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**Avaliação da indução de autofagia em células de câncer de pulmão em
resposta ao tratamento com cisplatina.**

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LISTA DE ABREVIATURAS

3MA	3-metiladenina (<i>3-methyladenine</i>)
Akt/PKB	proteína cinase B (<i>kinase protein B</i>)
AMP	adenosina monofosfato
AMPK	proteína cinase ativada por AMP (<i>AMP-activated protein kinase</i>)
AO	laranja de acridina (<i>acridine orange</i>)
ATG	genes relacionados à autofagia (<i>autophagy-related genes</i>)
Atg	proteínas relacionadas à autofagia (<i>autophagy-related proteins</i>)
ATP	adenosina trifosfato
AVOs	organelas ácidas vacuolares (<i>acidic vacuolar organelles</i>)
Bcl-2	proteína 2 de linfoma de células B (<i>B-cell lymphoma 2</i>)
Bcl-xL	proteína anti-apoptótica de linfoma de células B extra grande (<i>B-cell lymphoma-extra large</i>)
CDDP	cisplatina ou cis-diaminodicloroplatina (II)
CQ	cloroquina (<i>chloroquine</i>)
DMEM	<i>Dulbecco's Modified Eagle Medium</i>
DMSO	dimetilsulfóxido
DNA	ácido desoxirribonucleico (<i>deoxyribonucleic acid</i>)
DRAM	gene modulador de autofagia induzido por dano (<i>damage-regulated autophagy modulator</i>)
EDTA	ácido etilenodiaminotetracético (<i>ethylenediamine tetraacetic acid</i>)
FIP200	proteína de interação com a família FAK de 200 kD (<i>FAK family-interacting protein of 200 kDa</i>)
GβL	subunidade β -like da proteína G (<i>G protein β-subunit-like protein</i>)
INCA	Instituto Nacional do Câncer

LC3I/LC3II	formas I e II da cadeia leve 3 das proteínas associadas ao microtúbulo 1 (<i>microtubule-associated protein 1 light chain 3-I and II</i>)
MAP1-LC3/LC3	cadeia leve 3 das proteínas associadas ao microtúbulo 1 (<i>microtubule-associated protein 1 light chain 3</i>)
MEK	proteína cinase da cinase ativada por mitógeno ou MAPKK (<i>mitogen-activated protein kinase kinase</i>)
mTOR	proteína alvo da rapamicina em mamíferos (<i>mammalian target of rapamycin</i>)
mTORC1	complexo da mTOR 1 (<i>mTOR complex 1</i>)
mTORC2	complexo da mTOR 2 (<i>mTOR complex 2</i>)
MTT	sal de tetrazólio (<i>thiazolyl blue tetrazolium bromide</i>)
PE	fosfatidiletanolamina (<i>phosphatidylethanolamine</i>)
PI3KI	fosfatidilinositol 3-cinase classe I (<i>phosphoinositide 3-kinase class I</i>)
PI3KIII	fosfatidilinositol 3-cinase classe III (<i>phosphoinositide 3-kinase class III</i>)
PI3P	fosfatidilinositol 3,4,5-fosfato (<i>phosphatidylinositol 3,4,5-bisphosphate</i>)
PRAS40	substrato de Akt de 40 kD rico em prolina (<i>proline-rich Akt substrate of 40 kDa</i>)
Rap	rapamicina
Raptor	proteína regulatória associada a mTOR (<i>regulatory associated protein of mTOR</i>)
SDS	dodecil sulfato de sódio (<i>sodium dodecyl sulfate</i>)
Ulk1	cinase Unc-51-like 1 (<i>Unc-51-like 1 kinase</i>)

RESUMO

A autofagia tem sido alvo de extensas investigações nas pesquisas oncológicas devido ao seu papel dual na tumorigênese. Estudos recentes têm demonstrado que a autofagia pode ser ativada por agentes quimioterápicos e que esta ativação pode influenciar na resposta celular e afetar o resultado da terapia. Os papéis exercidos pela autofagia em resposta à quimioterapia são controversos e dependem do tipo e do contexto celular. Por um lado, a autofagia poderia atuar como um mecanismo de defesa na tentativa de evitar o estresse causado pelo dano ao DNA, porém, poderia também levar à morte celular dependendo da extensão do dano. A cisplatina é um agente quimioterápico amplamente utilizado no tratamento de diversos tipos de tumores, incluindo os cânceres de pulmão de células não-pequenas (NSCLC). Neste estudo foi investigado o papel da indução de autofagia pela cisplatina em células de câncer de pulmão H460. Para este fim, a cisplatina foi combinada com o indutor de autofagia rapamicina, o que resultou numa hiperestimulação da via autofágica, diminuindo consideravelmente a viabilidade celular. Além disso, foram utilizados os inibidores farmacológicos da autofagia 3-metiladenina e cloroquina. Em combinação com o tratamento da cisplatina, a inibição da autofagia pela 3-metiladenina não teve efeito na viabilidade celular. No entanto, o tratamento com cloroquina aumentou significativamente a viabilidade celular em relação ao tratamento isolado com a cisplatina. Em conjunto, estes resultados sugerem que a autofagia induzida pela cisplatina contribui para a eliminação das células de câncer de pulmão.

ABSTRACT

Autophagy has been the target of extensive investigation in oncology researches due to its dual role in tumorigenesis. Recent findings have demonstrated that autophagy can be activated by chemotherapeutical agents and that this activation may influence cellular responses and affect the results of therapy. The roles played by autophagy in response to chemotherapy are controversial and dependent on cell type and context. Autophagy could act as a defense mechanism in an attempt to avoid the stress caused by DNA damage, but it could also lead to cell death depending on damage extent. Cisplatin is one of the most effective chemotherapeutical agents used to treat several types of tumors including non-small cell lung cancer (NSCLC). In the present study we investigated the role of autophagy induction by cisplatin in H460 lung cancer cells. For this purpose, cisplatin was combined with the autophagy inducer rapamycin which resulted in autophagy overstimulation, considerably decreasing cell viability. Besides, the pharmacological inhibitors of autophagy 3-methyladenine and chloroquine were also used. Autophagy inhibition by 3-methyladenine had no effect on cell viability. However the treatment with chloroquine increased cell viability in comparison to cisplatin treatment only. Together these results suggest that autophagy induced by cisplatin contributes to lung cancer cells elimination.

1. INTRODUÇÃO

1.1. Autofagia

A autofagia é um processo catabólico altamente conservado evolutivamente que ocorre em todas as células eucarióticas. Existem pelo menos três tipos de autofagia: macroautofagia, microautofagia e autofagia mediada por chaperonas. A macroautofagia (posteriormente mencionada como autofagia) é caracterizada pela formação de uma estrutura de membrana dupla conhecida como autofagossomo. A microautofagia é um processo no qual componentes celulares são degradados diretamente pelo lisossomo pela invaginação da membrana desta organela. Na autofagia mediada por chaperonas, ocorre a translocação direta de proteínas do citosol pela membrana do lisossomo, o que requer a ação de chaperonas para o desdobramento destas proteínas (Jiang & Mizushima, 2014).

A autofagia é responsável pela homeostasia da célula, eliminando organelas danificadas e patógenos (Johansen & Lamark, 2011). Além disso, durante períodos de estresse ou escassez de nutrientes, a autofagia degrada organelas e proteínas celulares, fornecendo energia (ATP) e biomoléculas essenciais para a célula.

A via autofágica possui como característica principal o sequestro de componentes celulares em uma vesícula de membrana dupla conhecida como autofagossomo. Inicialmente forma-se o fagóforo, também conhecido como membrana de isolamento (Tooze *et al.*, 2010). Em seguida, as membranas desta estrutura se alongam e são fusionadas, levando à formação do autofagossomo. Na etapa seguinte, o autofagossomo funde-se com o lisossomo. A estrutura resultante desta fusão é o autofagolisossomo, local onde ocorre a degradação e reciclagem dos constituintes celulares pela ação das hidrolases ácidas lisossomais (Choi *et al.*, 2013; **Figura 1**).

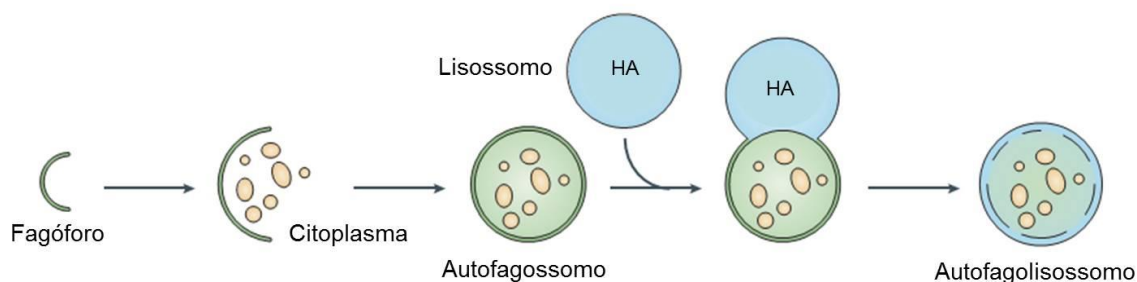


Figura 1. Fases da via autofágica. Inicialmente, o fagóforo ou membrana de isolamento é formado. Ocorre o sequestro de constituintes celulares e forma-se o autofagossomo. Posteriormente, ocorre a fusão desta vesícula de membrana dupla com o lisossomo. O compartimento fusionado é conhecido como autofagolisossomo, local onde os componentes celulares serão degradados pela ação das hidrolases ácidas (HA) lisossomais. Adaptado de Rubinsztein *et al.* (2007).

1.1.1. Maquinaria Molecular e Regulação da Via Autofágica

A autofagia é regulada por diversas vias de sinalização (**Figura 2A**). Na presença de fatores de crescimento e nutrientes, a via fosfatidilinositol-3 cinase de classe I (PIKI)-Akt é ativada. Neste caso, a autofagia é regulada negativamente, pois ocorre a ativação da mTOR (*mammalian target of rapamycin*), uma proteína cinase central no controle do metabolismo celular e uma das principais reguladoras de autofagia. A mTOR faz parte dos complexos proteicos mTORC1 e mTORC2, no entanto, apenas mTORC1 é capaz de regular negativamente a autofagia. Este complexo é composto por mTOR, Raptor (proteína regulatória associada à mTOR), GβL e PRAS40 (Benjamin *et al.*, 2011). mTORC1 inibe a cinase Unc-51-like 1 (Ulk1) e impede o início da autofagia. Ulk1 é o ortólogo de Atg1 em leveduras e faz parte do complexo Ulk1-Atg13-Atg101-FIP200. A fosforilação deste complexo é essencial para a fase de nucleação do autofagossomo, passo crucial para o início do processo autofágico (Alers *et al.*, 2012a).

Em condições de depleção de nutrientes os níveis intracelulares de AMP ficam elevados e, em contrapartida, os níveis de ATP decaem. Deste modo, a proteína cinase ativada por AMP (AMPK) regula a autofagia positivamente, pois controla

de forma negativa a mTOR, além de fosforilar a Ulk1 (**Figura 2A**; Kim *et al.*, 2011).

Outro complexo de cinases crucial para o início da autofagia é o complexo Beclina1-fosfatidilinositol 3 cinase classe III (PI3K classe III ou PI3KIII)-Atg14. Beclina 1 compartilha homologia de sequência com Atg6 de leveduras e é um importante marcador para avaliar a indução de autofagia. A estimulação deste complexo estimula a fosforilação do fosfatidilinositol (PI), levando à formação de fosfatidilinositol 3 fosfato (PI3P), o qual, por sua vez, induz a nucleação do autofagossomo (**Figura 2A**; He & Levine, 2010).

A fase subsequente à de nucleação do autofagossomo é a fase de alongamento desta vesícula, a qual necessita de dois sistemas de conjugação ubiquitina-*like* (**Figura 2B**). No primeiro, a proteína Atg12 é conjugada à Atg5. O conjugado Atg12-Atg5 liga-se à Atg16L1 para formar um supercomplexo multimérico o qual é recrutado ao fagóforo. Este complexo é essencial para o alongamento do autofagossomo, mas se dissocia do autofagossomo totalmente formado (Noda *et al.*, 2009). O segundo sistema de conjugação ubiquitina-*like* envolve a proteína Map1-LC3 (*microtubule-associated protein 1 light chain 3*), também conhecida como LC3 (homóloga à proteína Atg8 em leveduras). A LC3 é sintetizada na sua forma precursora e após é clivada resultando na sua forma citosólica LC3-I. Em presença de um sinal pré-autofágico, LC3-I conjuga-se com a fosfatidiletanolamina (PE) em uma reação envolvendo Atg7 e Atg3 para formar LC3-II, um passo chave na regulação da formação do autofagossomo. A LC3-II é recrutada para a fase de alongamento e permanece ligada ao autofagossomo até a fusão deste com o lisossomo. Esta associação específica entre o autofagossomo e a isoforma LC3-II torna esta proteína um excelente marcador de ativação de autofagia (Ravikumar *et al.*, 2010).

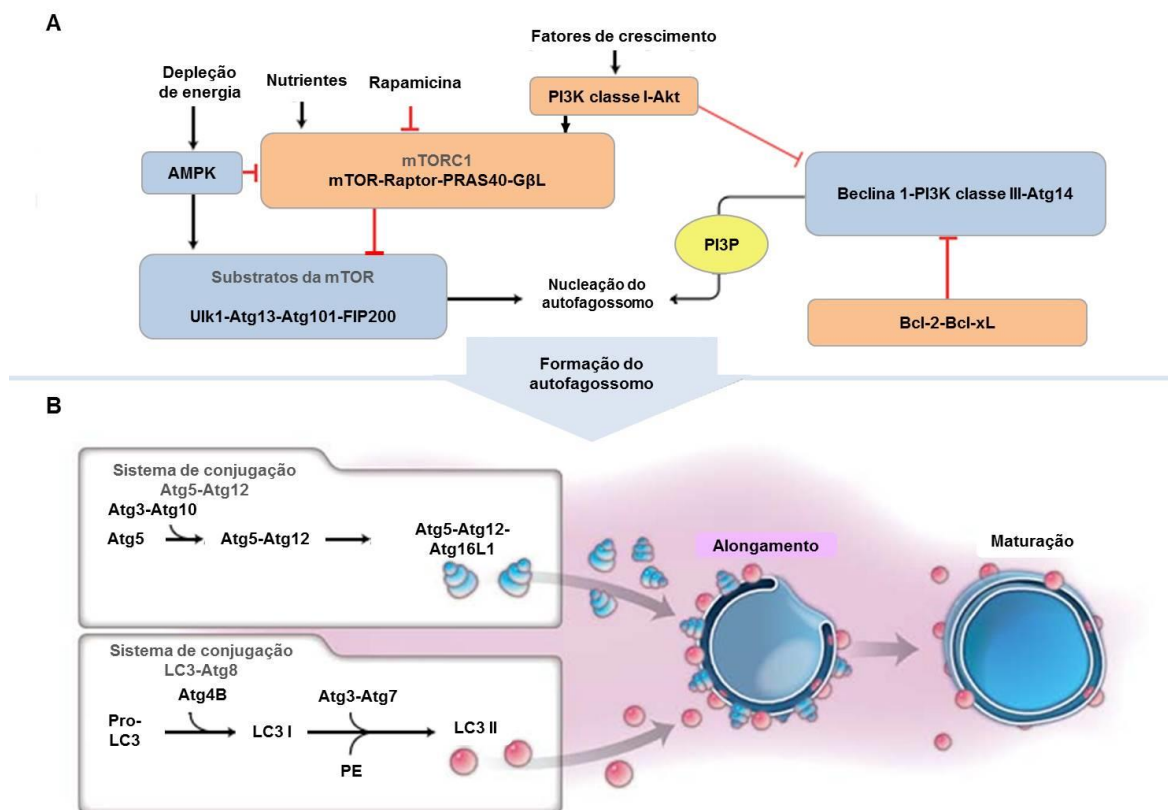


Figura 2. Maquinaria molecular e regulação da via autofágica. (A) PI3K classe I-Akt regula a autofagia negativamente ao ativar a mTOR em resposta a fatores de crescimento. Akt também regula negativamente a autofagia através da fosforilação de Beclina 1. O complexo mTORC1 inibe a Ulk1 quando existe disponibilidade de nutrientes e impede a autofagia. Em resposta a níveis aumentados de AMP, a cinase AMPK controla negativamente a mTOR e fosforila a Ulk1. A fosforilação do complexo Ulk1-Atg13-Atg101-FIP200 é essencial para a fase de nucleação do autofagossomo. A estimulação do complexo Beclina 1-PI3KIII também é crucial para o início da autofagia, pois gera PI3P e promove a nucleação da membrana do autofagossomo. As proteínas Bcl-2 e Bcl-xL são inibidoras da autofagia, pois sequestram Beclina 1. **(B)** Dois sistemas de conjugação ubiquitina-*like* são necessários para o alongamento do autofagossomo. O primeiro sistema leva à formação do complexo Atg5-Atg12-Atg16L1 e o segundo envolve a conversão de LC3-I em LC3-II. Adaptado de Choi *et al.* (2013).

1.1.2. A Degradação Autofágica Ocorre de Maneira Seletiva

Nos últimos anos tem sido reportada a seletividade da via autofágica em relação aos componentes degradados (Alers *et al.*, 2012b). Termos específicos são atribuídos para a degradação de diferentes alvos, tais como ER-fagia, pexofagia, mitofagia, ribofagia e xenofagia em referência à degradação do retículo

endoplasmático, peroxissomos, mitocôndrias, ribossomos e patógenos, respectivamente (Ravikumar *et al.*, 2010). Esta seletividade permite diversas regulações moleculares através de adaptadores autofágicos (Green & Levine, 2014). Um destes adaptadores é a proteína p62 (sequestossomo 1 ou SQSTM1), um substrato seletivo da autofagia. A p62 possui um domínio N-terminal (PB1) que a torna capaz de se auto-oligomerizar e um domínio C-terminal (UBA) capaz de interagir com proteínas ubiquitinadas (Johansen & Lamark, 2011). A p62 interage com a proteína LC3 através de uma região específica LRS (LC3-*recognition sequence*) (Komatsu & Ichimura, 2010) também conhecida como LIR (LC3-*interacting region*) (Johansen & Lamark, 2011). Esta interação faz com que a p62 seja dirigida até o autofagossomo em formação e direcione os substratos ubiquitinados para a degradação autofágica (Moscat & Diaz-Meco, 2009; **Figura 3**). Pelo fato de ser seletivamente degradada, a p62 também é um excelente marcador para a ativação de autofagia. No momento em que a via autofágica está ativa, é possível observar uma correlação inversa entre os níveis de expressão de p62 e LC3-II, pois enquanto os níveis de p62 ficam reduzidos, os níveis de LC3-II tornam-se elevados.

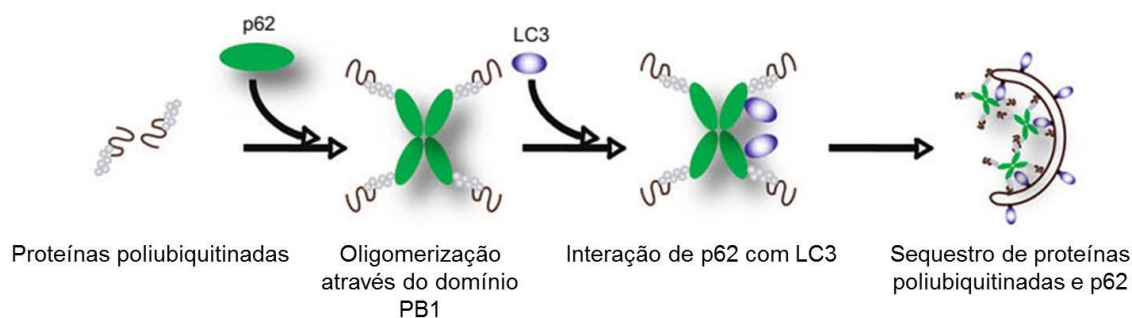


Figura 3. Modelo de p62 como receptor para proteínas poliubiquitinadas. p62 interage com as proteínas através do domínio UBA. Ocorre a auto-oligomerização de p62 através do domínio PB1. O complexo é seletivamente sequestrado para o autofagossomo através da interação com LC3. Adaptado de Komatsu & Ichimura (2010).

1.1.3. Autofagia e sua Associação com a Tumorigênese

Para que as células possam manter o equilíbrio homeostático é necessário que exista um balanço entre a biossíntese e a degradação de componentes

celulares. A autofagia, através da sua capacidade de degradar organelas inteiras, proteínas e patógenos, exerce um papel crítico nas células em resposta a vários estímulos. Além disso, tem um papel essencial na manutenção da integridade genômica nuclear e mitocondrial (Vessoni *et al.*, 2013). Desta forma, falhas nesta via estão associadas a diversas patologias como infecções, doenças neurodegenerativas, doenças metabólicas e câncer (Tooze & Yoshimori, 2010; Jiang & Mizushima, 2014).

A autofagia tem um importante papel como supressora tumoral, o qual está fortemente relacionado com a defesa antioxidante exercida por esta via na célula. A ativação da autofagia é essencial para a remoção de mitocôndrias danificadas, principais geradores de espécies reativas de oxigênio (White, 2012). Desta forma, a inativação da autofagia resulta na indução de estresse oxidativo e no acúmulo de p62, o qual aumenta ainda mais a produção de espécies reativas de oxigênio (Levy & Thorburn, 2011). Como consequência, defeitos na via autofágica estão associados à instabilidade genômica, provocada pelo estresse oxidativo, e à inflamação, promovendo um ambiente propício ao surgimento de tumores (Vessoni *et al.*, 2013).

A ativação da autofagia em células normais, portanto, age como supressora tumoral. No entanto, na célula tumoral, a ativação desta via possui papéis distintos, dependendo do estágio da tumorigênese (Rosenfeldt & Ryan, 2011). Nos estágios iniciais da tumorigênese, a ativação da autofagia atua como um mecanismo protetor, evitando a progressão tumoral. No entanto, em fases mais avançadas da doença, a autofagia dificulta a eliminação do tumor e leva à resistência ao tratamento, uma vez que contribui para a sobrevivência da célula tumoral (Rubinsztein *et al.*, 2007).

Inúmeros estressores podem levar à ativação da via autofágica. No microambiente tumoral, as células estão sujeitas ao estresse causado pela depleção de nutrientes e oxigênio. A ativação de autofagia nestes casos contribui para a manutenção dos níveis energéticos intracelulares, permitindo a sobrevivência da célula tumoral (Yang *et al.*, 2011). É possível que a autofagia também esteja envolvida na promoção da metástase, pois quando as células

tumorais perdem o contato com a matriz extracelular, o que normalmente levaria à morte celular apoptótica conhecida como *anoikis*, a autofagia é ativada para promover a sobrevivência da célula e, desta forma, contribui para a invasão para outros tecidos (Xu *et al.*, 2013). Apesar do papel citoprotetor em células de câncer, quando a autofagia é hiperestimulada ou está ativa por períodos prolongados, esta pode contribuir para a morte da célula. Acredita-se que este envolvimento com a sinalização letal não seja um indicativo de que exista um tipo de morte celular programada autofágica. Segundo Shen *et al.* (2012), a célula mantém a via ativada numa incansável tentativa de promover a sobrevivência, mas devido ao estresse, outras vias de morte também são ativadas, como apoptose ou necrose. Nesse caso, a célula morta pode demonstrar também aspectos autofágicos.

