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AVALIAÇÃO DA ATIVIDADE ANTITUMORAL DO EXTRATO DE *ACHYROCLINE SATUREIOIDES*

Porto Alegre

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de doutora em Bioquímica.

Orientador: Prof. Dr. José Claudio Fonseca Moreira

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A Comissão Examinadora, abaixo assinada, aprova a Tese de Doutorado

Avaliação da atividade antitumoral do extrato de *Achyrocline satureioides*

elaborada por

Priscila Oliveira de Souza

Como requisito à obtenção do título de **Doutora em Bioquímica**

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Sumário

Resumo

Glioblastoma multiforme é um câncer do sistema nervoso central agressivo e invasivo. Apesar dos avanços terapêuticos com cirurgia, radiação e quimioterapia, o tempo de sobrevida média permanece reduzido. Os polifenóis têm sido relatados por apresentarem várias atividades biológicas, incluindo atividade anticâncer. Nesse estudo, foi avaliada a potencial atividade antiglioma do extrato de *Achyrocline satureioides* (marcela) e seus principais flavonoides em modelos de glioblastoma *in vitro* e *in vivo*. O extrato de *A. satureioides* (100 µg/mL) assim como os flavonoides isolados (quercetina, 3-O-metil-quercetina, luteolina e achyrobichalcona; em 10 µM ou doses menores) reduziram a proliferação de três linhagens celulares de glioma (C6, U87 e U251). A combinação do extrato de *A. satureioides* com temozolomida (TMZ) demonstrou elevada atividade antiproliferativa comparada com o quimioterápico sozinho em uma menor concentração (50 µg/mL). Cabe ressaltar que nenhuma toxicidade foi observada nas células sadias do cérebro (astrócitos primários, neurônios primários e culturas organotípicas de fatias do hipocampo) na concentração que afetou as células de gliomas. Entretanto, a administração do extrato de *A. satureioides* no modelo tumoral em ratos não apresentou o mesmo efeito conforme observado *in vitro*. Além disso, ele foi capaz de bloquear o efeito do TMZ. Esses resultados geram preocupação uma vez que extratos de plantas poderiam afetar a atividade de fármacos anticâncer, especialmente considerando que o chá de marcela é amplamente consumido pela população da América do Sul e indicado para alívios de sintomas como dores de cabeça e náuseas. Portanto, mais estudos *in vivo* usando os flavonoides isolados são necessários para investigar a potencial atividade antiglioma observada *in vitro*.

Palavras-chaves: *Achyrocline satureioides*; Efeito antiproliferativo; Flavonoides; Glioblastoma multiforme.

Abstract

Glioblastoma multiforme is an aggressive and invasive central nervous system cancer. Despite the therapeutic improvements with surgery, radiation, and chemotherapy, the median survival times remain dismal. Polyphenols have been reported to have numerous biological activities, including anticancer activity. In this study, the potential antiglioma activity of *Achyrocline satureioides* (marcela) extract and its main flavonoids was evaluated both *in vitro* and *in vivo* in glioblastoma models. *A. satureioides* extract (100 µg/mL) as well as the isolated flavonoids (quercetin, 3-O-methyl-quercetin, luteolin and achyrobichalcone; at 10 µM or lower doses) reduced proliferation of three glioma cell lines (C6, U87 and U251). Combination of *A. satureioides* extract with temozolomide (TMZ) showed enhanced antiproliferative activity compared to the chemotherapeutic alone at a lower concentration (50 µg/mL). Importantly, no toxicity was observed in healthy cells of the brain (primary astrocytes, primary neurons, and organotypic hippocampal slice cultures) at the concentration that affected glioma cells. However, administration of *A. satureioides* extract did not show the same effect in a rat tumor model as observed *in vitro*. In addition, it blocked the effect of TMZ. These results raise concern since plant extracts could affect the activity of anticancer drugs, especially considering that marcela tea is widely consumed by the South American population and indicated for relief of symptoms such as headaches and nausea. Therefore, further *in vivo* studies using the isolated flavonoids are required to investigate their potential antiglioma activity observed *in vitro*.

Keywords: Antiproliferative effect; *Achyrocline satureioides*; Flavonoids; Glioblastoma multiforme.

Lista de Abreviaturas

ALT – Alanina aminotransferase

AP-1 – Ativador de proteína-1

AST – Aspartato aminotransferase

CAT – Catalase

DNA-PKcs – Proteínas quinases dependentes de DNA

EGFR – Receptor do fator de crescimento epidermal

GST – Glutationa S-transferase

IDH – Isocitrato desidrogenase

MAPK – Proteína quinase ativada por mitógeno

NFκB – Fator nuclear kappa B

OMS – Organização Mundial da Saúde

PI3K ou AKT – Fosfatidilinositol 3-quinase

PKC – Proteína quinase C

PTEN – Fosfatase homóloga à tensina deletada no cromossomo 10

SNC – Sistema Nervoso Central

SOD – Superóxido dismutase

TBARS – Substâncias reativas ao ácido tiobarbitúrico

TMZ – Temozolomida

TP53 – Gene para a proteína p53

INTRODUÇÃO

1.   Câncer

1.1   Aspectos epidemiológicos

O câncer é uma desordem caracterizada pelo aumento da atividade metabólica, resultando no aumento da proliferação e crescimento celular. Assim, células de câncer apresentam características metabólicas diferentes daquelas células consideradas saudáveis, como consequência direta ou indireta de mutações oncogênicas (HANAHAN; WEINBERG, 2011; PAVLOVA; THOMPSON, 2016). A prognose do câncer está intimamente ligada ao estágio da doença. Nos estágios mais avançados, quando ocorre metástase, ou seja, o deslocamento das células tumorais do sítio primário (onde iniciou o crescimento) para um sítio secundário no corpo do indivíduo, a prognose se agrava e os tratamentos tendem a falhar (TAURIELLO et al., 2017). O câncer é o principal problema de saúde mundial e lidera as causas de morte, sendo superado apenas pelas doenças cardíacas, cujo cenário espera-se reverter num futuro próximo (SIEGEL; MILLER; JEMAL, 2017).

Anualmente, a Sociedade Americana do Câncer estima o número de novos casos de câncer e mortes que ocorrerão nos Estados Unidos no referido ano e registra os dados mais recentes de incidência do câncer, mortalidade e sobrevivência. Segundo ela, as estimativas para 2018 nos Estados Unidos são de 1.735.350 novos casos de câncer e 609.640 mortes. A incidência de câncer é 20% maior em homens que em mulheres, apresentando uma taxa de mortalidade também superior (aproximadamente 208 mortes a cada 100.000 homens enquanto 145 a cada 100.000 mulheres). Cabe salientar que essas diferenças entre os sexos variam também conforme o tipo de câncer (AMERICAN CANCER SOCIETY, 2018; SIEGEL; MILLER; JEMAL, 2017).

Os tipos de câncer com maior ocorrência e causas mais comuns de morte são câncer de pulmão e brônquios, colorretal, próstata em homens e mama em mulheres. Eles representam 46% de todas as mortes registradas por câncer, sendo mais de um quarto (26%) devido ao câncer de pulmão. Enquanto câncer do cérebro e do sistema nervoso central representa 1,4% dos casos. Segundo projeções, o número de pessoas vivendo com diagnóstico de câncer atingiu 14,5 milhões em 2014, sendo esperado que alcance 19 milhões em 2024 (NATIONAL CANCER INSTITUTE, 2018; SIEGEL; MILLER; JEMAL, 2017).

A estimativa para o Brasil no período de 2016-2017, segundo o Instituto Nacional de Câncer José Alencar Gomes da Silva (INCA), apontou a ocorrência de aproximadamente 600.000 novos casos de câncer. Constatou-se similaridade do perfil epidemiológico em relação à América Latina e ao Caribe, onde os cânceres de próstata (61.000) em homens e mama (58.000) em mulheres foram os mais frequentes. Os tipos de câncer com maior ocorrência no Brasil, desconsiderando os casos de câncer de pele não melanoma (aproximadamente 180 mil casos novos), em homens são próstata (28,6%), pulmão (8,1%), intestino (7,8%), estômago (6,0%) e cavidade oral (5,2%). Enquanto nas mulheres, os principais são cânceres de mama (28,1%), intestino (8,6%), colo do útero (7,9%), pulmão (5,3%) e estômago (3,7%) (INCA, 2016).

