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O PAPEL DA AUTOFAGIA NA RESISTÊNCIA DE GLIOMAS AO TRATAMENTO COM TEMOZOLOMIDA E INIBIDOR DE HISTONAS DESACETILASES

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Dissertação submetida ao Programa de Pós Graduação em Ciências Biológicas: Bioquímica, como um pré-requisito parcial para obtenção do título de Mestre em Bioquímica.

Orientador: Prof. Dr. Alfeu Zanotto-Filho

Co-Orientador: Prof. Dr. José Cláudio Fonseca Moreira

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Sumário

PARTE I

RESUMO

Glioblastoma multiforme é o tipo mais frequente de tumor cerebral primário, sendo caracterizado por uma alta agressividade e um prognóstico bastante limitado. O ácido hidroxâmico suberoilanilida (SAHA) é um inibidor específico de histonas desacetilases aprovado para o tratamento de linfoma cutâneo de células T, e em fase de crescente investigação clínica e pré-clínica em tumores sólidos. Neste estudo, avaliamos a eficácia do ácido hidroxâmico suberoilanilida em tratamento combinado com temozolomida, o agente alquilante já utilizado em glioblastomas. Através de testes de viabilidade e analises por citometria de fluxo em células tumorais das linhagens U251MG e C6, observamos que não houve sinergismo de potenciação entre temozolomida e ácido hidroxâmico suberoilanilida, apenas efeito sinergismo de adição O tratamento combinado inicialmente promoveu parada do ciclo celular em fase G2/M (≥48 h) ao passo que a apoptose foi detectada apenas em exposição prolongada (≥96 h) aos fármacos em estudo. Ainda, as células tratadas com TMZ/SAHA apresentaram fenótipo autofágico, como determinado por citometria de fluxo e imunodetecção de proteínas marcadoras de autofagia como LC3 e o p62/SQSTM1. A autofagia temporalmente precedeu a apoptose e exerceu função citoprotetora, uma vez que o bloqueio da terminação autofágica com cloroquina promoveu uma significante redução na viabilidade celular, a qual foi associada a um aumento de apoptose em células de glioma tratadas com TMZ/SAHA. Portanto, os dados apresentados neste trabalho demonstram que a autofagia é um processo que diminui a eficácia antiglioma do temozolomida e do ácido hidroxâmico suberoilanilida, e a inibição deste fenômeno pode ser uma estratégia para aperfeiçoar a terapia com esses fármacos.

ABSTRACT

Glioblastoma multiforme (GBM) is the most frequent and aggressive type of primary brain tumor which has been associated with a dismal prognosis. In this study, we tested the efficacy of combining temozolomide (TMZ) with suberoylanilide hydroxamic acid (SAHA) - an inhibitor of HDACs 1, 2, 3, and 6 approved for the treatment of cutaneous T-cell lymphoma - in the viability of tumor cells. The data showed that potentiation synergism between TMZ e SAHA was not achieved due to activation of protective autophagy *in vitro*. The SAHA/TMZ treatment promoted arrest in the G2/M phase of the cell cycle as soon as 48 h after drug exposure whereas apoptosis was only detected after long-lasting exposure (≥96 h). In addition, SAHA and TMZ induced autophagy as detected by flow cytometry of acridine orange stained cells and immunodetection of the lipidated form of LC3 as well as decreases in p62/SQSTM1. Autophagy preceded apoptosis, and by blocking the termination step of autophagy with chloroquine promoted a significant reduction in the viability of glioma cells which was accompanied by increased apoptosis in SAHA/TMZ treatment. Overall, the herein presented data demonstrate that autophagy impairs the efficacy of combined TMZ/SAHA, and inhibiting this phenomenon could provide novel opportunities to improve the therapeutic potential of these compounds.

LISTA DE ABREVIATURAS

AMPK: Proteína cinase ativada por AMP

ATG: Genes relacionados à autofagia

ATM: Ataxia-telangiectasia mutada

bcl-2: Regulador da apoptose bcl2

bcl-xL: Proteína 1 tipo Bcl-2

BHE: Barreira hematoencefálica

CMA: Autofagia mediada por chaperonas

CQ: Cloroquina

DDR: Resposta ao dano em DNA

DR: Receptores de morte

DSB: Quebras da dupla-fita de DNA

EGFR: Receptor para o fator de crescimento epidermal

ERBB2: Receptor 2 do fator de crescimento epidermal humano

ERO: Espécies reativas de oxigênio

FDA: Food and Drug Administration

GBM: Glioblastoma multiforme

HAT: Histona acetiltransferase

HDAC: Histona desacetilases

HR: Recombinação Homóloga

INCA: Instituto Nacional do Câncer

LC3: Cadeia leve 3 da proteína 1 associada a microtúbulos

MAPK: Cinases ativadas por mitógenos

MGMT: O6-metilguanina-DNA-metiltransferase

MMR: Sistema de Reparo de Incompatibilidades

mPTP: Poro de permeabilidade transitória

MTIC: (5-(3-dimetil-1-triazenil)imidazol-4-carboxamida)

mTOR: Proteína alvo da rapamicina em mamíferos

MTT: (3-(4,5-dimetil)-2,5-difenil tetrazólio)

NAC: N-acetilcisteína

NHEJ: Junção Terminal Não Homóloga

OMS: Organização Mundial de Saúde

p62/SQSTM1: Sequestossoma-1

PI: Iodeto de propídio

PI3K/Akt: Fosfatidilinositol-3-cinase/serina-treonina cinase

PTEN: Fosfatase homóloga a tensina

Ras: Proteína Homóloga do Oncogene viral de Sarcoma de rato

SAHA: Ácido hidroxâmico suberoilanilida

SNC: Sistema nervoso central

TMZ: Temozolomida

TNFR: Receptor do fator de necrose tumoral (TNF)

ULK1: Cinase ativadora de autofagia tipo unc51

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1 INTRODUÇÃO

1.1 Gliomas

Gliomas são tumores primários que atingem o sistema nervoso central (SNC). Originados de precursores gliais/astrocíticos, eles são divididos em subtipos histológicos, os quais possuem diferentes graus de malignidade. A malignidade é determinada seguindo o sistema de classificação de tumores cerebrais da Organização Mundial de Saúde (OMS), que divide os gliomas em quatro graus, com base em algumas características específicas como malignidade celular, invasividade e capacidade de desenvolver necrose (Chintala *et al.*, 1999). Os tumores de grau I (astrocitoma pilocítico) são benignos e podem ser eliminados por cirurgia; os tumores de grau II (astrocitoma de baixo grau) são de baixa malignidade, porém, devido a sua característica infiltrativa, são de difícil remoção; os tumores de grau III (astrocitoma anaplásico) são altamente proliferativos; e os tumores de grau IV são os mais malignos e são mais conhecidos como glioblastoma multiforme (GBM). O GBM é letal e está associado à alta morbidade, uma vez que os pacientes apresentam um grande comprometimento do tecido nervoso periférico ao tumor com ocorrência de sintomas como cefaleia, além de alterações cognitivas e na fisiologia de órgãos/sistemas periféricos (Chintala *et al.*, 1999; Holland *et al.*, 2001; Mercer *et al*., 2009; Koukourakis *et al*., 2009).

Neste estudo focaremos nossa atenção no GBM. No Brasil, os cânceres de SNC, dos quais os gliomas possuem maior incidência (~60% dos diagnósticos), ocupam entre a 8ª e 10ª posição, dependendo da região. Segundo dados do Instituto Nacional do Câncer (INCA), para o Brasil, no ano de 2016, estimaram-se 5.440

casos novos de câncer do SNC em homens e 4.830 em mulheres. Esses valores correspondem a um risco estimado de 5,50 casos novos a cada 100 mil homens e 4,68 para cada 100 mil mulheres. Relevantemente, na região sul, os índices desses cânceres são mais altos que a média nacional, sendo o oitavo mais frequente em homens (10,44/ 100 mil) e o sexto (8,45/100 mil) entre as mulheres (dados do INCA) (Fig. 1). O GBM é o tumor primário mais comum, sendo altamente proliferativo, com infiltração difusa no parênquima cerebral, presença de necrose, intensa resistência a apoptose induzida por radio- e quimioterapia (Laws e Shaffrey, 1999). A dificuldade de tratamento se dá devido a sua localização no cérebro, que é protegido pela barreira hematoencefálica (BHE). Enquanto muitos tipos de câncer têm sido beneficiados pelo desenvolvimento de novas terapias, o tratamento de glioblastoma apresentou pouco progresso na última década. Em geral a terapia empregada se baseia na cirurgia para retirada do tumor seguida por radioterapia e quimioterapia com o alquilante temozolomida (TMZ) (Weller *et al*., 2005; Mercer *et al*., 2009). Mesmo com esses métodos de tratamento, a mediana de sobrevida dos pacientes é de aproximadamente 14 meses (Krakstad e Chekenya, 2010). Esse cenário se agravou quando os dados de estudos clínicos de fase II e III com inibidores do receptor para o fator de crescimento epidermal (EGFR) (erlotinib e gefitinib) (Karpel-Massler *et al*., 2011; Wen *et al*., 2014), receptor 2 do fator de crescimento epidermal humano ERBB2 (Reardon *et al*., 2012); inibidores de fosfatidilinositol-3-cinase/serina-treonina cinase (PI3K/Akt) (Pitz *et al*., 2015) e inibidores de Ras/MEK/ERK1/2 (TLN-4601 e sorafenib) (Mason *et al*., 2012; Karajannis *et al*., 2014) - estes considerados alvos promissores a partir de estudos pré-clínicos desenvolvidos nas últimas duas décadas – mostraram uma eficácia muito limitada ou

ausente. Isso renova a necessidade do desenvolvimento de novas estratégias terapêuticas para o tratamento dos GBM.

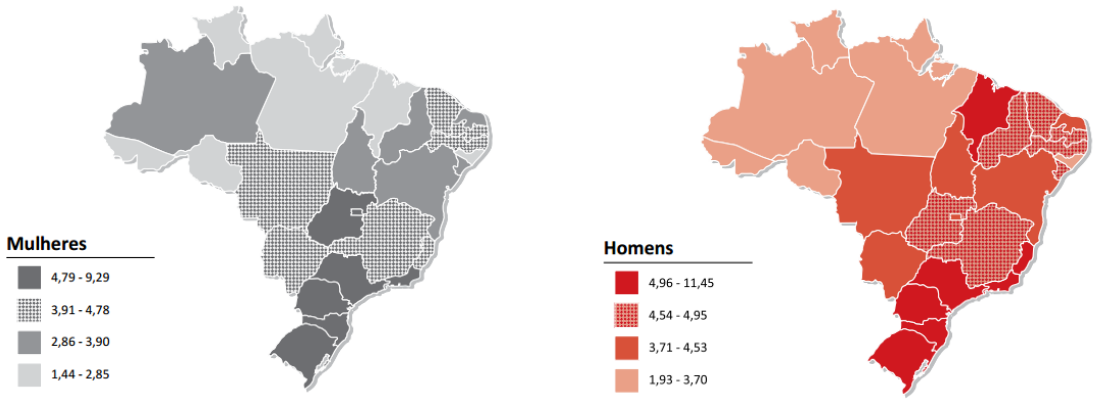

Figura 1. Representação espacial das taxas brutas de incidência por 100 mil, estimadas para o ano de 2016, segundo Unidade da Federação (neoplasia maligna do Sistema Nervoso Central). Dados retirados do site do INCA (Instituto Nacional de Câncer José de Alencar Gomes da Silva).

1.2 Temozolomida

Disponível comercialmente desde o ano 2000, a temozolomida (TMZ) é um pró-fármaco de baixo peso molecular (194,1 g/mol) (Fig. 2), que é ~100% absorvida por via oral, e é praticamente toda eliminada após 8 h da ingestão ($T_{1/2}$: 1,8 h). Por ser uma substância lipofílica, pode passar a BHE chegando aos tumores cerebrais (Friedman *et al*., 2000; Johnson e O'Neill, 2012). O agente citotóxico da TMZ, na verdade, é o metabólito 5-(3-dimetil-1-triazenil)imidazol-4-carboxamida (MTIC), um agente alquilante produzido por hidrolização em pH fisiológico. O mecanismo de ação proposto para o MTIC é a metilação dos resíduos de guanina da molécula de DNA, resultando na formação de O^6 e N^7-metilguanina (Friedman *et al*., 2000). A O^6-

metilguanina é responsável pela citotoxicidade do TMZ, pois a metilação por ela originada causa a formação de quebras em fita dupla na molécula de DNA as quais, dependendo da magnitude do dano e da capacidade dos sistemas de reparo, podem levar à morte celular. A N^7-metilguanina é menos tóxica que a O^6-metilguanina (Wesolowski *et al.*, 2010; Johnson e O'Neill, 2012) (Fig. 3). O tratamento quimioterápico com TMZ consiste na administração via oral de TMZ por 5 dias a cada 28 dias. Além da mielossupressão típica de agentes alquilantes (não acumulativa e rapidamente reversível no caso do TMZ; segundo Friedman *et al*., 2000), os efeitos colaterais não hematológicos mais frequentes são náuseas, cefaleia, vômito e fadiga (grau 1 e 2; leves), sendo estes controlados com o uso de antieméticos e analgésicos (Friedman *et al*., 2000; Koukourakis *et al.*, 2009; Wesolowski *et al.*, 2010; Johnson e O'Neill, 2012).

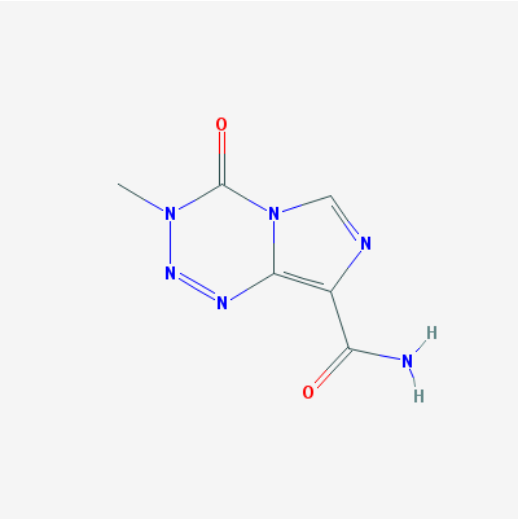

Figura 2. Estrutura química da temozolomida. Retirada de https://pubchem.ncbi.nlm.nih.gov/compound/5394.