Diante destes diferentes papéis que a autofagia exerce na tumorigênese, existe um grande interesse em modular a via autofágica como tentativa de melhorar o prognóstico de pacientes com câncer. A administração de agentes indutores ou inibidores da autofagia em combinação com fármacos antineoplásicos pode ser uma alternativa interessante para a obtenção de melhores respostas ao tratamento quimioterápico (Levy & Thorburn, 2011).

1.1.4. Agentes Moduladores da Via Autofágica

1.1.4.1. Rapamicina

A rapamicina é um composto macrolídeo produzido pela bactéria *Streptomyces hygroscopicus* e apresenta inúmeras aplicações farmacológicas. A primeira característica farmacológica descrita da rapamicina foi a ação antifúngica contra espécies patogênicas de *Candida*. Mais tarde, suas propriedades como imunossupressora, neuroprotetora, antienvhecimento e antitumoral, foram reveladas (Park *et al.*, 2010). Ademais, o composto também pode ser utilizado no tratamento de doenças autoimunes e em casos de rejeição de órgãos transplantados.

A rapamicina tem como alvo a proteína cinase TOR (*target of rapamycin*), a qual é conservada em eucariotos desde leveduras até humanos. O alvo da rapamicina em células de mamífero é a proteína mTOR. No entanto, apenas o complexo da mTORC1 pode ser inibido pela ação da rapamicina. Os substratos mais bem caracterizados de mTORC1 são a cinase S6 ribossomal e a 4E-BP1 (proteína de ligação ao fator de iniciação da tradução eucariótico eIF4E) (**Figura 4**). Desta forma, a inibição de mTORC1 leva a um decréscimo na síntese proteica (Abraham & Gibbons, 2007).

O complexo da mTORC1 atua como um sensor de nutrientes e energia: além de ativar a síntese de proteínas e a biogênese dos ribossomos, também participa do transporte de nutrientes na célula, na síntese de lipídeos e em outros processos que envolvem fatores de crescimento e demanda de energia. Quando ativo, o complexo da mTORC1 inibe Ulk1 impedindo a ativação da autofagia. No entanto, em presença da rapamicina, mTOR é inativada e permite que a via autofágica seja ativada (Kim *et al.*, 2011).

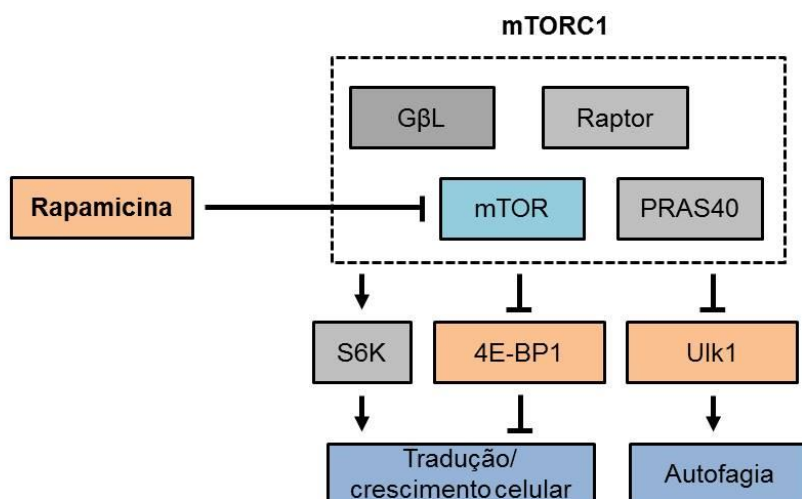


Figura 4. Inibição do complexo mTORC1 pela rapamicina e ativação da autofagia. O complexo mTORC1 ativa S6K a fim de promover a biogênese dos ribossomos. Em contrapartida, 4E-BP1 é inibida e, desta forma, permite a tradução dependente de cap. mTORC1 inibe a autofagia pela inativação de Ulk1. Na presença de rapamicina, mTORC1 é inibido ativando a autofagia. Adaptado de Benjamin *et al.* (2011).

Estudos recentes têm sugerido que a rapamicina possui a capacidade de inibir a proliferação de células tumorais. Dependendo do tipo tumoral é possível que a rapamicina contribua para a morte celular por diferentes mecanismos. Jiang *et al.* (2014) verificaram que a indução de autofagia pela rapamicina contribui para a morte de células de câncer de pulmão e que a adição de 3-metiladenina (inibidor de autofagia) em combinação com a rapamicina anula o efeito desta última, aumentando viabilidade celular. No entanto, também tem sido demonstrado que a utilização da rapamicina pode contribuir para a sobrevivência de células tumorais. Nakamura *et al.* (2014) mostraram que a rapamicina induz autofagia de forma citoprotetora em células Nara-H (histiocitoma fibroso). Porém, a adição de U0126 (inibidor de autofagia pela inibição de MEK) faz com que o tratamento com rapamicina leve à morte celular por apoptose.

1.1.4.2. Cloroquina

Há várias décadas a cloroquina tem sido utilizada como o principal tratamento contra a malária devido à sua eficácia, segurança, boa tolerabilidade e baixo custo (Solomon & Lee, 2009). Hoje são conhecidas várias outras propriedades farmacológicas da cloroquina, como atividade anti-inflamatória e capacidade de imunomodulação (Thomé *et al.*, 2013). Mais recentemente, tem sido dada atenção ao seu papel na terapia anticâncer. A cloroquina pode sensibilizar as células tumorais a outros fármacos e à radioterapia (Liu *et al.*, 2014; Schonewolf *et al.*, 2014). Inúmeros mecanismos estão associados à sua eficácia como agente anticâncer, entretanto, um dos mecanismos que se destaca é o de inibição da autofagia.

Dentro das células, a forma não protonada da cloroquina se acumula nos lisossomos e passa a sequestrar prótons levando ao aumento do pH lisossomal. Esta perda nas condições ácidas promove uma disfunção do lisossomo, uma vez que muitas de suas enzimas são inibidas. O aumento do pH lisossomal causado pela atividade da cloroquina impede que o lisossomo se fusione com os autofagossomos formados, impedindo a degradação do conteúdo autofagossomal (Kimura *et al.*, 2013; **Figura 5**).

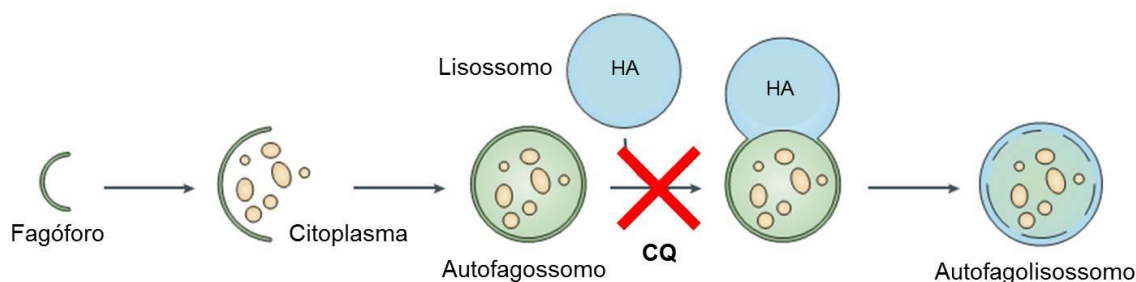


Figura 5. Inibição da via autofágica pela cloroquina. A cloroquina (CQ) impede a fusão do lisossomo com o autofagossomo, pois impede a acidificação do lisossomo. Adaptado de Rubinsztein *et al.* (2007).

Diversos autores têm demonstrado que o tratamento combinado da cloroquina com fármacos quimioterápicos impede a proliferação de células tumorais. Muitos fármacos utilizados no tratamento de diferentes tipos de cânceres, tais como, 5-fluorouracil, bortezomib e cisplatina, ativam a via autofágica. Esta ativação pode promover a resistência a estes fármacos e impedir a morte celular. Ao inibir a autofagia, a cloroquina auxilia na sensibilização das células tumorais e pode reverter a resistência ao agente quimioterápico (Liang *et al.*, 2014).

1.1.4.3. 3-Metiladenina

A 3-metiladenina (3MA) é um conhecido inibidor da autofagia. A capacidade da 3MA de inibir a degradação de proteínas endógenas e de impedir a formação de autofagossomos foi primeiramente evidenciada em hepatócitos de rato por Seglen & Gordon (1982). A 3MA tem como alvo a inibição da atividade da enzima PI3K classe III. Desta forma, PI3P não é formado, o que impede a fase de nucleação do autofagossomo (Petiot *et al.*, 2000; Wu *et al.*, 2010).

A 3MA também bloqueia PI3K de classe I, no entanto a PI3KI atua como um regulador negativo da autofagia, pois ativa a mTOR em resposta a fatores de crescimento (Choi *et al.*, 2013). Acredita-se que a 3MA bloqueia a PI3K classe I de forma persistente enquanto que a PI3K classe III é bloqueada de forma transiente, desta forma o tratamento com 3MA não deve ser feito por períodos prolongados quando existe a intenção de inibir a autofagia (Wu *et al.*, 2010).

A combinação de 3MA com quimioterápicos que ativam a via autofágica tem sido feita na tentativa de sensibilizar células de câncer (Sheng *et al.*, 2013; Palmeira-Dos-Santos *et al.*, 2014). No entanto, alguns autores têm reportado que a inibição da autofagia também pode estar relacionada ao aumento da viabilidade celular em alguns casos. No estudo feito por Qi *et al.* (2013), por exemplo, os autores demonstram que a adição de 3MA reduziu a morte por apoptose em células de linfoma tratadas com fluvastatina (fármaco da classe das estatinas).

1.2. Cisplatina

A cisplatina (cis-diaminodicloroplatina (II) ou CDDP) foi sintetizada em 1845 e começou a ser extensamente utilizada na clínica oncológica a partir da década de 1970. Na atualidade, a cisplatina continua sendo amplamente empregada no tratamento de diversos tipos de cânceres como ovário, bexiga, testículo, cabeça e pescoço, cérvix e pulmão (Gonzalez *et al.*, 2001; Macciò & Madeddu, 2013). Este composto platinado é formado por um átomo central de platina ligado a dois grupos cloreto e dois grupos amino. Uma vez dentro da célula, a cisplatina pode interagir com vários componentes celulares como, por exemplo, DNA, RNA, proteínas e fosfolípidos de membrana.

No DNA, o átomo de platina liga-se covalentemente à posição N⁷ das purinas e ocorre a formação de vários tipos de adutos de platina, os quais caracterizam a citotoxicidade do composto. Pontes intercadeia e intracadeia podem ser formadas, sendo as pontes intracadeia entre guaninas adjacentes o tipo de aduto mais frequente. Monoadutos e pontes entre DNA e proteínas também podem ser formados (Gonzalez *et al.*, 2001) (**Figura 6**). Várias respostas celulares ocorrem como consequência da formação dos adutos, tais como: parada na replicação, inibição da transcrição, parada no ciclo celular, reparo do DNA e morte celular (Wang & Lippard, 2005).

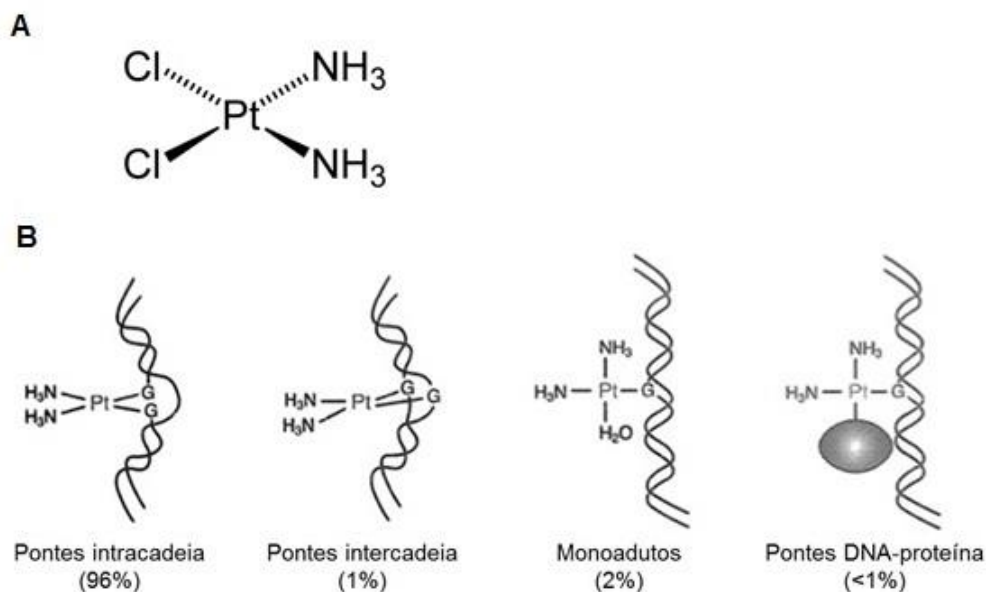


Figura 6. Estrutura da cisplatina e sua ação no DNA. (A) Estrutura molecular da cisplatina. **(B)** Lesões no DNA induzidas pela cisplatina. Adaptado de: <http://www.conconilab.ca/projects/> (acesso em 24/04/2014).

A cisplatina pode induzir morte celular por apoptose e também por necrose, dependendo da concentração intracelular do fármaco. Entretanto, a quimioresistência à cisplatina é muito comum, prejudicando a terapia. Mecanismos que influenciam na captação da cisplatina pelas células ou na execução da apoptose estão envolvidos nesta resistência (Galluzzi *et al.*, 2012). Além do mais, o aumento no efluxo da cisplatina através da membrana plasmática, o aumento nos níveis de glutatona bem como o aumento nos níveis de reparo de DNA também favorecem a resistência ao fármaco (Stathopoulos, 2013).

Em células tratadas com cisplatina ou outros tipos de compostos platinados, pode ocorrer a ativação de autofagia como uma tentativa de prevenir a morte celular (Bordin *et al.*, 2013). O aumento na expressão de Beclina 1 e LC3-II, assim como o aumento da quantidade autofagossomos, estão relacionados ao tratamento com cisplatina. Esta ativação pode impedir a morte celular por apoptose e contribuir para a quimioresistência. Em um estudo de 2013, Shen *et al.* constataram que a autofagia está ativa em células de osteossarcoma

resistentes à cisplatina e que a adição de cloroquina acelera a morte celular induzida pela cisplatina. Resultados semelhantes foram encontrados por Zhang *et al.* (2013) em células de câncer gástrico, sugerindo que a inibição da autofagia sensibiliza as células tratadas com cisplatina. A inibição da autofagia através do silenciamento de genes relacionados à via autofágica também comprova que a autofagia esteja envolvida na resistência à cisplatina, como demonstrado no estudo de Zhu *et al.* (2013) em que foi constatado um aumento na sensibilidade à quimioterapia em células de carcinoma esofágico pelo silenciamento do gene ATG7.

1.2.1. Cisplatina no Tratamento Contra o Câncer de Pulmão

O câncer de pulmão é a principal causa de morte por câncer na população mundial. No Brasil, de acordo com estimativas do Instituto Nacional do Câncer (INCA), são esperados 27 mil novos casos de câncer de pulmão para o ano de 2014 e, excetuando-se o câncer de pele não melanoma, o câncer de pulmão será o segundo tipo de câncer mais incidente em homens e o quarto em mulheres (consulte a publicação em INCA Estimativa 2014).

Diferentes classificações podem ser atribuídas ao câncer de pulmão de acordo com o tipo histológico do tumor. As principais classes morfológicas são os carcinomas de pequenas células, de células escamosas, de grandes células e os adenocarcinomas, cada uma apresentando diferentes perfis de expressão gênica (Meyerson *et al.*, 2004). Dentre as classes citadas, os cânceres de células não pequenas (*non-small cell lung cancer* ou NSCLC) constituem mais de 80% dos cânceres de pulmão (Stathopoulos, 2013). A identificação do tipo histológico é crucial para que o tratamento adequado seja indicado ao paciente.

A utilização da cisplatina no tratamento de pacientes diagnosticados com NSCLC começou no início da década de 1980. Ao final da década de 90, após a publicação de inúmeros estudos que mostravam a efetividade da cisplatina na quimioterapia, o tratamento de primeira linha mais aceito para NSCLC avançado era baseado em cisplatina (Giaccone, 2004). Testes em modelos pré-clínicos mostravam o sinergismo da cisplatina com paclitaxel (Rosell *et al.*, 2002). Outros

compostos, tais como, inibidores de desacetilases de histonas e inibidores de tirosina cinase, também cooperam de maneira sinérgica com a cisplatina (Lopergolo *et al.*, 2014; Zhou *et al.*, 2014). A combinação com outros fármacos também tem sido feita na tentativa de minimizar os efeitos colaterais, pois, quanto maior a dose de cisplatina utilizada, maiores são os efeitos indesejados. Entre estes, destacam-se a neurotoxicidade e nefrotoxicidade (Wang & Lippard, 2005).

A associação do câncer de pulmão com irregularidades na via autofágica tem sido relatada nos últimos anos. Zhou *et al.*, (2013) propuseram que a baixa expressão de Beclina 1 é um risco para o surgimento deste tipo de câncer e está associada ao mau prognóstico para pacientes com NSCLC. Em um modelo murino para câncer de pulmão, Rao *et al.* (2014) demonstraram que o silenciamento do gene ATG5 impede a progressão do tumor, todavia, este silenciamento também está associado ao início da tumorigênese, atribuindo um papel dual para ATG5 no câncer de pulmão. Assim como para outros tipos de cânceres, tanto a inibição quanto a superativação de autofagia poderiam conferir um aumento da morte celular e impedir a progressão do tumor (Ryter & Choi, 2010). Diante destes fatos, a manipulação da atividade da via autofágica pode tornar-se uma aliada na terapia contra o câncer de pulmão.

2. OBJETIVOS

2.1. Objetivo geral

No presente estudo pretende-se avaliar o papel da autofagia induzida pela combinação do fármaco antineoplásico cisplatina com agentes indutores e inibidores da via autofágica em células de câncer de pulmão da linhagem H460.

2.2. Objetivos específicos

- Revisar os dados publicados sobre o papel da autofagia na resposta ao tratamento com agentes alquilantes.
- Avaliar a citotoxicidade e a indução de autofagia em células H460 após a exposição ao agente alquilante cisplatina.
- Determinar o papel da hiperestimulação da autofagia na viabilidade de células H460 utilizando a rapamicina em combinação com a cisplatina.
- Analisar a resposta de células H460 após o tratamento com cisplatina em combinação com a rapamicina, sendo esta administrada em pré-tratamento, cotratamento e pós-tratamento.
- Avaliar o efeito da inibição da autofagia sobre a viabilidade de células H460 tratadas com cisplatina em presença dos inibidores farmacológicos da via autofágica 3-metiladenina e cloroquina.

3. CAPÍTULO I

DNA ALKYLATION DAMAGE AND AUTOPHAGY INDUCTION

Artigo de revisão publicado na revista Reviews in Mutation Research

APRESENTAÇÃO

Neste capítulo será apresentado o artigo de revisão publicado em julho de 2013 na revista *Reviews in Mutation Research*, do qual sou segunda autora. O artigo revisa os principais agentes alquilantes, os quais são divididos em três categorias: agentes clássicos, nos quais estão incluídas as mostardas nitrogenadas, os alquilsulfonados, as arizidinas e as nitrozureas; agentes não-clássicos, que compreendem as triazinas; compostos platinados, também conhecidos como agentes alquilantes-*like*.

O artigo também aborda o papel da autofagia na homeostase celular e faz uma breve revisão sobre a maquinaria molecular da autofagia. Por fim, discute o papel da autofagia na quimioterapia com agentes alquilantes baseado em resultados de estudos realizados com modelos *in vitro* e *in vivo*.



Review

DNA alkylation damage and autophagy induction

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ABSTRACT

Many alkylating agents are used as chemotherapeutic drugs and have a long history of clinical application. These agents inflict a wide range of DNA damage resulting in a complex cellular response. After DNA damage, cells trigger a series of signaling cascades promoting cellular survival and cell cycle blockage which enables time for DNA repair to occur. More recently, induction of autophagy has been observed in cancer cells after treatment with different DNA-targeted anticancer drugs, including alkylating agents. Several studies have demonstrated that induction of autophagy after DNA damage delays apoptotic cell death and may therefore lead to chemoresistance, which is the limiting factor for successful chemotherapy. On the other hand, depending on the extent of damage and the cellular context, the induction of autophagy may also contribute to cell death. Given these conflicting results, many studies have been conducted to better define the role of autophagy in cancer cells in response to chemotherapy. In this review, we describe the main alkylating agents used in clinical oncology as well as the cellular response they evoke with emphasis on autophagy.

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1. Introduction

Alkylating agents were first used in cancer chemotherapy in the 1940s when nitrogen mustard was used to treat Hodgkin's

lymphoma [1]. Since then, alkylating agents have been included in practically all drug regimens used for cancer treatment. From a chemical point of view, alkylating agents act by transferring an alkyl group to a wide variety of biological molecules. They preferentially react with the N⁷-position of guanine in DNA and can directly react with DNA or undergo chemical activation [2]. During the last decade, the expression "alkylating agent" has increasingly been used to denote any compound able to bind covalently to DNA, which is particularly prominent in the

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medical literature. The modified DNA interferes with essential processes in cancer cells such as DNA replication and protein synthesis. Because these agents are also toxic to non-cancer cells, especially highly proliferative cells present in the bone marrow, the digestive system, ovaries and testicles, the design of novel alkylating agents that more specifically target cancer cells is needed to improve the outcome of cancer patients.

The regulation of cell death mechanisms plays an important role in both the pathogenesis of cancer and the response to therapy. In chemotherapy, the destruction of neoplastic cells is achieved not only by apoptosis, but also by necrosis, mitotic catastrophe and autophagy [3–6]. Autophagy seems to play a dual role in the cellular response to antineoplastic drugs. In some cases, activation of autophagy serves as a pro-death mechanism blocking tumor progression. Alternatively, autophagy may also contribute to tumor growth and survival. In this review, we discuss the role of autophagy in response to treatment with some of the most important alkylating agents used in clinical oncology.

2. Alkylating agents

Alkylating agents covalently modify DNA and are often mutagenic and cytotoxic. These agents act by transferring an alkyl, chloroethyl or, in the case of platinum compounds, a platinum group, to DNA in chemical reactions called nucleophilic substitutions. Nitrogen, oxygen and phosphates are common targets for alkylation, although the specificity of the reaction may vary widely for different alkylating agents. The N^7 -atom of guanine is particularly susceptible to alkylation. In addition, other atoms in the purine and pyrimidine bases of DNA, such as the N^1 - or N^3 -atoms of adenine, the N^3 - of cytosine, and the O^6 - of guanine, may be modified. RNA adducts and DNA-protein cross-links can also be formed and contribute to the cytotoxicity of alkylating agents [7].