O câncer é uma doença multifatorial, podendo ter origem na combinação de fatores genéticos e ambientais, além de determinados hábitos de vida. Menos de 15% dos casos de câncer são hereditários. Os fatores ambientais com maior relevância são a radiação ultravioleta (causando melanoma, carcinoma de célula escamosa e de célula basal da pele), viroses e outras infecções (como exemplo a infecção em longo prazo com os vírus da hepatite B ou C pode resultar no câncer do fígado), e a exposição a carcinógenos ocupacionais (dentre

os quais alcatrões de carvão e seus derivados como benzeno, alguns hidrocarbonetos, anilina utilizada em corantes e amianto) (AMERICAN CANCER SOCIETY, 2014; IARC, 2012).

Dentre os hábitos de vida destacam-se o tabagismo (relacionado com os cânceres de pulmão, nasofaringe, da cavidade oral, nasal e seios paranasais, do esôfago, estômago, rim, ureter, cérvix uterino, leucemia mielóide, colorretal e do ovário/mucinoso); o consumo excessivo do álcool (associado com cânceres da cavidade oral, faringe, laringe, esôfago, colorretal, fígado e mama); a obesidade (fator de risco para cânceres do esôfago/adenocarcinoma, do cólon e reto, de vesícula biliar, do pâncreas, do rim, de próstata avançado, de mama após a menopausa, de endométrio e do ovário); o sedentarismo (relacionado com câncer de cólon, do endométrio e de mama); e alimentação inadequada como ingestão insuficiente de vegetais sem amido (associada com cânceres na cavidade oral/faringe, esôfago, estômago e laringe), de frutas (cânceres da cavidade oral/faringe, esôfago, estômago, laringe e pulmão), de fibras, carnes vermelhas e processadas (colorretal), de sal (estômago) (WHITEMAN; WILSON, 2016; WORLD CANCER RESEARCH FUND/AMERICAN INSTITUTE FOR CANCER RESEARCH, 2011; WORLD CANCER RESEARCH FUND / AMERICAN INSTITUTE FOR CANCER RESEARCH, 2007).

1.2 Marcadores biológicos do câncer

Em 2000, Hanahan e Weinberg estabeleceram os marcadores do câncer, os quais compreendem seis características biológicas adquiridas durante as múltiplas etapas de desenvolvimento dos tumores. Os marcadores constituem os pilares fundamentais para racionalizar a complexidade da doença neoplásica. Estes incluem sustentação da sinalização proliferativa, evasão dos supressores de crescimento, resistência à morte celular, imortalidade replicativa, indução à angiogênese, e ativação da invasão e metástase. A instabilidade

genômica e as mutações foram consideradas os principais mecanismos delineando esses marcadores (HANAHAN; WEINBERG, 2000).

Recentemente, esses marcadores biológicos foram revisados e acrescidos da capacidade de reprogramação do metabolismo energético celular (a fim de manter o contínuo crescimento e proliferação celular do tumor) e escape da destruição por células do sistema imune (particularmente linfócitos B e T, macrófagos e células natural killer). Além disso, os tumores exibem outra dimensão de complexidade, tanto o parênquima quanto o estroma dos tumores contem um repertório de células recrutadas e normais do organismo (pericito, células endoteliais, células inflamatórias do sistema imune e fibroblastos associados às células de câncer), as quais contribuem para a aquisição dos tipos de marcadores, criando assim o "microambiente tumoral" (HANAHAN; WEINBERG, 2011).

Durante as últimas décadas tem sido demonstrado que para melhor compreensão da biologia do tumor, precisa-se considerar a contribuição de cada tipo celular que compõe o microambiente tumoral. Em estágios avançados do câncer, o microambiente é considerado um modulador central das características como invasão celular, intravasão (passagem das células do câncer através das paredes dos vasos sanguíneos ou vasos linfáticos próximos) e extravasão, além da capacidade de se estabelecer e colonizar novos nichos, formando metástases (TAURIELLO; BATLLE, 2016). O microambiente induz mudanças temporárias no perfil gênico, influenciando nas características metastáticas das células tumorais por promover alterações metabólicas, proliferativas, migratórias ou de diferenciação (CALON et al., 2012, 2015). Particularmente, o microambiente tumoral também participa da regulação das metástases quiescentes, de modo que as alterações das células do estroma e dos componentes da matriz extracelular inibem a eliminação das células tumorais pelo sistema imune e permite o escape da vigilância imunológica, promovendo a saída do estágio de dormência (GIANCOTTI, 2013).

Nos estágios iniciais do desenvolvimento do câncer, o microambiente é capaz de exercer uma pressão negativa, que através da interação com células cancerosas, é frequentemente superada, deslocando o equilíbrio de um ambiente antitumorigênico para um microambiente pró-tumorigênico (BISSELL; HINES, 2011). Dessa forma, células do câncer e o microambiente tumoral são considerados unidades interativas que evoluem juntas (POLYAK; HAVIV; CAMPBELL, 2009). Nesse cenário, as alterações das células tumorais adquirem um papel fundamental ao conferir vantagem em um determinado ambiente ou fornecendo ferramentas a fim de modular o ambiente repressivo circundante.

2.   Câncer do Sistema Nervoso Central

O câncer cerebral primário consiste de tumores que se originam do sistema nervoso central (SNC) e compreende uma variedade de tipos tumorais os quais podem apresentar-se como benignos ou malignos (LOUIS et al., 2016). Ao contrário da disseminação metastática dos cânceres para o SNC, os quais são de ocorrência mais comum, pacientes com câncer cerebral primário tipicamente permanecem assintomáticos até o aparecimento da manifestação clínica do tumor, em geral nos estágios mais avançados da doença. Dentre as manifestações destacam-se dores de cabeça, convulsão, náusea/emese, síncope, disfunção neurocognitiva, mudanças de personalidade, perda sensorial, desequilíbrio da marcha, incontinência urinária, hemiplegia, afasia, negligência unilateral e disfunção do campo visual (LOUIS DN, OHGAKI H, WIESTLER OD, CAVENEE, 2016).

Por anos a classificação dos tumores baseou-se na histogênese e suas similaridades microscópicas. A classificação dos tumores do SNC, de acordo com a Organização Mundial da Saúde (OMS) em 2007, agrupava todos os tumores com fenótipo astrocitário (aqueles originados dos astrócitos, células estreladas do SNC) separadamente daqueles com fenótipo

oligodendroglial (neoplasias derivadas dos oligodendrócitos, células responsáveis pela mielinização do SNC), sem considerar se a variedade de tumores astrocíticos apresentavam similaridades ou divergências clínicas (LOUIS DN, OHGAKI H, WIESTLER OD, 2007).

Em 2016, a OMS definiu uma nova classificação dos tumores do SNC utilizando parâmetros moleculares em conjunto com a histologia a fim de melhor definir os diferentes tipos tumorais, estabelecendo um novo conceito segundo o qual o diagnóstico de tumores do SNC deve ser reestruturado na era molecular. A nova classificação dos tumores do sistema nervoso central compreende: tumores astrocítico e oligodendroglial difusos, ependimal, plexo coroide, neuronal e mix neuronal-glial, região pineal, embrionários, dos nervos cranianos e paravertebral, meningiomas, mesequimal e não meningotelial, melanocíticos, linfomas, histiocíticos, de células germinativas, de região selar e metastáticos (LOUIS et al., 2016).

A análise do ponto de vista patogênico possibilita uma classificação dinâmica baseada tanto no fenótipo quanto no genótipo; do ponto de vista prognóstico, esses grupos de tumores compartilham marcadores prognósticos similares; do ponto de vista de gestão do paciente, ela guia o uso de terapias (convencionais ou direcionadas) para subtipos biologicamente e geneticamente similares (LOUIS et al., 2016).