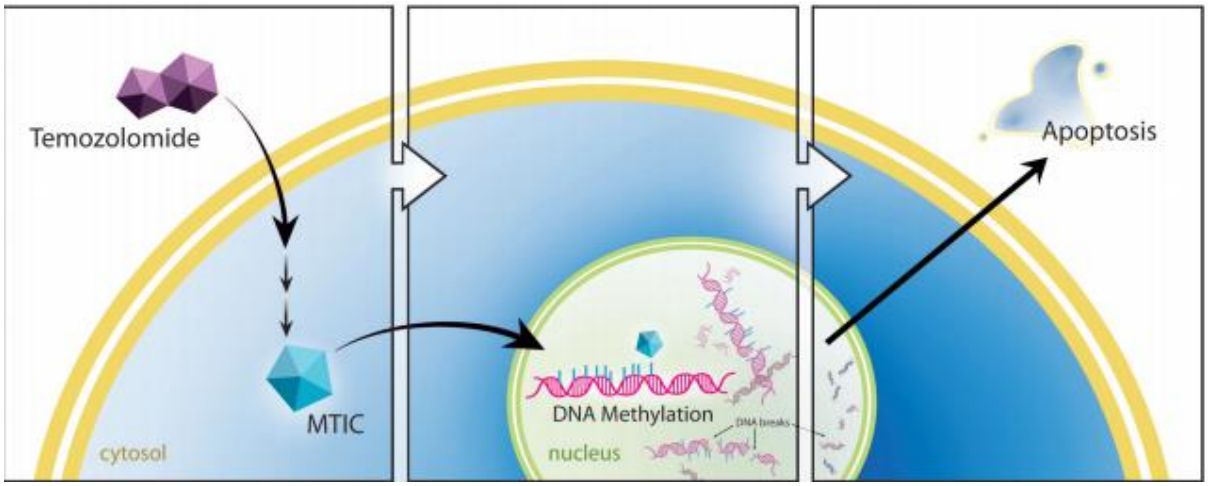

Figura 3. Ilustração esquemática do mecanismo proposto para a ação da temozolomida. A temozolomida é convertida intracelularmente em MTIC, que metila o DNA. Na ausência de reparo celular eficiente, a metilação pode resultar em ruptura do DNA e consequentemente em apoptose. Retirado de Wesolowski *et al.*, 2010. MTIC: (5-(3-dimetil-1-triazenil)imidazol-4-carboxamida).

A quimioterapia com TMZ proporciona um aumento médio de 5 a 6 meses na sobrevida de pacientes, e muito desse efeito limitado se deve ao fato de o GBM rapidamente desenvolver mecanismos de resistência à TMZ (Johnson e O'Neill, 2012). A combinação de TMZ com radioterapia também apresenta resultados favoráveis, no entanto o aumento de sobrevida é pouco maior. Um dos mecanismos mais relevantes na clínica de GBM é a expressão da enzima O^6-methylguanine-DNA-methyltransferase (MGMT), a qual remove o grupamento da O^6-metilguanina à custa de sua inativação por metilação (chamada de “enzima suicida”). Estudos mostram que pacientes de GBM com metilação do promotor de MGMT (ou seja, com menor expressão da enzima) são mais responsivos ao TMZ que a pacientes com expressão normal ou aumentada desta enzima (Hegi *et al*., 2005; Knizhnik *et al*, 2013; Molenaar *et al*., 2014). Neste contexto, o uso combinado de TMZ com inibidores de MGMT, como a O^6-benzilguanina, foi testado clinicamente, mas os dados pré-clínicos não se reproduziram nos pacientes nos protocolos testados até o

momento (Quinn *et al*., 2009; Blumenthal *et al*., 2015). Outra alteração encontrada nos tumores é a deficiência de MMR (reparo de incompatibilidade do DNA) quando essa via está alterada não ocorre o efeito de O^6-MG, a replicação do DNA prossegue sem parada no ciclo ou apoptose (Koukourakis *et al.*, 2009).

1.3 Ácido hidroxâmico suberoilanilida (SAHA)

Além dos mecanismos genéticos, o processo tumoral depende também de alterações nos mecanismos epigenéticos responsáveis pelo controle da expressão gênica, os quais sofrem grande influência do microambiente celular (Adcock *et al.*, 2006). Entre as modificações epigenéticas que ocorrem podemos destacar a acetilação de histonas, que desempenha um importante papel na modulação da expressão de genes que atuam no controle do ciclo celular e contribuem para o desenvolvimento e progressão de neoplasias (Masseti *et al.,* 2011; Sharma, Kelly, Jones, 2010).

A acetilação das histonas é mediada por um grupo de enzimas chamadas histonas acetiltransferases (HATs) que adicionam radicais acetil aos resíduos de lisina das proteínas histonas, resultando em aumento da densidade eletrônica, diminuição da interação entre DNA/histonas e, consequentemente, na descompactação da cromatina associada aos nucleossomas e aumento da atividade transcricional de um determinado gene. Já as histonas desacetilases (HDACs) atuam removendo os radicais acetil e recrutando complexos co-repressores, o que resulta na compactação da cromatina e diminuição da expressão gênica (Minucci, Pelicci, 2006; Ropero e Esteller, 2007).

Atualmente, as HDACs são consideradas um importante alvo para o desenvolvimento de novas drogas para o tratamento de cânceres (Minucci e Pelicci, 2006). Os inibidores de HDAC são agentes que atuam sobre a regulação da expressão gênica e mostram diversos efeitos como regulação da expressão gênica e indução de apoptose em células tumorais, enquanto células sadias se apresentam mais resistentes aos efeitos dessa classe de compostos (Minucci e Pelicci, 2006; Park *et al.*, 2004; Romanski *et al.*, 2004).

O ácido hidroxâmico suberoilanilida (SAHA; ou Vorinostat) (Fig. 4) é um inibidor das HDACs 1, 2, 3 e 6 que, até o momento, está aprovado pela *Food and Drug Administration* (FDA) para o tratamento de linfoma cutâneo de células-T (Gammoh *et al., 2012*). O SAHA também tem efeito no câncer de próstata, ovários, mama e GBM - testes em fase II identificaram boa tolerância em pacientes, onde 26% deles apresentaram algum sinal de toxicidade, quando usado como monoterapia (Galanis *et al.*, 2009; Marks *et al.*, 2007). O mecanismo de ação do SAHA consiste na ligação ao sítio ativo das HDACs, agindo como um quelante de íons zinco. Essa inibição resulta num aumento de histonas e outras proteínas acetiladas, incluindo fatores de transcrição importantes para a expressão de genes envolvidos na diferenciação celular, proliferação, apoptose e resposta ao dano celular (Minucci e Pelicci, 2006). Alguns estudos, de fase clínica I e II, vêm sendo realizados com GBM tratado com SAHA sozinho ou combinado com outras drogas; e os resultados até então encontrados sugerem que SAHA é bem tolerado embora os dados de eficácia ainda não sejam conclusivos, dado o número de pacientes recrutados até o momento (Galanis *et al.*, 2009;).

Alguns estudos demonstram o envolvimento da produção de ERO e depleção de glutationa como envolvidas na morte celular induzida por SAHA (Chiaradona *et al*., 2015), embora ainda não se saiba se esses efeitos ocorrem como consequência da inibição de HDACs ou pela modulação de acetilação de outras proteínas celulares.

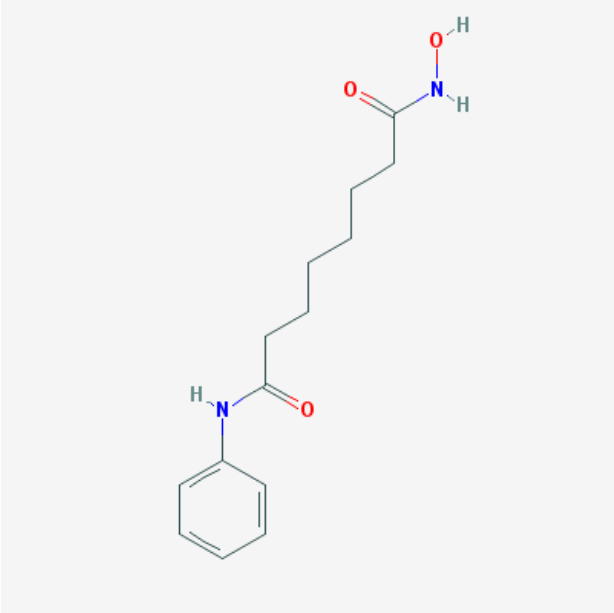

Figura 4. Estrutura química do ácido hidroxâmico suberoilanilida (SAHA). Retirado de https://pubchem.ncbi.nlm.nih.gov/compound/5311#section=2D-Structure.

1.4 Morte celular

1.4.1 Apoptose

A morte celular por apoptose representa um importante papel durante o desenvolvimento do organismo, bem como na regulação do sistema imunológico e na defesa contra doenças. Ela é conhecida como morte celular programada do tipo I.

A apoptose é caracterizada por uma condensação e fragmentação da cromatina, que gera um encolhimento de toda a célula. A fosfatidilserina, um fosfolipídio de membrana, é exposta para o lado de fora da célula, sinalizando para

os macrófagos que a célula deve ser fagocitada (Elmore, 2007). O interessante desse processo de morte é que ele ocorre sem que haja rompimento da membrana plasmática, não ocorrendo extravasamento de citocinas, impedindo que ocorra um processo inflamatório.

A morte por apoptose é descrita como induzida por dois mecanismos um por via intrínseca e outro por via extrínseca, que divergem na forma de ativação. A via intrínseca é ativada por permeabilidade da mitocôndria. Os estímulos que iniciam a via intrínseca podem ser mediados por sinais positivos ou negativos. O sinal positivo pode ser toxinas, hipóxia, infecções virais ou radicais livres. Já o sinal negativo envolve a ausência de fatores de crescimento, hormônios e citocinas que suprimiriam a morte, desencadeando uma falha na supressão de sinal e levando a apoptose (Elmore, 2007). A via intrínseca envolve a perda de polarização do potencial de membrana mitocondrial, abertura do poro de permeabilidade transitória (mPTP), o que leva à liberação de citocromo c da mitocôndria para o compartimento citoplasmático. O citocromo c liga e ativa proteínas como a Apaf-1 e a caspase-9, formando um complexo chamado apoptossoma. Caspase-9 ativa, por fim, cliva e ativa as caspases-3 e 7 efetoras, iniciando o processor de degradação de proteínas chave para a fragmentação do DNA e formação dos corpos apoptóticos (Elmore, 2007). A via extrínseca é ativada por sinais externos à célula, através de receptores transmembrana. Os receptores de morte são chamados de *death receptors* (DR), membros da superfamília *tumor necrosis factor receptors* (TNFR) e FAS/Fas-ligante. Após a ativação do receptor, é ativada uma cascata de caspases através da dimerização da caspase-8, que cliva e ativa as caspases 3 e 7, ponto de encontro das duas vias, para efetivação da apoptose (Elmore, 2007). Em células tumorais, a

apoptose muitas vezes é suprimida tanto pela superexpressão de proteínas antiapoptóticas (como bcl-xl e bcl2) tanto pela inativação de supressores tumorais, como a p53. Em gliomas, a p53 é mutada em aproximadamente ~ 42 % dos tumores (Brennan *et al*., 2013). De modo indireto, a ativação de rotas mitogênicas também favorece a supressão da apoptose. Em gliomas, as vias de PI3K e EGFR são constitutivas (por mutação/deleção da fosfatase homóloga a tensina (PTEN) e mutação/amplificação de EGFR, respetivamente) causando hiperativação do sinal mitótico e supressão da apoptose (Brennan *et al*., 2013).

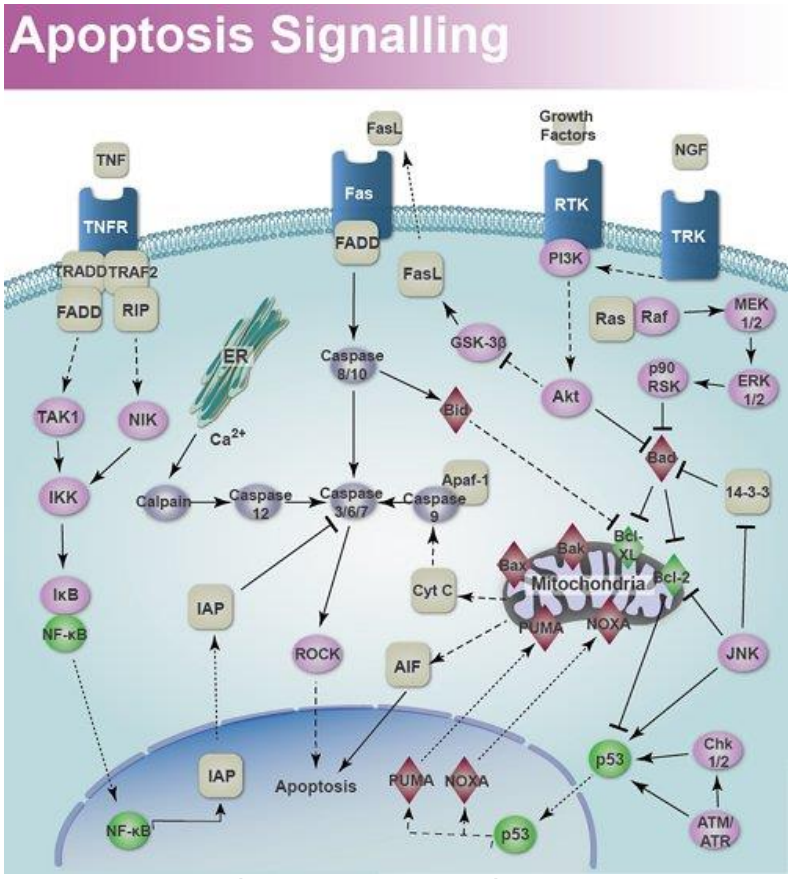

Figura 5. Ilustração esquemática das vias intrínsecas (mitocondrial) e extrínseca (via receptor de membrana) do processo de apoptose. Retirado de https://www.tocris.com/pharmacologicalBrowser.php?ItemId=187886#.WHyoslMrKM8.