Alkylating agents can be divided into three categories: classical, nonclassical and platinum compounds (Fig. 1), which can inflict a variety of lesions in the DNA molecule (Fig. 2). Moreover, these agents can be monofunctional or bifunctional. Monofunctional alkylating agents transfer a single alkyl group to DNA and the

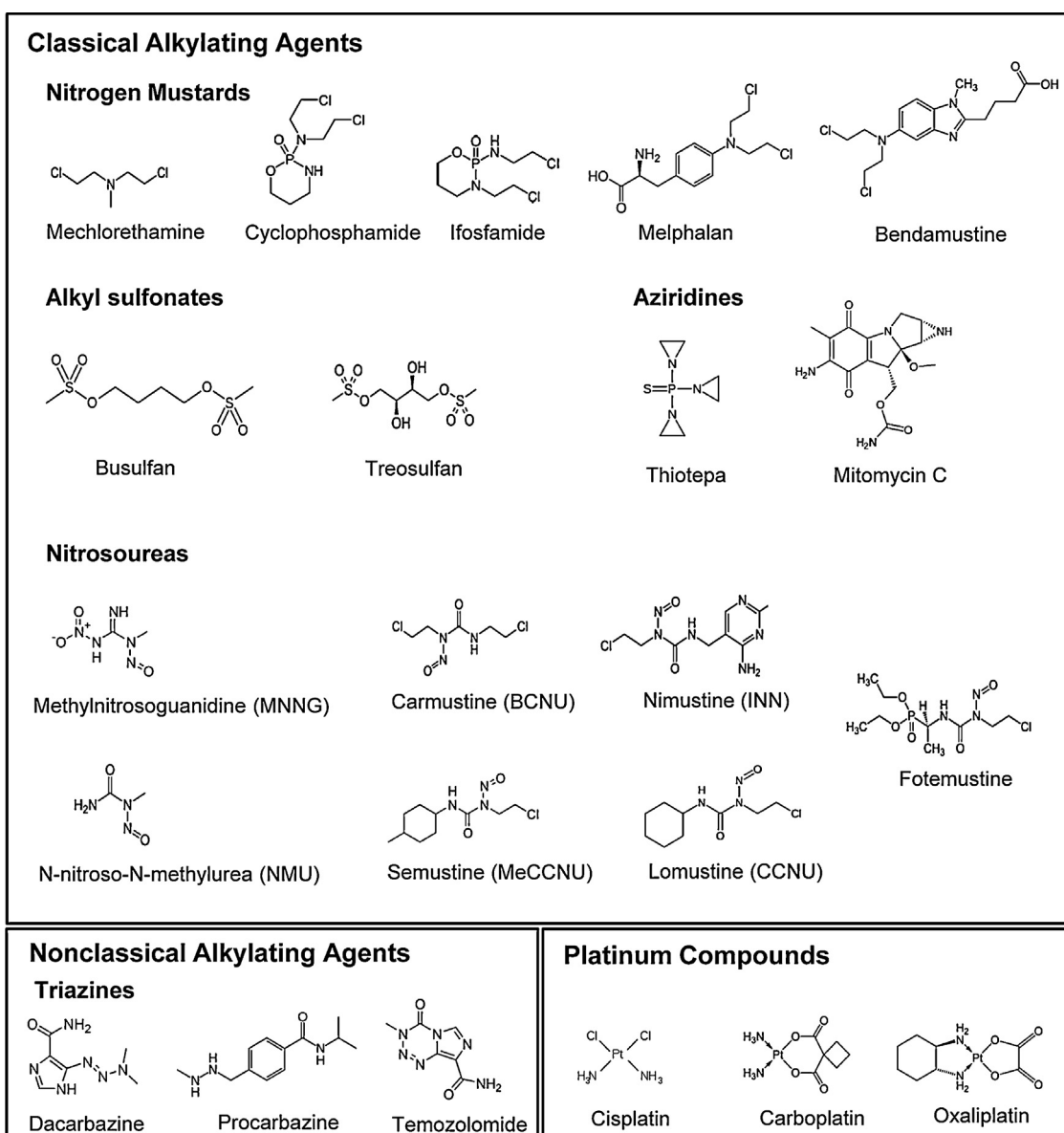


Fig. 1. Chemical structures of the main alkylating agents used in cancer therapy.

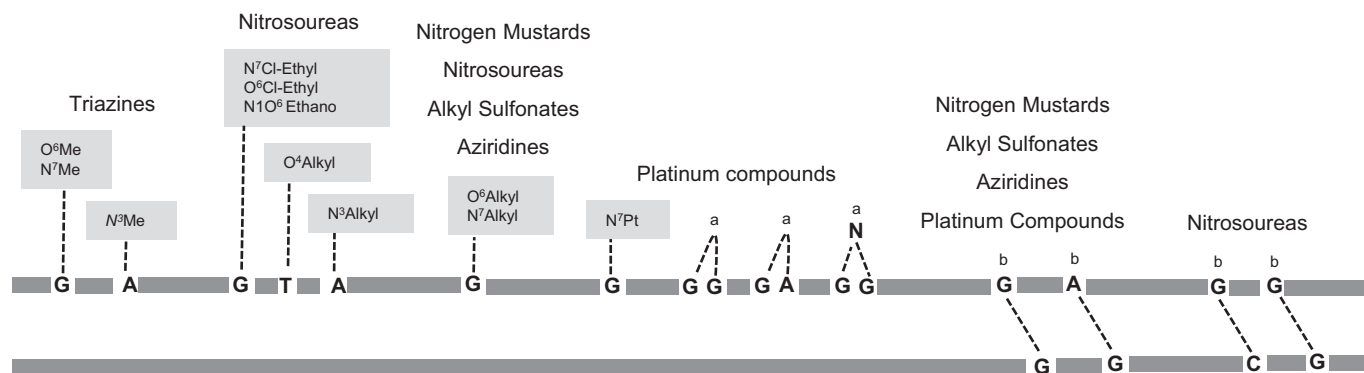


Fig. 2. Major lesions caused in DNA by different classes of alkylating agents. a: intrastrand cross-links; b: interstrand cross-links.

major adducts formed are N^7 -alkylguanine (N^7 -alkylG) and O^6 -alkylguanine (O^6 -alkylG). Bifunctional alkylators, on the other hand, can react at two different sites in the DNA resulting in the generation of a cross-link. If the resulting cross-link is between two complementary strands of DNA it is called an interstrand DNA crosslink (ICL) and if it is within the same strand of DNA it is known as an intrastrand DNA crosslink. Some natural compounds, as well as synthetic derivatives of natural alkylating agents, are able to form bulky covalent DNA adducts and therefore exhibit anticancer activity. These monofunctional alkylating agents represent an attractive alternative for the treatment of tumors resistant to conventional alkylating drugs since these compounds are able to bind covalently to the N^2 -atom of guanine in the minor groove of DNA and induce double strand breaks (DSBs) [8–12]. Although there are several compounds in clinical trials, some of them deserve special mention. These are: trabectedin, (ET-743, Yondelis[®]), an alkaloid isolated from the marine tunicate *Ecteinascidia turbinata* [13], lurbinectedin (PM01183) and Zalypsis[®] (PM00104), a jorumycin synthetic derivative [14,15], and S23906, a synthetic anticancer agent derived from acronycine [16]. These agents display therapeutic efficacy against human tumor xenografts and several tumor cell lines resistant to cisplatin, synergise with cisplatin when used to treat some tumor types [11,15,17].

In agreement with the diversity of DNA damage caused by alkylating agents, the cellular response to these drugs is quite complex. After DNA damage, cells orchestrate a series of responses that include the triggering of DNA damage checkpoints and cell cycle blockade. This cell cycle arrest enables additional time for DNA repair. If the DNA repair is not successful or if the DNA damage is too abundant, signaling cascades that lead to cell death will be activated.

In addition to apoptosis, which is considered the main cell death pathway activated in response to damage caused by alkylating agents, autophagy can also direct cells to death. There is currently no consensus on whether autophagic death should be considered as a form of programmed cell death or if it only represents dying cells with autophagic features [18]. Although cell death with autophagic features can occur at the same time as other cell death mechanisms, especially in cases where a massive amount of cells are going to be eliminated, autophagy is particularly associated with defective apoptosis [19,20]. Current data suggest that autophagy can have both cytoprotective and cytotoxic mechanism dependent on the tumor type, the type of chemotherapeutic drug used and the extent of DNA damage.

3. Alkylating agents and autophagy

Although three types of autophagic processes are known, namely microautophagy, chaperone-mediated autophagy and

macroautophagy, only the latter type will be discussed here and will be referred to as autophagy. However, it is worth noting that the common feature among the three types of autophagy is the delivery of cellular constituents to lysosomes for degradation.

Autophagy is a highly conserved evolutionary process that destroys cellular components producing ATP and macromolecules derived from the destroyed components. Stimuli such as hypoxia [21], infections caused by viruses and bacteria [22], oxidative stress [23], starvation [24] and DNA damage [25] are responsible for triggering autophagy. Both autophagy and the proteasome system are able to degrade proteins, but only autophagy is capable of degrading entire organelles. For this reason, when compared to the proteasome system, autophagy is normally considered a less selective process of degrading both biomolecules and more complex cellular components. However, some organelles such as mitochondria, endoplasmic reticulum and peroxisome can be selectively degraded in processes called mitophagy, reticulophagy and peroxophagy, respectively [26].

3.1. Role of autophagy in cellular homeostasis

In the first instance, activation of autophagy serves as a cellular mechanism for quality control by promoting the degradation and elimination of damaged cellular constituents, intracellular pathogens, apoptotic cells and long-lived proteins [27,28]. In addition to recycling cellular components, autophagy is involved in the development, differentiation and remodeling of tissues of various organisms [29]. Failure to remove damaged cellular components leads to tissue damage and genetic instability, which can promote and accelerate tumorigenesis [30]. The role of autophagy as a tumor suppressor was first demonstrated in mice with a monoallelic deletion of Beclin 1 [31]. Beclin 1 is an essential protein recruited early during the autophagic process. A higher incidence of spontaneous tumors, such as liver cancer, lung cancer and lymphomas, was observed in *Beclin 1*^{+/-} compared with *Beclin 1*^{+/+} mice. Interestingly, there was no loss of heterozygosity in tumor cells derived from *Beclin 1*^{+/-} mice, suggesting that a reduced level, but not an absence of autophagy, is pro-tumoral. Accordingly, and confirming the essentiality of the gene, *Beclin 1*^{-/-} mice die early in embryogenesis [32].

Furthermore, autophagy can be induced by changes in environmental conditions, such as nutrient depletion. In this case, autophagy provides energy and amino acids necessary to allow cell survival under metabolic stress [33]. Hence, autophagy can be considered as a form of protection against various types of injuries and stress. However, persistent stress and therefore the prolonged activation of autophagic pathway can lead to excessive degradation of intracellular constituents and cell death with autophagic features [18].

3.2. Molecular machinery of autophagy

The molecular machinery involved in the control of autophagy is modulated by different signaling pathways. The major regulator of autophagy is the mammalian target of rapamycin complex I (mTORC1), which is regulated by the class I phosphoinositide 3-kinase/Akt (PI3K/Akt) pathway. PI3K/Akt responds to nutrient availability in the environment through growth factors activating mTORC1 thereby controlling cell growth and autophagy. AMPK, another protein kinase involved in the regulation of autophagy, acts by sensing the decrease in the ratio of intracellular ATP/AMP and is a positive regulator of autophagy [34].

In response to genotoxic stress, autophagy can be induced through the signaling pathways summarized in Fig. 3. These pathways link the DNA damage response involving ATM, Chk1/2 and p53 to the autophagy-regulating machinery, mainly AMPK and mTORC1. p53 can either activate AMPK, which inhibits mTORC1 thereby inducing autophagy, or induce the expression of the damage-regulated autophagy modulator (DRAM), a lysosomal protein facilitating the autophagic process. Interestingly, p53, one of the most important tumor suppressor genes, has been described as both a positive and a negative regulator of autophagy depending on its subcellular localization. In contrast to nuclear p53, cytoplasmic p53 prevents autophagy in a poorly characterized manner [35].

Autophagy starts with the nucleation of membranes that are expanded in an elongation step to form double-membrane vesicles called autophagosomes, a morphological hallmark of autophagy. The formation and expansion of the autophagosomes are mediated by autophagy-specific genes (Atg), and their products have been described as markers for autophagy. Beclin-1 and LC3 (the mammalian orthologs of the yeast genes Atg 6 and Atg 8, respectively) are the most widely autophagic proteins studied.

Beclin 1 is one of the first proteins to be recruited to the formation of autophagosomes whereas LC3 is integrated in the membrane of autophagosomes during the elongation step. Autophagosomes envelop the cellular material to be degraded and fuse it with lysosomes forming the autolysosomes. Once in contact with lysosomal hydrolases, proteins and organelles contained in autolysosomes are degraded and free amino acids are released.

3.3. Role of autophagy in alkylating chemotherapy

Chemotherapeutic drugs that target the DNA molecule frequently initiate autophagy in cancer cells. Although autophagy has a beneficial effect as a tumor suppressor in healthy cells, induction of autophagy may promote survival of tumor cells leading to resistance to cell death. On the other hand, depending on the extent of damage and the cellular context, autophagy may also be the mediator of a non-apoptotic form of cell death called autophagic cell death. Several studies focusing in elucidating the role of autophagy in cancer cells treated with DNA damaging agents are shown in Table 1. Most of the results obtained with alkylating agents in different cancer cell lines indicate that autophagy plays a cytoprotective role. Although the activation of autophagy and apoptosis can occur simultaneously, the activation of autophagy can reduce and/or delay cell death by apoptosis. Despite the complexity of the relationship between apoptotic and autophagic pathways, an important mechanism that appears to be involved in the reduction of apoptosis by activation of autophagy is the removal of damaged mitochondria [36]. Therefore, in a scenario where the apoptotic pathway is functional, elimination of mitochondria would avoid the release of proapoptotic molecules, such as cytochrome c and apoptosis inducing factor (AIF). In contrast, where apoptosis is inhibited or defective, autophagy can be activated and contribute to cell death [37].

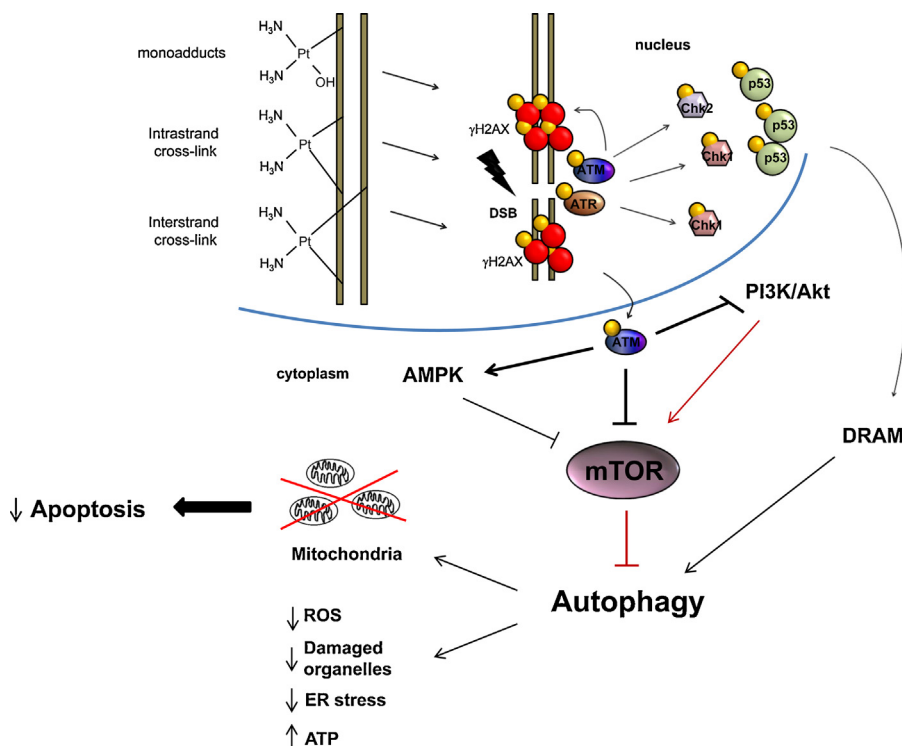


Fig. 3. DNA alkylating damage and autophagy induction. Different DNA damage caused by alkylating agents (here represented by cisplatin adducts) induce double-strand breaks (DSBs) in DNA. In response to DSB, the PI3K-like kinases ATM and ATR kinases are activated. Then, H2AX is rapidly phosphorylated by ATM and ATR, to form γH2AX. Other downstream targets of activated ATM and ATR include the checkpoint kinases Chk1 and Chk2 which in turn phosphorylate and stabilize p53. ATM, in addition to its central role in the nucleus, is able to activate AMPK and repress PI3K/Akt and mTOR to stimulate autophagy in the cytoplasm. p53 can also affect key regulatory components of the autophagic pathway by inducing the expression of DRAM thereby activating AMPK and/or inhibiting mTOR.

Table 1
Alkylating agents and autophagy in different *in vivo* and *in vitro* models.

Class of alkylating agent	Alkylating agent	Mechanism of DNA damage	Tumor type/cell line	Autophagy inhibitor	Apoptosis detection	Role of autophagy	Reference
Nitrogen mustard	Cyclophosphamide	Interstrand crosslink	Mouse Myc-induced xenograft model of lymphoma	CQ ^a	Positive	Cytoprotective	[38]
	Melphalan	Interstrand crosslink	Multiple myeloma cells	3MA ^b /HCQ ^c /siRNA Atg5/siRNA Beclin-1	Positive	Cytoprotective	[39]
	N-mustard BO-1051	Interstrand crosslink	Hepatocellular carcinoma cells	E64d/pepstatin A/BafA1 ^d /shRNA Beclin-1/siRNA p62/SQSTM1	Positive	Cytoprotective	[40]
	N-mustard BO-1051	Interstrand crosslink	Malignant glioma cells	3MA/BafA1/shRNA Beclin 1	Positive	Cytoprotective	[41]
Nitrosourea	MNNG	Monoadducts and interstrand crosslink	Mouse Myc-induced model of lymphoma cells	CQ	Positive	Cytoprotective	[38]
Platinum compound	Cisplatin	Intrastrand and interstrand crosslink	Hepatocellular carcinoma cells	3MA/siRNA Atg5	Positive	Cytoprotective	[42]
			Hepatocellular carcinoma cells	3MA/CQ/siRNA Beclin-1	Positive	Cytoprotective	[43]
			Cervical cancer cells	3MA/CQ	Positive	Cytoprotective	[44]
			Colorectal cancer, breast adenocarcinoma and cervical carcinoma cells	CQ/andrographolide	Positive	Cytoprotective	[45]
			Esophageal cancer cells	3MA	Positive	Cytoprotective	[46]
			Skin carcinoma cells	3MA/siRNA Atg5	Positive	Cytoprotective	[47]
	E-platinum	Intrastrand and interstrand crosslink	Lung adenocarcinoma cells	3MA	Positive	Cytoprotective	[48]
			Malignant glioma cells	BafA1/CQ/wortmannin	Positive	Cytoprotective	[49]
			Gastric carcinoma cells	3MA/CQ	Not verified	Cytotoxic	[50]
			Hepatocellular carcinoma cells	CQ/siRNA Atg7	Positive	Cytoprotective	[51]
Oxaliplatin	Intrastrand and interstrand crosslink	Hepatocellular carcinoma cells	CQ/siRNA Atg5	Positive	Cytoprotective	[52]	
		Hepatocellular carcinoma cells	CQ	Positive	Cytoprotective	[53]	
		Gastric cancer cells	CQ	Positive	Cytoprotective	[53]	
		Gastric cancer cells	CQ	Positive	Cytoprotective	[53]	
Triazine	Temozolomide	Monoadducts	Malignant glioma cells and xenograft model	3MA/BafA1/CQ/resveratrol	Positive	Cytoprotective	[54]
			Apoptosis-resistant malignant glioma cells	siRNA Beclin-1/siRNA Atg5	Negative	Cytotoxic	[55]
			Malignant glioma cells	3MA/siRNA Beclin-1	Positive	Cytoprotective	[56]
			Malignant glioma cells	Baf A1/3MA	Positive	Cytoprotective	[57]
			Resistant malignant glioma cells	3MA	Negative	Cytotoxic	[58]

^a Chloroquine (CQ).

^b 3-Methyladenine (3MA).

^c Hydrochloroquine (HCQ).

^d Bafilomycin A1 (BafA1).

To determine the physiological relevance of autophagy, a variety of autophagy inhibitors have been employed in combination with alkylating treatments, since inhibition of autophagy at different steps of the pathway may produce different cellular responses [57]. Besides the knockdown/knockout of autophagic genes (Atg 5, Atg 7 and Beclin 1, for example), several pharmacological inhibitors have been employed to investigate autophagy. The most commonly used inhibitors are 3-methyladenine (3MA), wortmannin and LY294002; these inhibitors block autophagy at an early stage by blocking class III PI3K activity (required to initiate autophagy) and interfering with the formation of autophagosomes. However, these compounds are not specific autophagy inhibitors and may also inhibit class I PI3K activity (which inhibits autophagy), therefore promoting autophagy in cells exposed to these inhibitors for long periods [59]. Chloroquine (CQ), an antimalarial drug, is capable of inhibiting the lysosomal function resulting in accumulation of autophagosomes. Similar to CQ, pepstatin A and E64d are also inhibitors of lysosomal proteases whereas Bafilomycin A1 (BafA1) is a specific inhibitor of vacuolar-type H⁺-ATPase. It should be emphasized that autophagy inhibition

at the lysosomal stage leads to the accumulation of autophagosomes, whereas inhibition at the earlier steps reduces the amount of autophagosomes in the cell. The accumulation of autophagosomes resulting from the use of inhibitors of lysosomal function can be cytotoxic. Large amounts of ineffective autophagosomes lead to mitochondrial and lysosomal membrane permeabilization causing activation of caspase-3 and apoptotic cell death [57]. On the other hand, inhibition of the initial steps of the autophagic pathway, which prevents the formation of autophagosomes, leads to the accumulation of polyubiquitinated protein aggregates and unfolded/misfolded proteins in the endoplasmic reticulum [60]. Additionally, inhibition of autophagy reduces ATP levels leading to mitotic catastrophe in gliomas treated with temozolomide and restoration of ATP levels with a permeable analog of pyruvate prevented mitotic catastrophe, indicating that autophagy-generated ATP is important for cell survival [56].

As shown in Table 1, the majority of studies show that in the presence of alkylating agents, autophagy has a cytoprotective role. For example, treatment of a Myc-induced model of lymphoma with the nitrosourea MNNG combined with CQ lead to enhanced cell

death, when compared with MNNG treatment alone, showing that autophagy served as a survival mechanism in response to DNA damage [38]. Results for multiple myeloma treated with low doses of melphalan and hepatocarcinoma treated with BO-1051, a newly synthesized nitrogen mustard with high DNA affinity, also indicated that adding two lysosomal protease inhibitors, E64d and pepstatin A, potentiates the effects of DNA alkylating therapy by increasing the fraction of apoptotic cells [39,40].

