S., Smith, Q. R., Bhat, G. J., and Srivenugopal, K. S. 2007. Increased expression of the MGMT repair protein mediated by cysteine prodrugs and chemopreventative natural products in human lymphocytes and tumor cell lines. *Carcinogenesis* 28: 378–389.

Oliboni, L. S., Dani, C., Funchal, C., Henriques, J. A., and Salvador, M. 2011. Hepatoprotective, cardioprotective, and renal-protective effects of organic and conventional grapevine leaf extracts on Wistar rat tissues. *Ann. Acad. Bras. Cienc*. 83: 1403–1411.

Orhan, N., Aslan, M., Orhan, D. D., Ergun, F., and Yesilada, E. 2006. In-vivo assessment of antidiabetic and antioxidant activities of grapevine leaves (*Vitis vinifera*) in diabetic rats. *J. Ethnopharmacol*. 108: 280–286.

Pacifico, S., D'Abrosca, B., Scognamiglio, M., Gallicchio, M., Potenza, N., Piccolella, S., Russo, A., Monaco, P., and Fiorentino, A. 2011. Metabolic profiling of strawberry grape (*Vitis x labruscana* cv. 'Isabella') components by nuclear magnetic resonance (NMR) and evaluation of their antioxidant and antiproliferative properties. *J. Agric. Food Chem*. 59: 7679–7687.

Rafehi, H., Orlowski, C., Georgiadis, G. T., Ververis, K., El-Osta, A., and Karagiannis, T. C. 2011. Clonogenic assay: Adherent cells. *J. Vis. Exp*. 49: 2573.

Pastrana-Bonilla, E., Akoh, C. C., Sellappan, S., and Krewer, G. 2003. Phenolic content and antioxidant capacity of muscadine grapes. *J. Agric. Food Chem*. 51: 5497–5503.

Ramos, A. A., Marques, F., Fernandes-Ferreira, M., and Pereira-Wilson, C. 2013. Water extracts of tree Hypericum sps. protect DNA from oxidative and alkylating damage and enhance DNA repair in colon cells. *Food Chem. Toxicol*. 51: 80–86.

Rockenbach, I. I., Rodrigues, E., Gonzaga, L.V., Caliari, V., Genovese, M.I., Goncalves, A. E. S. S., and Fett, R. 2011. Phenolic compounds content and antioxidant activity in pomace from selected red grapes (*Vitis vinifera* L. and *Vitis labrusca* L.) widely produced in Brazil. *Food Chem*. 127: 174–179.

Santos, V. da Silva, Bisen-Hersh, E., Yu, Y., Cabral, I. S., Nardini, V., Culbreth, M., Teixeira, J. B. da Rocha, BarbosaF., Jr., and Aschner, M. 2014. Anthocyanin-rich açaí (*Euterpe oleracea* Mart.) extract attenuates manganese-induced oxidative stress in rat primary astrocyte cultures. *J. Toxicol. Environ. Health A* 77: 390–404.

Sato, M., Bagchi, D., Tosaki, A., and Das, D. K. 2001. Grape seed proanthocyanidin reduces cardiomyocyte apoptosis by inhibiting ischemia/reperfusion-induced activation of JNK-1 and C-JUN. *Free Radical Biol. Med*. 31: 729–737.

Scalbert, A., Manach, C., Morand, C., Rémésy, C., and Jiménez, L. 2005. Dietary polyphenols and the prevention of diseases. *Crit. Rev. Food Sci. Nutr*. 45: 287–306.

Singleton, V. L., and Rossi, J. A. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Viticult*. 16: 144–158.

Singh, N. P., McCoy, M. T., Tice, R. R., and Schneider, E. L. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res*. 175: 184–191.

Soleas, G. J., Diamandis, E. P., and Goldberg, D. M. 1997. Resveratrol: A molecule whose time has come? And gone? *Clin. Biochem* 30: 91–113.

Thomas, P., and Fenech, M. 2011. Cytokinesis-block micronucleus cytome assay in lymphocytes. *Methods Mol. Biol*. 682: 217–234.

Trindade, C., Juchem, A. L., de Albuquerque, N. R., de Oliveira, I. M., Rosa, R. M., Guecheva, T. N., Saffi, J., and Henriques, J. A. 2015. Antigenotoxic and antimutagenic effects of diphenyl ditelluride against several known mutagens in Chinese hamster lung fibroblasts. *Mutagenesis* 30: 799–809.

Wang, S. W., Wang, Y. J., Su, Y. J., Zhou, W. W., Yang, S. G., Zhang, R., Zhao, M., Li, Y. N., Zhang, Z. P., Zhan, D. W., and Liu, R. T. 2012. Rutin inhibits beta-amyloid aggregation and cytotoxicity, attenuates oxidative stress, and decreases the production of nitric oxide and proinflammatory cytokines. *Neurotoxicology* 33: 482–490.

Watjen, W., Michels, G., Steffan, B., Niering, P., Chovolou, Y., Kampkotter, A., Tran-Thi, Q. H., Proksch, P., and Kahl, R., 2005. Low concentrations of flavonoids are protective in rat H4IIE cells whereas high concentrations cause DNA damage and apoptosis. *J. Nutr*. 135: 525–531.