1.4.2 Autofagia

Autofagia é um processo de reciclagem que é ativada quando a célula sofre algum tipo de privação de nutrientes ou ainda um dano pequeno, esse mecanismo é desencadeado para proteger a célula de uma morte desnecessária, visto que a injuria sofrida pode ser reversível. Durante o processo, organelas e material citosólico serão degradados em vesículas e os aminoácidos serão retornados ao microambiente celular para serem reutilizados na produção de novas proteínas (Levine *et al*, 2004).

A autofagia pode ser classificada como macroautofagia, microautofagia e autofagia mediada por chaperonas (Mizushima e Komatsu, 2011). Na microautofagia ocorre o engolfamento do citoplasma por invaginação da membrana lisossomal (Rodriguez-Rocha *et al*., 2011), já na autofagia mediada por chaperonas (CMA) o lisossomo degrada proteínas citoplasmáticas que foram encaminhadas pela hsp70 e co-chaperonas (Majeski *et al.*, 2004). A macroautofagia (também chamada apenas de autofagia) é responsável pela degradação de organelas e proteínas de longa duração (Levine *et al.*, 2004). Ela se caracteriza por formar autofagossomos, que se fusionam com o lisossomo, e esta estrutura irá degradar e reciclar os componentes celulares em um ambiente ácido composto por hidrolases lisossomais (Kroemer, 2005).

Para que a autofagia ocorra é necessário que um grupo de proteínas da família dos genes relacionados à autofagia (ATG) sejam ativadas. Tais proteínas são os principais constituintes dessa maquinaria, as ATG5, ATG7, ATG8, ATG10 e a ATG12 são as primeiras a serem recrutadas e formam o autofagossomo. A ATG8 é conhecida como *light chain 3* (LC3), ela pode ser encontrada em duas formas a LC3-I (localizada no citosol) e a forma proteolítica LC3-II (localizada na membrana do autofagossomo) (Kögel *et al.*, 2010). A proteína LC3 é clivada formando LC3-I e então é conjugada a fosfatidiletanolamina (PE) formando a LC3-II, nessa forma ela se liga ao autofagossomo, a conversão LC3-I para LC3-II é utilizada como marcador positivo para autofagia.

Outra importante proteína deste processo é ATG6 também chamada de beclina-1, ela é uma reguladora do processo autofágico, formando um complexo com a PI3K atua na formação do autofagossomo e facilita a fusão desse com o

lisossomo, onde finalmente acontecerá a degradação dos materiais capturados (Pirtoli *et al*, 2009; Qiu *et al*, 2014).

No contexto do câncer, a autofagia tem sido frequentemente associada com a resistência celular ao estresse causado pelos agentes citotóxicos usados em quimioterapia. O processo autofágico parece ser induzido como uma tentativa de aumento de catabolismo de biomoléculas e organelas danificadas, de modo a tanto minimizar sinais apoptóticos mediados por tais estruturas (ex.: liberação de citocromo c em mitocôndrias disfuncionais) quanto aproveitar os esqueletos carbonados resultantes para a formação de novas moléculas necessárias em resposta ao estresse. Por outro lado, embora menos frequente em estudos com modelos tumorais, a autofagia pode promover morte celular, uma vez que inibidores da autofagia podem reverter a perda de viabilidade em alguns modelos de citotoxicidade (Hippert *et al*, 2006; He e Klinsky, 2009; Jiang e Mizushima, 2013; Sui *et al*., 2013). Nesse contexto, inúmeros estudos clínicos estão em andamento na tentativa de elucidar se inibidores da autofagia como a cloroquina e hidroxicloroquina são capazes de trazer benefícios terapêuticos em diferentes canceres (detalhados em Sui *et al*., 2013). Em GBM, os estudos clínicos NCT00224978 e NCT00486603 (www.clinicaltrials.gov; acesso em 02/2017) são alguns exemplos do uso de inibidores de autofagia em terapia adjuvante de GBM e, no nosso conhecimento, apenas o estudo de fase I/II de Rosenfeld *et al* (2014) foi publicado até o momento.

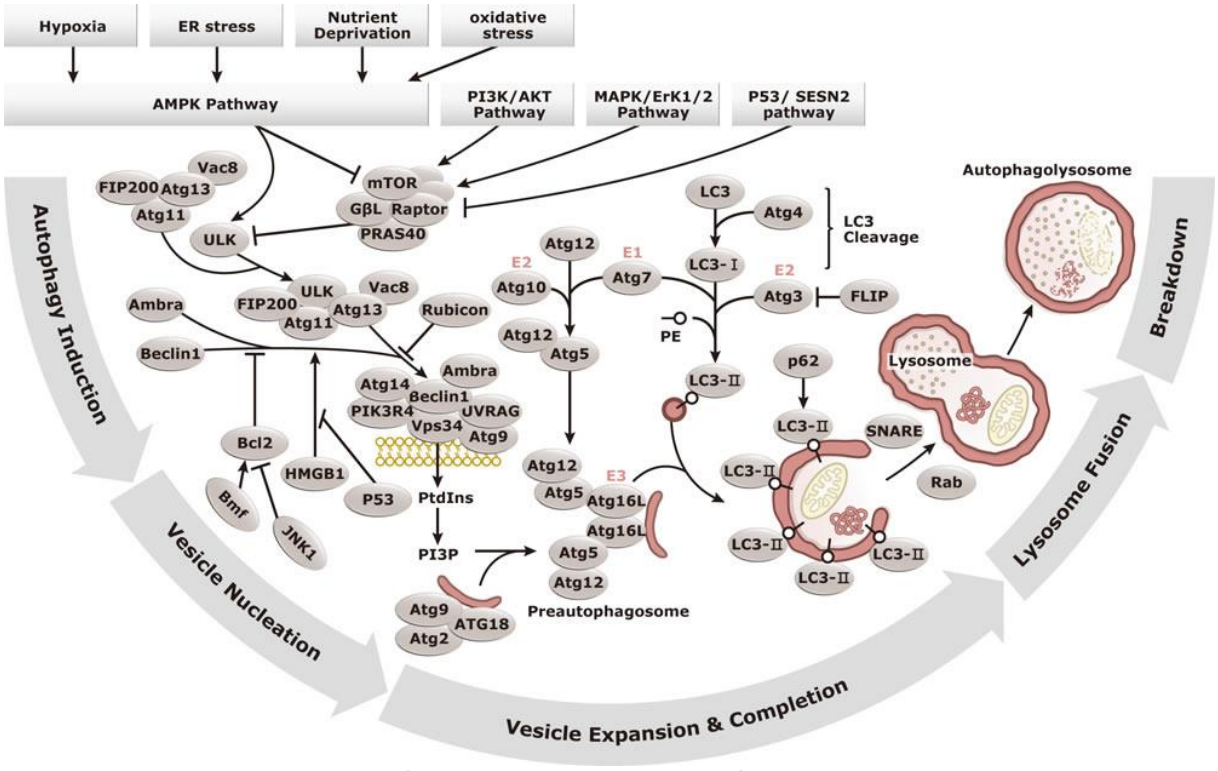

Figura 6. Ilustração esquemática do processo autofágico. A figura mostra alguns dos principais reguladores positivos e negativos da rota assim como as famílias de proteínas (como ATG, Beclina-1, ULK, LC3) envolvidas nas diferentes etapas de formação do autofagolisossoma. Retirado de http://www.genetex.com/Web/Pathway/Autophagy-Pathway-39. ATG: genes relacionados à autofagia; ULK: Cinase ativadora de autofagia; LC3: Cadeia leve 3 da proteína 1 associada a microtúbulos.

2 JUSTIFICATIVA E HIPÓTESE

Com base no contexto atual da terapêutica de GBM, vê-se a necessidade óbvia de buscar novos tratamentos para melhorar o prognóstico deste tipo tumoral. Sabendo que até o momento o fármaco com melhores resultados no tratamento dos GBM é o TMZ, uma das vertentes de pesquisa visa à validação de moléculas que possam melhorar a eficácia deste agente alquilante. Neste projeto, investigaremos a associação do TMZ com outro fármaco, o SAHA, que se encontra em ampla investigação pré-clínica e clínica em diferentes tipos de câncer. O SAHA, como descrito anteriormente, age no mecanismo de acetilação de histonas, inibindo as

HDACs e com isso alterando o padrão de expressão gênica celular. Hipotetizamos se essa inibição das HDACs resultando na alteração da expressão gênica poderia sensibilizar as linhagens de glioma ao tratamento com TMZ, driblando assim os mecanismos de resistência, e promovendo um sinergismo de potenciação.

3 OBJETIVOS

Objetivo geral

O objetivo geral desta dissertação foi determinar se a inibição das histonas desacetilases (HDAC) pelo ácido hidroxâmico suberoilanilida (SAHA) é capaz de potencializar os efeitos citotóxicos da temozolomida (TMZ) em linhagens celulares de glioma, e os mecanismos envolvidos na morte e resistência celular nesse contexto.

Objetivos específicos

1) Avaliar os efeitos citotóxicos de SAHA e da combinação SAHA/TMZ em linhagens de glioma e a seletividade em modelo de células gliais sadias (astrócitos).
2) Determinar as alterações de ciclo celular e dos marcadores fenotípicos de apoptose e autofagia em linhagens de glioma tratadas com SAHA e TMZ.
3) Determinar o papel da inibição da autofagia, pelo uso de cloroquina (CQ), na morte ou sobrevivência de células de glioma tratadas com SAHA e TMZ.

PARTE II

4 ARTIGO CIENTÍFICO

Autophagy promotes survival in temozolomide/SAHA-treated glioma cells

A ser submetido à BBACTA – Molecular Basis of Disease. (Modelo da revista retirado de: https://www.elsevier.com/journals/bba-general-subjects/0304-4165/guide-for-authors)

Autophagy promotes cell survival in temozolomide/SAHA-treated glioma cells

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Running title: Autophagy promotes TMZ/SAHA survival

Keywords: autophagy; temozolomide; SAHA; gliomas.

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Abstract

Glioblastoma multiforme (GBM) is the most frequent and aggressive type of primary brain tumor which has been associated with a dismal prognosis. In this study, we tested the efficacy of combining temozolomide (TMZ) with suberoylanilide hydroxamic acid (SAHA) - a inhibitor of histone deacetylases 1, 2, 3, and 6 approved for the treatment of cutaneous T-cell lymphoma - in the viability of tumor cells. The data showed that synergy between TMZ and SAHA was not achieved due to activation of protective autophagy *in vitro*. The SAHA/TMZ treatment promoted arrest in the G2/M phase of the cell cycle after 48 h drug exposure whereas apoptosis was only detected after long-lasting exposure (≥96 h). Autophagy preceded apoptosis as detected by flow cytometry of acridine orange stained cells and immunodetection of the lipidated form of LC3 as well as decreases in p62/SQSTM1. By blocking the termination step of autophagy with chloroquine we were capable of promoting a significant reduction of viability of glioma cells which was accompanied by increased apoptosis in SAHA/TMZ treatment. Overall, the herein presented data demonstrate that autophagy impairs the efficacy of combined TMZ/SAHA, and inhibiting this phenomenon could provide novel opportunities to improve the therapeutic potential of these compounds.

Introduction

Glioblastoma multiforme (GBM), or grade IV glioma, is the most aggressive type of primary brain tumor. This type of tumor is particularly difficult to treat because of its particular location, the selective permeability offered by the blood-brain barrier (BBB) as well as its the fast growing and capability of developing chemoresistance. While many types of cancers have been benefited by development of targeted therapies and versatile therapeutic regimens, GBM therapy remains basically the same over the last decade. In general, this includes neurosurgery followed by radiation and adjuvant chemotherapy with the temozolomide (TMZ) [1]. Due to this limited number of possibilities, patient prognosis remains dismal, with a median survival of ~14 months.

TMZ is an alkylating agent whose toxicity is mediated by addition of methyl groups at N^7 and O^6 sites on guanines and the O^3 site on adenines in DNA. Alkylation of the O^6 site on guanine leads to the insertion of a thymine instead of a cytosine opposite the methylguanine during subsequent DNA replication, and this can result in DNA strand breaks, DNA damage

repair system activation, cell cycle arrest at G2/M [22] leading to mitotic catastrophe [28], autophagy and/or apoptosis [18, 22], which seem to depend on the cell line, time exposure and drug concentration used in the different studies.

Autophagy is conserved process which cytoplasmic components are targeted for lysosomal degradation aiming to recycle macromolecular building blocks and provide energy under stressor conditions which, in the context of tumors, seem to offer a survival advantage [10-13, 16, 19, 22]. Autophagy initiates with the activation of ULK1 and class III PI3K complexes which signal downstream to a group of proteins belonging to the ATG family, such as ATG5 and ATG12 among others. Once active, these proteins are anchored to the autophagosome membrane causing lipidation of the LC3-I protein into LC3-II; the later may be recruit long-lived protein and organelles to recycle. Upon maturation, the autophagosome fuses with the lysosome, leading to macromolecule degradation [4]. In the context of gliomas, it has been shown that TMZ-induced autophagy involves classical upstream regulators such as inhibition of mTOR [29] and PI3K [5], and activation of AMPK [31] as well as of some DDR proteins such as ATM and MSH6 [18] and MAPKs [30] thus characterizing an intricate network. While most of the studies show a protective role, TMZ-induced autophagy decreases the rate of apoptosis, causing tumor recurrence [2], while some studies point out to an autophagy-mediated cell death.