Chen et al. [40] demonstrated that autophagy induced by BO-1051 is a downstream target of the ATM signaling pathway. Alkylating agents, such as nitrogen mustards, generate DSBs during DNA repair recruiting DNA damage activated kinases. Faced with these lesions, ATM, together with ATR and their signal-transducer molecules Chk1/2 are engaged to arrest the cell cycle. Moreover, a cytoplasmic role of ATM has been described, showing that ATM activation suppresses mTORC1 via LKB1/AMPK metabolic pathway, which in turn induces autophagy [61]. This cytoplasmic pathway linking DNA damage response and autophagy, and more precisely ATM and autophagy, may have important therapeutic implications in cancer. However, the connection seems to be much more complex considering the variety of proteins and steps involved in each signaling pathway. In this case, the kinase ATM, as well as the transducers in the DNA damage signaling, Chk1 and Chk2, may be involved in autophagy induction in cancer cells treated with chemotherapeutic agents.

Platinum derived compounds primarily induce the formation of DNA intrastrand crosslinks, but can also induce the formation of ICLs. As listed in Table 1, cells treated by platinum compounds can induce autophagy as a way to prevent cell death. Due to their clinical importance, many studies using different cell types have been carried out to elucidate the role of autophagy in response to platinum agents. As an example, autophagy has been shown to play a role in the resistance of cancer cells to cisplatin, the first platinum drug ever used in cancer treatment. Different studies have shown increased expression of LC3II and Beclin 1 and an increase in the number of autophagosomes after cisplatin exposure [46]. Cisplatin triggers autophagy by activation of AMPK and downregulation of mTOR, thereby protecting glioma cells from oxidative stress, caspase activation and DNA fragmentation [49]. In HCT116 colon cancer cells treated with cisplatin, CQ inhibits the prosurvival function of autophagy by increasing PARP and caspase 3 cleavage [45]. Furthermore, it has been shown that cisplatin can induce the accumulation of misfolded proteins leading to endoplasmic reticulum stress that can cause cell death mainly by apoptosis. However, Xu et al. [44] demonstrated that while

treatment with cisplatin induces endoplasmic reticulum stress, cisplatin also activates autophagy, which is implicated in reducing the ubiquitinated proteins thereby preventing apoptosis induced by endoplasmic reticulum stress in HeLa cells.

Oxaliplatin is reported to induce autophagy in certain tumor cell lines. Hepatocellular carcinoma and gastric cancer cells treated with oxaliplatin show conversion of the LC3 cytoplasmic form (LC3I) to the autophagosome-integrated form (LC3II) indicating the induction of autophagy [51,52]. As well as cisplatin, oxaliplatin seems to induce autophagy as a protective mechanism. Although less is known about the role of autophagy in the cytotoxicity exerted by oxaliplatin, the mechanism of autophagy induction seems to be very similar to the one induced by cisplatin. Oxaliplatin prevents phosphorylation of Akt and mTOR and may induce autophagy by inhibiting PI3K/Akt/mTOR pathway [53]. Autophagy inhibition induced by oxaliplatin with CQ or 3MA enhances the production of reactive oxygen species (ROS), which contributes to death of hepatocarcinoma cells [52].

In clinical treatment protocols, different regimens are usually combined in order to prevent drug resistance and to achieve better survival outcomes. Combining high-LET radiation with oxaliplatin to treat glioma cells, Benzina et al. [62] found an increase in the number of DSBs in DNA (when compared to each single treatment), as detected by increased phosphorylation of the H2AX histone (γ H2AX), a marker of DSB formation. The increase in DNA damage correlated with the induction of autophagy, and only very low levels of apoptosis were observed. More recently, Altemeyer et al. [63] used hepatocellular carcinoma cells treated with oxaliplatin and high-LET radiation. The authors showed a positive correlation between DSB formation and autophagy activation when DNA repair was blocked by concomitant administration of an inhibitor for PARP, a protein essential for efficient single-strand break repair. Combining oxaliplatin with high-LET radiation markedly increased autophagy and reduced cell growth.

A study with E-platinum, a newly synthesized platinum drug with a structure similar to oxaliplatin, showed that autophagy induction in gastric cancer *in vitro* and *in vivo* plays an important role in the inhibition of cell growth [50]. The mechanism that leads to induction of autophagy by E-platinum is not well understood, but inhibition of mTOR seems to be the key event to trigger the autophagic pathway. In addition, E-platinum suppressed the phosphorylation of Akt, ERK1/2 and p38MAPK in a time-dependent manner.

More recently, the alkylating agent temozolomide was shown to induce autophagy in malignant glioma cells [57,64]. Different

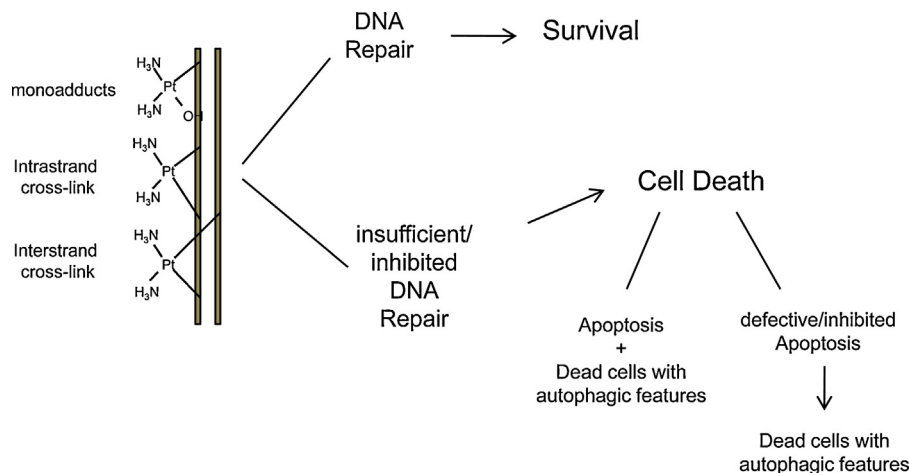


Fig. 4. DNA alkylating damage (here represented by cisplatin adducts) leads to cell death with autophagic features in cases of insufficient/inhibited DNA repair and defective/inhibited apoptosis.

studies have demonstrated a protective role of autophagy in cells treated with temozolomide [56]. The autophagy induced in response to treatment with temozolomide degrades cellular biomolecules and provides metabolic substrates to the mitochondria. This results in increasing of intracellular ATP levels, which protects cells from death characterized by multimicronucleation [56]. Temozolomide mediated-autophagy seems to happen through an increase of ROS levels and ERK activation. Inhibiting ROS production with resveratrol is synergistic with temozolomide most likely by reducing the autophagic flux and increasing apoptotic cell death [54].

Exceptions for this pattern of cytoprotection have been seen in treatments where autophagy served as a mechanism of cell death as represented in Fig. 4. Apoptosis-resistant glioma cells treated with temozolomide and (–)-gossypol, a pan-Bcl2 inhibitor, showed a significant increase in cell death compared to cells that received only temozolomide. Cell death was characterized as caspase-independent and presented increased autophagosome formation which led the authors to conclude that the cells underwent autophagic cell death [55]. In order to control autophagy, the anti-apoptotic Bcl-2 and Bcl-x_L proteins sequester Beclin 1 preventing the assembly of proteins involved in vesicle nucleation that initiate the autophagosome formation [65]. Therefore, using a pan-Bcl2 inhibitor, Beclin 1 is released and can initiate the autophagosome formation which can explain the high number of autophagosomes and consequently cell death. In other cases, autophagy is induced, but has no significant role in the outcome for cells treated with temozolomide [66].

MGMT is the enzyme responsible to repair monoadducts induced by temozolomide in DNA, which is the major cause of resistance in the treatments of gliomas. The autophagic cell death was potentiated when MGMT negative cells received temozolomide and (–)-gossypol, indicating that DNA repair inhibition can be an important aspect that contributes to autophagic cell death [55]. Inhibiting DNA repair with O⁶-Benzylguanine, a MGMT inhibitor, Kanzawa et al. [58] demonstrated that resistant glioma cells treated with temozolomide underwent autophagic cell death. Although the studies with temozolomide seem conflicting, it is worthwhile to emphasize that autophagy seems to serve as a cell death mechanism only in cases where DNA repair and apoptosis are inhibited or defective.

4. Concluding remarks

Alkylating agents is a major class of cancer chemotherapeutic drugs. Since their initial use in clinical oncology, many new compounds with higher specificity and lower toxicity have been discovered and are currently used to treat a wide variety of tumors. Due to their crucial importance in cancer chemotherapy, there is great interest in investigating the mechanisms of cellular response to alkylation employed by cancer cells.

Alkylating agents react with DNA and other macromolecules, producing a broad range of cellular damage. The cellular responses to alkylation are, therefore, complex and can result in the activation of cell cycle arrest, survival signaling and DNA repair, or of cell death pathways, depending on the context and amount of cellular damage. Recently, autophagy has been described as an important factor in the response to DNA damage. Whether this response contributes or not to the survival of cancer cells is still a matter of debate.

Induction of autophagy in cancer cells during chemotherapy has been reported to prolong cell survival by delaying apoptosis and leading to chemoresistance. However, chemoresistance can be prevented in the presence of pharmacological or genetic inhibitors of the autophagic pathway. Several reported studies indicate that co-treatment with autophagy inhibitors and alkylating agents

leads to an increase in alkylation sensitivity of tumor cells in culture. However, studies demonstrating the efficacy of inhibition of autophagy combined with alkylating drugs in *in vivo* models are still scarce. However, among the few *in vivo* data available, the co-treatment of HCQ or CQ with alkylating agents was shown to significantly increase tumor suppression and delay tumor recurrence [38,39]. Furthermore, these inhibitors are well tolerated and not toxic. In humans, only HCQ and CQ inhibitors have been tested in combination with conventional chemotherapy. Several studies now in preclinical phase are being conducted combining these inhibitors with chemotherapeutic drugs [30]. However, temozolomide is the only alkylating agent so far tested. Currently, clinical phase I/II trials combining temozolomide and HCQ are being conducted in order to find the concentration of HCQ able to inhibit autophagy and improve treatment as well as to identify potential side effects of the combined use of both drugs [30]. In addition, it would be interesting to assess if the efficacy of autophagy inhibition is modified whether it is administered before, during or post-alkylating agent treatment.

Treatment with alkylating agents seems, in general, to induce autophagy to support cell survival during genotoxic stress by maintaining sufficiently high intracellular ATP levels to allow DNA repair, by reducing ubiquitinated proteins and subsequent endoplasmic reticulum stress, and by clearing damaged organelles. Selective autophagy of damaged organelles, like mitochondria, may prevent the production of ROS and the release of cytochrome c, a potent apoptotic trigger. However, in association with DNA repair inhibition and defective apoptosis, autophagy is associated with cell death. In these cases, the use of autophagy inducers combined with DNA repair inhibitors and chemotherapeutic agents might provide a new and attractive approach in cancer treatment. Some studies suggest a link between the DNA damage response and autophagy, demonstrating that DNA lesions, such as intrastrand and interstrand DNA cross-links, induce autophagy. Although this connection is not fully elucidated, the investigation of new chemotherapeutic regimens that can simultaneously modulate DNA damage signaling and recruitment of repair machinery, as well as the induction of autophagy, especially in apoptosis defective cells, provides a new and exciting avenue for cancer research and treatment. However, more experimental data are needed before we can identify potential therapeutic targets that integrate the process of autophagy, DNA repair and/or DNA damage response in chemotherapy.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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References

- [1] L.H. Hurley, DNA its associated processes as targets for cancer therapy, *Nat. Rev. Cancer* 2 (2002) 188–200.
- [2] J.A. Hartley, J.P. Bingham, R.L. Souhami, DNA sequence selectivity of guanine-N7 alkylation by nitrogen mustards is preserved in intact cells, *Nucleic Acids Res.* 20 (1992) 3175–3178.

- [3] A. Notte, L. Leclere, C. Michiels, Autophagy as a mediator of chemotherapy-induced cell death in cancer, *Biochem. Pharmacol.* 82 (2011) 427–434.
- [4] W.X. Zong, D. Ditsworth, D.E. Bauer, Z.Q. Wang, C.B. Thompson, Alkylating DNA damage stimulates a regulated form of necrotic cell death, *Genes Dev.* 18 (2004) 1272–1282.
- [5] R.R. Gordon, P.S. Nelson, Cellular senescence and cancer chemotherapy resistance, *Drug Resist. Updat.* 15 (2012) 123–131.
- [6] M.S. Ricci, W.X. Zong, Chemotherapeutic approaches for targeting cell death pathways, *Oncologist* 11 (2006) 342–357.
- [7] E.D. Michaelson-Richie, X. Ming, S.G. Codreanu, R.L. Loeber, D.C. Liebler, C. Campbell, N.Y. Tretyakova, Mechlorethamine-induced DNA-protein cross-linking in human fibrosarcoma (HT1080) cells, *J. Proteome Res.* 10 (2011) 2785–2796.
- [8] D.G. Soares, A.E. Escargueil, V. Poindessous, A. Sarasin, A. de Gramont, D. Bonatto, J.A. Henriques, A.K. Larsen, Replication and homologous recombination repair regulate DNA double-strand break formation by the antitumor alkylator ecteinascidin 743, *Proc. Natl. Acad. Sci. U.S.A.* 104 (2007) 13062–13067.
- [9] S. Leonce, L. Kraus-Berthier, R.M. Golsteyn, M.H. David-Cordonnier, C. Tardy, A. Lansiaux, V. Poindessous, A.K. Larsen, A. Pierre, Generation of replication-dependent double-strand breaks by the novel N2-G-alkylator S23906-1, *Cancer Res.* 66 (2006) 7203–7210.
- [10] J. Guirouilh-Barbat, S. Antony, Y. Pommier, Zalypsis (PM00104) is a potent inducer of gamma-H2AX foci and reveals the importance of the C ring of trabectedin for transcription-coupled repair inhibition, *Mol. Cancer Ther.* 8 (2009) 2007–2014.
- [11] D.G. Soares, M.S. Machado, C.J. Rocca, V. Poindessous, D. Ouaret, A. Sarasin, C.M. Galmarini, J.A. Henriques, A.E. Escargueil, A.K. Larsen, Trabectedin its C subunit modified analogue PM01183 attenuate nucleotide excision repair and show activity toward platinum-resistant cells, *Mol. Cancer Ther.* 10 (2011) 1481–1489.
- [12] M.H. David-Cordonnier, W. Laine, A. Lansiaux, F. Rosu, P. Colson, E. de Pauw, S. Michel, F. Tillequin, M. Koch, J.A. Hickman, A. Pierre, C. Bailly, Covalent binding of antitumor benzoacronycines to double-stranded DNA induces helix opening and the formation of single-stranded DNA: unique consequences of a novel DNA-bonding mechanism, *Mol. Cancer Ther.* 4 (2005) 71–80.
- [13] Y. Pommier, G. Kohlhaagen, C. Bailly, M. Waring, A. Mazumder, K.W. Kohn, DNA sequence- and structure-selective alkylation of guanine N2 in the DNA minor groove by ecteinascidin 743, a potent antitumor compound from the Caribbean tunicate *Ecteinascidia turbinata*, *Biochemistry* 35 (1996) 13303–13309.
- [14] A. Vidal, C. Munoz, M.J. Guillen, J. Moreto, S. Puertas, M. Martinez-Iniesta, A. Figueras, L. Padullés, F.J. Garcia-Rodríguez, M. Berdiel-Acer, M.A. Pujana, R. Salazar, M. Gil-Martin, L. Marti, J. Ponce, D.G. Mollevi, G. Capella, E. Condom, F. Vinals, D. Huertas, C. Cuevas, M. Esteller, P. Aviles, A. Villanueva, Lurbinecetin (PM01183) a new DNA minor groove binder, inhibits growth of orthotopic primary graft of cisplatin-resistant epithelial ovarian cancer, *Clin. Cancer Res.* 18 (2012) 5399–5411.
- [15] E.M. Ocio, P. Maiso, X. Chen, M. Garaya, S. Alvarez-Fernandez, L. San-Segundo, D. Vilanova, L. Lopez-Corral, J.C. Montero, T. Hernandez-Iglesias, E. de Alava, C. Galmarini, P. Aviles, C. Cuevas, J.F. San-Miguel, A. Pandiella, Zalypsis: a novel marine-derived compound with potent antimyeloma activity that reveals high sensitivity of malignant plasma cells to DNA double-strand breaks, *Blood* 113 (2009) 3781–3791.
- [16] M.H. David-Cordonnier, W. Laine, A. Lansiaux, M. Kouach, G. Briand, A. Pierre, J.A. Hickman, C. Bailly, Alkylation of guanine in DNA by S23906-1, a novel potent antitumor compound derived from the plant alkaloid acronycine, *Biochemistry* 41 (2002) 9911–9920.
- [17] M. D'Incalci, T. Colombo, P. Ubezio, I. Nicoletti, R. Giavazzi, E. Erba, L. Ferrarese, D. Mecco, R. Riccardi, C. Sessa, E. Cavallini, J. Jimeno, G.T. Faircloth, The combination of yondelis and cisplatin is synergistic against human tumor xenografts, *Eur. J. Cancer* 39 (2003) 1920–1926.
- [18] S. Shen, O. Kepp, G. Kroemer, The end of autophagic cell death? *Autophagy* 8 (2012) 1–3.
- [19] E. Buytaert, G. Callewaert, J.R. Vandenheede, P. Agostinis, Deficiency in apoptotic effectors Bax and Bak reveals an autophagic cell death pathway initiated by photodamage to the endoplasmic reticulum, *Autophagy* 2 (2006) 238–240.
- [20] K.N. Dalby, I. Tekedereli, G. Lopez-Berestein, B. Ozpolat, Targeting the prodeath and prosurvival functions of autophagy as novel therapeutic strategies in cancer, *Autophagy* 6 (2010) 322–329.
- [21] N.M. Mazure, J. Pouyssegur, Hypoxia-induced autophagy: cell death or cell survival? *Curr. Opin. Cell Biol.* 22 (2010) 177–180.
- [22] A. Orvedahl, B. Levine, Eating the enemy within: autophagy in infectious diseases, *Cell Death Differ.* 16 (2009) 57–69.
- [23] J. Lee, S. Giordano, J. Zhang, Autophagy, mitochondria and oxidative stress: cross-talk and redox signalling, *Biochem. J.* 441 (2012) 523–540.
- [24] L. Shang, S. Chen, F. Du, S. Li, L. Zhao, X. Wang, Nutrient starvation elicits an acute autophagic response mediated by Ulk1 dephosphorylation and its subsequent dissociation from AMPK, *Proc. Natl. Acad. Sci. U.S.A.* 108 (2011) 4788–4793.
- [25] H. Rodriguez-Rocha, A. Garcia-Garcia, M.I. Panayiotidis, R. Franco, DNA damage and autophagy, *Mutat. Res.* 711 (2011) 158–166.
- [26] S. Alers, A.S. Loffler, S. Wesselborg, B. Stork, The incredible ULKs, *Cell Commun. Signal.* 10 (2012) 7.
- [27] B. Levine, G. Kroemer, Autophagy in the pathogenesis of disease, *Cell* 132 (2008) 27–42.
- [28] E. White, C. Karp, A.M. Strohecker, Y. Guo, R. Mathew, Role of autophagy in suppression of inflammation and cancer, *Curr. Opin. Cell Biol.* 22 (2010) 212–217.
- [29] B. Levine, D.J. Klionsky, Development by self-digestion: molecular mechanisms and biological functions of autophagy, *Dev. Cell* 6 (2004) 463–477.
- [30] Z.J. Yang, C.E. Chee, S. Huang, F.A. Sinicrope, The role of autophagy in cancer: therapeutic implications, *Mol. Cancer Ther.* 10 (2011) 1533–1541.
- [31] X. Qu, J. Yu, G. Bhagat, N. Furuya, H. Hibshoosh, A. Troxel, J. Rosen, E.L. Eskelinen, N. Mizushima, Y. Ohsumi, G. Cattoretti, B. Levine, Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene, *J. Clin. Invest.* 112 (2003) 1809–1820.
- [32] Z. Yue, S. Jin, C. Yang, A.J. Levine, N. Heintz, Beclin 1 an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor, *Proc. Natl. Acad. Sci. U.S.A.* 100 (2003) 15077–15082.
- [33] G. Kroemer, G. Marino, B. Levine, Autophagy and the integrated stress response, *Mol. Cell* 40 (2010) 280–293.
- [34] Y. Li, J. Zhang, X. Chen, T. Liu, W. He, Y. Chen, X. Zeng, Molecular machinery of autophagy and its implication in cancer, *Am. J. Med. Sci.* 343 (2012) 155–161.
- [35] M.C. Maiuri, L. Galluzzi, E. Morselli, O. Kepp, S.A. Malik, G. Kroemer, Autophagy regulation by p53, *Curr. Opin. Cell Biol.* 22 (2010) 181–185.
- [36] D. Gozuacik, A. Kimchi, Autophagy as a cell death and tumor suppressor mechanism, *Oncogene* 23 (2004) 2891–2906.
- [37] M.C. Maiuri, E. Zalckvar, A. Kimchi, G. Kroemer, Self-eating and self-killing: crosstalk between autophagy and apoptosis, *Nat. Rev. Mol. Cell Biol.* 8 (2007) 741–752.
- [38] R.K. Amaravadi, D. Yu, J.J. Lum, T. Bui, M.A. Christophorou, G.I. Evan, A. Thomas-Tikhonenko, C.B. Thompson, Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma, *J. Clin. Invest.* 117 (2007) 326–336.
- [39] Y. Pan, Y. Gao, L. Chen, G. Gao, H. Dong, Y. Yang, B. Dong, X. Chen, Targeting autophagy augments in vitro and in vivo antimyeloma activity of DNA-damaging chemotherapy, *Clin. Cancer Res.* 17 (2011) 3248–3258.
- [40] L.H. Chen, C.C. Loong, T.L. Su, Y.J. Lee, P.M. Chu, M.L. Tsai, P.H. Tsai, P.H. Tu, C.W. Chi, H.C. Lee, S.H. Chiou, Autophagy inhibition enhances apoptosis triggered by BO-1051, an N-mustard derivative, and involves the ATM signaling pathway, *Biochem. Pharmacol.* 81 (2011) 594–605.
- [41] P.M. Chu, L.H. Chen, M.T. Chen, H.I. Ma, T.L. Su, P.C. Hsieh, C.S. Chien, B.H. Jiang, Y.C. Chen, Y.H. Lin, Y.H. Shih, P.H. Tu, S.H. Chiou, Targeting autophagy enhances BO-1051-induced apoptosis in human malignant glioma cells, *Cancer Chemother. Pharmacol.* 69 (2012) 621–633.
- [42] R. Chen, R.Y. Dai, C.Y. Duan, Y.P. Liu, S.K. Chen, D.M. Yan, C.N. Chen, M. Wei, H. Li, Unfolded protein response suppresses cisplatin-induced apoptosis via autophagy regulation in human hepatocellular carcinoma cells, *Folia Biol. (Praha)* 57 (2011) 87–95.
- [43] X.L. Guo, D. Li, F. Hu, J.R. Song, S.S. Zhang, W.J. Deng, K. Sun, Q.D. Zhao, X.Q. Xie, Y.J. Song, M.C. Wu, L.X. Wei, Targeting autophagy potentiates chemotherapy-induced apoptosis and proliferation inhibition in hepatocarcinoma cells, *Cancer Lett.* 320 (2012) 171–179.
- [44] Y. Xu, H. Yu, H. Qin, J. Kang, C. Yu, J. Zhong, J. Su, H. Li, L. Sun, Inhibition of autophagy enhances cisplatin cytotoxicity through endoplasmic reticulum stress in human cervical cancer cells, *Cancer Lett.* 314 (2012) 232–243.
- [45] J. Zhou, S.E. Hu, S.H. Tan, R. Cao, Y. Chen, D. Xia, X. Zhu, X.F. Yang, C.N. Ong, H.M. Shen, Andrographolide sensitizes cisplatin-induced apoptosis via suppression of autophagosome-lysosome fusion in human cancer cells, *Autophagy* 8 (2012) 338–349.
- [46] D. Liu, Y. Yang, Q. Liu, J. Wang, Inhibition of autophagy by 3-MA potentiates cisplatin-induced apoptosis in esophageal squamous cell carcinoma cells, *Med. Oncol.* 28 (2011) 105–111.
- [47] S. Claerhout, L. Verschouten, S. Van Kelst, R. De Vos, C. Proby, P. Agostinis, M. Garmyn, Concomitant inhibition of AKT and autophagy is required for efficient cisplatin-induced apoptosis of metastatic skin carcinoma, *Int. J. Cancer* 127 (2010) 2790–2803.
- [48] J.H. Ren, W.S. He, L. Nong, Q.Y. Zhu, K. Hu, R.G. Zhang, L.L. Huang, F. Zhu, G. Wu, Acquired cisplatin resistance in human lung adenocarcinoma cells is associated with enhanced autophagy, *Cancer Biother. Radiopharm.* 25 (2010) 75–80.
- [49] L. Harhaji-Trajkovic, U. Vilimanovich, T. Kravic-Stevovic, V. Bumbasirevic, V. Trajkovic, AMPK-mediated autophagy inhibits apoptosis in cisplatin-treated tumour cells, *J. Cell. Mol. Med.* 13 (2009) 3644–3654.
- [50] C. Hu, M.J. Zou, L. Zhao, N. Lu, Y.J. Sun, S.H. Gou, T. Xi, Q.L. Guo, E. Platinum, a newly synthesized platinum compound, induces autophagy via inhibiting phosphorylation of mTOR in gastric carcinoma BGC-823 cells, *Toxicol. Lett.* 210 (2012) 78–86.
- [51] H. Du, W. Yang, L. Chen, M. Shi, V. Seewoo, J. Wang, A. Lin, Z. Liu, W. Qiu, Role of autophagy in resistance to oxaliplatin in hepatocellular carcinoma cells, *Oncol. Rep.* 27 (2012) 143–150.
- [52] Z.B. Ding, B. Hui, Y.H. Shi, J. Zhou, Y.F. Peng, C.Y. Gu, H. Yang, G.M. Shi, A.W. Ke, X.Y. Wang, K. Song, Z. Dai, Y.H. Shen, J. Fan, Autophagy activation in hepatocellular carcinoma contributes to the tolerance of oxaliplatin via reactive oxygen species modulation, *Clin. Cancer Res.* 17 (2011) 6229–6238.
- [53] L. Xu, X.J. Qu, Y.P. Liu, Y.Y. Xu, J. Liu, K.Z. Hou, Y. Zhang, Protective autophagy antagonizes oxaliplatin-induced apoptosis in gastric cancer cells, *Chin. J. Cancer* 30 (2011) 490–496.
- [54] C.J. Lin, C.C. Lee, Y.L. Shih, T.Y. Lin, S.H. Wang, Y.F. Lin, C.M. Shih, Resveratrol enhances the therapeutic effect of temozolomide against malignant glioma in vitro and in vivo by inhibiting autophagy, *Free Radic. Biol. Med.* 52 (2012) 377–391.
- [55] V. Voss, C. Senft, V. Lang, M.W. Ronellenfitch, J.P. Steinbach, V. Seifert, D. Kogel, The pan-Bcl-2 inhibitor (–)-gossypol triggers autophagic cell death in malignant glioma, *Mol. Cancer Res.* 8 (2010) 1002–1016.
- [56] M. Katayama, T. Kawaguchi, M.S. Berger, R.O. Pieper, DNA damaging agent-induced autophagy produces a cytoprotective adenosine triphosphate surge in malignant glioma cells, *Cell Death Differ.* 14 (2007) 548–558.
- [57] T. Kanzawa, I.M. Germano, T. Komata, H. Ito, Y. Kondo, S. Kondo, Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells, *Cell Death Differ.* 11 (2004) 448–457.