Xia, E. Q., Deng, G. F., Guo, Y. J., and Li, H. B., 2010. Biological activities of polyphenols from grapes. *Int. J. Mol. Sci*. 11: 622–646.

Yamaguchi, T., Takamura, H., Matoba, T., and Terao, J. 1998. HPLC method for evaluation of the free radical-scavenging activity of foods by using 1,1-diphenyl-2-picrylhydrazyl. *Biosci. Biotechnol. Biochem*. 62: 1201–1204.

Zhou, K., and Raffoul, J. J. 2012. Potential anticancer properties of grape antioxidants. *J. Oncol*. 2012: 803294.

Anexo II

Currículo Vitae - resumido

Cristiano Trindade

Possui graduação em Biomedicina pela Universidade Luterana do Brasil (2009) e mestrado em Biologia Celular e Molecular pela Universidade Federal do Rio Grande do Sul (2012). Doutorando em Genética e Biologia Molecular pela Universidade Federal do Rio Grande do Sul. Tem experiência na área de Genética Toxicologica; mutagênese/antimutagênese e estresse oxidativo. **(Texto informado pelo autor)**

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Formação Complementar

2013 – 2013 Curso de Sistema de Qualidade BPL. (Carga horária: 20h).
Associação Rede de Metrologia e Ensaios do Rio Grande do Sul, REDE METROLOGICA, Brasil.

2013 – 2013 Workshop HUMN and HUMNxl. (Carga horária: 28h).
Universidade Luterana do Brasil, ULBRA, Brasil.

2012 – 2012 Survival Analysis. Theory and Application". (Carga horária: 12h).
Associação Latino Americana de Genética, ALAG, Argentina.

2011 – 2011 Extensão universitária em Virologia Molecular: o HIV como modelo de estudo. (Carga horária: 15h).
Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

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Instituto de Educação para pesquisa, Desenvolvimento e Inovação Tecnológica Royal, GENOTOX, Brasil

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Artigos completos publicados em periódicos

Trindade, Cristiano; BORTOLINI, GIOVANA VERA ; COSTA, BÁRBARA SEGALOTTO; ANGHINONI, JOANNA CARRA ; GUECHEVA, TEMENOUGA NIKOLOVA ; ARIAS, XIMENA ; CÉSIO, MARIA VERÓNICA ; HEINZEN, HORÁCIO ; MOURA, DINARA JAQUELINE ; SAFFI, JENIFER ; SALVADOR, MIRIAN ; HENRIQUES, JOÃO ANTONIO PÊGAS . Antimutagenic and antioxidant properties of the aqueous extracts of organic and conventional grapevine cv. Isabella leaves in V79 cells. Journal of Toxicology and Environmental Health. Part A, v. 79, p. 825-836, 2016.

GONÇALVES, TATIANA SIQUEIRA ; MENEZES, LUCIANE MACEDO DE ; Trindade, Cristiano ; THOMAS, PHILIP ; FENECHC, MICHAEL ; HENRIQUES, JOÃO ANTONIO PÊGAS . In vivo evaluation of the genotoxic effects of Hyrax auxiliary orthodontic appliances containing silver-soldered joints. Mutation Research. Genetic Toxicology and Environmental Mutagenesis, v. 791, p. 25-29, 2015.

TRINDADE, C.; JUCHEM, A. L. M. ; DE ALBUQUERQUE, N. R. M. ; DE OLIVEIRA, I. M. ; ROSA, R. M. ; GUECHEVA, T. N. ; SAFFI, J. ; HENRIQUES, J. A. P. . Antigenotoxic and antimutagenic effects of diphenyl ditelluride against several known mutagens in Chinese hamster lung fibroblasts. Mutagenesis, v. 30, p. 1-11, 2015.

BORTOLOTTO, IRANEZ ; BRUM, ANA PAULA S. ; DE SOUZA, LARISSA M. ; Trindade, Cristiano ; GUECHEVA, TEMENOUGA N. ; LUIZ, FABIANO M ; DE PAULA-RAMOS, ANA LIGIA L ; CONSIGLIO, ANGELICA R. . DNA damage Evaluation in a

Nursing Team Occupationally Exposed to Ionizing Radiation. Brazilian Journal of Radiation Sciences, v. 3, p. 1-21, 2015.

GONÇALVES, TATIANA SIQUEIRA ; MENEZES, LUCIANE MACEDO DE ; Trindade, Cristiano ; MACHADO, MIRIANA DA SILVA ; THOMAS, PHILIP ; FENECH, MICHAEL ; HENRIQUES, JOÃO ANTONIO PÊGAS . Cytotoxicity and genotoxicity of orthodontic bands with or without silver soldered joints. Mutation Research. Genetic Toxicology and Environmental Mutagenesis, v. 762, p. 1-8, 2014.

Greggio, Samuel ; de Paula, Simone ; de Oliveira, Iuri M. ; TRINDADE, C. ; Rosa, Renato M. ; Henriques, João A.P. ; DaCosta, Jaderson C. . NAP prevents acute cerebral oxidative stress and protects against long-term brain injury and cognitive impairment in a model of neonatal hypoxia ischemia. Neurobiology of Disease, v. 44, p. Pages 152-159, 2011.