Suberoylanilide hydroxamic acid (SAHA, vorinostat) is an inhibitor of HDAC 1, 2, 3, and 6 approved for the treatment of cutaneous T-cell lymphoma [10]. SAHA also showed modest effects on cancers of the prostate, ovary, breast and GBM when used as monotherapy [20,21]. Even though its expected mechanism of action affects chromatin, various studies have shown that HDAC inhibitors may impair cell survival through both chromatin-dependent and -independent mechanisms. In the context of gliomas, SAHA promotes cell death by G2 checkpoint abrogation leading to mitotic catastrophe [23] and radiosensitivity potentiation [24]. SAHA also induced protective autophagy in GBM stem cells [4].

In this study, we tested whether glioma cell responsiveness to TMZ/SAHA is impacted by autophagy, and the mechanisms involved. With two glioma cell lines, U251MG and C6, we observed that TMZ/SAHA increased the autophagic flux as a survival response which was accompanied by G2/M arrest and low levels of apoptosis. Autophagy inhibition re-sensitized the cells thereby increasing in TMZ/SAHA-treatments.

Materials and methods

Reagents

Vorinostat/SAHA, Temozolomide, propidium iodide (PI), acridine orange (AO), MTT (3-(4,5-dimethyl)-2,5-diphenyl tetrazolium), RNAse A, Chloroquine (CQ) and N-acetylcysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, USA), Anti-Histone 3 (#4499), Anti-Histone 4 (#13919), Anti-acetyl-H3 (#9649), Anti-acetyl-H4 (#2594), Anti-HDAC2 (#5113), Anti-LC3A/B (#3868), Anti-beclin 1 (#8679), Anti-p62/SQSTM1 (#5114) and HRP-conjugated secondary antibodies were from Cell Signaling Technologies. SDS-PAGE and immunoblot reagents were from Biorad.

Cell lines and treatments

C6 (p53wt/PTEN deficient), U251MG (p53mut/PTEN-deficient), U87MG (p53wt/PTEN deficient) cell lines were purchased from American Type Culture Collection (ATCC; Rockville, Maryland, USA) and were grown in DMEM supplemented with 10% FBS plus 1X antibiotic/antimycotic solution (Sigma-Aldrich) in a humidified incubator as recommended. Primary astrocytes were isolated from cortex of 2-days old Wistar rats by mechanical dissociation with Ca^{+2}/Mg^{+2} – free Hank's balanced salt solution, and plated in 96-well plates. The use of animals was approved by Institutional Animal Care and Use Committee (project #27686). The cells were maintained in high-glucose DMEM plus antibiotics (Gibco BRL, Carlsbad, USA) in a humidified incubator, and treated after reaching a 90% confluence (12-15 days). TMZ and SAHA stock solutions were prepared in DMSO at 150 mM and 20 mM concentrations, respectively. SAHA and TMZ were co-treated and, when used, inhibitors/antioxidants were pre-incubated for 30 min prior to TMZ/SAHA.

Cell viability assays

MTT reduction by cellular dehydrogenases was used as an estimation of cellular viability. For PI uptake, treated cells were incubated with 6 µM PI in DMEM for 1 h, and images were obtained using a Nikon Eclipse TE 300 inverted microscope setup with rhodamine filter [5].

Cell cycle analysis

Treated cells were trypsinized, centrifuged and resuspended in 500 µL permeabilization buffer containing 10 mM PBS, 0.1% v/v Nonidet P-40, 1.2 mg/mL spermine, 100 µg/mL RNAse, and 2.5 µg/mL propidium iodide/PI, pH 7.4. The cells were vortexed and incubated for 10 min on ice. The DNA content was determined by FACS, and analyzed by CellQuest® software (BD Biosciences, USA) [5].

Western blot

Total cell lysates (~30 µg protein) were separated by SDS-PAGE and electrotransferred onto nitrocellulose membranes. The membranes were stained with Ponceau, rinsed with TBS-T, blocked with 5% non-fat dry milk in TBS-T (1 h), and then incubated with primary antibodies (Anti-Histone 3 (#4499), Anti-Histone 4 (# 13919), Anti-acetyl-H3 (#9649), Anti-acetyl-H4 (#2594), Anti-HDAC2 (#5113), Anti-LC3A/B (#3868), Anti-beclin 1 (#8679), Anti-p62/SQSTM1(#5114) (1:1000; 4ºC, overnight). Afterwards, the membranes were incubated with secondary antibodies (HRP-conjugated (rabbit or mouse)) (1:2000, 2h/room temperature), washed with TBS-T, and the signals were visualized using luminescent image analyzer (ImageQuant LAS 4000).

Annexin-V PE staining

The annexin-V-PE/phycoerithrin conjugated reagent (Sigma-Aldrich) was used for quantification of apoptosis. After trypsinization, the cell pellets were suspended in 500 uL binding buffer containing 1x binding buffer in water, and externalized phosphatidylserine was labeled with 10 µL annexin-PE for 15 min on ice. Viable (annexin-) and apoptotic (early+late apoptosis; annexin+) cell populations were determined by FACS and analyzed by CellQuest® software (BD, Biosciences, USA) [5].

Caspase-3/7 activity

The caspase-3/7 activity was assessed following CASP3F Fluorimetric kit instructions (Sigma, Saint Louis/MI). After whole-cell extracts preparation, 150 µg proteins (Bradford method) were mixed with 200 µL of assay buffer containing Ac-DEVD-AMC, a caspase-3/7-specific substrate. Ac-DEVD-AMC cleavage was monitored for 1 h at 37 ºC at: 360/460 nm

in a fluorescence reader. Delta 1 h of fluorescence was calculated and expressed as fold-change as compared to control/untreated cells [5].

Acridine orange staining

Acridine orange (AO) is a probe that fluoresces green in the whole cell except in acidic compartments, where it fluoresces red. Vacuolar acidification of autophagosomes is a characteristic of efficient autophagy, thus red fluorescence is proportional to autophagic flux. At the end of treatments, the cells were incubated with AO (5 µg/mL) for 15 min in a humidified incubator, trypsinized, spun down and resuspended in PBS. The green/red (FL1-H/FL3-H) fluorescence was detected using a FACSCalibur, and acridine orange positive cells (AO+; FL3-H channel) were quantified by CellQuest® software (BD, Biosciences, USA) [adap. 5].

DCF assay

The 2,7- dichlorofluorescein diacetate (DCFH–DA) probe diffuses to intracellular compartment where it undergoes the esterase action becoming dichlorofluorescein (DCFH) which, in turn, when in the presence of reactive oxygen species (ROS) is oxidized to dichlorofluorescein (DCF), thus emitting fluorescence. The DCF fluorescence intensity is proportional to the amount of intracellular ROS. Briefly, U251MG cells were seeded in 24-well plates at a density of 3×10^4 cells/well in DMEM supplemented with 10 % FBS. One day after plating, the cells were incubated with TMZ and SAHA. After 6 and 24h treatment, the cells were then incubated with 5 µM of 2,7-DCFH-DA (Sigma-aldrich, USA) in DMEM for 30 min. The cells were then trypsinized, centrifuged, resuspended in PBS, and the fluorescence was estimated using a flow cytometer (FACSCalibur). DCF positive cells and their fluorescence median intensities were quantified by CellQuest® software (BD, Biosciences, USA) [5].

Generation of stable cell lines with retroviral or lentiviral infection

BECN1 (beclin-1) gene was knocked down by transduction of C6 cells with lentivirus vectors produced with the plasmid clone NM_019584.2-970s1c1 and NM_028835.3-1655s21c1, respectively, from the Mission RNAi library from SigmaAldrich. Non-target (pLKO.1-puro; hereafter named PLKO) sequence was used as a control. Lentiviruses were produced as described previously (Tamajusuku *et al*., 2010). Knockdown was confirmed by

western blotting. Cell viability in knockdown cells in relation to wild-type and PLKO shRNA control cells did not differ (data not shown).

Statistical analysis

The experiments were repeated at least two times in duplicates or triplicates. Data were expressed as average ± SD. ANOVA followed by Tukey or Bonferroni post-hoc were used as appropriate (GraphPad Prism 5) at a $p<0.05$.

Results

TMZ/SAHA combination exerts additive and cancer cell selective toxicity in glioma cell lines.

We first tested the impact of SAHA (a specific inhibitor of class I and II HDACs) upon the viability of GBM cell lines harboring different mutations. At the end of 72 h treatment, SAHA cytotoxicity was similar irrespective of the mutational background of the glioma cell lines evaluated: C6 (p53wt/PTEN deficient), U251MG (p53mut/PTEN-deficient) and U87MG (p53wt/PTEN-deficient) (Fig. 1A). On the other hand, sirtinol (class III HDAC sirtuin 1 inhibitor) showed no toxicity from 10 to 100 µM at least up to 72 h incubation (data not shown). The range of SAHA concentrations was determined experimentally (0.5 – 4 µM); agreeing with other studies [4, 6, 24]. We then carried out SAHA combined with 2 doses of TMZ (100 e 200 µM – as determined in previous studies [5, 30]) and found that combination exerted more toxicity than either drugs alone in both C6 and U251MG cells (Fig 1C e D). In addition, SAHA and TMZ alone or in combination were less toxic to astrocytes if compared to gliomas as assessed by the cell viability parameter at the end of 72 h exposure (Fig. 1B). While IC50 (72 h) of SAHA in C6 and U251MG were ~2 µM and 3 µM, respectively, it was > 4 µM in astrocytes (Fig. 1A). With a 72 h exposure, glioma cells showed increased incorporation of PI (% PI+ cells), decreases in cell density and morphological changes in both SAHA and TMZ treatments (Fig. 1E); these effects were more pronounced in SAHA/TMZ combination (Fig. 1C-E). We then sought to confirm whether SAHA, but not TMZ, increases the acetylation of histones from the 0.5 to 2 µM range tested, by means of acetyl-H3 detection, in both U251MG gliomas and astrocytes (Fig. 1F and G). The amount of acetylation by SAHA was not altered in the presence of combined TMZ (Fig. 1F). Neither total H3 nor total HDAC2 levels were affected by SAHA, corroborating the yet described

specificity of SAHA to inhibition of HDAC enzymes, and also indicating that the SAHA mechanism does not overlap TMZ effects, at least at the level of HDAC (Fig 1F).

SAHA/TMZ induces G2/M arrest that precedes apoptosis.

From figure 1 data, it was observed that the effect of combined SAHA/TMZ is no more than the sum of the fractional effects of each drugs alone, therefore indicating that additive synergism but not potentiation synergism toxicity occurred

To further explore the cell response to SAHA/TMZ, the possible changes in the cell cycle distribution of GBM cell lines (U251MG, C6) were examined. At 24h treatment, both SAHA and TMZ induced accumulation of cells in G2/M, and this effect was more pronounced in drug combinations (Fig. 2A). By extending drug incubation to longer periods (96 h) it was possible to determine significant accumulation of sub-G1 phenotype in cell cycle analysis (Fig. 2A) as well as phosphatydilserine externalization as determined by annexin-V binding assay (Fig. 2B) which were increased in SAHA/TMZ as compared to both drugs alone. At 24 h, annexin-V binding was <10% cells and did not differ between groups (data not shown). Time course of caspase-3/7 activity confirmed that apoptosis is a later event occurring from 72 h incubation in SAHA/TMZ treated gliomas (Fig. 2C) and it was enhanced in combined treatments (Fig. 2D). These data imply that G2/M checkpoint activation – and thus cell proliferation inhibition - precedes apoptosis in our model (Fig. 2A).

Autophagy as a protective event in SAHA/TMZ treated cells

Previous evidence has shown that gliomas are prone to respond via activation of protective autophagy thereby limiting the apoptosis/toxicity caused by some types of chemotherapeutics [4, 5, 18]. By flow cytometry, we observed that both SAHA and TMZ increased acidic vacuole formation which was further stimulated by SAHA/TMZ combination (Fig 3A-B). While apoptosis detection occurred at later time-points (>72 h), autophagy occurred earlier, being evident from 24-48 h treatment across the cell lines (Fig. 3A-B). In keeping with acridine orange assays, we observed an increase in the conversion of LC3-I to LC3-II (lipidated form) by SAHA and TMZ which is even more enhanced in drug combinations (Fig. 3C-D). We also observed decreases in the p62/SQSTM1 protein (a major hallmark of autophagy) but this was only observed in TMZ/SAHA combinations unlike drugs alone, irrespective of the concentrations and time-exposure tested, therefore indicating that autophagic flux is accelerated in combined TMZ/SAHA (Fig. 3C-D). These

data altogether confirm that SAHA and TMZ redundantly lead to the same cell response, i.e. autophagy, before activating apoptosis.

Blocking autophagic flux with low doses of Chloroquine (CQ, 10 μM) - an inhibitor of autophagosome acidification and fusion with lysosomes (i.e., late phase autophagy inhibitor) [25] - promotes robust LC3-II accumulation (Fig. 4A) leading a significant increment in the cytotoxic effect of SAHA, TMZ and combined SAHA/TMZ, as assessed by cell viability assay (Fig. 4A). CQ also incremented the percentage of sub-G1 events (Fig. 4B), phosphatidylserine externalization (Fig. 4C) and caspase-3/7 activation (Fig. 4D) compared to either drugs alone or combinations as assessed by cell cycle analysis, annexin-V-PE and caspase-3/7 activity assays, respectively, in both cell lines tested. Besides the pharmacological inhibition of autophagy termination with CQ, we also used beclin-1 silenced cells (by adenoviral expression of shRNA) in order to inhibit the earlier steps of the process. Interestingly, we observed that the cell viability impact of beclin-1 depletion, although significant if compared to PLKO control, was smaller than those observed with CQ despite the good knockdown efficiency (Fig. 4E). Different from SAHA groups, beclin-1 depletion had no effect upon TMZ alone toxicity suggesting that SAHA- and TMZ-induced autophagy may differ in a mechanism not investigated herein. However, it is key to note that beclin-1 deficiency only dictated a partial inhibition of acidic vacuole formation thereby explaining its less pronounced effect on SAHA/TMZ toxicity if compared to CQ (Fig. 4E, right panel).