- [58] T. Kanzawa, J. Bedwell, Y. Kondo, S. Kondo, I.M. Germano, Inhibition of DNA repair for sensitizing resistant glioma cells to temozolomide, *J. Neurosurg.* 99 (2003) 1047–1052.
- [59] Y.T. Wu, H.L. Tan, G. Shui, C. Bauvy, Q. Huang, M.R. Wenk, C.N. Ong, P. Codogno, H.M. Shen, Dual role of 3-methyladenine in modulation of autophagy via different temporal patterns of inhibition on class I and III phosphoinositide 3-kinase, *J. Biol. Chem.* 285 (2010) 10850–10861.
- [60] M. Ogata, S. Hino, A. Saito, K. Morikawa, S. Kondo, S. Kanemoto, T. Murakami, M. Taniguchi, I. Tani, K. Yoshinaga, S. Shiosaka, J.A. Hammarback, F. Urano, K. Imaizumi, Autophagy is activated for cell survival after endoplasmic reticulum stress, *Mol. Cell. Biol.* 26 (2006) 9220–9231.
- [61] A. Alexander, S.L. Cai, J. Kim, A. Nanez, M. Sahin, K.H. MacLean, K. Inoki, K.L. Guan, J. Shen, M.D. Person, D. Kusewitt, G.B. Mills, M.B. Kastan, C.L. Walker, ATM signals to TSC2 in the cytoplasm to regulate mTORC1 in response to ROS, *Proc. Natl. Acad. Sci. U.S.A.* 107 (2010) 4153–4158.
- [62] S. Benzina, A. Altmeyer, F. Malek, P. Dufour, J.M. Denis, J. Gueulette, P. Bischoff, High-LET radiation combined with oxaliplatin induce autophagy in U-87 glioblastoma cells, *Cancer Lett.* 264 (2008) 63–70.
- [63] A. Altmeyer, A.C. Jung, M. Ignat, S. Benzina, J.M. Denis, J. Gueulette, G. Noel, D. Mutter, P. Bischoff, Pharmacological enhancement of autophagy induced in a hepatocellular carcinoma cell line by high-LET radiation, *Anticancer Res.* 30 (2010) 303–310.
- [64] H. Aoki, Y. Kondo, K. Aldape, A. Yamamoto, E. Iwado, T. Yokoyama, E.F. Hollingsworth, R. Kobayashi, K. Hess, N. Shinojima, T. Shingu, Y. Tamada, L. Zhang, C. Conrad, O. Bogler, G. Mills, R. Sawaya, S. Kondo, Monitoring autophagy in glioblastoma with antibody against isoform B of human microtubule-associated protein 1 light chain 3, *Autophagy* 4 (2008) 467–475.
- [65] M.C. Maiuri, G. Le Toumelin, A. Criollo, J.C. Rain, F. Gautier, P. Juin, E. Tasdemir, G. Pierron, K. Troulinaki, N. Tavernarakis, J.A. Hickman, O. Geneste, G. Kroemer, Functional and physical interaction between Bcl-X(L) and a BH3-like domain in Beclin-1, *EMBO J.* 26 (2007) 2527–2539.
- [66] E.C. Filippi-Chiela, M.P. Thome, E.S.M.M. Bueno, A.L. Pelegrini, P.F. Ledur, B. Garicochea, L.L. Zamin, G. Lenz, Resveratrol abrogates the Temozolomide-induced G2 arrest leading to mitotic catastrophe and reinforces the Temozolomide-induced senescence in glioma cells, *BMC Cancer* 13 (2013) 147.

4. CAPÍTULO II

Autophagy induction in non-small cell lung cancer cells contributes to cell death induced by cisplatin treatment

Manuscrito a ser submetido para publicação

APRESENTAÇÃO

Neste capítulo será apresentado um manuscrito que aborda o papel da indução de autofagia pelo agente alquilante cisplatina em células de câncer de pulmão de células não-pequenas (NSCLC) da linhagem humana H460. Através da utilização do indutor farmacológico de autofagia rapamicina foi possível provocar uma hiperestimulação desta via e reduzir significativamente a viabilidade celular.

Além do mais, na tentativa de verificar a papel da autofagia induzida pela cisplatina foram utilizados os fármacos inibidores de autofagia 3-metiladenina e cloroquina. A inibição pela 3-metiladenina não influenciou na viabilidade celular, no entanto, a adição de cloroquina levou a um pequeno aumento da viabilidade, sugerindo que a ativação de autofagia pela cisplatina contribui para a morte celular.

Autophagy induction in non-small cell lung cancer cells contributes to cell death induced by cisplatin treatment

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Abstract

Cisplatin (CDDP) is still one of the most effective agents against various types of cancer, including non-small cell lung cancer (NSCLC). Recently, it has been shown that CDDP and other chemotherapeutic compounds that cause DNA damage activate the autophagic pathway, which may influence cellular responses and affect the result of therapy. CDDP activated autophagy in H460 lung cancer cells, as assessed through the quantification of AVOs (acidic vacuolar organelles) and levels of LC3-I/II and p62 proteins. The combination of rapamycin with CDDP lead to overstimulation of autophagy and considerably decreased the clonogenic capacity of treated cells. Moreover, the early step autophagy inhibitor 3-methyladenine did not affect the capacity of colony formation after CDDP treatment, while chloroquine, which inhibits autophagy completion, significantly increased cell viability, suggesting that autophagy completion contributes to cell death.

1. Introduction

Lung cancer is the leading cause of cancer deaths in the world (www.inca.gov.br; www.cancer.org). Among the lung cancer subtypes, non-small cell lung cancer (NSCLC) constitutes more than 80% of all lung cancer cases (Stathopoulos, 2013). The main options to treat NSCLC include surgery, radiotherapy and chemotherapy, depending on tumor stage. One of the most employed drugs in NSCLC chemotherapy treatment is cisplatin (*cis*-diamminedichloroplatinum (II) or CDDP) (Giaccone, 2004). CDDP acts as an alkylating-like agent and its cytotoxicity is characterized by the formation of platinum adducts on DNA, including monoadducts, intrastrand and interstrand crosslinks, and DNA protein crosslink (Gonzalez *et al.*, 2001). Adducts formation activates several cellular responses such as transcription inhibition, cell cycle arrest, DNA repair or apoptosis when DNA is highly damaged and repair is not possible (Wang & Lippard, 2005).

Cells exposed to CDDP and other chemotherapeutic agents induce autophagy, but the role of the induction of this mechanism is variable (Bordin *et al.*, 2013; Vessoni *et al.*, 2013). Autophagy is an evolutionarily conserved process that takes part in the degradation of cellular constituents and is essential for the maintenance of cellular homeostasis (Jiang & Mizushima, 2014). During periods of starvation, autophagy provides energy (ATP) and important biomolecules to the cell. This process also participates in the cellular quality control through the degradation of damaged organelles and pathogens (Johansen & Lamark, 2011). Autophagy induction by chemotherapeutic agents prevents cell death and contributes to chemoresistance in some cases (Zhang *et al.*, 2013; Zhu *et al.*, 2013). On the other hand, autophagy activation may also lead to cell death and cooperate in tumor elimination (Qi *et al.*, 2013). Given these controversial functions of autophagy in chemotherapy, it is fundamental to evaluate different autophagy inducers and/or inhibitors in combination with chemotherapeutic compounds in an effort to sensitize tumor cells (Pan *et al.*, 2011; Liu *et al.*, 2013; Selvakumaran *et al.*, 2013). In the present study, we modulated autophagy and explored its role in NSCLC cells subjected to CDDP treatment. For this purpose,

we combined CDDP with the autophagy inducer rapamycin (Rap), which inhibits mTORC1 (mammalian target of rapamycin complex 1). We also made a combination of CDDP with the autophagy inhibitors 3-methyladenine (3MA) and chloroquine (CQ). 3MA inhibits PI3K class III and therefore prevents the autophagosomal formation while CQ impairs lysosome acidification and its fusion with the autophagosome, thus blocking the late stages of autophagy.

In our results, CDDP was able to induce autophagy in H460 NSCLC cells. We observed a greater induction of autophagy when cisplatin was combined with Rap, especially when a pretreatment with Rap followed by treatment with CDDP was made. This result was associated with a significant reduction in cell viability. On the contrary, treatment with CDDP combined with CQ slightly enhanced cell survival.

2. Materials and methods

2.1. Reagents

Cisplatin (Fauldcispla) was obtained from Libbs. Rapamycin (Rap), chloroquine (CQ), 3-methyladenine (3MA), MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), acridine orange (AO) and Krebs-Ringer Bicarbonate Buffer were all purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture

H460 (NCI-H460 ATCC) cells were grown in DMEM high glucose (Dulbecco's modified Eagle's medium; Invitrogen, Carlsbad, CA). Medium was supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin, and 100 mg/mL streptomycin. Cells were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO₂.

2.3. MTT viability assay

Cells were seeded in 24 wells plates at a density of 7×10^3 /well and treated with CDDP (doses varying from 0 to 33 μ M) for 48h. Cotreatment with Rap was

also performed. Following treatment, cells were incubated with 0.4 mg/ml MTT salt for 3h. Absorbance was measured at a wavelength of 540 nm.

2.4. *Clonogenic assay*

We assessed the ability of cells to form colonies on a monolayer surface into a well of a six-well plate. Cells were plated at a density of 200 cells/well and allowed to adhere. The next day cells were incubated with 0.2 and 0.9 μM of CDDP (IC_{10} and IC_{25} values obtained by MTT assay, respectively) for 48h. Combined treatments using CDDP, the autophagy inducer Rap (100 nM), and the autophagy inhibitors 3MA (1 mM) or CQ (10 μM) were performed. After the treatment period, cells were maintained in drug free medium for 7 days. After that, cells were fixed in methanol and stained with crystal violet. Colonies were counted and are presented as the mean of colonies \pm SD from three independent experiments.

2.5. *Flow cytometric quantification of acidic vesicular organelles (AVOs)*

Cells were plated in 24 wells plates at a density of 3×10^4 /well and incubated with IC_{10} and IC_{25} of CDDP for 48h. After combined treatments with CDDP and Rap or CDDP and 3MA, cells were harvested by trypsinization, washed in PBS, and then stained with 1 $\mu\text{g/ml}$ acridine orange for 15 min. AO fluorescence was measured by flow cytometry.

2.6. *Western blot analysis*

Cells were washed with PBS and collected from plates with lysis buffer containing M-PER mammalian protein extraction reagent and protease inhibitor mini tablets EDTA-free (both from Pierce, Rockford, IL). Proteins were quantified using the BCA assay (Pierce, Rockford, IL). The same amount (30 μg) of proteins was resolved in a SDS-PAGE gel and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). After transfer, membranes were blocked in 5% skimmed milk powder with 0.1% Tween 20. The blots were then probed at 4°C overnight with the primary antibodies for LC3 B (1:1000) and p62/SQSTM1 (1:1000) both from Cell Signaling Technology (Beverly, MA, USA).

Secondary antibodies were goat anti-rabbit IgG-HRP or goat anti-mouse IgG-HRP (1:5000) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Signal was detected using ECL western blotting substrate system or Supersignal chemiluminescence (Pierce, Rockford, IL).

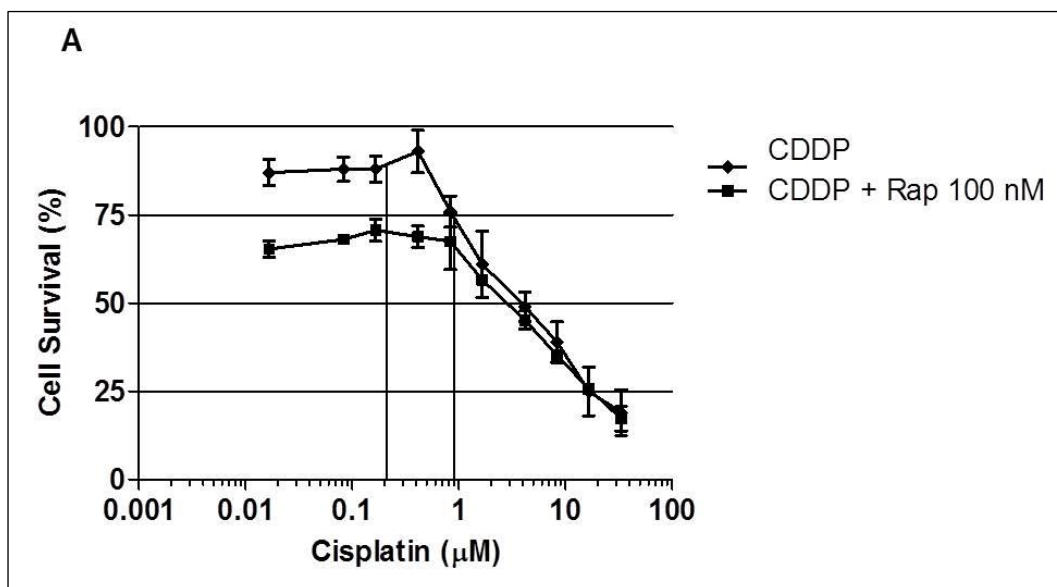
2.7. Statistical analysis

Each experiment was carried out at least in triplicate. Statistical analysis was conducted by ANOVA followed by Tukey post-hoc test to multiple comparisons. 'p' value under 0.05 was considered significant. The GraphPad Prism 5.0 software (GraphPad Software Inc., Philadelphia, U.S.A.) was employed.

3. Results

3.1. Rapamycin combined with cisplatin decreases the viability of H460 cells

Rapamycin (100 nM) potentiated the effects of CDDP on cell viability measured by MTT assay after 48h, mainly at the lower doses of CDDP (**Fig. 1A**). Noteworthy is the fact that rapamycin alone did not elicit any cytotoxicity at concentrations ranging from 1 to 10000 nM (**Fig. 1B**).



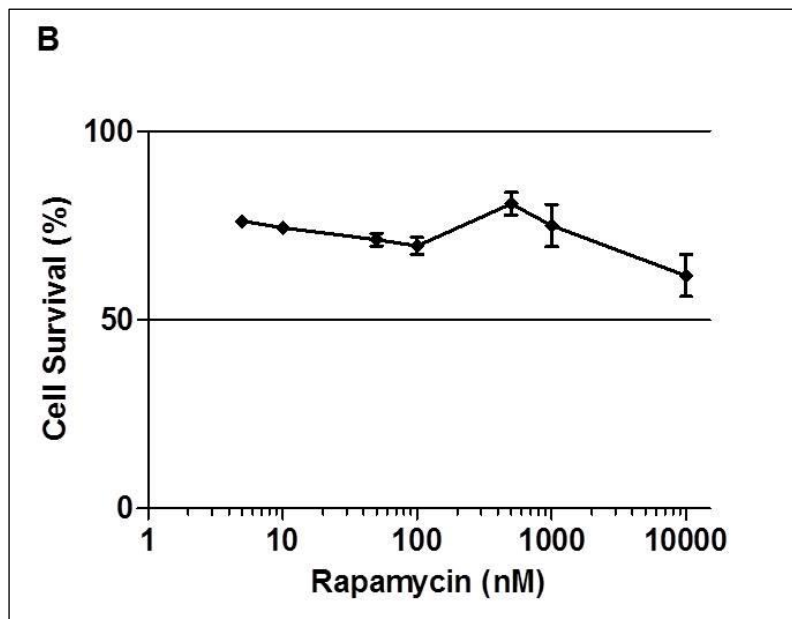


Figure 1. Effect of rapamycin and cisplatin on the viability of H460 cells. Cell viability was determined by MTT assay after 48h of treatment. **(A)** CDDP alone or combined with rap 100 nM. **(B)** Rap alone.

Based on these results, we decided to use low concentrations of CDDP in the following experiments in order to confirm that the combination of CDDP and rapamycin contributes to cell death. Thus, concentrations of 0.2 μM and 0.9 μM , equivalent to the IC_{10} and IC_{25} values of cisplatin, respectively, were used. In addition, we also found important to evaluate whether the addition of rapamycin, before (pretreatment), during (cotreatment) or shortly after (post-treatment) exposition to CDDP, could influence the response of H460 cells. Thus, different treatments were applied to H460 cells, which are explained in Figure 2. Experimental procedures using autophagy inhibitors are also included in Figure 2.

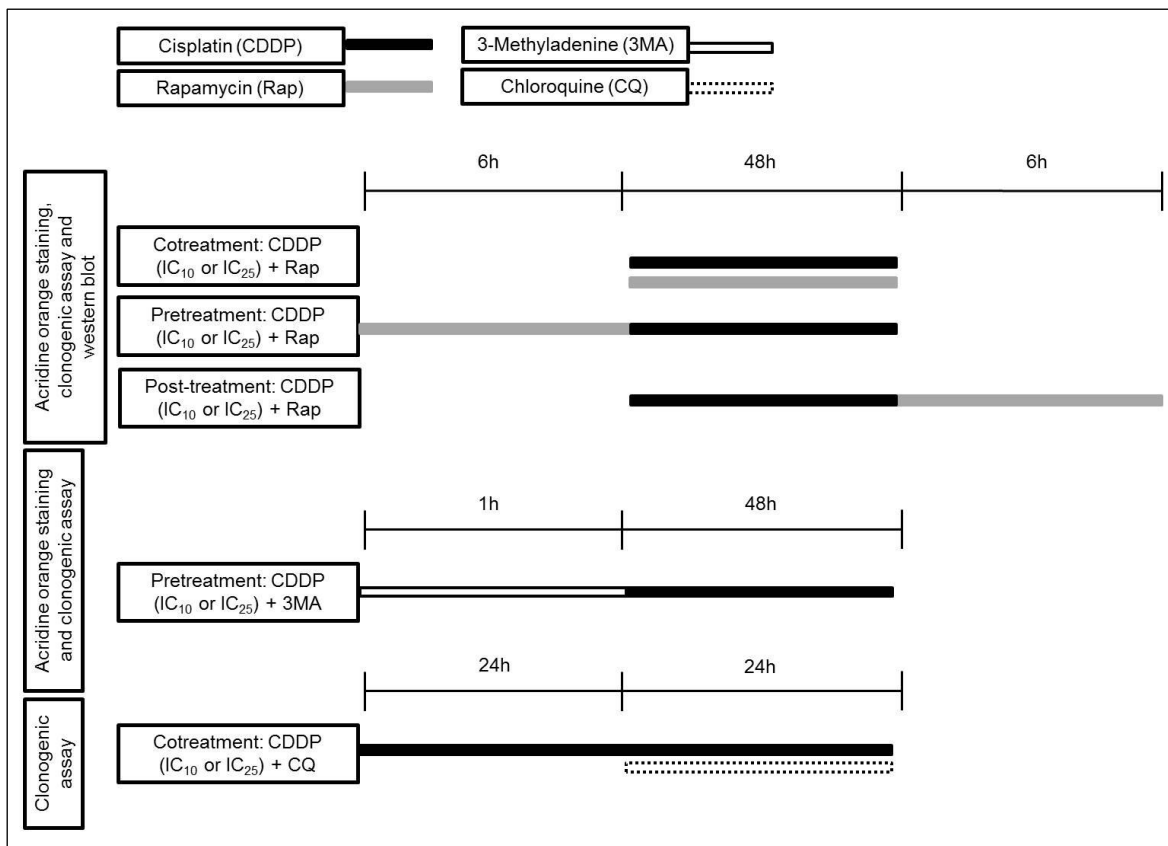


Figure 2. Experimental design for combined treatments among CDDP and Rap or CDDP and autophagy inhibitors.