2.1   Gliomas

Glioma é o mais comum e agressivo tumor primário do SNC, representando 32% de todos os tumores cerebrais e aproximadamente 80% dos tumores malignos do cérebro. De acordo com a OMS, os gliomas são classificados segundo o grau de malignidade (I-IV) (OSTROM et al., 2014).

Os gliomas tipo I são considerados benignos e ocorrem frequentemente em crianças e jovens, sendo curado após ressecção cirúrgica completa (astrocitoma pilocítico é o mais frequente). Os gliomas grau II, também denominados gliomas difusos de baixo-grau,

apresentam crescimento lento, estão associados com melhor prognóstico, porém podem progredir para o glioma de alto grau ao longo do tempo. Os gliomas de grau III ou gliomas anaplásicos acometem indivíduos na faixa dos 49 anos e incluem o oligodendroglioma anaplásico, oligoastrocitoma anaplásico e astrocitoma anaplásico, os quais apresentam cursos clínicos heterogêneos. O glioma de grau IV ou glioblastoma multiforme é o mais comum e agressivo, representando 75% dos casos de gliomas diagnosticados em adultos (ALENTORN et al., 2015).

Segundo a nova classificação dos tumores do SNC da OMS (2016), os glioblastomas ainda podem ser divididos em (1) glioblastoma, IDH[1]- tipo selvagem (90% dos casos) e acomete pacientes com média de 55 anos; (2) glioblastoma, IDH-mutante (10% dos casos), chamado de glioblastoma secundário (progrediu de um glioma de menor grau) e se estabelece em pacientes jovens; (3) glioblastoma não especificado, assim classificados aqueles tumores cuja análise completa do IDH não pode ser realizada (LOUIS et al., 2016).

O glioblastoma apresenta características alterações genéticas, dentre as mais comuns destacam-se amplificação dos oncogenes (EGFR[2] e CDK4) e deleção dos genes supressores do tumor, mais frequentemente PTEN[3] e TP53[4] (FURGASON et al., 2015). Além disso, a crescente agressividade dos tumores com deficiência em PTEN e p53 sugere que a sua ausência combinada pode resultar em um crescente potencial tumorigênico (MEMMEL et al., 2014). O gene EGFR está mutado ou amplificado em 40% dos glioblastomas primários e altamente expresso em mais de 60% dos tumores, estando

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[1] IDH é o gene da enzima isocitrato desidrogenase, mudanças na função dessa enzima leva à produção de 2-hidroxiglutarato, um possível metabólito oncogênico, ao invés de produzir NADPH.

[2] EGFR (receptor do fator de crescimento epidermal) é uma proteína encontrada na superfície de algumas células, quando ligada ao fator de crescimento epidermal envia sinais para a célula continuar crescendo.

[3] PTEN (fosfatase homóloga à tensina deletada no cromossomo 10) é um suppressor tumoral via inativação de PI3K.

[4] TP53 é o gene para a proteína p53, mais frequentemente mutada em glioblastoma, a qual normalmente suprime o crescimento tumoral.

relacionado à proliferação, invasão e resistência à indução terapêutica da morte celular apoptótica (MELLINGHOFF et al., 2005; WELLER et al., 2014).

Devido ao padrão de crescimento rápido e infiltrativo, o glioblastoma apresenta uma progressiva deterioração neurológica, levando à morte em um curto período de tempo (WU et al., 2013). Apesar dos recentes avanços no tratamento do tumor cerebral, incluindo a máxima ressecção cirúrgica seguida por radioterapia, quimioterapia adjuvante com o agente alquilante temozolomida, o prognóstico para o glioblastoma permanece muito limitado (MARSH et al., 2013; WACHSBERGER et al., 2014). Outro aspecto relevante nos tratamentos quimioterápicos dos gliomas é a existência da barreira hematoencefálica, a qual se constitui de células vasculares endoteliais do sistema nervoso central e atuam restringindo o transporte de substâncias e fármacos para o cérebro (MU et al., 2017).

As causas do prognóstico desfavorável para o glioblastoma incluem sua típica invasividade agressiva, elevada angiogênese e proliferação (OTANI et al., 2018). Recentemente, tratamentos angiogênicos foram realizados em testes clínicos, entretanto nenhum deles resultou em prolongamento da sobrevida (CHINOT et al., 2014; GILBERT et al., 2014). Piao e colaboradores demonstraram que a terapia com o anti-fator de crescimento vascular endotelial induziu mais invasividade e fenótipos resistentes ao tratamento (PIAO et al., 2012).

A sobrevida média dos pacientes diagnosticados com glioblastoma atualmente encontra-se entre 15-18 meses, especialmente após o tratamento associado à temozolomida. Contudo, esses tumores frequentemente recorrem no sítio primário após 7 meses do tratamento e, considerando a natureza infiltrativa desses tumores, a limitação da terapia local e a resistência, o prognóstico e co-morbidades associadas à recidiva acaba sendo bastante desanimadora (BEAL; ABREY; GUTIN, 2011; KIM et al., 2014; STUPP et

al., 2009). Estimativas do Instituto Nacional do Câncer de 2016 para o Brasil revelaram 10.270 novos casos de câncer do Sistema Nervoso Central (SNC), sendo que os gliomas representam 40-60%, apresentando maior incidência em homens (INCA, 2016). Considerando esse contexto clínico, há uma crescente busca por novas formas terapêuticas que apresentem potencial atividade no tratamento dessa doença.

2.2 Temozolomida

Durante três décadas (1980-2013), 570 testes clínicos foram realizados com aproximadamente 33.000 pacientes, os quais foram tratados com diferentes formas terapêuticas a fim de melhor compreender e tratar o glioblastoma (GROSSMAN; ELLSWORTH, 2016). Desses estudos, um agente quimioterápico, temozolomida (TMZ), foi capaz de melhorar moderadamente a sobrevida dos pacientes (STUPP et al., 2005). TMZ, uma pequena molécula lipofílica (194 Da) capaz de atravessar a barreira hematoencefálica, é um agente alquilante de DNA monofuncional disponível oralmente, pertencente à classe do imidazotetrazina (ZHANG; STEVENS; BRADSHAW, 2012).

TMZ atua como um pró-fármaco, estável em pH ácido, uma propriedade que permite sua administração oral (NEWLANDS et al., 1992), porém instável em pH maior que 7, com tempo de meia-vida de 1,8 horas em pH 7,4 no plasma (TSANG et al., 1990). Assim, TMZ é rapidamente absorvido intacto e sofre hidrólise espontânea (pH > 7) para formar seu metabólito ativo monometil triazeno, 5-(3-metiltriazen-1-il)-imidazol-4-carboxamida (MTIC). MTIC ainda reage com a água (pH < 7) para liberar 5-aminoimidazol-4-carboxamida (AIC) e um cátion metil diazônio altamente reativo (Fig. 1). As espécies ativas do cátion metildiazonio metila preferencialmente o DNA nas posições *N7* da guanina (*N7*-MeG; 70%), mas também pode metilar *N3* da adenina (*N3*-MeA; 9%) e *O6* de resíduos de guanina (*O6*-MeG; 6%) (DENNY et al., 1994; TISDALE, 1987).
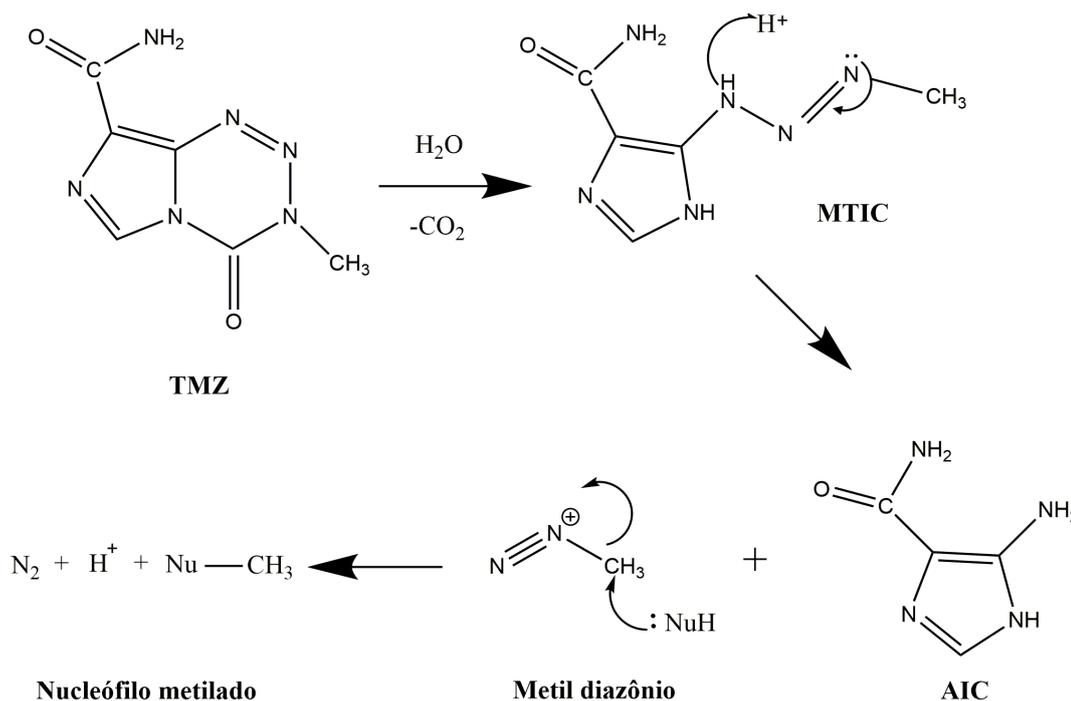
Figura 1. Mecanismo de ação da temozolomida e seus metabólitos. MTIC (5-(3-metiltriazen-1-il)-imidazol-4-carboxamida); AIC (5-aminoimidazol-4-carboxamida); Nu (centro nucleofílico no DNA) (ZHANG; F.G. STEVENS; D. BRADSHAW, 2012).