Augmented ROS production mediates SAHA cytotoxicity

Because ROS production has been suggested as a key component of SAHA [6] and TMZ [26] toxicity (the later with minor evidence), we asked whether quenching ROS could modulate the antiglioma effects of TMZ/SAHA combination. Firstly, we monitored ROS production by DCF assay, and observed a dose-dependent increase in ROS at cytotoxic levels of SAHA, TMZ and a greater effect in drug combination after 24 h treatment (Fig. 5B), agreeing with [27, 32]. ROS production did not differ from controls at 6 h treatment (data not shown). While both SAHA and TMZ increased ROS, pre-treatment of cells with the antioxidant N-acetylcysteine (NAC) only inhibited the toxicity of both SAHA alone and SAHA/TMZ combination whereas TMZ-induced losses in cell viability were not altered by NAC (Fig. 5A). NAC pre-treatment attenuated SAHA and SAHA/TMZ-induced autophagy (Fig. 5C; but not in SAHA/TMZ treated U251MG), sub-G1 accumulation (Fig. 5D-E), annexin-V-PE binding (Fig. 5F) and caspase 3/7 activity (Fig. 5G) and (Fig. 4C), indicating

that ROS production is mainly involved in SAHA cytotoxicity whereas cell survival to TMZ is ROS-independent, at least in the time-exposure and concentration ranges tested herein.

Discussion

TMZ is the chemotherapy with the best results in the treatment of GBM and, despite it increases patient survival, the median overall survival does not exceed 14 months [33]. Given that there is consensus about the needs of increasing the effectiveness of GBM treatments, the use of drugs in combination with TMZ appears to be a viable alternative. SAHA is a HDAC inhibitor that showed potential results at *in vitro* and clinical settings in different cancers [10, 15, 20, 21, 27]. In fact, the combination of SAHA with TMZ promoted a significant increase of cell death if compared to each drug alone, even though the mechanism does not seem to involve potentiation synergism but yet synergism additive effects. This raised question whether either SAHA is not reversing some key mechanism required for TMZ survival or the cells are triggering some antiapoptotic mechanism which could be limiting to achieve synergy in combined treatments. Here we identified autophagy as at least one of these protective cell responses to TMZ/SAHA. We found that both drugs caused autophagy either alone or in combination, although the magnitude effect was greater with combined drugs. This redundant cell response phenotype was also observed in the G2/M blockage of cell cycle progression. There is previous studies from our group and others showing that TMZ and SAHA, studied as monotherapy, may induce autophagy in glioma cells [5, 18, 4], but there is none research that has tested the two drugs combined.

Autophagy is a process associated with both apoptosis and survival, depending on the genetic background and origin of the cells and the stressor stimuli features [18]. It has been matter of controversy given that some studies have associated autophagy with chemoresistance whereas other defend that autophagy is an alternative mechanism of cell death when apoptosis machinery is failed [10, 16, 19, 30, 34]. In addition, autophagy has been described in drug-resistant cancer cells [12] and can be blocked to promote apoptosis in different models [2, 4, 5, 11-13, 30]. In GBM, autophagy seems to be protective, contributing to tumor resistance to chemotherapeutics such as TMZ [2, 3, 5, 22]. In our experiments autophagy occurrence concomitantly with G2/M arrest, and these events preceded apoptosis. TMZ/SAHA treatments in combination with CQ resulted in decreased cell viability, increased the apoptotic machinery as detected by annexin V-PE and sub-G1 cells as well as caspase 3/7 activity. While CQ - which promotes extensive blockage of

autophagy termination leading to robust accumulation of inactive autophagic vacuoles in the cytoplasm (data not shown) [agreeing with 11, 25] – potentiated TMZ/SAHA toxicity. On the other hand, beclin-1 depletion conferred a minor effect upon TMZ/SAHA toxicity and was not capable of blocking autophagy as assessed by acridine orange assays. It suggests that either other mechanisms may be compensating the lack of beclin-1 (given that beclin-1 deficient cells were not capable of completely blocking autophagy) or other variations of the autophagic process may take place.

Diverse pathways are differentially modulated by TMZ and SAHA to promote autophagy. TMZ, as an alkylating agent, activated DDR-dependent autophagy, which involved DNA strand break sensor and mismatch repair proteins ATM and MSH6 [18]. SAHA induction autophagy seems to involve inactivation of mTOR [4]. Noteworthy, mTOR inhibition allows ULK1 (unc51 like autophagy activating kinase 1) activation and its bind to phagophore and therefore recruitment of ATG (autophagy-related-genes) proteins [8]. How the increased histone acetylation induces autophagy remains unknown, although our data suggest that changes in redox homeostasis of cells treated with SAHA may be involved in autophagy induction and toxicity by SAHA [agreeing with 6]. SAHA treatment increased ROS production, and this seems to be play a partial role in SAHA-induced cell death since the presence of NAC partially decreased cell death in both SAHA and SAHA/TMZ. Despite the observed increases in ROS production with TMZ alone, it does not dictate TMZ toxicity given that NAC neither affected cell viability nor autophagy/apoptosis in TMZ alone- treated cells. If the effect of NAC is only attributed to its antioxidant role or by its effects on GSH pools (therefore improving GSH-dependent detoxification machinery) remains to be tested by using GSH-unrelated antioxidants.

In summary, our data indicate that the efficacy of TMZ and SAHA combination is not achieved due to the autophagic response of glioma cells. Autophagy accompanied G2/M arrest and these events preceded apoptosis. Blockage of autophagy, especially at later steps of the processes, as exemplified with CQ, may offer a strategy of potentiating TMZ/SAHA toxicity therefore optimizing the therapy with these compounds either alone or in combination. Preclinical models of glioma growth are required to provide further evidence on the therapeutic potential of these *in vitro* observable mechanisms.

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Figure legends

Fig. 1. SAHA and TMZ induce additive cytotoxicity in GBM. (A) MTT viability experiments showing the effect of SAHA alone in U251MG, U87MG, C6 as well as primary astrocytes (72h). (B) MTT viability experiments showing the effect of TMZ and SAHA alone and TMZ/SAHA combination in primary astrocytes (72h). (C, D) MTT viability experiments showing the effect of TMZ and SAHA alone and TMZ/SAHA combination in U251MG and C6 (72h). (E) Representative phase contrast and PI uptake microphotographs in TMZ/SAHA-treated cells for 72h. (F) Representative immunoblots showing histone acetylated (acH3, acH4) and total histone (H3, H4) in U251MG cells at 24 h treatment. (G) Representative immunoblots showing acH3 and HDAC2 in primary astrocytes (24 h treatment). If not otherwise specified, the cells were treated with 200 µM TMZ and 2 µM SAHA. *Different from untreated control; $^{\#}$Different from TMZ and SAHA alone at equivalent concentrations (n=3 in quadruplicate, $p<0.05$, ANOVA). Legends: acH3 – acetylated histone 3; HDAC2 – histone deacetylase 2.

Fig. 2. SAHA and TMZ effects on cell cycle distribution and apoptosis in glioma cells. (A) Cell cycle analysis of C6 and U251MG cells treated for 24 and 96 h with SAHA and TMZ. Representative histograms of C6 treated cells are also shown. (B) Annexin-V-PE flow cytometry assays showing TMZ and SAHA-induced apoptosis in glioma cells after 96 h treatment. Representative histograms of C6 treated cells are also shown; (C) Time course effect of TMZ and SAHA and combination upon caspase-3/7 activity in U251MG cells. (D) Caspases-3/7 activity in U251MG cells treated with differing doses of SAHA and TMZ 200 µM (72 h treatment). If not otherwise specified, the cells were treated with 200 µM TMZ and 2 µM SAHA *Different from untreated control; $^{\#}$Different from TMZ and SAHA alone at equivalent concentrations (n=3, $p<0.05$, ANOVA).

Fig. 3. Autophagy induction in TMZ and SAHA treated glioma cells. (A) Representative dot-plot graphs of acridine orange (AO) flow cytometry assay in TMZ/SAHA-treated C6 and U251MG cells. Increased FL3-H fluorescence denotes AO positive cells (autophagic); (B) Time-course and the effect of different SAHA concentrations upon autophagy in acridine orange assays. (C-D) Representative immunoblots showing the effect of TMZ and SAHA on the immunocontent of LC-3, acH4, H4, HDAC2 and p62/SQSTM1 in U251MG cells were

treated for 24 and 48 h. Unless otherwise specified, the cells were treated with TMZ: 200 µM and SAHA: 2 µM. *Different from untreated control; #Different from TMZ and SAHA alone at equivalent concentrations/time-point (n=3, p<0.05, ANOVA). Legends: acH4 – acetylated histone 4; AO – acridine orange; H4 – total histone 4; HDAC2 - histone deacetylase 2; LC3 – light chain 3.

Fig. 4. Autophagy inhibition sensitizes TMZ and SAHA treated cells to apoptosis. (A) MTT cell viability assays and representative LC3 immunoblot showing the effect of CQ on SAHA/TMZ toxicity in C6 and U251MG cells. (B) Quantification of cell cycle distribution in C6 and U251MG cells treated with SAHA/TMZ in the presence/absence of CQ. (C-D) Annexin V-PE flow cytometry and caspase-3/7 activity assay showing the impact of autophagy inhibition with CQ on apoptosis of U251MG cells. (E) MTT cell viability experiments showing the effect of TMZ and SAHA alone and TMZ/SAHA combination in C6 cells expressing scrambled shRNA control (PLKO) or beclin-1 shRNA. The panel also shown immunoblot confirmation of beclin-1 knockdown efficiency in beclin-1 shRNA compared to wild-type (wt), PLKO shRNA expressing C6 cells. The Ponceau staining was used as loading control across the different C6 clones. If not otherwise specified, the cells were treated for 72 h; CQ (chloroquine) was at 10 µM; TMZ at 200 µM and SAHA at2 µM. *Different from untreated control or at indicated comparisons; #Different from TMZ and SAHA alone at equivalent concentrations (n=3, p<0.05, ANOVA).

Fig. 5. Antioxidant treatment inhibits SAHA but not TMZ toxicity. (A) MTT cell viability assay showing the effect of NAC on the viability of U251MG cells treated with TMZ and SAHA (72 h). (B) DCF assay showing the effect of TMZ and SAHA on ROS production in U251MG (24 h). (C) Acridine orange (AO) staining (48 h treatment) and (D) cell cycle analysis (96 h treatment) showing the effect of NAC on TMZ/SAHA-induced autophagy and cell cycle profiling of C6 and U251MG cells; in (E) representative dot-plot graphs of cell cycle of C6 cells treated in the presence of NAC. (F) Annexin V-PE flow cytometry assays and (G) caspase-3/7 activity assay showing the effect of NAC on TMZ/SAHA-induced apoptosis in U251MG. Unless otherwise specified, the cells were treated with 200 µM TMZ and 2 µM SAHA. *Different from untreated control or at indicated comparisons; #Different from TMZ and SAHA alone at equivalent concentrations (n=3, p<0.05, ANOVA). Legends: NAC (N-acetylcysteine), ROS (reactive oxygen species).

Figures:

Figure 1

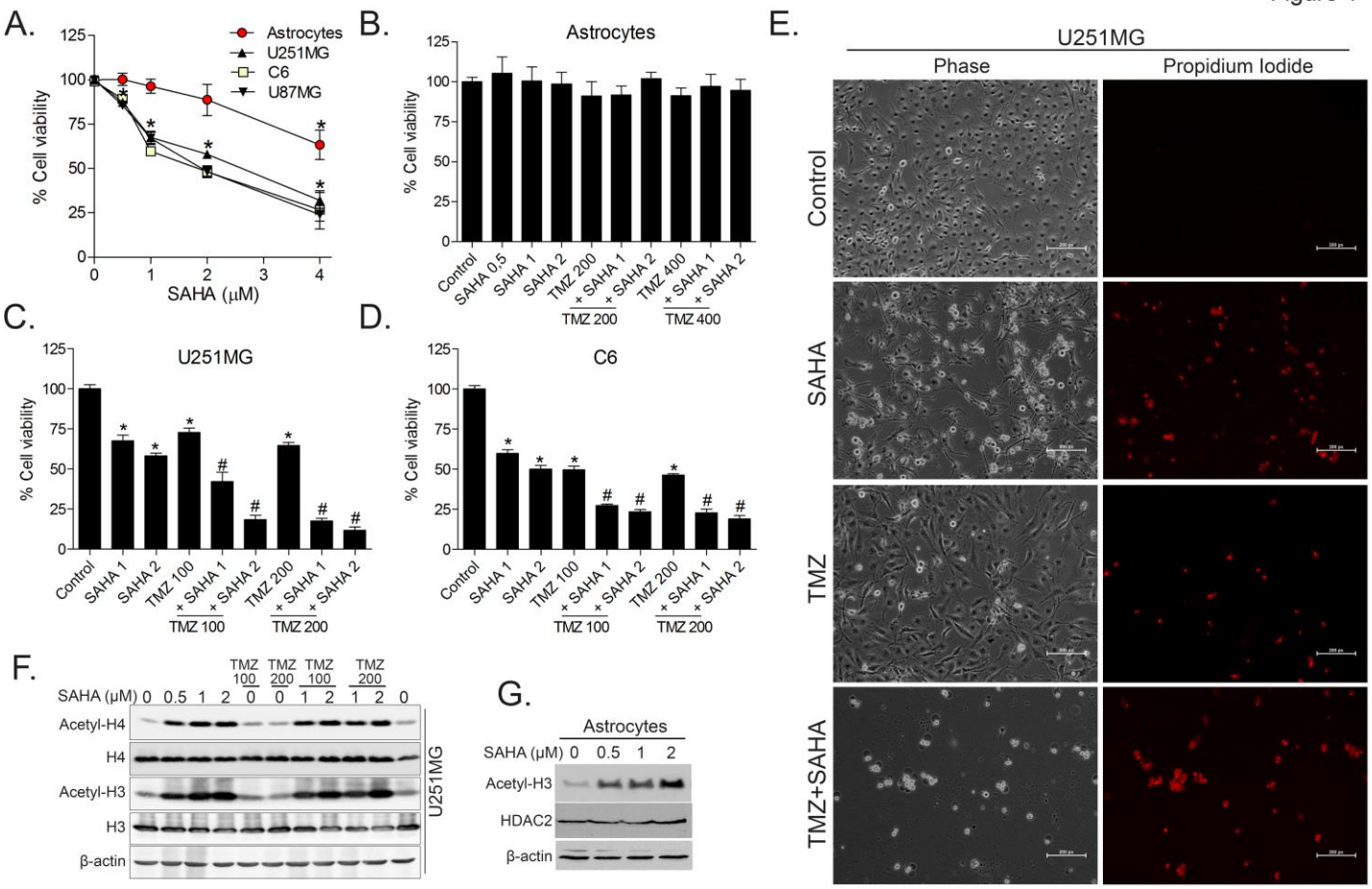


Figure 2

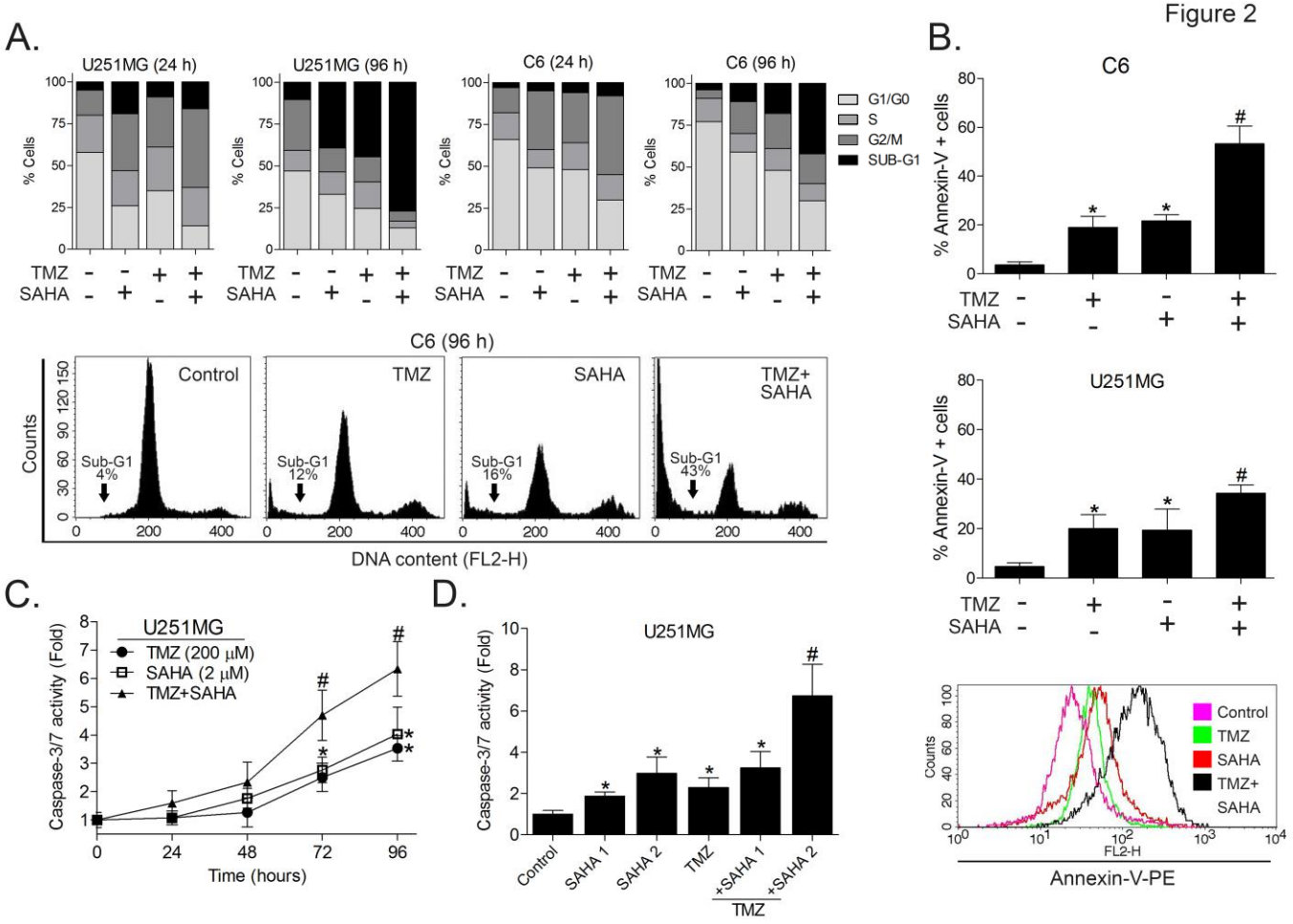

A.
U251MG (24 h)
U251MG (96 h)
C6 (24 h)
C6 (96 h)
% Cells
G1/G0
S
G2/M
SUB-G1
TMZ
SAHA
C6 (96 h)
Control
TMZ
SAHA
TMZ+ SAHA
Counts
Sub-G1 4%
Sub-G1 12%
Sub-G1 16%
Sub-G1 43%
DNA content (FL2-H)
B.
C6
% Annexin-V + cells
U251MG
Control
TMZ
SAHA
TMZ+ SAHA
FL2-H
Annexin-V-PE
C.
U251MG
TMZ (200 μM)
SAHA (2 μM)
TMZ+SAHA
Caspase-3/7 activity (Fold)
Time (hours)
D.
U251MG
Caspase-3/7 activity (Fold)
Control
SAHA 1
SAHA 2
TMZ
+SAHA 1
+SAHA 2

Figure 3

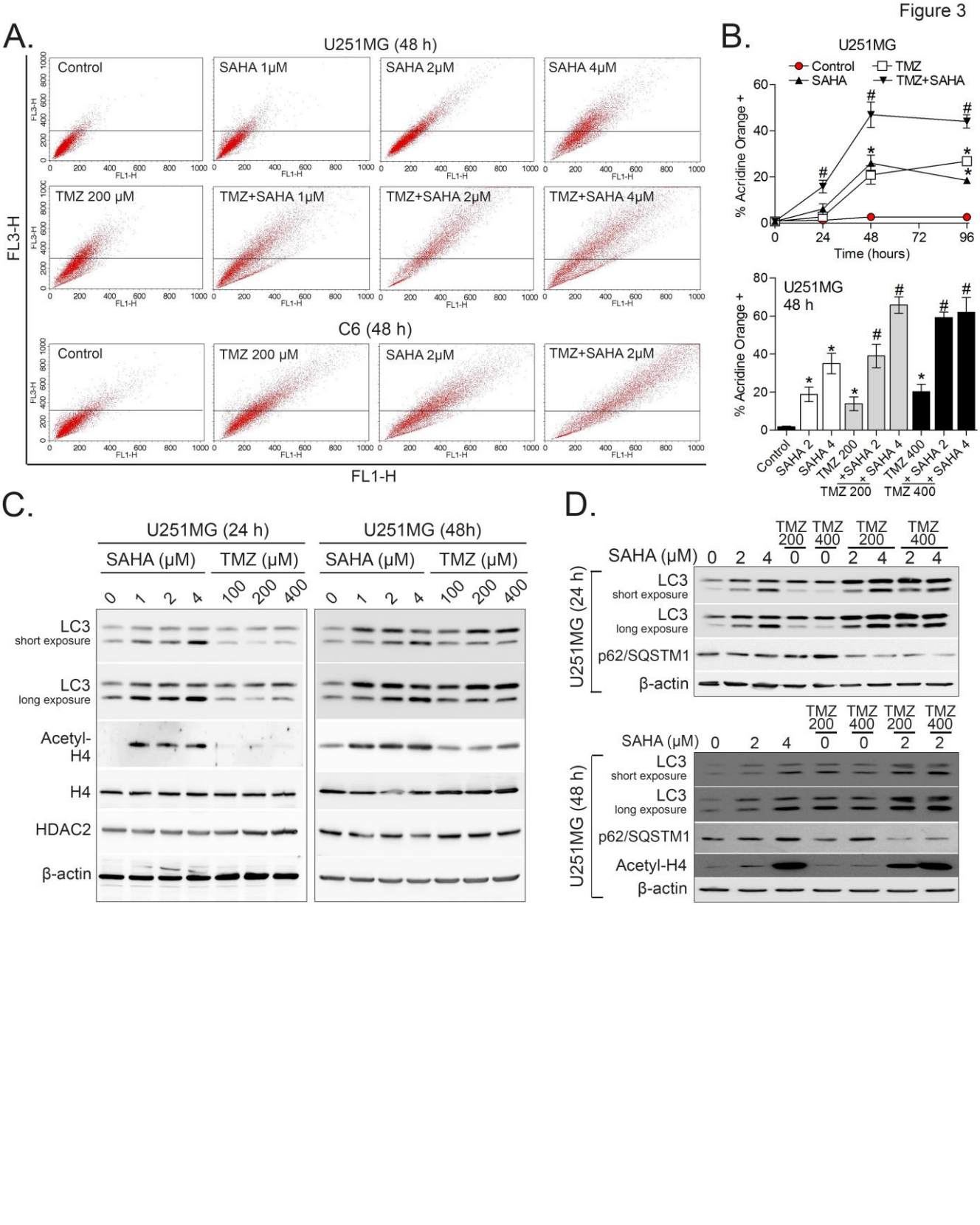

A.
U251MG (48 h)
Control
SAHA 1μM
SAHA 2μM
SAHA 4μM
TMZ 200 μM
TMZ+SAHA 1μM
TMZ+SAHA 2μM
TMZ+SAHA 4μM
C6 (48 h)
Control
TMZ 200 μM
SAHA 2μM
TMZ+SAHA 2μM
FL3-H
FL1-H
B.
U251MG
Control
SAHA
TMZ
TMZ+SAHA
% Acridine Orange +
Time (hours)
U251MG
48 h
Control
SAHA 2
SAHA 4
TMZ 200
TMZ 200 +SAHA 2
TMZ 200 +SAHA 4
TMZ 400
TMZ 400 +SAHA 2
TMZ 400 +SAHA 4
C.
U251MG (24 h)
U251MG (48h)
SAHA (μM)
TMZ (μM)
LC3 short exposure
LC3 long exposure
Acetyl-H4
H4
HDAC2
β-actin
D.
SAHA (μM)
TMZ 200
TMZ 400
U251MG (24 h)
U251MG (48 h)
LC3 short exposure
LC3 long exposure
p62/SQSTM1
Acetyl-H4
β-actin