3.2. Rapamycin increases autophagy induction in H460 cells after combined treatments with cisplatin

The formation of acidic vacuolar organelles (AVOs) is a characteristic of autophagy. Acridine orange is a fluorescent dye which stains the cell and emits green fluorescence. However, in contact with acidic compartments, such as autolysosomes, it emits bright red fluorescence, serving as an indicator of autophagic cells.

To quantify the formation of AVOs, H460 cells were exposed to IC₁₀ and IC₂₅ of CDDP for 48h and submitted to pre-, co- or post-treatment with rapamycin. Cisplatin at the IC₂₅ dose was able to induce autophagy compared to untreated cells, whereas IC₁₀ did not induce the formation of AVOs (**Fig. 3 A-B**). However, rapamycin combined with CDDP significantly contributed to autophagy induction in

comparison to CDDP IC₁₀ and IC₂₅ treatment only (**Fig. 3 C-D**). We observed a greater formation of AVOs in cotreatment and pretreatment in comparison with post-treatment, suggesting that the addition of rapamycin before or together with CDDP is more efficient to activate autophagy.

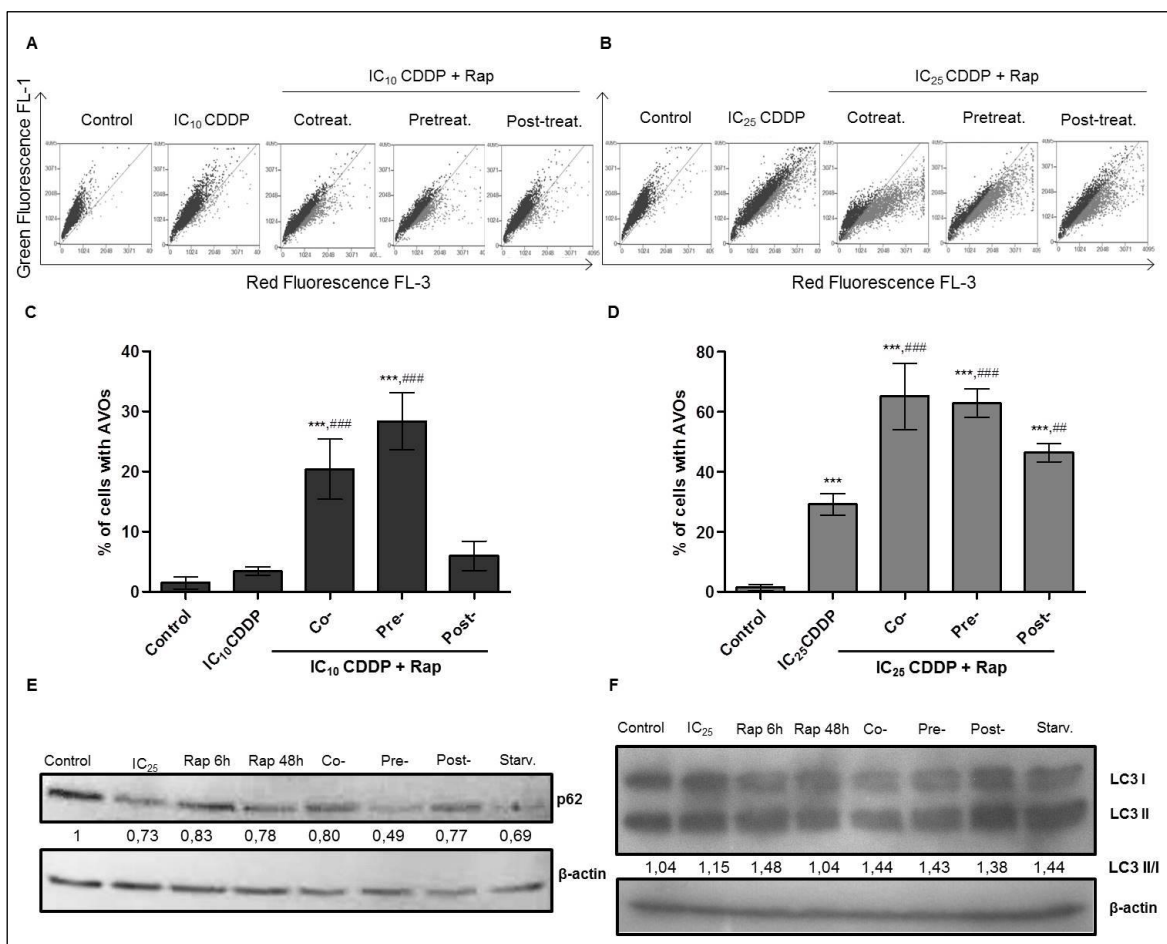


Figure 3. Autophagy induction in H460 cells after CDDP and Rap treatment. Cells were treated with CDDP alone or in combination with Rap in pre-, co- and post-treatment. **(A)** and **(B)** representative flow cytometry plots of acridine orange staining of cells treated with CDDP and different combinations with Rap. **(C)** Quantification of AVOs in cells treated with IC₁₀ or **(D)** IC₂₅ CDDP values. *** Statistically different from the negative control, $P \leq 0.001$; #### statistically different from CDDP treatment alone, $P \leq 0.001$; ## $P \leq 0.01$. **(E)** p62 and **(F)** LC3 expression determined by western blot. Cells were treated with IC₂₅ value of CDDP and Rap (100 nM). Starv.: starvation induced using Krebs-Ringer Buffer during 24h.

To confirm these results, the expression of LC3 (microtubule-associated protein 1 light chain 3) and p62 (SQSTM1) proteins was measured by western blot. LC3 is synthesized in its precursor form and is cleaved resulting in the

cytosolic form LC3-I. When LC3-I receives a pre-autophagic signal it is converted in LC3-II which is recruited to the autophagosome and remains attached until the fusion with the lysosome. This conversion of LC3-I in LC3-II is a proper marker in autophagic assays (Ravikumar *et al.*, 2010). The p62 protein is a selective substrate for autophagy which interacts with ubiquitinated substrates and with the regulator LC3 (Komatsu & Ichimura, 2010). This interaction with LC3 mediates p62 to carry the ubiquitinated substrates to the autophagosome for degradation (Moscat & Diaz-Meco, 2009). There is an inverse correlation between p62 and LC3-II in active autophagic pathway: while p62 is selectively degraded, the levels of LC3-II become elevated.

The western blot analysis demonstrated that the IC_{25} of CDDP decreased the p62 levels, indicating activation of autophagy in this condition. Low levels of p62 were also detected in cells treated with different combinations of CDDP and Rap. However, a greater induction of autophagy was observed in cells pretreated with Rap (**Fig. 3E**). In agreement, the analysis of LC3-II expression suggests a slightly higher induction of autophagy in pretreatment and cotreatment in comparison to post-treatment (**Fig. 3F**). These results are in accordance with the autophagy induction profile seen in the AVOs quantification assay.

3.3. Autophagy overstimulation by Rap decreases clonogenic capacity in cells treated with CDDP

Combined treatments among CDDP and Rap were able to increase autophagy induction significantly, especially in pretreatment. Therefore, we decided to evaluate if the overstimulation of autophagy induced by Rap could influence cell survival. For this purpose, we performed clonogenic assay in H460 cells treated with CDDP alone and in association with Rap. We observed a decrease in clonogenic formation when cells were exposed to combined treatments in comparison to cisplatin treatment only. However, we did not observe a difference in colony formation capacity among co-, pre- and post-treatment with Rap (**Fig. 4**). These results suggest that the overstimulation of autophagy may contribute to death of cells treated with CDDP. Nevertheless, the order in which Rap is added to the treatment does not seem to influence the capacity of colony formation.

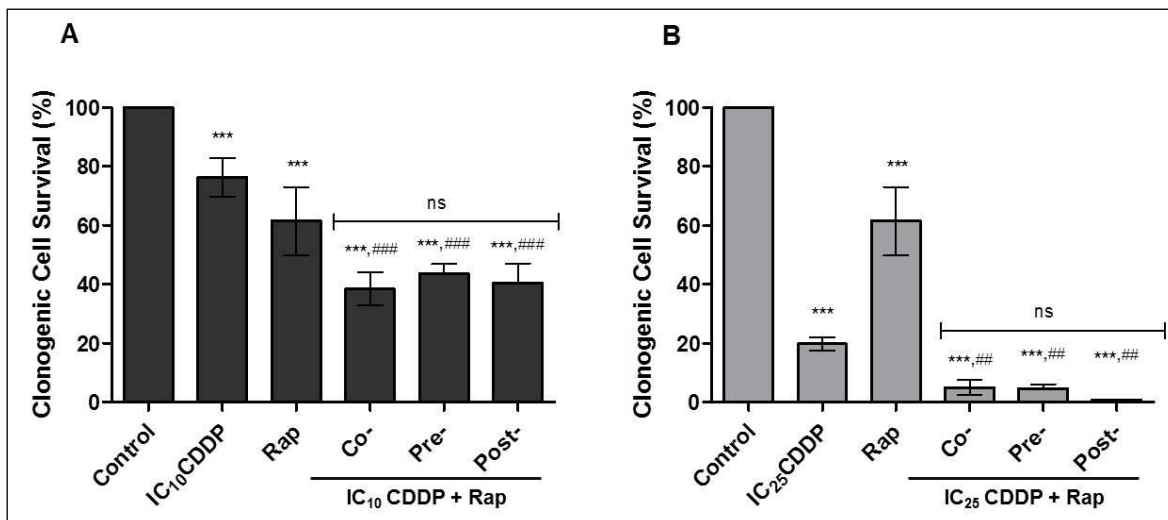


Figure 4. Clonogenic assay of H460 cells after CDDP and Rap treatment. Cells were treated with CDDP alone during 48h or Rap (100 nM) alone during 6h. Three different combined treatments between CDDP and Rap were also performed. **(A)** IC₁₀ and **(B)** IC₂₅ CDDP concentration. *** Statistically different from the negative control, $P \leq 0.001$; ### statistically different from the CDDP treatment alone; # $P \leq 0.01$; ns: non-significant.

3.4. Inhibition of autophagy protects H460 cells from cell death induced by cisplatin

The pharmacological inhibitors of autophagy 3MA and CQ were chosen to block this pathway. After pretreatment with 3MA, cells were treated with cisplatin and the quantification of AVOs was performed by flow cytometry. We observed that 3MA pretreatment was able to decrease autophagy induction in cells treated with cisplatin (**Fig. 5**).

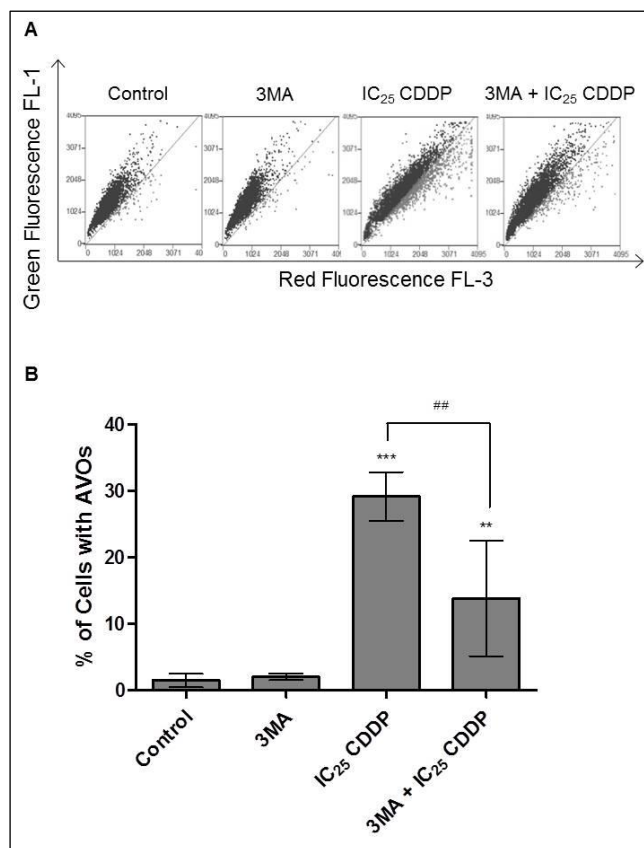


Figure 5. Autophagy inhibition in H460 cells after CDDP and 3MA treatment. Cells were treated with CDDP alone or after a pre-treatment with 3MA for 1h (1 mM). **(A)** Representative flow cytometry plots of acridine orange staining. **(B)** Quantification of AVOs in cells treated with IC₂₅ CDDP value. *** Statistically different from the negative control, $P \leq 0.001$; ** $P \leq 0.01$; ## statistically different from CDDP treatment alone, $P \leq 0.01$.

Next, we evaluated the influence of 3MA and CQ on cell viability, when combined with cisplatin. Through clonogenic assay, no difference was observed in the capacity to form colonies between 3MA pretreatment and cisplatin treatment only (**Fig. 6**). Nonetheless, we could notice an increase in colony formation when cells received cisplatin in cotreatment with CQ, in comparison to cisplatin treatment alone.

All the tests performed to evaluate the clonogenic capacity were conducted based on 200 cells/well plating density. In figure 6A, there is a significant increase in the capacity to form colonies in treatment with IC₁₀ CDDP value together with CQ. In order to increase the sensitivity of the assay, we used 800 cells when analyzing the treatment with IC₂₅ CDDP (**Fig. 6B**). In this experiment we observed

an increase in colonies formation capacity in the presence of CQ, following the same profile observed in IC₁₀ CDDP value treatment. Therefore, the results obtained for IC₁₀ and IC₂₅ concentration in the presence of CQ suggest that autophagy inhibition contributes to cell survival.

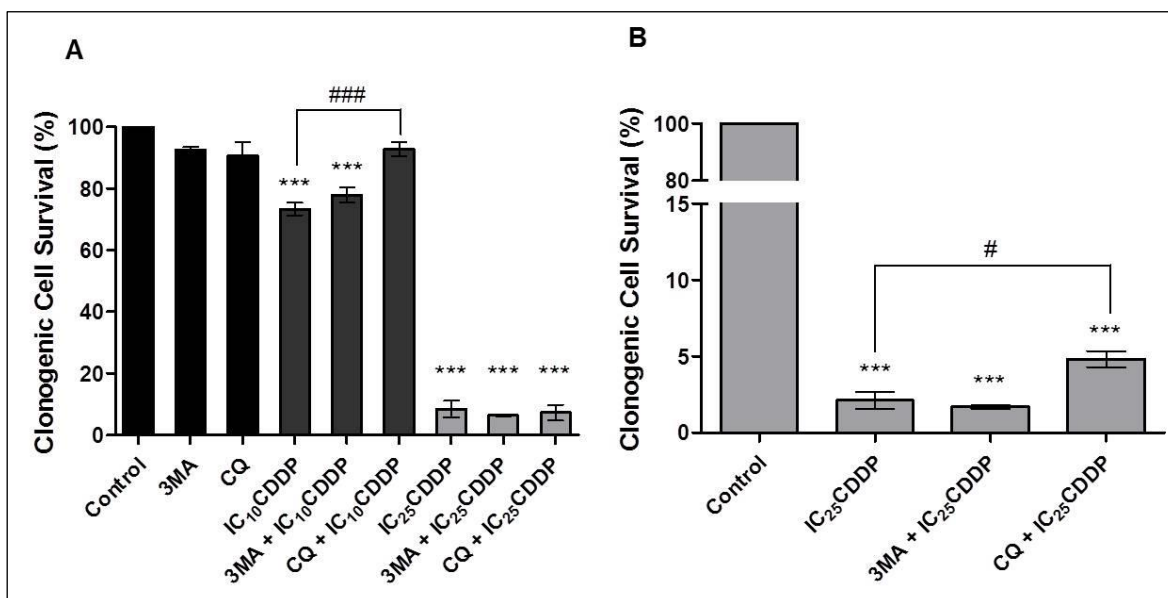


Figure 6. Clonogenic assay of H460 cells after CDDP and 3MA or CQ treatment. Cells were treated with CDDP alone or in combination with 3MA and CQ. **(A)** IC₁₀ and IC₂₅ CDDP concentration. **(B)** IC₂₅ CDDP concentration. Cells were plated at a density of 800 cells in petry dishes. *** Statistically different from control group; $P \leq 0.001$. ### Statistically different from the CDDP treatment alone; $P \leq 0.001$; # $P \leq 0.05$.

4. Discussion

In the past few years, autophagy has been the subject of great debate in the oncology field. In healthy cells, autophagy acts as a cytoprotective mechanism preventing the beginning of tumorigenesis (White, 2012). Despite that, in tumor cells autophagy plays different roles depending on tumorigenesis stage: in initial stages it has a protective role avoiding tumor progression, but in more advanced tumors autophagy may contribute to cell survival and impair tumor elimination (Rosenfeldt & Ryan, 2011).

Many stressors can activate autophagy in tumor cells such as nutrient deprivation, hypoxia and genotoxic stress (Dalby *et al.*, 2010; Ravikumar *et al.*,

2010). In our study we showed that the alkylating-like agent CDDP, which causes various types of DNA damage, was able to activate the autophagic pathway in H460 lung cancer cells. Autophagy induction by chemotherapeutic drugs that causes DNA damage has been shown in several studies (Notte *et al.*, 2011; Rodriguez-Rocha *et al.*, 2011; Sui *et al.*, 2013). DNA damage leads to complex responses within the cell and depending on damage extent, cell cycle arrest and DNA repair can be triggered or, if the damage is more severe, cell death pathways are initiated (Surova & Zhivotovsky, 2013). Depending on cellular type and context, autophagy activation in response to DNA damage may have different roles. Autophagy can be activated as a defense mechanism against chemotherapy and lead to treatment resistance, in these cases the inhibition of autophagy may help in the elimination of tumor cells (Guo *et al.*, 2012). However, when sustained for a longer period or overstimulated, autophagy can lead to cell death (Yang *et al.*, 2011).

In order to evaluate the effects of autophagy overstimulation in NSCLC cells we used Rap in combination with CDDP. Our results show that Rap significantly enhances autophagy induction in combination with CDDP. It was also demonstrated that the addition of Rap before or together with CDDP treatment is more efficient in autophagy activation in comparison to the post-treatment. We also found a significant decrease in cell viability through clonogenic capacity method when these two compounds were combined. These results confirm that autophagy overstimulation contributes to cell death in our conditions.

There is still no consensus on whether autophagy acts as an independent mechanism of cell death or only contributes to another form of cell death such as apoptosis or necrosis. It is possible that autophagy may act as a futile attempt to adapt to stress caused by severe damage and lead to cell death with autophagic characteristics but not to autophagic cell death itself (Shen *et al.*, 2012).

Recent studies have suggested that if autophagy occurs in an attempt to scape lethal pathways, autophagy inhibition may sensitize cells to cisplatin and promote cell death. Zhang *et al.* (2013) showed that autophagy prevents apoptosis in gastric cancer cells treated with cisplatin and that the addition of CQ recovers the

sensitivity leading to apoptotic cell death. Through the silencing of an autophagy related gene (ATG7) in esophageal carcinoma cells, Zhu *et al.* (2013) also found an increase in cell sensibility to cisplatin suggesting that autophagy impairs apoptosis. Nevertheless, some authors have reported that the combination of autophagic inhibitors with chemotherapeutical compounds may increase cell viability instead of promoting cell death (Qi *et al.*, 2013).

In this study we found that the addition of 3MA decreased autophagy induction by CDDP although no significant effect of this inhibition was seen in cell viability. Meanwhile, the addition of CQ in combination with CDDP was able to slightly, but significantly increase cell viability in comparison to CDDP alone. 3MA prevents the initiation of autophagy by blocking the production of PI3P and the recruitment of Atg proteins for the isolation membrane. This mechanism impairs the autophagosomes formation and enables the presence of damaged organelles and biomolecules in the cytoplasm. On the other side, CQ acts in the later stages of autophagy and permits the cytotoxic components to be restrained within the autophagosomes, which could explain the increase in cell viability. However, when present in excess or for long periods of time, the autophagosomes may become toxic for the cell. Thereby, it is important to confirm and evaluate this result with additional experiments to determine the effect of CQ treatment for longer periods of time.

Our data suggest that the activation of autophagy by CDDP contributes to cell death in H460 cells. Furthermore, overstimulation of autophagy with Rap contributed to the elimination of cancer cells which implies that the combination of autophagy inducers with cisplatin may be a good strategy to improve chemotherapy for NSCLC.

References

BORDIN, D. L.; LIMA, M.; LENZ, G.; SAFFI, J.; MEIRA, L. B.; MÉSANGE, P.; SOARES, D. G.; LARSEN, A. K.; ESCARGUEIL, A. E.; HENRIQUES, J. A. P. DNA alkylation damage and autophagy induction. **Mutation research**, v. 753, n. 2, p. 91–9, 2013.

DALBY, K. N.; TEKEDERELI, I.; LOPEZ-BERESTEIN, G.; OZPOLAT, B. Targeting the prodeath and prosurvival functions of autophagy as novel therapeutic strategies in cancer. **Autophagy**, v. 6, n. 3, p. 322–9, abr. 2010.

GIACCONE, G. Twenty-five years of treating advanced NSCLC: what have we achieved? **Annals of oncology: official journal of the European Society for Medical Oncology / ESMO**, v. 15 Suppl 4, n. Supplement 4, p. iv81–3, jan. 2004.

GONZALEZ, V. M.; FUERTES, M. A.; ALONSO, C.; PEREZ, J. M. Is cisplatin-induced cell death always produced by apoptosis? **Molecular pharmacology**, v. 59, n. 4, p. 657–63, abr. 2001.