Existe uma estreita janela de pH próximo ao pH fisiológico no qual todo o processo de ativação pró-fármaco do TMZ para transferência do grupo metil possa ocorrer. O tumor cerebral possui um pH mais alcalino comparado com o tecido saudável ao seu redor, situação que favorece a ativação do pró-fármaco preferencialmente no tecido tumoral (TIEK et al., 2018). Assim, TMZ é usado para tratar tumores cerebrais (mas não exclusivamente), proporcionando benefícios terapêuticos significativos para pacientes com glioblastoma (STUPP et al., 2009).

A citotoxicidade da temozolomida é mediada primariamente através de *O6*-MeG, um carcinogênico, mutagênico e indutor de lesões tóxicas (DRABLØS et al., 2004; WEDGE et al., 1996; WEDGE; NEWLANDS, 1996; WEDGE; PORTEOUS; NEWLANDS, 1996). O

reparo direto de *O6*-MeG pela enzima suicida metilguanina-DNA metiltransferase (MGMT) remove o aduto metil, restaurando a guanina. *O6*-MeG não reparada se liga erroneamente com timina (ao invés de citosina) durante a replicação do DNA, alertando o reparo de erro de combinação do DNA (MMR) (KYRTOPOULOS et al., 1997; MARGISON; SANTIBÁÑEZ-KOREF, 2002). MMR reconhece exclusivamente timina pareada erroneamente na fita filha e a retira, assim *O6*-MeG persiste na fita molde. Portanto, ciclos fúteis de reinserção de timina e excisão resulta em constantes quebras da fita de DNA, resultando no colapso da forquilha de replicação (MOJAS; LOPES; JIRICNY, 2007). A parada do ciclo celular na fase G2/M é desencadeada, ocorrendo no segundo ciclo celular após o tratamento (CEJKA, 2003; ROOS; BAUMGARTNER; KAINA, 2004; ZHUKOVSKAYA et al., 1994) via sinalização ATR/CHK1-dependente (STOJIC; BRUN; JIRICNY, 2004), por fim, levando à apoptose (D'ATRI et al., 1998).

Dessa forma, uma boa resposta ao TMZ requer MMR funcional e baixos níveis de MGMT. As lesões em *N7*-MeG e *N3*-MeA, quantitativamente mais abundantes, são reparadas rapidamente pelo reparo por excisão de bases do DNA (BER). *N7*-MeG não é marcadamente citotóxico, entretanto lesões em *N3*-MeA são fatais se não interceptadas (HORTON; WILSON, 2007). Apesar do tratamento com TMZ aumentar a sobrevida dos pacientes em média 4 meses, o rápido desenvolvimento de resistência ao quimioterápico indica uma necessidade de buscar novas terapias para o futuro (TIEK et al., 2018).

3.   Produtos Naturais e Câncer

Produtos naturais são substâncias produzidas por organismos vivos (plantas, animais, organismos marinhos ou microrganismos) como metabólitos secundários, os quais não são essenciais para sobrevivência, porém proporcionam vantagens evolutivas aos seres (KOEHN,

2013). Devido às inúmeras pressões ecológicas e evolutivas, os produtos naturais são moléculas complexas que apresentam uma grande diversidade química e, frequentemente, diferentes propriedades biológicas. Essas características tornaram essas moléculas adequados alvos para a descoberta de fármacos, além de apresentarem potenciais aplicações terapêuticas (BERNARDINI et al., 2017).

Historicamente, extratos de produtos naturais foram as principais fontes de medicamentos, posteriormente tornando-se possível purificar moléculas ativas. Um clássico exemplo foi a descoberta da aspirina derivada da casca do salgueiro *Salix* sp. Linnaeus (família Salicaceae) pelo químico Felix Hoffman na Bayer em 1897, cujos registros de aplicação empírica da planta por egípcios e gregos datam de 1934 AC, utilizando tanto folhas quanto a casca para tratamento de dores (DESBOROUGH; KEELING, 2017).

De acordo com a OMS, atualmente mais de 80% da população mundial prioriza os tradicionais medicamentos etnobotânicos para suas necessidades básicas de saúde. Na área da terapêutica do câncer, 75% dos fármacos descobertos e aprovados entre 1940 e 2014 são produtos naturais ou derivados deles (KOEHN, 2013; NEWMAN; CRAGG, 2016). Destacam-se na classe dos quimioterápicos o paclitaxel (Taxol®, da planta *Taxus brevifolia*), camptotecina (da *Camptotheca acuminata*), podofilotoxina (Podofilox®, obtido de raízes e rizomas de espécies de *Podophyllum*) e alcaloides da vinca, como vincristina e vimblastina (*Catharanthus roseus*), além de outros agentes anticâncer em estudo como magnolol, resveratrol e curcumina (KHAN; GURAV, 2018).

3.1   Paclitaxel

O paclitaxel é um taxano obtido da casca da planta alcalóide *Taxus brevifolia* Nuttall (família Taxaceae), também conhecida como Teixo do Pacífico. Ele age desestabilizando a função dos microtúbulos, essencial para a divisão celular e outras funções celulares normais, eventualmente causando morte celular. Nas células tumorais reduz a proliferação do tumor.

Paclitaxel também se demonstrou capaz de inibir autofagia através de dois mecanismos dependentes do estágio no ciclo celular. Em células mitóticas, paclitaxel bloqueou a ativação de PI3KC3, um iniciador crítico da formação do autofagossomo. Em células não mitóticas, paclitaxel inibiu o movimento e a maturação dos autofagossomos. Entretanto, bloqueando química ou geneticamente a formação do autofagossomo diminui a morte celular induzida pelo paclitaxel, sugerindo que a autofagia aumenta a sensibilidade das células à toxicidade do paclitaxel (VELDHOEN et al., 2013). Esse quimioterápico é indicado no tratamento do câncer de pulmão, ovário, próstata, mama e Sarcoma de Kaposi (DU et al., 2018; ERCOLAK et al., 2015).