Figure 4

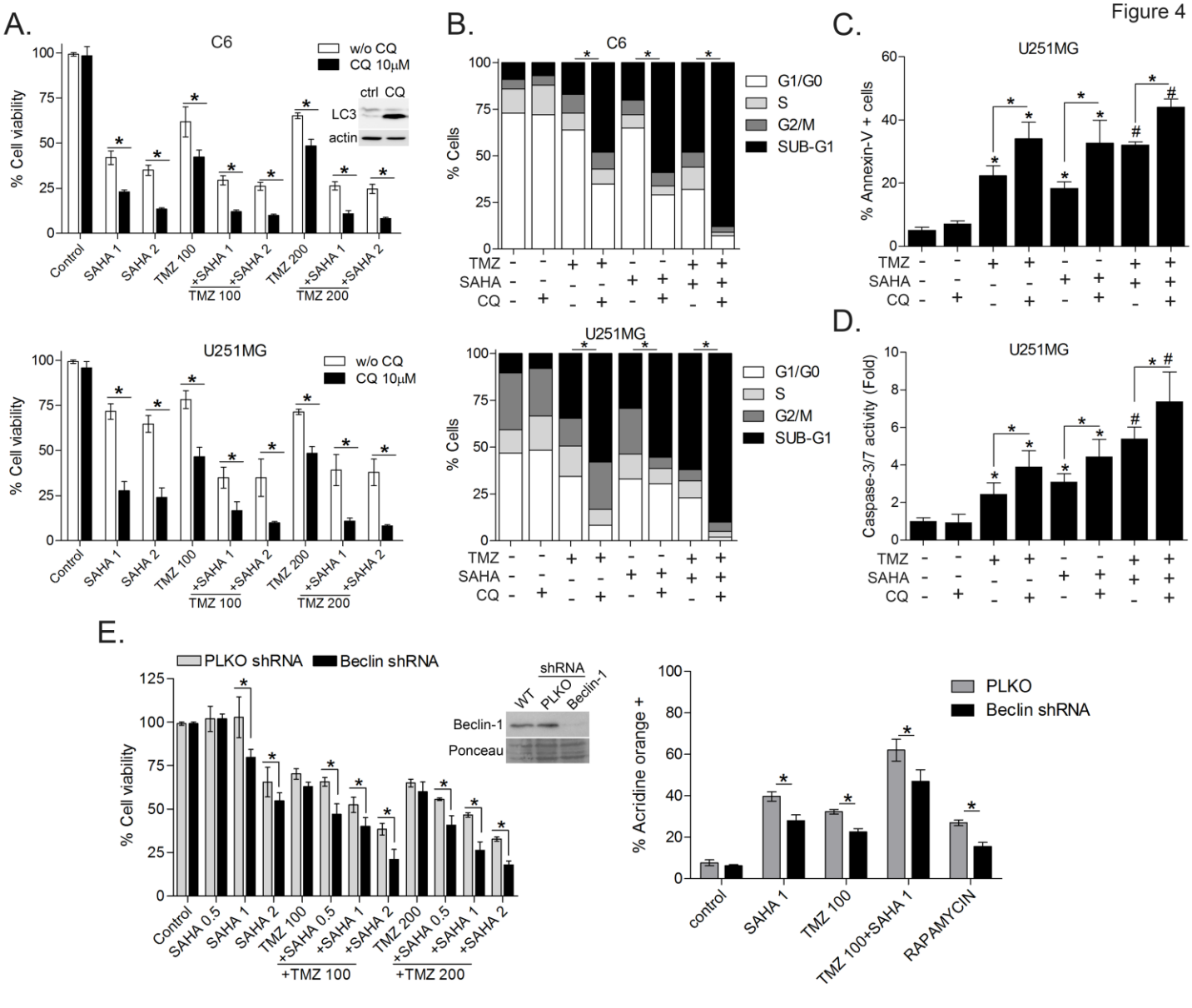
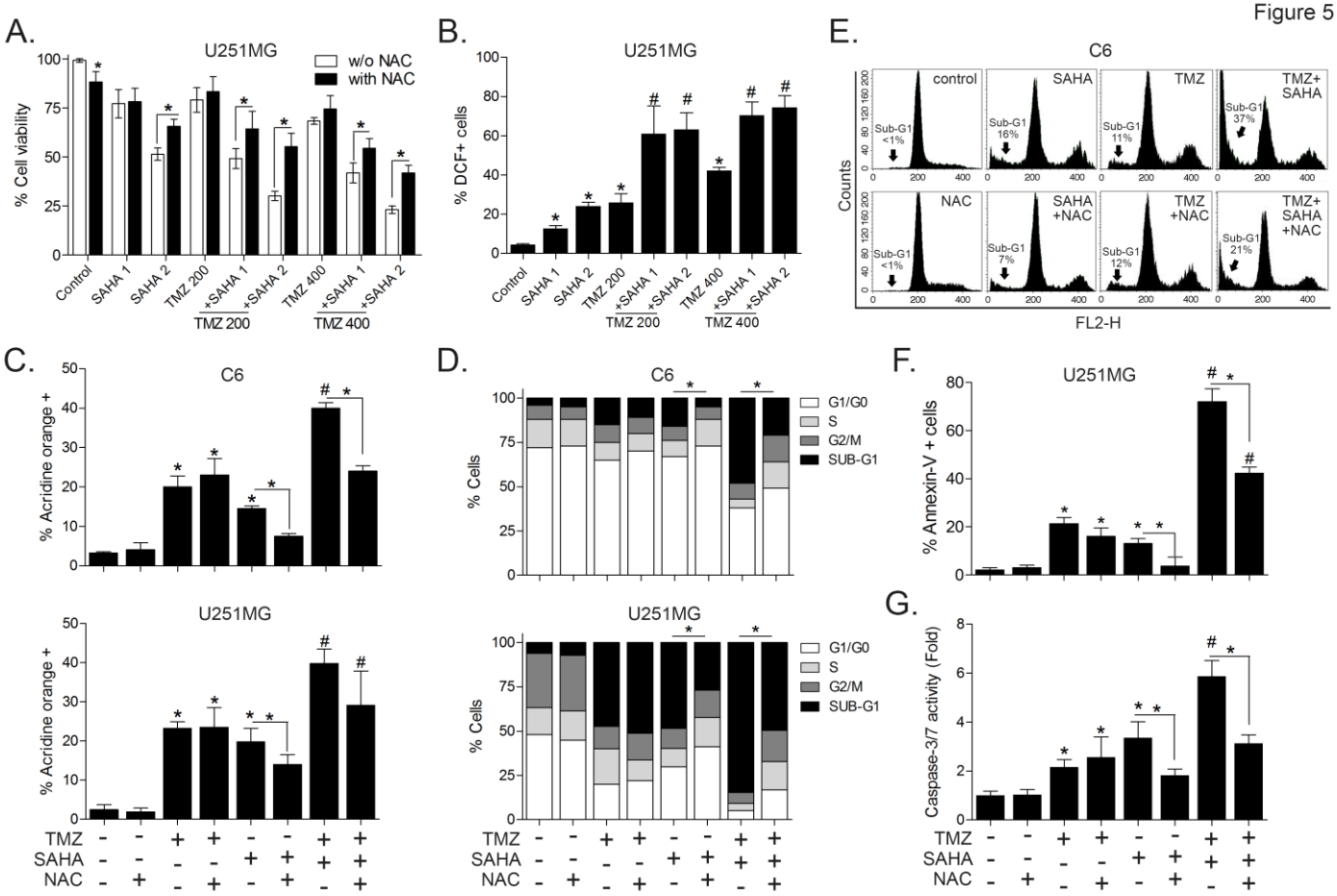

A.
C6
w/o CQ
CQ 10μM
% Cell viability
ctrl CQ
LC3
actin
Control
SAHA 1
SAHA 2
TMZ 100
+SAHA 1
+SAHA 2
TMZ 200
U251MG
B.
C6
% Cells
G1/G0
S
G2/M
SUB-G1
TMZ
SAHA
CQ
U251MG
C.
U251MG
% Annexin-V + cells
D.
U251MG
Caspase-3/7 activity (Fold)
E.
PLKO shRNA
Beclin shRNA
% Cell viability
shRNA
WT
PLKO
Beclin-1
Beclin-1
Ponceau
Control
SAHA 0.5
SAHA 1
SAHA 2
TMZ 100
+SAHA 0.5
+SAHA 1
+SAHA 2
TMZ 200
+TMZ 100
+TMZ 200
% Acridine orange +
PLKO
Beclin shRNA
control
SAHA 1
TMZ 100
TMZ 100+SAHA 1
RAPAMYCIN

Figure 5


A.
U251MG
w/o NAC
with NAC
% Cell viability
Control
SAHA 1
SAHA 2
TMZ 200
+SAHA 1
+SAHA 2
TMZ 400
+SAHA 1
+SAHA 2
TMZ 200
TMZ 400
B.
U251MG
% DCF+ cells
E.
C6
control
SAHA
TMZ
TMZ+ SAHA
NAC
SAHA +NAC
TMZ +NAC
TMZ+ SAHA +NAC
Sub-G1 <1%
Sub-G1 16%
Sub-G1 11%
Sub-G1 37%
Sub-G1 7%
Sub-G1 12%
Sub-G1 21%
Counts
FL2-H
C.
C6
% Acridine orange +
U251MG
% Acridine orange +
TMZ
SAHA
NAC
D.
C6
% Cells
G1/G0
S
G2/M
SUB-G1
U251MG
% Cells
F.
U251MG
% Annexin-V + cells
G.
Caspase-3/7 activity (Fold)

PARTE III

5 DISCUSSÃO GERAL

Os tumores cerebrais estão entre os mais agressivos, e com uma grande dificuldade de tratamento, principalmente por sua delicada localização e principalmente pela presença da BHE que, por sua função fisiológica de proteger o cérebro dificultando o acesso de químicos acaba dificultando a chegada de muitos quimioterápicos em concentrações farmacologicamente ativas no SNC (Friedman *et al*., 2000; Johnson e O'Neill, 2012). Consequentemente, o arsenal de quimioterápicos para o tratamento de tumores cerebrais acaba sendo bem mais restrito.

O quimioterápico com melhores resultados até o momento é o agente alquilante TMZ, o qual é um dos poucos capazes de cruzar a BHE atingindo as células tumorais em concentrações terapêuticas (revisado em Friedman *et al*., 2000; Johnson e O'Neill, 2012). No entanto, o benefício proporcionado pelo tratamento adjuvante com TMZ ainda é pequeno, uma vez que a sobrevida média dos pacientes com GBM fica em torno de 14 meses, mesmo com o tratamento combinado de cirurgia/radioterapia/TMZ (Johnson e O'Neill, 2012). A falha terapêutica do TMZ se dá, na maioria das vezes, em consequência da seleção de clones resistentes, contexto no qual a indução da enzima MGMT (detalhado na introdução deste trabalho) é um dos mecanismos identificados até o momento. Entretanto, a indução de MGMT ocorre apenas em parte dos gliomas resistentes, sugerindo a existência de outras vias. Em um dos trabalhos que motivaram a elaboração desta dissertação, Kitange e colaboradores (2012) demonstraram que alterações epigenéticas promovendo diminuição da acetilação da histona H3 no promotor do gene de MGMT poderiam contribuir para a resistência ao TMZ em modelos de resistência induzida

em animais (Kitange *et al*., 2012). Neste contexto, a identificação e validação de fármacos capazes de potencializar e/ou eliminar a resistência ao TMZ e, assim, prolongar a sobrevida dos pacientes, é objeto de muito interesse no tratamento de GBM.

O objetivo deste estudo foi avaliar se a inibição de HDAC poderia melhorar a eficácia da TMZ. Em nosso trabalho utilizamos um fármaco com mecanismo de ação em destaque nas recentes pesquisas com diversos tipos de tumores, o SAHA (Marks e Breslow, 2007; Liu *et al.*, 2010; Guo *et al.*, 2012; Sampson, 2015). O SAHA possui a vantagem de já ter sido aprovado pela FDA para o tratamento de um tipo tumoral que atinge as células T (Li *et al*, 2012). Ele também possui comprovada identificação de eficiência na passagem pela BHE, o que, como já citado, é de grande importância para os tumores cerebrais, além de uma baixa incidência de efeitos tóxicos graves (Yin *et al*, 2007).

Nosso estudo comparou os efeitos do SAHA e TMZ em duas linhagens de glioma, U251MG e C6. Inicialmente, as células foram tratadas com SAHA sozinho em diferentes concentrações e percebemos que o mesmo possui toxicidade *per se* em gliomas (IC50 ~ 2 μM; 72 h) . Outros estudos utilizaram doses um pouco maiores que as utilizadas por nós (Chiao *et al*, 2013), tais como 4 μM de SAHA. Na faixa de 4 μM, o nosso modelo não permitiu a manutenção das células por tempo de incubação maior que 24 h, especialmente no tratamento combinado com TMZ, visto que a viabilidade celular diminuiu significantemente, tornando difícil a obtenção de células para os experimentos de imunoblot e citometrias de fluxo (Fig. 1, parte II). Para testar a citotoxicidade de SAHA em células sadias, utilizamos cultivos primários de astrócitos. Neste modelo, foi observada ausência de toxicidade até 2 μM SAHA, e

uma diminuição de ~25% em 4 μM (fig. 1; parte II), o que poderia ser atribuído a uma fração de células proliferativas – e potencialmente mais sensíveis a esse tipo de fármaco - presentes na cultura primária de astrócitos. *In vivo*, os experimentos mostram boa tolerância e aumento da sobrevida em modelos animais de implante de glioma tratados com SAHA em monoterapia (Yin *et al*, 2007; Kitange *et al*., 2012). Através da determinação do imunoconteúdo de histonas acetiladas, foi possível detectar que concentrações tão baixas quanto 0.5 μM SAHA aumentaram a acetilação de histonas em gliomas e astrócitos sem afetar o imunoconteúdo total de histonas e HDACs avaliadas, comprovando o mecanismo esperado de inibição da atividade enzimática de HDACs independente da malignidade celular.

A boa relação eficácia/segurança do SAHA já foi demonstrada em alguns tipos tumorais como linfoma de células-T (Li *et al*, 2012), carcinoma hepatocelular (Liu *et al*, 2010), câncer de pulmão (Pan *et al*, 2016). Em pacientes com GBM, apenas um estudo clínico de fase I/II foi apresentado (Lee *et al*, 2012). O SAHA foi considerado bem tolerado, e apresentou doses máximas em torno de 400-500 mg nos diferentes protocolos (Lee *et al*, 2012). Tal dose foi estabelecida devido à anorexia (grau 3) e hemorragia (grau 5) em um grupo de pacientes que apresentou trombocitopenia de grau 4; este último sendo o fator majoritário no escalonamento de dose. Nenhuma interação farmacocinética com TMZ foi observada, e a hiperacetilação de histonas foi validada em leucócitos extraídos de sangue periférico (Lee *et al*, 2012). Dados de eficácia antitumoral e sobrevida ainda não estão disponíveis.

Em nosso modelo, os dados de viabilidade celular não mostraram um perfil de sinergismo de potenciação na combinação SAHA e TMZ. Pode se ver claramente

que o efeito das combinações, muitas vezes, não foi maior que a soma das toxicidades individuais das drogas, o que sugere efeito de sinergismo aditivo (figuras 1 e 2 apresentadas na parte II deste trabalho). No experimento de incorporação de PI (figura 1E, parte II), observamos alterações na morfologia das células tratadas com TMZ/SAHA, a qual foi caracterizada por uma grande redução na quantidade total de células e aumento de marcação com PI no co-tratamento, o que indica um rompimento e/ou perda de função da membrana celular, no tempo de 72 h, indicando apoptose tardia ou necrose. Para melhor elucidação do fenótipo das células tratadas, os experimentos de ciclo celular mostraram que, embora a população celular tenha apresentado bloqueio em G2/M quando tratada com TMZ/SAHA em tempos curtos, foi possível determinar a ativação da apoptose através da quantificação de caspase-3/7, formação de sub-G1 e a externalização de fosfatidilserina. Entretanto, os marcadores de apoptose só foram detectados em tempos prolongados de tratamento (72-96 h), indicando um processo tardio de colapso celular e, possivelmente, a existência de mecanismos de resistência em tempos mais iniciais de exposição aos fármacos teste. Baseados em tais resultados, interpretamos que o SAHA e TMZ poderiam estar desencadeando respostas redundantes de proteção celular, embora desencadeadas por diferentes mecanismos de ação destes compostos.

Perguntando o porquê da não ocorrência de sinergismo de potenciação, e sabendo da ocorrência de mecanismos de resistência envolvendo autofagia já identificados em estudos prévios realizados por nosso grupo com o TMZ e a curcumina em modelos *in vitro* e *in vivo* de gliomas (Zanotto-Filho *et al*, 2015), assim como por outros grupos para com o SAHA (Liu *et al.*, 2010, Chiao *et al.*, 2013) e com

TMZ (Fillipi-Chiela *et al.*, 2015) em monoterapia, decidimos caracterizar a morte celular e verificar o papel da autofagia como resposta de sobrevivência celular no contexto.

Inicialmente realizamos experimentos para identificar a presença de marcadores autofágicos. Por citometria de fluxo, identificamos a presença dos autofagolisossomos que são organelas ácidas, formadas para a degradação e reciclagem dos componentes do citosol e de organelas celulares danificadas, que por essa acidez podem ser marcadas com laranja de acridina (Fig. 3 parte II). Por meio da citometria de fluxo identificamos um grande percentual de autofagia acontecendo nos mono-tratamentos de TMZ e SAHA, sendo que, quando combinamos os dois fármacos obtivemos um aumento ainda mais significativo de autofagia, o que pode ser visualizado na figura 3 da parte II.