GUO, X.-L.; LI, D.; HU, F.; SONG, J.-R.; ZHANG, S.-S.; DENG, W.-J.; SUN, K.; ZHAO, Q.-D.; XIE, X.-Q.; SONG, Y.-J.; WU, M.-C.; WEI, L.-X. Targeting autophagy potentiates chemotherapy-induced apoptosis and proliferation inhibition in hepatocarcinoma cells. **Cancer letters**, v. 320, n. 2, p. 171–9, 28 jul. 2012.

JIANG, P.; MIZUSHIMA, N. Autophagy and human diseases. **Cell research**, v. 24, n. 1, p. 69–79, jan. 2014.

JOHANSEN, T.; LAMARK, T. Selective autophagy mediated by autophagic adapter proteins. **Autophagy**, v. 7, n. 3, p. 279–296, 1 mar. 2011.

KOMATSU, M.; ICHIMURA, Y. Physiological significance of selective degradation of p62 by autophagy. **FEBS letters**, v. 584, n. 7, p. 1374–8, 2 abr. 2010.

LIU, F.; LIU, D.; YANG, Y.; ZHAO, S. Effect of autophagy inhibition on chemotherapy-induced apoptosis in A549 lung cancer cells. **Oncology letters**, v. 5, n. 4, p. 1261–1265, abr. 2013.

NOTTE, A.; LECLERE, L.; MICHIELS, C. Autophagy as a mediator of chemotherapy-induced cell death in cancer. **Biochemical pharmacology**, v. 82, n. 5, p. 427–34, 1 set. 2011.

PAN, Y.; GAO, Y.; CHEN, L.; GAO, G.; DONG, H.; YANG, Y.; DONG, B.; CHEN, X. Targeting autophagy augments in vitro and in vivo antimyeloma activity of DNA-damaging chemotherapy. **Clinical cancer research: an official journal of the American Association for Cancer Research**, v. 17, n. 10, p. 3248–58, 15 maio 2011.

QI, X.-F.; KIM, D.-H.; LEE, K.-J.; KIM, C.-S.; SONG, S.-B.; CAI, D.-Q.; KIM, S.-K. Autophagy contributes to apoptosis in A20 and EL4 lymphoma cells treated with fluvastatin. **Cancer cell international**, v. 13, n. 1, p. 111, jan. 2013.

RAVIKUMAR, B.; SARKAR, S.; DAVIES, J. E.; FUTTER, M.; GARCIA-ARENCIBIA, M.; GREEN-THOMPSON, Z. W.; JIMENEZ-SANCHEZ, M.; KOROLCHUK, V. I.; LICHTENBERG, M.; LUO, S.; MASSEY, D. C. O.; MENZIES, F. M.; MOREAU, K.; NARAYANAN, U.; RENNA, M.; SIDDIQI, F. H.; UNDERWOOD, B. R.; WINSLOW, A. R.; RUBINSZTEIN, D. C. Regulation of Mammalian Autophagy in Physiology and Pathophysiology. **Physiological Reviews**, v. 90, p. 1383–1435, 2010.

RODRIGUEZ-ROCHA, H.; GARCIA-GARCIA, A.; PANAYIOTIDIS, M. I.; FRANCO, R. DNA damage and autophagy. **Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis**, v. 711, n. 1-2, p. 158–166, 2011.

ROSENFELDT, M. T.; RYAN, K. M. The multiple roles of autophagy in cancer. **Carcinogenesis**, v. 32, n. 7, p. 955–63, jul. 2011.

SELVAKUMARAN, M.; AMARAVADI, R. K.; VASILEVSKAYA, I. A; O'DWYER, P. J. Autophagy inhibition sensitizes colon cancer cells to antiangiogenic and cytotoxic therapy. **Clinical cancer research : an official journal of the American Association for Cancer Research**, v. 19, n. 11, p. 2995–3007, 1 jun. 2013.

SHEN, S.; KEPP, O.; KROEMER, G. The end of autophagic cell death? **Autophagy**, n. January, p. 1–3, 2012.

STATHOPOULOS, G. P. Cisplatin: process and future. **Journal of B.U.ON. : official journal of the Balkan Union of Oncology**, v. 18, n. 3, p. 564–9, 2013.

SUI, X.; CHEN, R.; WANG, Z.; HUANG, Z.; KONG, N.; ZHANG, M.; HAN, W.; LOU, F.; YANG, J.; ZHANG, Q.; WANG, X.; HE, C.; PAN, H. Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. **Cell death & disease**, v. 4, p. e838, jan. 2013.

SUMANTRAN, V. N. Cellular chemosensitivity assays: an overview. **Cancer cell culture: methods and protocols**, Methods in Molecular Biology. v. 731, 2011.

SUROVA, O.; ZHIVOTOVSKY, B. Various modes of cell death induced by DNA damage. **Oncogene**, v. 32, n. 33, p. 3789–97, 15 ago. 2013.

VESSONI, A T.; FILIPPI-CHIELA, E. C.; MENCK, C. F.; LENZ, G. Autophagy and genomic integrity. **Cell death and differentiation**, v. 20, n. 11, p. 1444–54, nov. 2013.

WANG, D.; LIPPARD, S. J. Cellular processing of platinum anticancer drugs. **Nature reviews. Drug discovery**, v. 4, n. 4, p. 307–20, abr. 2005.

WHITE, E. Deconvoluting the context-dependent role for autophagy in cancer. **Nature reviews. Cancer**, v. 12, n. 6, p. 401–10, jun. 2012.

YANG, Z. J.; CHEE, C. E.; HUANG, S.; SINICROPE, F. A. The role of autophagy in cancer: therapeutic implications. **Molecular cancer therapeutics**, v. 10, n. 9, p. 1533–41, set. 2011.

ZHANG, H.-Q.; HE, B.; FANG, N.; LU, S.; LIAO, Y.-Q.; WAN, Y.-Y. Autophagy inhibition sensitizes cisplatin cytotoxicity in human gastric cancer cell line SGC7901. **Asian Pacific journal of cancer prevention : APJCP**, v. 14, n. 8, p. 4685–8, jan. 2013.

ZHU, L.; DU, H.; SHI, M.; CHEN, Z.; HANG, J. ATG7 deficiency promote apoptotic death induced by Cisplatin in human esophageal squamous cell carcinoma cells. **Bulletin du cancer**, v. 100, n. 7-8, p. 15–21, 2013.

5. DISCUSSÃO GERAL

Nos últimos anos, o fenômeno autofagia tem recebido grande atenção nas pesquisas oncológicas devido ao seu papel dual na tumorigênese. Recentemente, tem sido demonstrada a capacidade dos agentes alquilantes, comumente utilizados na quimioterapia, em induzir autofagia. A expressão “agente alquilante” tem sido utilizada para designar qualquer composto capaz de se ligar covalentemente ao DNA. No entanto, os agentes alquilantes platinados não transferem um grupamento alquil, mas sim o grupo platina para o DNA, por este motivo também podem ser conhecidos como agentes alquilantes-*like*. A cisplatina é um fármaco alquilante capaz de provocar vários tipos de lesões no DNA, como monoadutos, pontes intercadeia, pontes intracadeia e pontes entre DNA e proteína (Boulikas, 2007). Na presença destas lesões ocorre a ativação da reposta de danos ao DNA o que leva à indução de várias vias de transdução de sinal. Em seguida, ocorre a parada no ciclo celular através da ativação dos pontos de checagem para permitir o reparo do DNA (Fu *et al.*, 2012). Todavia, quando o dano é mais severo e não reparável pode ocorrer a indução de senescência ou de mecanismos de morte celular como apoptose, necrose e catástrofe mitótica (Surova & Zhivotovsky, 2013; Hosoya & Miyagawa, 2014).

Mais recentemente, tem sido demonstrado que, em resposta aos danos causados no DNA por agentes alquilantes, pode ocorrer a indução de autofagia. A ativação de autofagia em resposta ao dano no DNA pode apresentar diferentes papéis dependendo do contexto e do tipo celular. Acredita-se que a ativação desta via possa ocorrer como um mecanismo de proteção contra o estresse causado pelo dano e contribuir para a resistência ao fármaco. Nestes casos, a utilização de inibidores de autofagia poderia reverter a resistência e auxiliar na eliminação das células tumorais (Ren *et al.*, 2010). Entretanto, quando sustentada por longos períodos, a autofagia pode colaborar para a morte da célula. Nos resultados apresentados no Capítulo II foi demonstrado que a CDDP levou à indução de autofagia em células de câncer de pulmão da linhagem H460. Uma das proteínas envolvidas na resposta de danos ao DNA e na ativação de

autofagia é a p53. Em um dos estudos realizados no Laboratório de Reparação de DNA de Eucariotos com a linhagem H460 (dados não publicados), foi constatado um aumento da expressão de p53 após 48 horas de tratamento com CDDP, conforme demonstrado no ensaio de imunofluorescência apresentado na **Figura 7**. A ativação de p53 leva ao aumento da expressão de genes relacionados à autofagia como DRAM (*damage-related autophagy modulator*), uma proteína localizada na membrana do lisossomo que facilita a autofagia (Crighton *et al.*, 2007). Outro importante alvo da p53 é a proteína cinase AMPK, a qual medeia a inibição de mTORC1 e ativa a autofagia (Rodriguez-Rocha *et al.*, 2011).

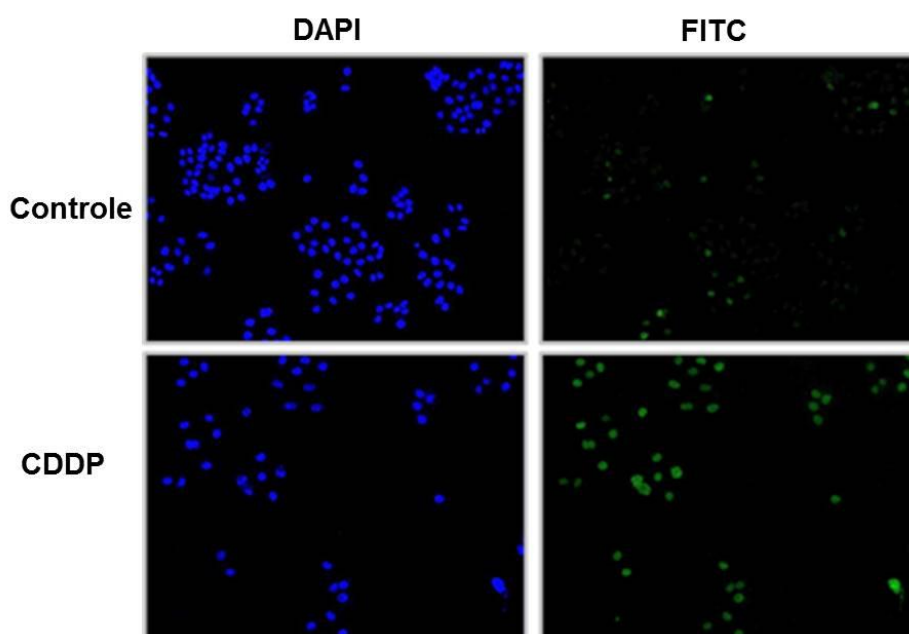


Figura 7. Expressão de p53 em células H460 tratadas com cisplatina. As células foram incubadas com CDDP na dose correspondente ao valor de IC_{50} obtido pelo ensaio MTT (3 μ M). A expressão de p53 foi avaliada pelo ensaio de imunofluorescência. Foram utilizados o anticorpo primário anti-p53 e o anticorpo secundário conjugado ao composto fluorescente FITC.

A combinação de Rap com CDDP aumentou significativamente a indução de autofagia em comparação ao tratamento com CDDP somente, como demonstrado no capítulo II. Estes resultados foram obtidos pela realização do ensaio de quantificação de compartimentos ácidos vacuolares (AVOs), detectados por

citometria de fluxo pela coloração com laranja de acridina. A quantificação dos níveis das proteínas LC3 e p62, as quais apresentam uma correlação inversa na indução de autofagia, corroborou estes resultados. É importante salientar que as células foram submetidas a diferentes combinações dos dois fármacos para avaliar em que momento a adição de Rap poderia influenciar na resposta celular. A indução de autofagia foi significativamente maior quando a Rap foi adicionada antes (pré-tratamento) ou no mesmo momento (cotratamento) em relação à adição de Rap após o tratamento com CDDP.

Foi constatado que esta hiperestimulação da via autofágica tem influência na viabilidade de células H460. Este resultado foi obtido através do ensaio clonogênico, que avalia a capacidade de formação de colônias e mede o efeito prolongado de um fármaco. A capacidade de formação de colônias foi significativamente menor em todos os tratamentos combinados de CDDP e Rap em relação ao tratamento somente com CDDP. Estes resultados sugerem que, nestas condições, a hiperestimulação de autofagia contribui para a morte celular. No entanto, é importante salientar, que o tratamento com Rap pode favorecer a morte celular não somente pela indução de autofagia. Isto se deve ao fato de que a cinase mTOR está envolvida em vias centrais de regulação do crescimento e proliferação celular, sendo que sua inibição leva à parada no ciclo celular, podendo estimular também a morte celular por apoptose. (Notte *et al.*, 2011).

Alguns autores consideram a autofagia como um tipo de morte celular programada (Dalby *et al.*, 2010; Shimizu *et al.*, 2014). Entretanto, segundo Shen *et al.*, (2012) a autofagia atua como uma tentativa fútil de proteger a célula contra o estresse causado por dano severo. Neste caso a célula morreria por outro mecanismo, mas apresentaria características autofágicas. Em nosso estudo, para avaliar a contribuição da autofagia induzida por CDDP na viabilidade celular ou morte celular, foram utilizados os inibidores 3-metiladenina e cloroquina. Pela quantificação de AVOs foi possível observar uma inibição da autofagia quando as células foram submetidas ao tratamento com 3MA antes do tratamento com CDDP. Esta inibição não mostrou influência na viabilidade celular. Em contrapartida, a adição de CQ ao tratamento com CDDP levou ao aumento da

viabilidade celular. Este resultado, em conjunto com o observado com a Rap, sugere que a ativação da autofagia em decorrência do tratamento com CDDP, contribui para a morte de células H460. Uma explicação plausível para a 3MA não ter influenciado na viabilidade de células H460 é que este composto inibe as etapas iniciais da via autofágica. Este mecanismo de ação bloqueia a formação de autofagossomos impedindo que organelas danificadas sejam eliminadas do citoplasma. Por outro lado, o aumento da viabilidade celular observado no tratamento com CQ permite que organelas permaneçam englobadas nos autofagossomos protegendo a célula da toxicidade destes componentes. Apesar disso, diversos estudos relatam que a inibição da autofagia por CQ sensibiliza células de tumores. Uma das explicações para a sensibilização de células tumorais ao tratamento com cloroquina seria devido ao acúmulo de autofagossomos na célula, os quais podem se tornar citotóxicos quando em excesso ou presentes por períodos prolongados. Este acúmulo de vacúolos autofágicos pode levar à indução de apoptose via ativação de caspase-3 devido à permeabilização das membranas lisossomal e mitocondrial. Desta forma, embora tenha sido observado o aumento da viabilidade celular na presença de CQ nesta condição e período de tratamento, é importante avaliar e confirmar este resultado em experimentos adicionais, os quais verificariam o efeito da presença de CQ por período prolongado.

Nossos resultados confirmam que a autofagia é um importante mecanismo na terapia alquilante e sugerem que a ação deste, combinada com um estimulador da autofagia pode ser uma aliada na eliminação de células de câncer de pulmão.

6. CONCLUSÕES

Durante o período do mestrado, foi elaborado um artigo de revisão que aborda o papel da autofagia na quimioterapia alquilante. A partir desta revisão é possível concluir que grande parte dos estudos publicados nesta área sugere que a autofagia atua como um mecanismo citoprotetor em células submetidas ao tratamento com agentes alquilantes.

Para determinar qual o papel da autofagia em células de câncer de pulmão tratadas com cisplatina foram realizados experimentos utilizando moduladores farmacológicos da via autofágica. A partir dos resultados experimentais obtidos neste trabalho é possível concluir que:

- CDDP é capaz de induzir autofagia em células de câncer de pulmão H460.
- Ocorre um aumento na indução de autofagia, principalmente em regime de pré-tratamento e cotratamento ao utilizar Rap em combinação com CDDP.
- O tratamento combinado de CDDP e Rap contribui para a eliminação das células de câncer de pulmão.
- A inibição da autofagia pelo tratamento com 3MA não tem influência na viabilidade celular.
- A utilização de CQ em combinação com CDDP aumenta a viabilidade celular de células H460.

7. PERSPECTIVAS

No decorrer do período de mestrado foram obtidos resultados importantes sobre a indução de autofagia em células de câncer de pulmão tratadas com cisplatina. Algumas questões relevantes foram levantadas durante o desenvolvimento desta dissertação. Desta forma, com o intuito de dar continuidade ao projeto, pretende-se:

- Avaliar a indução de apoptose nas células H460 e a sua correlação com a indução ou inibição da autofagia.
- Determinar a persistência da ativação da via autofágica após o tratamento com cisplatina, na presença ou ausência de rapamicina correlacionando a indução de autofagia com a viabilidade de células H460.
- Confirmar os resultados obtidos com inibidores farmacológicos da autofagia utilizando células H460 silenciadas para os genes essenciais da via autofágica, ATG4 e ATG5.
- Avaliar a indução e o papel da autofagia em células H460 resistentes ao tratamento com cisplatina.

8. REFERÊNCIAS BIBLIOGRÁFICAS

ABRAHAM, R. T.; GIBBONS, J. J. The mammalian target of rapamycin signaling pathway: twists and turns in the road to cancer therapy. **Clinical cancer research: an official journal of the American Association for Cancer Research**, v. 13, n. 11, p. 3109–14, 1 jun. 2007.

ALERS, S.; LÖFFLER, A. S.; WESSELBORG, S.; STORK, B. Role of AMPK-mTOR-Ulk1/2 in the regulation of autophagy: cross talk, shortcuts, and feedbacks. **Molecular and cellular biology**, v. 32, n. 1, p. 2–11, jan. 2012a.

ALERS, S.; LÖFFLER, A. S.; WESSELBORG, S.; STORK, B. The incredible ULKs. **Cell communication and signaling : CCS**, v. 10, n. 1, p. 7, jan. 2012b.

BENJAMIN, D.; COLOMBI, M.; MORONI, C.; HALL, M. N. Rapamycin passes the torch: a new generation of mTOR inhibitors. **Nature reviews. Drug discovery**, v. 10, n. 11, p. 868–80, nov. 2011.

BORDIN, D. L.; LIMA, M.; LENZ, G.; SAFFI, J.; MEIRA, L. B.; MÉSANGE, P.; SOARES, D. G.; LARSEN, A. K.; ESCARGUEIL, A. E.; HENRIQUES, J. A P. DNA alkylation damage and autophagy induction. **Mutation research**, v. 753, n. 2, p. 91–9, 2013.

BOULIKAS, T. Molecular mechanisms of cisplatin and its liposomally encapsulated form , Lipoplatin TM . Lipoplatin TM as a chemotherapy and antiangiogenesis drug Review Article. **Cancer Therapy**, v. 5, p. 351–376, 2007.

CHOI, A. M. K.; RYTER, S. W.; LEVINE, B. Autophagy in human health and disease. **The New England journal of medicine**, v. 368, n. 7, p. 651–62, 14 fev. 2013.

CRIGHTON, D.; WILKINSON, S.; RYAN, K. M. DRAM links autophagy to p53 and programmed cell death. **Autophagy**, v. 3, n. 1, p. 72–4, 2007.

DALBY, K. N.; TEKEDERELI, I.; LOPEZ-BERESTEIN, G.; OZPOLAT, B. Targeting the prodeath and prosurvival functions of autophagy as novel therapeutic strategies in cancer. **Autophagy**, v. 6, n. 3, p. 322–9, abr. 2010.

FU, D.; CALVO, J. A.; SAMSON, L. D. Balancing repair and tolerance of DNA damage caused by alkylating agents. **Nature reviews. Cancer**, v. 12, n. 2, p. 104–20, mar. 2012.

GALLUZZI, L.; SENOVILLA, L.; VITALE, I.; MICHELS, J.; MARTINS, I.; KEPP, O.; CASTEDO, M.; KROEMER, G. Molecular mechanisms of cisplatin resistance. **Oncogene**, v. 31, n. 15, p. 1869–83, 12 abr. 2012.

GIACCONE, G. Twenty-five years of treating advanced NSCLC: what have we achieved? **Annals of oncology: official journal of the European Society for Medical Oncology / ESMO**, v. 15 Suppl 4, n. Supplement 4, p. iv81–3, jan. 2004.

GONZALEZ, V. M.; FUERTES, M. A; ALONSO, C.; PEREZ, J. M. Is cisplatin-induced cell death always produced by apoptosis? **Molecular pharmacology**, v. 59, n. 4, p. 657–63, abr. 2001.

GREEN, D. R.; LEVINE, B. To Be or Not to Be? How Selective Autophagy and Cell Death Govern Cell Fate. **Cell**, v. 157, n. 1, p. 65–75, mar. 2014.

HE, C.; LEVINE, B. The Beclin 1 interactome. **Current opinion in cell biology**, v. 22, n. 2, p. 140–9, abr. 2010.

HOSOYA, N.; MIYAGAWA, K. Targeting DNA damage response in cancer therapy. **Cancer science**, v. 1, 31 jan. 2014.

Instituto Nacional de Câncer, disponível em <http://www.inca.gov.br/estimativa/2014/>, acesso em 10/03/14.

JIANG, P.; MIZUSHIMA, N. Autophagy and human diseases. **Cell research**, v. 24, n. 1, p. 69–79, jan. 2014.

JIANG, R.-Y.; PEI, H.-L.; GU, W.-D.; HUANG, J.; WANG, Z.-G. Autophagic inhibitor attenuates rapamycin-induced inhibition of proliferation in cultured A549 lung cancer cells. **European review for medical and pharmacological sciences**, v. 18, n. 6, p. 806–10, mar. 2014.

JOHANSEN, T.; LAMARK, T. Selective autophagy mediated by autophagic adapter proteins. **Autophagy**, v. 7, n. 3, p. 279–296, 1 mar. 2011.

KANZAWA, T.; GERMANO, I. M.; KOMATA, T.; ITO, H.; KONDO, Y.; KONDO, S. Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. **Cell death and differentiation**, v. 11, n. 4, p. 448–57, abr. 2004.

KIM, J.; KUNDU, M.; VIOLLET, B.; GUAN, K.-L. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. **Nature cell biology**, v. 13, n. 2, p. 132–41, mar. 2011.

KIMURA, T.; TAKABATAKE, Y.; TAKAHASHI, A.; ISAKA, Y. Chloroquine in cancer therapy: a double-edged sword of autophagy. **Cancer research**, v. 73, n. 1, p. 3–7, 1 jan. 2013.

KOMATSU, M.; ICHIMURA, Y. Physiological significance of selective degradation of p62 by autophagy. **FEBS letters**, v. 584, n. 7, p. 1374–8, 2 abr. 2010.