3.2   Camptotecina

Camptotecina é um alcaloide natural encontrado na casca, caule e folhas da árvore *Camptotheca acuminata* Decne (família Nyssaceae) nativa da China. Ela é um potente inibidor da topoisomerase I e apresenta atividades antitumorais contra leucemia, câncer de cólon humano e uma variedade de tumores sólidos. Camptotecina foi capaz de induzir autofagia e morte celular apoptótica nas células HeLa. Além disso, o tratamento de células tumorais com camptotecina combinada com dois potentes inibidores de autofagia (miR-15a e miR-16) demonstrou aumento da eficácia terapêutica da camptotecina (HUANG et al., 2015).

Estudos clínicos da camptotecina demonstraram propriedades desfavoráveis, dentre as quais baixa solubilidade em água, substancial toxicidade e rápida inativação via hidrólise da lactona (GOTTLIEB; LUCE, 1972). Devido a essas particularidades, topotecano e irinotecano derivados da camptotecina foram estudados e atualmente são utilizados na clínica. Em comparação à camptotecina, o topotecano contém uma cadeia lateral de amina terciária (C9) e um grupamento hidroxil (C10), aumentando sua hidrosolubilidade. O topotecano é usado no tratamento de câncer de ovário em estágio avançado, de câncer de pulmão de células pequenas sensíveis ao tratamento e tumores malignos na infância. Enquanto o irinotecano é

uma formulação de pró-fármaco do metabólito ativo SN-38 (7-etil-10-hidroxi-camptotecina) e clinicamente usado como tratamento padrão no câncer coloretal metastático (MIURA et al., 2015).

3.3 Podofilotoxina (Podofilox®)

Podofilotoxina é uma lignana bem conhecida extraída das raízes de *Podophyllum* sp. Linnaeus (família Berberidaceae), essa molécula pode ser semi-sintetizada comercialmente, sendo utilizada como agente quimioterápico contra vários tipos de câncer como carcinoma cervical, osteosarcoma, carcinoma de nasofaringe, câncer de cólon, de mama, de próstata e de células pequenas do pulmão (ONG et al., 2016; SATHISH et al., 2018; YIN; GUO; TAO, 2013).

Podofilotoxina e seus derivados como etoposide e teniposide são potentes agentes anticâncer, os quais atuam por diferentes mecanismos, dentre eles inibem a formação de microtúbulos, interagem com DNA e inibem a topoisomerase (YOU, 2005). A topoisomerase tem se destacado na terapia do câncer, uma vez que ela catalisa a quebra e reconstrução do fosfodiéster na estrutura do DNA durante o ciclo celular normal. Além disso, podofilotoxina apresenta potencial atividade contra células tumorais resistentes a fármacos (ZHANG et al., 2018).

3.4 Vimblastina e Vincristina

Vimblastina e vincristina são alcaloides extraídos da vinca *Catharanthus roseus* (Linnaeus) G.Don. (família Apocynaceae)*,* os quais têm sido amplamente utilizados nos últimos 50 anos no tratamento de diversos tipos de câncer. Dentre eles destacam-se linfoma de Hodgkin e não Hodgkin, câncer testicular, de ovário, de mama, de cabeça e pescoço, os quais são tratados com vimblastina, enquanto a leucemia linfoide da infância e linfoma de Hodgkin são tratados com vincristina (SABA; SEAL, 2018; SEARS; BOGER, 2015).

Esses alcaloides inibem a formação de microtúbulos e assim a mitose, sendo a base da atividade anticâncer (CODERCH; MORREALE; GAGO, 2012). Embora vimblastina seja também efetiva em matar os gliomas *in vitro*, ela demonstrou-se ineficaz na quimioterapia na clínica devido à restrição da barreira hematoencefálica. Uma solução para esse obstáculo seria a incorporação da molécula em lipossomos (MU et al., 2017).

3.5   Magnolol

O magnolol é um composto bifenílico hidroxilado isolado da planta chinesa *Magnolia officinalis* Rehder & E.H.Wilson (família Magnoliaceae), que apresenta atividade anticâncer através da indução da apoptose ou autofagia. Magnolol foi capaz de induzir morte celular da linhagem de carcinoma de pulmão (H460) por autofagia através do bloqueio da via PI3K/PTEN/Akt (HUA; SHANG; HU, 2017).

3.6   Resveratrol

O resveratrol é um polifenol encontrado abundantemente em uvas, nozes, amendoins e pinheiros. Os efeitos anticâncer do resveratrol e seus derivados têm sido intensivamente estudados e evidenciados os mecanismos biológicos alvos do resveratrol para o tratamento de diferentes tipos de câncer, dentre os quais câncer de pele, mama, mielóides e linfoides, ovário, cérvix, estômago, próstata, cólon, fígado, pâncreas e tireóide (AZIZ; AZIZ, 2018; HADI et al., 2010; KO et al., 2017; SINHA et al., 2016).

O resveratrol pode afetar os diferentes tipos de câncer desde estágios de iniciação até progressão tumoral, atuando por variadas vias de transdução de sinal que controlam crescimento celular e divisão, inflamação, apoptose, metástase e angiogênese. O resveratrol também pode ativar a morte celular por autofagia, reduzindo os níveis de Akt fosforilada e mTOR na célula (HUA; SHANG; HU, 2017). Pesquisas indicaram que o resveratrol é capaz de reverter a resistência multifármacos de células de câncer e quando combinadas com

fármacos utilizados na clínica, ele pode sensibilizar as células tumorais aos agentes quimioterápicos (FAROOQI; KHALID; AHMAD, 2018). A resposta ao tratamento com resveratrol foi altamente heterogêneo nas células-tronco de glioma, em que o resveratrol inibiu a proliferação celular, aumentou a mortalidade e reduziu a motilidade dessas células, modulando a via de sinalização Wnt (CILIBRASI et al., 2017).

3.7 Curcumina

A curcumina (diferuloilmetano) é um polifenol derivado da raiz do rizoma *Curcuma longa* Linnaeus (família Zingiberaceae), popularmente conhecida como açafrão, o qual tem sido consumido pelo ser humano por séculos como agente anti-inflamatório e antioxidante. Na última década, evidências mostraram que a curcumina é ativa contra uma variedade de câncer *in vitro* e *in vivo* como de pulmão, pele, cabeça e pescoço, oral, mama, gliomas, carcinoma hepatocelular, linfomas, leucemias e polipose adenomatosa familiar (PRASAD; TYAGI; AGGARWAL, 2014; ZANOTTO-FILHO et al., 2012).

A capacidade de interferir em vias que são superexpressas nas células tumorais, como NFκB, STAT3, Sonic Hedgehog e PI3K/Akt, contribui para a seletividade da curcumina em induzir morte celular nos tumores, enquanto resguarda os tecidos saudáveis. Estudos em gliomas demonstraram que a curcumina foi capaz de inibir eficientemente o seu crescimento induzindo morte celular por autofagia por meio da simultânea inibição da via de sinalização Akt/mTOR e estimulação da via ERK1/2 (ZANOTTO-FILHO et al., 2015).

4. Flavonoides

Os polifenóis são os compostos mais numerosos e amplamente distribuídos nas plantas, contendo um ou mais grupos fenóis na sua estrutura química, sendo bem conhecidos por suas propriedades antioxidantes. Os polifenóis são classificados em quatro grupos

principais: flavonoides, estilbenos, lignanas e ácidos fenólicos. Os flavonoides representam a maior classe de polifenóis, contêm dois ou mais anéis aromáticos na sua estrutura (Fig. 2) e de acordo com as suas substituições são classificados nas subclasses flavanols, isoflavonas, flavanonas, flavonols, flavonas e antocianidinas (Fig. 3) (BRODOWSKA, 2017).
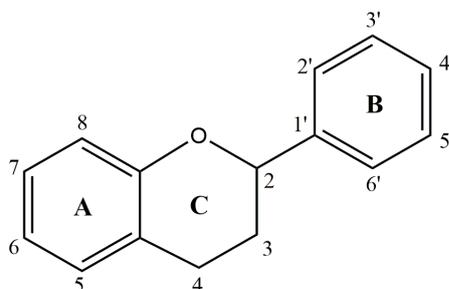

Figura 2. Estrutura química dos flavonoides e sistema de numeração.