Outra maneira de identificarmos a presença de autofagia foi através das proteínas marcadoras de autofagia, como a LC3 (cadeia leve 3 da proteína 1 associada a microtúbulos), que é uma proteína citosólica (na forma LC3-I) que sofre clivagem C-terminal quando recebe um sinal pró-autofágico sendo convertida para a forma II (LC3-II). Quando ativa, LC3-II se localiza na membrana dos autofagossomos. Para utilizar LC3 como marcadora de autofagia realizamos o ensaio de Western Blot com anticorpo que detecta as formas lipidada e não lipidada da proteína. Ou seja, o anticorpo marca o quanto de LC3-I foi convertido em LC3-II, sendo essa uma mudança molecular clássica na autofagia (He e Klionsky, 2009; Sui *et al*, 2013; Fillipi-Chiela *et al.*, 2015). Nos nossos experimentos obtivemos uma significativa conversão de LC3, a partir de 24 h de tratamento principalmente nos tratamentos de SAHA sozinho ao passo que o TMZ apresentou maior ocorrência de

conversão em 48 h. O co-tratamento SAHA/TMZ apresentou tanto maior imunoconteúdo total de LC3-I quanto da forma convertida LC3-II se comparado às monoterapias, evidenciando o mecanismo redundante de resposta autofágica promovida por SAHA e TMZ em gliomas. Outro indicativo de positividade para autofagia é a redução do imunoconteúdo da proteína p62/SQSTM1, um efeito já demonstrado em outros achados para TMZ e SAHA sozinhos em outros modelos tumorais (Knizhnik *et al*, 2013; Chiao *et al*, 2013). A p62/SQSTM1 é uma proteína ligante de ubiquitina presente no autofagossoma, a qual liga na LC3 presente na membrana dos autofagossomas e marca/liga/direciona proteínas a serem degradadas por autofagia. A degradação dos autofagossomas pelo lisossoma leva à diminuição dos níveis de p62 durante a autofagia. Um ponto interessante, e diferente do observado nos tratamentos combinados é que, enquanto ocorre uma acumulação/conversão de LC3 nas células tratadas com SAHA e TMZ sozinhos, a diminuição de p62 não foi detectada no mesmo contexto. Isso sugere que ou o fluxo autofágico é baixo – logo o balanço degradação/síntese de novo de p62/SQSTM1 não é detectado por imunoblot – ou a autofagia está deficiente. A hipótese de uma autofagia deficiente pode ser minimizada, uma vez que os experimentos com laranja de acridina comprovam a que a acidificação dos vacúolos autofágicos ocorreu. Esses dados corroboram a ideia de que as drogas sozinhas alteram o fluxo autofágico, o qual é exacerbado na combinação SAHA/TMZ, de modo que a p62 não é sintetizada na mesma taxa em que é degradada. Embora não investigado em nossos experimentos, a atuação de TMZ e SAHA na indução de autofagia parece ocorrer por diferentes vias. O TMZ é um agente alquilante, que ativa a autofagia de maneira dependente da ativação das proteínas de DDR, como ATM e MSH6; como

demonstrado em células U87 e LN229 (Knizhnik *et al*., 2013) e U251MG (Zanotto-Filho *et al*., 2015). SAHA induz autofagia inibindo a proteína mTOR e com isso possibilitando que o complexo ULK1(*unc51 like autophagy activating kinase 1*) seja ativado para se ligar a membrana do fagóforo e assim recrutar as demais proteínas da família ATG (Wang, Hu e Shen, 2016). Ainda referente aos mecanismos de ação, observamos que a inibição de HDAC (medida indiretamente pela acetilação de histonas H3 e H4) por SAHA ocorre igualmente na presença do TMZ/SAHA; TMZ sozinho não teve efeito (figura 1; parte II). (Kitange *et al*, 2012). Essas evidências indicam que, embora TMZ e SAHA atuem por mecanismos de ação diferentes, os gliomas respondem de modo similar a esses estressores celulares através da indução de autofagia.

Embora a autofagia, por muitos anos, tenha sido chamada de morte celular do tipo II (sendo a apoptose o tipo I), é necessário entender que este é um processo fisiológico na célula, uma vez que ela participa da degradação de estruturas não funcionais intracelulares, como a mitocôndria (mitofagia) entre outras (Hippert, *et al.*, 2006, Chiao *et al*., 2013; Fillipi-Chiela *et al.*, 2015). Ou seja, existe um fluxo autofágico basal constante na maioria das células que, no nosso experimento, pode ser visualizado na detecção basal de LC3 lipidada (LC3-II) em células de glioma não tratadas (figura 3, parte II). Em câncer, existe um crescente número de estudos mostrando que a autofagia atua de forma protetiva na resposta ao estresse celular e inibindo a ativação do processo apoptótico em células tumorais (Gammoh *et al*, 2012). No contexto de gliomas, estudos prévios do nosso grupo e outros mostram que o tratamento com 3-MA e/ou cloroquina, assim como o silenciamento de genes da família ATG (como ATG7), potencializam a morte celular causada por TMZ em

diferentes linhagens de glioma (Lin *et al*., 2012; Sui *et al*., 2013; Zanotto-Filho *et al*., 2015; Fillipi-Chiela *et al.*, 2015; Hori *et al*., 2015); efeito reproduzido neste estudo .

Embora esperássemos que os dois fármacos combinados obtivessem um efeito de sinergismo de potenciação que poderia compensar possíveis mecanismos de resistência, o efeito encontrado no co-tratamento foi maior, mas não o suficiente para ser considerado esse tipo de sinergismo, e uma alta porcentagem de células positivas para marcação com laranja de acridina (ou seja, autofágicas) foi observado nas combinações SAHA/TMZ. Assim optamos por bloquear a autofagia com cloroquina e determinar se isso poderia aumentar a citotoxicidade e a apoptose. A cloroquina age impedindo a ligação entre o lisossomo e o autofagossomo, através de mudanças de pH lisossomal (Thomé *et al*., 2013), o que inibe a fase de terminação da autofagia, gerando um acúmulo de vesículas (Kim *et al*, 2010; Makowska *et al*, 2016). Além disso, a cloroquina já é aprovada e utilizada no tratamento de malária (Homewood *et al.*, 1972) e encontra-se em fase de testes em pacientes com glioma (Rosenfeld *et al*., 2014; Sui *et al*., 2013). Nossos resultados indicaram que a CQ é pouco tóxica para as células de GBM, mas quando combinada ao SAHA ou TMZ e/ou SAHA/TMZ o efeito citotóxico das drogas é potencializado. Bloqueando a autofagia com CQ obtivemos uma redução na parada em G2/M e um aumento de Sub-G1, assim como aumento de células positivas para anexina-V externalizada e maior ativação de caspase-3/7, sugerindo uma aumentada taxa de apoptose nos cultivos de gliomas (Fig. 5 parte II).

Além da inibição farmacológica da autofagia com cloroquina, realizamos ensaios com células silenciadas para beclina-1, que é um gene envolvido nos passos iniciais da formação da vesícula autofágica (Pirtoli *et al*, 2009). Sua

expressão anormal é encontrada em vários cânceres humanos como melanoma (Pirtoli *et al*, 2009), cólon (Li *et al*, 2009), ovário (Shen *et al*, 2008), e tumores cerebrais (Miracco *et al*, 2007). Os experimentos de viabilidade em clones da linhagem de glioma C6 com silenciamento adenoviral para beclina-1 mostraram uma redução na viabilidade celular após tratamento com SAHA e SAHA/TMZ, mas não TMZ sozinho, quando comparado aos clones controles de silenciamento, PLKO (figura 5, parte II). Importante notar que, embora a eficiência de silenciamento de beclina-1 tenha sido bastante significativo, o impacto na sensibilização celular causado pela depleção de beclina-1 foi muito menor que o efeito da cloroquina (Figura 5, parte II). Isso provavelmente está atribuído ao efeito compensatório de outros genes autofágicos (como os vários genes da família das ATGs) ou, até mesmo, outros processos, como a senescência celular, ainda não avaliados até esta etapa do estudo. Sendo assim, embora se atribua diferentes papeis à autofagia no que se refere à decisão entre morte e sobrevida celular, em nosso estudo ela está envolvida com um mecanismo de proteção, visto que quando ela foi inibida por cloroquina - ou pelo silenciamento do gene de beclina-1 (em menor magnitude de efeito) - obtivemos um aumento de morte celular pelo SAHA/ TMZ sozinhos, assim como no tratamento combinado. Nossos resultados corroboram com outros estudos já realizados onde se verificou que a inibição da autofagia contribui para aumentar a sensibilização de células quimio e radio-resistentes (O'Donovan *et al*, 2011; Guo *et al*, 2012). Estendendo ao contexto clínico, o uso de inibidores de autofagia associados a quimioterápicos parece ser uma estratégia promissora para o aperfeiçoamento da terapia com TMZ, SAHA e provavelmente outros fármacos, ou

tipos tumores, que utilizam a resposta autofágica como mecanismo de sobrevivência celular.

6 CONCLUSÃO

A partir dos resultados obtidos nesta dissertação podemos sugerir que:

- SAHA sozinho tem bom efeito na redução de viabilidade celular em células de gliomas, e quando combinado ao quimioterápico de escolha, TMZ, o efeito citotóxico observado é mais pronunciado, embora o perfil gráfico aponte para um efeito de sinergismo aditivo, e não de sinergismo de potenciação.

- O tratamento de TMZ/SAHA, mesmo levando a um bloqueio em fase G2/M do ciclo celular, as células parecem resistir à indução de apoptose, uma vez os marcadores fenotípicos de apoptose (externalização de fosfatidilserina e ativação de caspase-3) só são alterados em tempos longos de tratamento.

- O tratamento com SAHA/TMZ induz autofagia em tempos tão curtos quanto 24 h de tratamento, e esse aumento é maior nos tratamentos combinados. A inibição do fluxo autofágico pelo uso de inibidor farmacológico cloroquina – e em menor magnitude pelo silenciamento de beclina 1 – promoveu um aumento na citotoxicidade do tratamento TMZ/SAHA com ativação do processo de apoptose (embora a ativação da caspase-3/7 efetora quantificada aqui não nos indique se a via apoptótica é intrínseca ou extrínseca). A autofagia exerce um papel protetor na resposta celular ao TMZ/SAHA, dificultando a ativação da cascata apoptótica.

- Diferentemente de TMZ, o aumento na produção de radicais livres mediante o tratamento com SAHA, parece ser necessário para a efetividade da droga, visto que quando utilizamos um antioxidante a toxicidade de SAHA foi reduzida.

Em suma, baseado na abordagem *in vitro* utilizada, concluímos que a resposta autofágica ao tratamento com TMZ/SAHA parece atuar como um

mecanismo de sobrevivência celular ao estresse causado por esses antitumorais, retardando e inibindo a indução de apoptose nas células de glioma estudadas. O uso de inibidores da autofagia, como aqui exemplificado pela cloroquina, pode ser promissor. Para uma melhor elucidação da real contribuição da autofagia na sobrevivência/resistência de gliomas tratados com TMZ e SAHA, se faz necessária a confirmação destes dados em modelos *in vivo* da doença.

7 PERSPECTIVAS

As principais perspectivas de seguimento deste trabalho são:

1) Avaliação do efeito de SAHA, em uma curva com doses acima de 4 µM, *in vitro*, com células sadias.

2) Avaliação dos efeitos antitumorais e pró-autofágicos da combinação TMZ/SAHA em modelo animal com implante de linhagem tumoral C6.

3) Avaliação da eficácia do uso de inibidores de autofagia em combinação com o tratamento TMZ/SAHA em modelo animal com implante de linhagem tumoral C6

4) Avaliação da eficácia antitumoral do SAHA e TMZ em ratos com implantes intracerebrais de linhagens C6 silenciadas permanentemente para beclina-1 (usada nesse estudo) e ATG5 (em validação)

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ANEXOS

Anexo I: Modelo de artigo solicitado pelo periódico BBACTA – Molecular Basis of Disease.

Anexo II: Carta de aprovação da Comissão de Ética no Uso de Animais (CEUA), para o uso de células sadias.

Anexo II – Modelo de artigo solicitado pelo periódico BBACTA – Molecular Basis of Disease.

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Examples:

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[1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, J. Sci. Commun. 163 (2010) 51–59.

Reference to a book:

[2] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.

Reference to a chapter in an edited book:

[3] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304.

Reference to a website:

[4] Cancer Research UK, Cancer statistics reports for the UK. http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/, 2003 (accessed 13.03.03).

Reference to a dataset:

[dataset] [5] M. Oguro, S. Imahiro, S. Saito, T. Nakashizuka, Mortality data for Japanese oak wilt disease and surrounding forest compositions, Mendeley Data, v1, 2015. http://dx.doi.org/10.17632/xwj98nb39r.1.

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Anexo II – Carta de aprovação do CEUA

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CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 27686
Título: AVALIAÇÃO DA EXPRESSÃO DO EIXO RAGE/NF-KB DURANTE O DESENVOLVIMENTO E NO CONTEXTO PATOLÓGICO (DOENÇA DE PARKINSON) EM MODELOS IN VITRO E IN VIVO.

Pesquisadores:

Equipe UFRGS:

JOSE CLAUDIO FONSECA MOREIRA - coordenador desde 01/01/2015

Comissão De Ética No Uso De Animais aprovou o mesmo, em reunião realizada em 22/12/2014, em seus aspectos éticos e metolodológicos, para a utilização de 25 ratos machos adultos e 50 ratas fêmeas adultas com idade de 90 dias e peso entre 200-250g e 170 filhotes com peso entre 200- 250g, de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008 que disciplina a criação e utilização de animais em atividades de ensino e pesquisa.

Porto Alegre, Quarta-Feira, 21 de Janeiro de 2015

CRISTIANE MATTE
Vice Coordenador da comissão de ética

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