LEVY, J. M. M.; THORBURN, A. Targeting autophagy during cancer therapy to improve clinical outcomes. **Pharmacology & therapeutics**, v. 131, n. 1, p. 130–41, jul. 2011.

LIANG, X.; TANG, J.; LIANG, Y.; JIN, R.; CAI, X. Suppression of autophagy by chloroquine sensitizes 5-fluorouracil-mediated cell death in gallbladder carcinoma cells. **Cell & bioscience**, v. 4, n. 1, p. 10, jan. 2014.

LIU, F.; SHANG, Y.; CHEN, S. Z. Chloroquine potentiates the anti-cancer effect of lidamycin on non-small cell lung cancer cells in vitro. **Acta Pharmacologica Sinica**, p. 645–652, 2014.

LOPERGOLO, A.; NICOLINI, V.; FAVINI, E.; DAL BO, L.; TORTORETO, M.; COMINETTI, D.; FOLINI, M.; PEREGO, P.; CASTIGLIONI, V.; SCANZIANI, E.; BORRELLO, M. G.; ZAFFARONI, N.; CASSINELLI, G.; LANZI, C. Synergistic cooperation between sunitinib and cisplatin promotes apoptotic cell death in human medullary thyroid cancer. **The Journal of clinical endocrinology and metabolism**, v. 99, n. 2, p. 498–509, fev. 2014.

MACCIÒ, A.; MADEDDU, C. Cisplatin: an old drug with a newfound efficacy -- from mechanisms of action to cytotoxicity. **Expert opinion on pharmacotherapy**, v. 14, n. 13, p. 1839–57, set. 2013.

MEYERSON, M.; FRANKLIN, W. A.; KELLEY, M. J. Molecular classification and molecular genetics of human lung cancers. **Seminars in Oncology**, v. 31, n. 1, p. 4–19, fev. 2004.

MOSCAT, J.; DIAZ-MECO, M. T. P62 At the Crossroads of Autophagy, Apoptosis, and Cancer. **Cell**, v. 137, n. 6, p. 1001–4, 12 jun. 2009.

NAKAMURA, O.; HITORA, T.; YAMAGAMI, Y.; MORI, M.; NISHIMURA, H.; HORIE, R.; YAMAGUCHI, K.; YAMAMOTO, T. The combination of rapamycin and MAPK inhibitors enhances the growth inhibitory effect on Nara-H cells. **International journal of molecular medicine**, v. 33, n. 6, p. 1491–7, jun. 2014.

NODA, T.; FUJITA, N.; YOSHIMORI, T. The late stages of autophagy: how does the end begin? **Cell death and differentiation**, v. 16, n. 7, p. 984–90, jul. 2009.

NOTTE, A.; LECLERE, L.; MICHIELS, C. Autophagy as a mediator of chemotherapy-induced cell death in cancer. **Biochemical pharmacology**, v. 82, n. 5, p. 427–34, 1 set. 2011.

PALMEIRA-DOS-SANTOS, C.; PEREIRA, G. J. S.; BARBOSA, C. M. V.; JURKIEWICZ, A.; SMAILI, S. S.; BINCOLETTO, C. Comparative study of autophagy inhibition by 3MA and CQ on Cytarabine-induced death of leukaemia cells. **Journal of cancer research and clinical oncology**, 22 mar. 2014.

PARK, S. R.; YOO, Y. J.; BAN, Y.-H.; YOON, Y. J. Biosynthesis of rapamycin and its regulation: past achievements and recent progress. **The Journal of antibiotics**, v. 63, n. 8, p. 434–41, ago. 2010.

PETIOT, A.; OGIER-DENIS, E.; EDWARD, F. C.; MEIJER, A. J.; CHEM, J. B.; BLOMMAART, E. F. C.; CODOGNO, P. Distinct Classes of Phosphatidylinositol 3'-Kinases Are Involved in Signaling Pathways That Control Macroautophagy in HT-29 Cells. **The Journal of biological chemistry**, p. 1–8, 2000.

QI, X.-F.; KIM, D.-H.; LEE, K.-J.; KIM, C.-S.; SONG, S.-B.; CAI, D.-Q.; KIM, S.-K. Autophagy contributes to apoptosis in A20 and EL4 lymphoma cells treated with fluvastatin. **Cancer cell international**, v. 13, n. 1, p. 111, jan. 2013.

RAO, S.; TORTOLA, L.; PERLOT, T.; WIRNSBERGER, G.; NOVATCHKOVA, M.; NITSCH, R.; SYKACEK, P.; FRANK, L.; SCHRAMEK, D.; KOMNENOVIC, V.; SIGL, V.; AUMAYR, K.; SCHMAUSS, G.; FELLNER, N.; HANDSCHUH, S.; GLÖSMANN, M.; PASIERBEK, P.; SCHLEDERER, M.; RESCH, G. P.; MA, Y.; YANG, H.; POPPER, H.; KENNER, L.; KROEMER, G.; PENNINGER, J. M. A dual role for autophagy in a murine model of lung cancer. **Nature communications**, v. 5, p. 3056, jan. 2014.

RAVIKUMAR, B.; SARKAR, S.; DAVIES, J. E.; FUTTER, M.; GARCIA-ARENCEBIA, M.; GREEN-THOMPSON, Z. W.; JIMENEZ-SANCHEZ, M.; KOROLCHUK, V. I.; LICHTENBERG, M.; LUO, S.; MASSEY, D. C. O.; MENZIES, F. M.; MOREAU, K.; NARAYANAN, U.; RENNA, M.; SIDDIQI, F. H.; UNDERWOOD, B. R.; WINSLOW, A. R.; RUBINSZTEIN, D. C. Regulation of Mammalian Autophagy in Physiology and Pathophysiology. **Physiological Reviews**, v. 90, p. 1383–1435, 2010.

REN, J.-H.; HE, W.-S.; NONG, L.; ZHU, Q.-Y.; HU, K.; ZHANG, R.-G.; HUANG, L.-L.; ZHU, F.; WU, G. Acquired cisplatin resistance in human lung adenocarcinoma cells is associated with enhanced autophagy. **Cancer biotherapy & radiopharmaceuticals**, v. 25, n. 1, p. 75–80, fev. 2010.

RODRIGUEZ-ROCHA, H.; GARCIA-GARCIA, A.; PANAYIOTIDIS, M. I.; FRANCO, R. DNA damage and autophagy. **Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis**, v. 711, n. 1-2, p. 158–166, 2011.

ROSELL, R.; GATZEMEIER, U.; BETTICHER, D. C.; KEPPLER, U.; MACHA, H. N.; PIRKER, R.; BERTHET, P.; BREAU, J. L.; LIANES, P.; NICHOLSON, M.; ARDIZZONI, A.; CHEMAISSANI, A.; BOGAERTS, J.; GALLANT, G. Phase III randomised trial comparing paclitaxel/carboplatin with paclitaxel/cisplatin in patients with advanced non-small-cell lung cancer: a cooperative multinational trial. **Annals of Oncology**, v. 13, n. 10, p. 1539–1549, 1 out. 2002.

ROSENFELDT, M. T.; RYAN, K. M. The multiple roles of autophagy in cancer. **Carcinogenesis**, v. 32, n. 7, p. 955–63, jul. 2011.

RUBINSZTEIN, D. C.; GESTWICKI, J. E.; MURPHY, L. O.; KLIONSKY, D. J. Potential therapeutic applications of autophagy. **Nature reviews. Drug discovery**, v. 6, n. 4, p. 304–12, abr. 2007.

RYTER, S. W.; CHOI, A. M. K. Autophagy in the lung. **Proceedings of the American Thoracic Society**, v. 7, n. 1, p. 13–21, fev. 2010.

SCHONEWOLF, C. A.; MEHTA, M.; SCHIFF, D.; WU, H.; HAFFTY, B. G.; KARANTZA, V.; JABBOUR, S. K. Autophagy inhibition by chloroquine sensitizes HT-29 colorectal cancer cells to concurrent chemoradiation. **World journal of gastrointestinal oncology**, v. 6, n. 3, p. 74–82, 15 mar. 2014.

SEGLER, P. O.; GORDON, P. B. 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. **Proceedings of the National Academy of Sciences of the United States of America**, v. 79, n. 6, p. 1889–92, mar. 1982.

SHEN, C.; WANG, W.; TAO, L.; LIU, B.; YANG, Z.; TAO, H. Chloroquine blocks the autophagic process in cisplatin-resistant osteosarcoma cells by regulating the expression of p62/SQSTM1. **International journal of molecular medicine**, v. 32, n. 2, p. 448–56, ago. 2013.

SHEN, S.; KEPP, O.; KROEMER, G. The end of autophagic cell death? **Autophagy**, n. January, p. 1–3, 2012.

SHENG, Y.; SUN, B.; GUO, W.-T.; ZHANG, Y.-H.; LIU, X.; XING, Y.; DONG, D.-L. 3-Methyladenine induces cell death and its interaction with chemotherapeutic drugs is independent of autophagy. **Biochemical and biophysical research communications**, v. 432, n. 1, p. 5–9, 1 mar. 2013.

SHIMIZU, S.; YOSHIDA, T.; TSUJIOKA, M.; ARAKAWA, S. Autophagic cell death and cancer. **International journal of molecular sciences**, v. 15, n. 2, p. 3145–53, jan. 2014.

SOLOMON, V. R.; LEE, H. Chloroquine and its analogs: a new promise of an old drug for effective and safe cancer therapies. **European journal of pharmacology**, v. 625, n. 1-3, p. 220–33, 25 dez. 2009.

STATHOPOULOS, G. P. Cisplatin: process and future. **Journal of B.U.ON. : official journal of the Balkan Union of Oncology**, v. 18, n. 3, p. 564–9, 2013.

SUROVA, O.; ZHIVOTOVSKY, B. Various modes of cell death induced by DNA damage. **Oncogene**, v. 32, n. 33, p. 3789–97, 15 ago. 2013.

THOMÉ, R.; LOPES, S. C. P.; COSTA, F. T. M.; VERINAUD, L. Chloroquine: modes of action of an undervalued drug. **Immunology letters**, v. 153, n. 1-2, p. 50–7, jun. 2013.

TOOZE, S. A.; JEFFERIES, H. B. J.; KALIE, E.; LONGATTI, A.; MCALPINE, F. E.; MCKNIGHT, N. C.; ORSI, A.; POLSON, H. E. J.; RAZI, M.; ROBINSON, D. J.; WEBBER, J. L. Trafficking and signaling in mammalian autophagy. **IUBMB life**, v. 62, n. 7, p. 503–8, jul. 2010.

TOOZE, S. A.; YOSHIMORI, T. The origin of the autophagosomal membrane. **Nature cell biology**, v. 12, n. 9, p. 831–5, set. 2010.

VESSONI, A T.; FILIPPI-CHIELA, E. C.; MENCK, C. F.; LENZ, G. Autophagy and genomic integrity. **Cell death and differentiation**, v. 20, n. 11, p. 1444–54, nov. 2013.

WANG, D.; LIPPARD, S. J. Cellular processing of platinum anticancer drugs. **Nature reviews. Drug discovery**, v. 4, n. 4, p. 307–20, abr. 2005.

WHITE, E. Deconvoluting the context-dependent role for autophagy in cancer. **Nature reviews. Cancer**, v. 12, n. 6, p. 401–10, jun. 2012.

WU, Y.-T.; TAN, H.-L.; SHUI, G.; BAUVY, C.; HUANG, Q.; WENK, M. R.; ONG, C.-N.; CODOGNO, P.; SHEN, H.-M. Dual role of 3-methyladenine in modulation of autophagy via different temporal patterns of inhibition on class I and III phosphoinositide 3-kinase. **The Journal of biological chemistry**, v. 285, n. 14, p. 10850–61, 2 abr. 2010.

XU, Y.; XIA, X.; PAN, H. Active autophagy in the tumor microenvironment: A novel mechanism for cancer metastasis. **Oncology letters**, v. 5, n. 2, p. 411–416, fev. 2013.

YANG, Z. J.; CHEE, C. E.; HUANG, S.; SINICROPE, F. A. The role of autophagy in cancer: therapeutic implications. **Molecular cancer therapeutics**, v. 10, n. 9, p. 1533–41, set. 2011.

ZHANG, H.-Q.; HE, B.; FANG, N.; LU, S.; LIAO, Y.-Q.; WAN, Y.-Y. Autophagy inhibition sensitizes cisplatin cytotoxicity in human gastric cancer cell line SGC7901. **Asian Pacific journal of cancer prevention : APJCP**, v. 14, n. 8, p. 4685–8, jan. 2013.

ZHOU, W.; YUE, C.; DENG, J.; HU, R.; XU, J.; FENG, L.; LAN, Q.; ZHANG, W.; JI, D.; WU, J.; LIU, Q.; LIU, A. Autophagic protein Beclin 1 serves as an independent positive prognostic biomarker for non-small cell lung cancer. **PloS one**, v. 8, n. 11, p. e80338, jan. 2013.

ZHOU, Y.; PAN, D.-S.; SHAN, S.; ZHU, J.-Z.; ZHANG, K.; YUE, X.-P.; NIE, L.-P.; WAN, J.; LU, X.-P.; ZHANG, W.; NING, Z.-Q. Non-toxic dose chidamide synergistically enhances platinum-induced DNA damage responses and apoptosis in Non-Small-Cell lung cancer cells. **Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie**, 18 mar. 2014.

ZHU, L.; DU, H.; SHI, M.; CHEN, Z.; HANG, J. ATG7 deficiency promote apoptotic death induced by Cisplatin in human esophageal squamous cell carcinoma cells. **Bulletin du cancer**, v. 100, n. 7-8, p. 15–21, 2013.

ANEXO I

Metodologia adicional

Imunofluorescência

As células foram semeadas em uma densidade de 8×10^4 células/poço em placas de 6 poços, sobre lamínulas de 18 mm de diâmetro pré-tratadas com polilisina para melhor aderência. No dia seguinte foi feita a incubação com cisplatina, utilizando a concentração correspondente ao valor de IC_{50} por 48 horas. Após este período, as células foram fixadas com paraformaldeído (4%) e permeabilizadas com PBS-Triton (0,5%). Em seguida, os antígenos foram revelados utilizando o anticorpo primário anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Foi utilizado o anticorpo secundário conjugado com FITC anti-mouse. As imagens foram coletadas utilizando aumento de 10x através do Pathfinder™ Cellscan SreenTox (IMSTAR, Paris, França) no Laboratório Genotox-Royal, UFRGS. Foram analisadas no mínimo 100 células por grupo de tratamento quanto à presença de marcação do anticorpo na célula.

ANEXO II

CURRICULUM VITAE RESUMIDO

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DADOS PESSOAIS

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FORMAÇÃO

2007 – 2011: Graduação em Ciências Biológicas. Universidade Federal do Rio Grande do Sul, Brasil. Título: Análise da Resposta Celular em Linhagens de Câncer Colorretal e Câncer de Pulmão Expostas ao Tratamento com Agentes Antineoplásicos Platinados e a Baixas Concentrações de Glicose.

2012 – 2014: Mestrado em Programa de Pós-Graduação em Biologia Celular e Molecular, Universidade Federal do Rio Grande do Sul, Brasil. Orientador: Prof. Dr. João Antonio Pêgas Henriques. Coorientador: Prof. Dr. Guido Lenz.

ESTÁGIOS

2009 – 2011: Bolsista de Iniciação Científica no Laboratório de Reparação de DNA de Eucariotos, UFRGS.

ARTIGOS COMPLETOS PUBLICADOS

Bordin, Diana L.; Lima, Michelle; Lenz, Guido; Saffi, Jenifer; Meira, Lisiane B.; Mésange, Paul; Soares, Daniele G.; Larsen, Annette K.; Escargueil, Alexandre E.; Henriques, João A.P.. DNA alkylating damage and autophagy induction. *Mutation Research. Reviews in Mutation Research*, v. 753, p. 91-99, 2013.

RESUMOS E TRABALHOS APRESENTADOS EM CONGRESSOS

Resumos em anais de congresso como autor principal

1. Lima, M.; Bordin, D.; Escargueil, A.; Larsen, A.; Soares, D; Lenz, G.; Henriques, J.A.P. Cellular Response Analysis in Colorectal Cancer Cells Induced by Alkylating Agents. FeSBE 2011. Rio de Janeiro, 2011.
2. Lima, M.; Machado, M.; Bordin, D.; Prá, D.; Arigony, A.L.; Henriques, J.A.P. Avaliação da Viabilidade e Proliferação Celular nas Linhagens HepG2 e MRC5 em Cultura Suplementada com Ferro. XIII Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular. Porto Alegre, 2011.
3. Lima, M.; Bordin, D.; Lenz, G.; Henriques, J.A.P. Cellular Response Analysis in Colorectal Cancer and Lung Cancer Cells Exposed to Treatment with Platinum Agents in Low Glucose Conditions. XVII Alexander Hollaender Course. Montevideo, 2012.
4. Lima, M.; Bordin, D.; Lenz, G.; Henriques, J.A.P. Cellular Response Analysis in Colorectal Cancer and Lung Cancer Cells Exposed to Treatment with Platinum Agents in Low Glucose Conditions. Workshop Cell Stress. Porto Alegre, 2012.
5. Lima, M.; Bordin, D.; Lenz, G.; Henriques, J.A.P. Cellular Response Analysis in Colorectal Cancer and Lung Cancer Cells Exposed to Treatment with Platinum Agents in Low Glucose Conditions. 10th International Congress on Cell Biology. Rio de Janeiro, 2012.

6. Lima, M.; Bordin, D.; Lenz, G.; Henriques, J.A.P. Avaliação do Papel da Autofagia em Células de Câncer de Pulmão Tratadas com o Agente Alquilante Cisplatina. XIV Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular. Porto Alegre, 2012.
7. Lima, M.; Bordin, D.; Soares, V.J.; Lenz, G.; Henriques, J.A.P. Autophagy Activation in Lung Cancer Cells Treated with the Alkylating Agent Cisplatin. 11th ICEM, International Conference on Environmental Mutagens. Foz do Iguaçu, 2013.
8. Lima, M.; Bordin, D.; Soares, V.J.; Lenz, G.; Henriques, J.A.P. Autophagy Activation in Lung Cancer Cells Treated with the Alkylating Agent Cisplatin. XV Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular. Porto Alegre, 2013.

Resumos em anais de congresso como co-autor

1. Bordin, D.; Lima, M.; Lenz, G.; Escargueil, A.; Soares, D.; Larsen, A.; Henriques, J.A.P. Efeito dos Agentes Antineoplásicos Oxaliplatina e Cisplatina em Linhagens de Câncer Colorretal Expostas a Diferentes Concentrações de Glicose. Congresso Brasileiro da SBMCTA. Ouro Preto, 2009.
2. Bordin, D.; Lima, M.; Lenz, G.; Escargueil, A.; Soares, D.; Larsen, A.; Henriques, J.A.P. Efeito dos Agentes Antineoplásicos Oxaliplatina e Cisplatina em Linhagens de Câncer Colorretal Expostas a Diferentes Concentrações de Glicose. XI Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular. Porto Alegre, 2009.
3. Bordin, D.; Lima, M.; Lenz, G.; Escargueil, A.; Soares, D.; Larsen, A.; Henriques, J.A.P. Avaliação da Resposta Celular em Linhagens de Câncer Colorretal por Agentes Alquilantes em Condições de Estresse Energético. XII Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular. Porto Alegre, 2010.
4. Arigony, A.L.V.; Lima, M.; Schuler, G.; Bordin, D.; Machado, M.; Prá, D.; Henriques, J.A.P. Efeitos da Suplementação com Ferro na Viabilidade Celular e Genotoxicidade em Linhagens HepG2 e MRC5. Toxsul, II Congresso Sul de Toxicologia Clínico-Laboratorial. Porto Alegre, 2011.

5. Bordin, D.; Lima, M.; Lenz, G.; Escargueil, A.; Soares, D.; Larsen, A.; Henriques, J.A.P. Avaliação da Indução de Autofagia por Agentes Alquilantes em Linhagens de Câncer Colorretal em Condições de Estresse Energético. XIII Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular. Porto Alegre, 2011.
6. Arigony, A.L.V.; Lima, M.; Schuler, G.; Juchem, A.L.; Trindade, C.; Bordin, D.; Machado, M.; Prá, D.; Henriques, J.A.P. Avaliação da Influência do Meio de Cultura Suplementado com Ferro na Viabilidade Celular e na Estabilidade Genômica das Linhagens HepG2 e MRC5. XIII Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular. Porto Alegre, 2011.
9. Bordin, D.; Lima, M.; Lenz, G.; Soares, D.; Escargueil, A.; Larsen, A.; Henriques, J.A.P. Autophagy Induction by Oxaliplatin in Colorectal Cancer Cells Treated in Energy Stress Conditions. XVII Alexander Hollaender Course. Montevideo, 2012.
10. Bordin, D.; Lima, M.; Lenz, G.; Henriques, J.A.P. Autophagy Activation in Colorectal Cancer Contributes to the Tolerance of Oxaliplatin Under Energy Stress Conditions. 10th International Congress on Cell Biology. Rio de Janeiro, 2012.
11. Arigony, A.L.V.; Milano, L.; Lima, M.; Schuler, G.; Juchem, A.L.; Trindade, C.; Machado, M.; Bordin, D.; Filippi-Chiela, E.; Lenz, G.; Prá, D.; Henriques, J.A.P. Evaluation of Iron Supplemented Media Culture in the Cellular Viability and Stability of Cell Lines MRC5 and HepG2. 10th International Congress on Cell Biology. Rio de Janeiro, 2012.
7. Bordin, D.; Lima, M.; Lenz, G.; Escargueil, A.; Soares, D.; Larsen, A.; Henriques, J.A.P. Ativação de Autofagia Contribui para a Resistência ao Tratamento com Oxaliplatina em Condições de Estresse Energético. XIV Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular. Porto Alegre, 2012.
8. Arigony, A.L.V.; Milano, L.; Lima, M.; Schuler, G.; Juchem, A.L.; Trindade, C.; Machado, M.; Bordin, D.; Filippi-Chiela, E.; Lenz, G.; Prá, D.; Henriques, J.A.P. Avaliação da Influência do Meio de Cultura Suplementado com Ferro na Viabilidade Celular e na Estabilidade Genômica das Linhagens HepG2 e

- MRC5. XIV Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular. Porto Alegre, 2012.
9. Bordin, D.; Lima, M.; Lenz, G.; Mésange, P.; Soares, D.; Larsen, A.; Escargueil, A.; Henriques, J.A.P. Autophagy Induction in Colorectal Cancer Contributes to the Tolerance of Oxaliplatin Under Low Glucose Condition. 11th International Conference on Environmental Mutagens. Foz do Iguaçu, 2013.
 10. Soares, V.J.; Lima, M.; Bordin, D.; Lenz, G.; Henriques, J.A.P. Criação de uma Linhagem de Câncer de Pulmão Humana Resistente à Cisplatina e Modulação da Via Autofágica nesta Resistência. XV Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular. Porto Alegre, 2013.