Os flavonoides estão presentes em vegetais, legumes, frutas e folhas verdes de ervas. Mais de 4.000 flavonoides já foram identificados em plantas, sendo eles parcialmente responsáveis pela coloração das flores, frutas e folhas (HARBORNE; WILLIAMS, 2000). Os flavonoides da dieta apresentam inúmeras atividades biológicas, podendo atuar como agentes antioxidante, anti-inflamatório, antiviral, antiproliferativo, pro-apoptótico e recentemente tem se destacado na quimioterapia do câncer (HUSSAIN et al., 2016).

Os alvos moleculares dos flavonoides geralmente são proteínas quinases ativadas por mitógeno (MAPK), proteína quinase C (PKC), fosfatidilinositol 3-quinase (PI3K ou AKT), e vias da β-catenina, cujas atividades têm sido associadas com transformação maligna e promoção do tumor. Os flavonoides podem também interferir no fator nuclear kappa B (NFκB) e ativador de proteína-1 (AP-1), induzindo parada do ciclo celular e apoptose (ANGELONI et al., 2015).

Flavanol

Catequina

Epigalocatequina

Epigalocatequina-3-galato

Isoflavona

Flavanona

Flavona

Genisteína

Hesperetina

Luteolina

Flavonol

Antocianidina
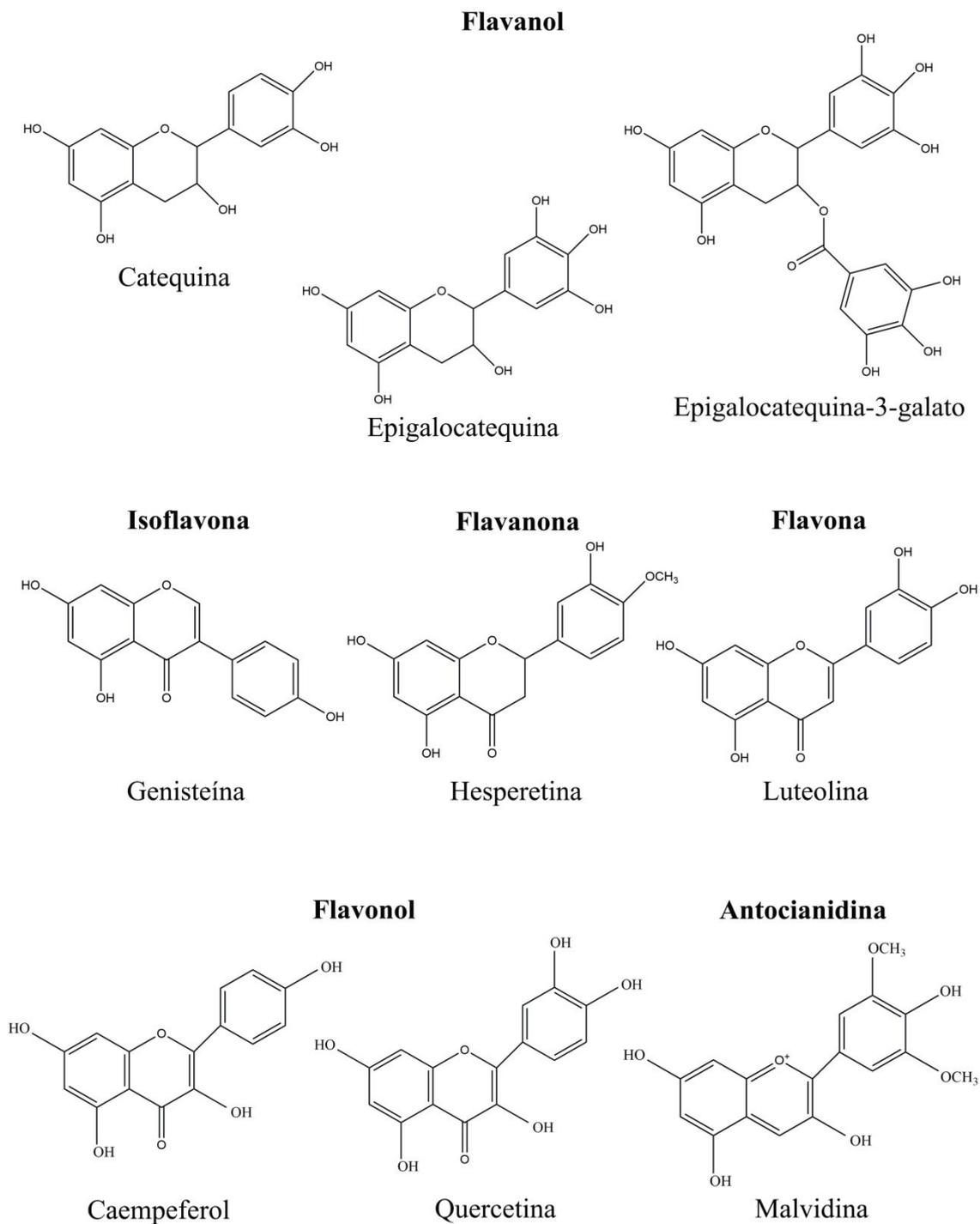
Caempeferol

Quercetina

Malvidina

Figura 3. Estruturas químicas dos representantes mais comuns das subclasses dos flavonoides (BRODOWSKA, 2017).

Quercetina (3, 3', 4', 5, 7-pentahidroxiflavona) é um flavonoide anticâncer bem estabelecido, que apresenta propriedade antiproliferativa contra diferentes linhagens tumorais e em modelos animais. Embora seu efeito de sensibilização aos quimioterápicos não tenha ainda sido analisado em condições clínicas, estudos pré-clínicos apoiam fortemente seu potencial como adjuvante na quimioterapia convencional, podendo aumentar potencialmente a resposta quimioterápica em muitos cânceres (HUSSAIN et al., 2016). Em linhagem de células humanas de câncer de mama (MCF-7), quercetina foi capaz de reverter a resistência ao quimioterápico doxorubicina através da *downregulation* da expressão da glicoproteína P e eliminando as células tronco do câncer pela translocação nuclear de YB-1 (LI et al., 2018).

Muitas flavonas, como acacetina, apigenina, crisina, diosmetina e luteolina, além de alguns flavonóis como campeferol e campeferide também já demonstraram alto potencial de reverter resistência multifármacos, com considerável segurança para ser utilizada na prática clínica (IMAI et al., 2004). Luteolina (3', 4', 5, 7-tetrahidroxiflavona) foi capaz de induzir apoptose nas células de leucemia humana (HL-60) por meio da ativação da via de sinalização JNK e da acetilação da histona H3 mediada pelas expressões de Fas/FasL (WANG et al., 2018). Galangina, luteolina e miricetina, flavonoides isolados da própolis, demonstraram atividade citotóxica e pró-apoptótica em linhagens celulares de câncer de cólon (HCT-116) e mama (MDA-MB-231) (VUKOVIC et al., 2018).

Genisteína (4', 5, 7-trihidroxiflavona ou 5, 7-dihidroxi-3-(4-hidroxifenil)-4H-cromen-4-ona) é uma das principais isoflavonas encontradas na soja. Muitos estudos tem demonstrado que genisteína inibe vários tipos de câncer como pancreático, do cólon, de tireóide, carcinoma hepatocelular, dentre outros, modulando diferentes vias de sinalização envolvidas na progressão do ciclo celular, apoptose, invasão, angiogênese e metástase (BI et al., 2018; OZTURK et al., 2018; SANAEI et al., 2018; ZHU; REN; TANG, 2018). Além disso, genisteína apresentou promissora atividade sensibilizando células de glioblastoma à

radioterapia, aumentando a eficácia do tratamento e inibindo a fosforilação de proteínas quinases dependentes de DNA (DNA-PKcs) e as vias responsáveis pelo reparo do DNA (LIU et al., 2018). Devido à sua potencial atividade anticâncer demonstrada por inúmeros estudos e/ou capacidade de sensibilizar tumores resistentes aos tratamentos convencionais, um análogo sintético (Fenoxodiol) está sendo comercializado como novo fármaco para tratamento do câncer (CARINI; KLAMT; BASSANI, 2014).

Outro flavonoide semi-sintético com potencial atividade anticâncer é o flavopiridol, uma flavona derivada da rohitukina, isolada das árvores indianas *Dysoxylum binectariferum* e *Amoora rohituka* (Meliaceae) (REN et al., 2003). Flavopiridol foi o primeiro inibidor quinase dependente de ciclina a ser testado em testes clínicos, devido às propriedades biológicas descritas em estudos *in vitro* e *in vivo* como parada do ciclo celular nas fases G1 e G2, citotoxicidade contra diversas linhagens tumorais em escala nanomolar, supressão do crescimento tumoral em animais e atividade sinérgica com fármacos anticâncer e radiação (BLAGOSKLONNY, 2004; MCINNES, 2008; REN et al., 2003). Apesar do flavopiridol ter sido ineficaz contra o câncer e apresentado elevada toxicidade aos pacientes nos testes clínicos de fases II (BLAGOSKLONNY, 2004; GUHA, 2012; MCINNES, 2008), 62 testes clínicos anticâncer estão em andamento com flavopiridol sozinho ou combinado com outros fármacos (UNITED STATE NATIONAL INSTITUTES OF HEALTH, 2018).

5. *Achyrocline satureoides*

Achyrocline satureioides (Lam.) DC. (Asteraceae), popularmente conhecida como "marcela" ou "macela", é uma erva anual aromática de tamanho médio, encontrada no Brasil, Paraguai, Uruguai e Argentina (GONZÁLEZ et al., 2015). No território brasileiro existem 25 espécies do gênero *Achyrocline* descritas (SALGUEIRO et al., 2016). *A. satureioides* é uma

planta medicinal amplamente empregada na medicina popular e reconhecida oficialmente pela Farmacopeia Brasileira (RETTA et al., 2012).

Na medicina popular infusões das inflorescências dessa espécie são utilizadas para tratar doenças do sistema nervoso central, respiratórias, gastrointestinais e desordens inflamatórias (BOLSON et al., 2015). Particularmente, existem inúmeros registros indicando que o chá preparado, principalmente pela população no sul do Brasil, das inflorescências de *A. satureioides* leva ao alívio de sintomas de úlcera gástrica e doenças inflamatórias do trato gastrointestinal, como a doença de Crohn (KADARIAN et al., 2002; TAYLOR, 2005).

Ensaios experimentais *in vivo* e *in vitro* tem confirmado a aplicação etnofarmacológica dos extratos obtidos da *A. satureioides*. Santin e colaboradores confirmaram o potencial antiúlcera gástrica dessa planta (SANTIN et al., 2010) e seu efeito antiespasmódico no músculo liso do trato gastrointestinal, além de ação analgésica e sedativa (SIMÕES et al., 1988). Da Silva e colaboradores demonstraram que o extrato hidroalcoólico das inflorescências *de A. satureioides* possui atividade anti-inflamatória no intestino, sendo capaz de reduzir a migração de neutrófilos e a ativação de macrófagos, consequentemente o dano oxidativo, e ajustar os níveis de citocinas pró- e anti-inflamatórias (DA SILVA et al., 2016). Estudo *in vitro* com células mononucleares do sangue periférico humano e leucócitos polimorfonucleares evidenciaram vários efeitos imunomodulatórios de extratos de *A. satureioides* (COSENTINO et al., 2008).

Estudos em camundongos demonstraram efeito hepatoprotetor de *A. satureioides*, sendo mediada pelas propriedades antioxidante da planta conforme já evidenciado em estudos *in vitro* (KADARIAN et al., 2002; POLYDORO et al., 2004). Devido à sua atividade antioxidante, *A. satureioides* também é capaz de conferir fotoproteção, apresentando potencial aplicação na área de cosmética (MORQUIO; RIVERA-MEGRET; DAJAS, 2005).

Casero e colaboradores comprovaram atividade antimicrobiana de compostos isolados de *A. satureioides* assim como de seus derivados semi-sintéticos diante de um quadro variado de bactérias gram-positivas e gram-negativas, além de leveduras (CASERO et al., 2015). Atividade antiviral de *A. satureioides* também foi reportada em infecções com herpes após aplicação tópica do extrato da planta (BIDONE et al., 2015). Estudo *in vitro* avaliou o potencial tripanocida de extratos de *A. satureioides* contra *Trypanosoma evansi*, sugerindo a utilização da planta como um tratamento alternativo (BALDISSERA et al., 2014). Além dessas atividades, estudos têm relatado propriedades anticâncer de *A. satureioides* em carcinoma hepatocelular (células Hep G2) e carcinoma epidermóide de orofaringe (células KB) (CARINI; KLAMT; BASSANI, 2014; MUNEHISA, 1994; RUFFA et al., 2002).

Investigações da composição química revelaram que o extrato obtido das inflorescências de *A. satureioides* é rico em flavonoides, predominando quercetina, 3-O-metil-quercetina, luteolina e achyrobichalcona, sendo a última molécula exclusiva dessa planta (CARINI et al., 2015; DE SOUZA; SCHAPOVAL; BASSANI, 2002; HOLZSCHUH et al., 2010). A presença de flavonoides nas inflorescências de *A. satureioides* é descrita como o componente responsável pelas propriedades biológicas dos extratos (RETTA et al., 2012). Assim, esse estudo busca avaliar a potencial aplicação dos flavonoides para terapia antiglioma com enfoque nos componentes presentes no extrato hidroetanólico de *A. satureioides*.

OBJETIVOS

1.   Objetivo Geral

- Verificar a atividade antitumoral do extrato hidroetanólico da planta medicinal *Achyrocline satureioides* assim como dos seus flavonoides isolados, avaliando tanto parâmetros antitumorais, de sinalização celular quanto a toxicidade sistêmica.

2.   Objetivos Específicos

- Determinar a composição do extrato hidroetanólico de *A. satureioides* por meio do CLAE (Cromatografia Líquida de Alta Eficiência);

- Testar a atividade antiproliferativa do extrato de *A. satureioides* e dos seus flavonoides isolados em modelos de cultivo celular de linhagens contendo as mutações mais frequentes dos gliomas (p53 e PTEN);

- Avaliar citotoxicidade do extrato de *A. satureioides* e dos seus flavonoides isolados nas culturas primárias de células sadias do cérebro (astrócitos, neurônios, co-cultura de astrócitos/neurônios e cultura organotípica de fatias do hipocampo);

- Testar possíveis alterações no ciclo celular das linhagens celulares de glioma;

- Elucidar possíveis vias de sinalização pelo qual os flavonoides inibem o crescimento dos gliomas, com foco nas principais vias alteradas no câncer;

- Avaliar a atividade antitumoral do extrato de *A. satureioides* em modelo animal de implantação de glioma;

- Investigar o efeito do tratamento com o quimioterápico TMZ associado ao extrato de *A. satureioides in vivo*;

- Analisar a potencial toxicidade sistêmica e alterações histopatológicas resultantes da administração do extrato de *A. satureioides in vivo*.

Capítulo I

Artigo aceito no periódico **"Toxicology in Vitro"**

Fator de Impacto: 2,866

Anticancer activity of flavonoids isolated from *Achyrocline satureioides* in gliomas cell lines

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ARTICLE INFO

ABSTRACT

Achyrocline satureioides, popularly known as "marcela", is a medicinal plant found in South America. This plant is rich in flavonoids, which have been reported to exert numerous biological activities. The aim of this study was to purify, identify and evaluate the mechanisms underlining anticancer activity of *A. satureioides* flavonoids in glioma cell lines (U87, U251 and C6) as well as their comparative toxicity in normal brain cells (primary astrocytes, neurons and organotypic hippocampal cultures). The main flavonoids present in *A. satureioides* are luteolin, quercetin, 3-*O*-methyl-quercetin and achyrobichalcone, the later a very unique metabolite present in this plant. Isolated flavonoids as well as *A. satureioides* extracts reduced proliferation and clonogenic survival, and induced apoptosis of glioma cell lines. In addition, *A. satureioides* flavonoids potentiated the cytotoxic effect and apoptosis induction by the glioma chemotherapeutic temozolomide (TMZ). Importantly, *A. satureioides* flavonoids were less cytotoxic to astrocytes, neuron:astrocytes co-cultures and hippocampal cultures if compared to gliomas. Investigation of 10 cancer-related pathways showed a reduced activation of MYC and the Map kinases ERK and JNK by *A. satureioides* flavonoid-enriched extract, an effect not observed when individual flavonoids were evaluated. Altogether, the herein presented results show that *A. satureioides* extract possesses a combination of flavonoids, some unique for this plant, which have synergistic anticancer activity and potential for further studies *in vivo*.

1. Introduction

Gliomas are the most common type of primary central nervous system tumors. The American Cancer Society reported an estimated 23,770 cases of brain and other nervous system cancers in 2016 (American Cancer Society, 2016). Gliomas are classified into four grades (I-IV) according to their molecular and histological features, with glioblastoma multiforme (GBM, grade IV) being the most aggressive, invasive and difficult-to-treat subtype (Louis et al., 2016). Therapeutic advances including combination of surgery and radiotherapy plus alkylation chemotherapy with temozolomide (TMZ) have only modestly increased the survival of glioma patients to a median 14.6 months (Rai et al., 2016). In this context, the lack of chemotherapeutic alternatives is still a major concern in the field.

Natural products are a major source of antitumoral agents. Of 175 small molecules approved for cancer treatment between 1940 and 2014, 49% were natural products or their derivatives. Plant-derived compounds including taxanes, vinca alkaloids, camptothecins and epipodophyllotoxins are still in the forefront of therapeutic protocols used to treat a variety of cancers (Newman and Cragg, 2016). Multifactorial properties of natural products can be advantageous to target-specific chemotherapeutic drugs, which usually result in resistance in cancer cells. Of particular interest are polyphenols. These are abundantly present in plants and show attractive properties such as structural complexity, chemical diversity, lack of substantial toxic effects, and biological activities, including antioxidant, anti-inflammatory, antimicrobial and anticancer (Chanput et al., 2016; Leclercq and Jacquot, 2014; Santos et al., 2016; Tsai et al., 2016; Xiao et al., 2016; Xiao and Lee, 2016; Ziaullah and Rupasinghe, 2015).

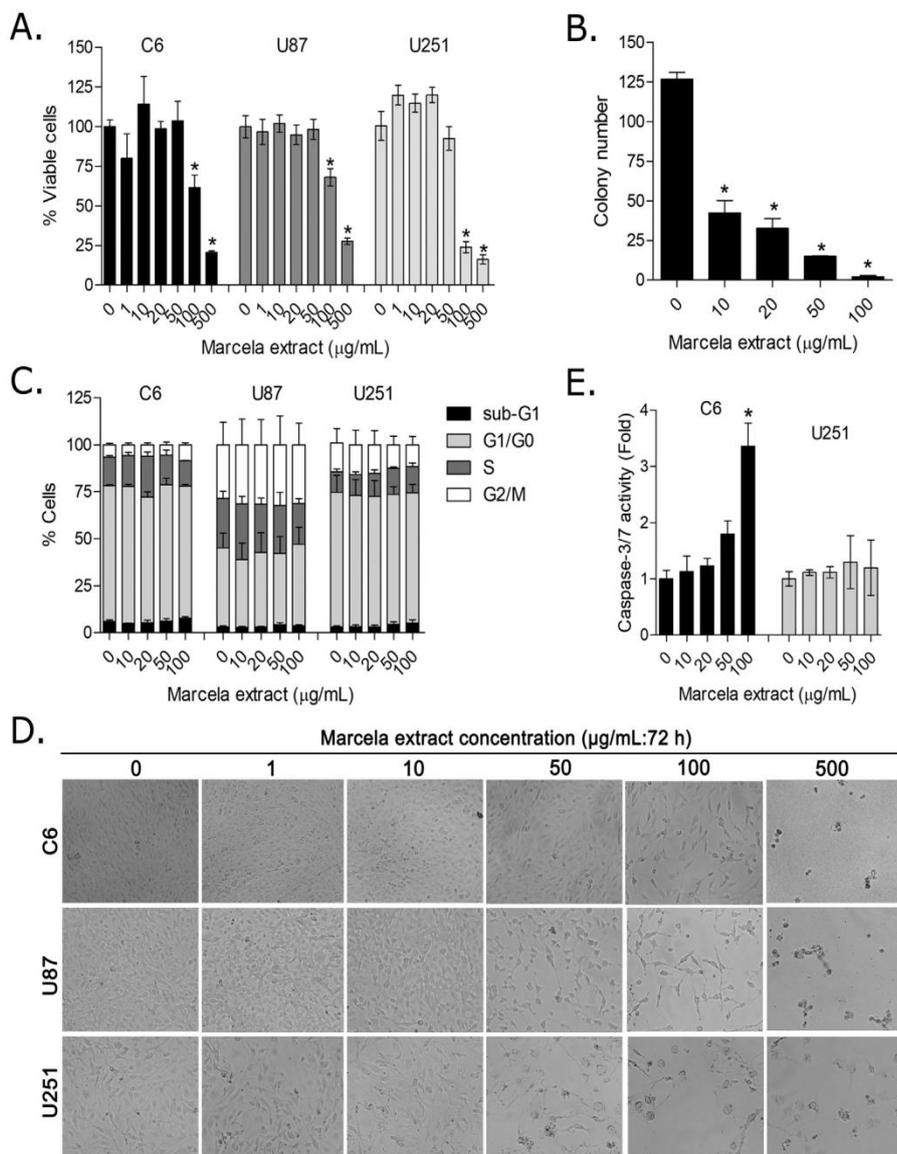*Achyrocline satureioides* (Lam.) DC, commonly known as "marcela"

Fig. 1. (A) Viability of C6, U87, and U251 glioma cell lines after 72 h treatment with *A. satureioides* (marcela) extract (μg/mL) as evaluated by SRB assay. (B) Long-term survival of U87 cells evaluated by clonogenic assay after 8 days. (C) Cell cycle in C6, U87 and U251 cells after 72 h treatment with marcela extract (10, 20, 50, 100 μg/mL). (D) Representative microphotographs of glioma cells after 72 h treatment with differing concentrations of marcela extract (10× magnification). (E) Caspase-3/7 activity in glioma cells after 72 h treatment with marcela extract. *Different from control ($n = 3$ in triplicate, $p < 0.05$, ANOVA).

or "macela", is a medicinal plant found in South America (Brazil, Argentina, Paraguay, Bolivia and Uruguay) (Retta et al., 2012). Traditionally, it has been used as a folk medicine for the treatment of respiratory diseases, inflammatory disorders, and central nervous system and gastrointestinal system disorders (Bolson et al., 2015). Several studies have reported its biological activities such as anti-inflammatory, hepatoprotective, antioxidant, immunomodulatory, antimicrobial, trypanocidal and photoprotective in different models (Baldissera et al., 2014; Balestrin et al., 2016; Carini et al., 2014; Casero et al., 2015; Cosentino et al., 2008; Ritter et al., 2017; Yamane et al., 2016; Zorzi et al., 2016). Its activities have been associated with the presence of high amounts of flavonoids; of which, luteolin, quercetin, 3-*O*-methyl-quercetin, and achyrobichalcone are the most abundant (Carini et al., 2015; Retta et al., 2012). Thus, the present study aimed to test whether *A. satureioides* extracts and its main constituent compounds exert antitumoral effects, cancer cell selectivity as well as to delineate some molecular mechanisms in *in vitro* models of glioma.

2. Material and methods

2.1. Chemicals and reagents

Dulbecco's modified eagle's medium - high glucose (DMEM), trypsin-EDTA, dimethyl sulfoxide (DMSO), propidium iodide (PI), trichloroacetic acid (TCA), sulforhodamine B, and TMZ were purchased from Sigma–Aldrich (St. Louis, MO, USA). Heat-inactivated fetal bovine serum (FBS) and neurobasal medium were obtained from Gibco (Life Technologies). All organic solvents used for High Performance Liquid Chromatography (HPLC) were HPLC grade. Quercetin (M.W. 302.24, purity ≥95%) and luteolin (M.W. 286.24, purity ≥98%) were
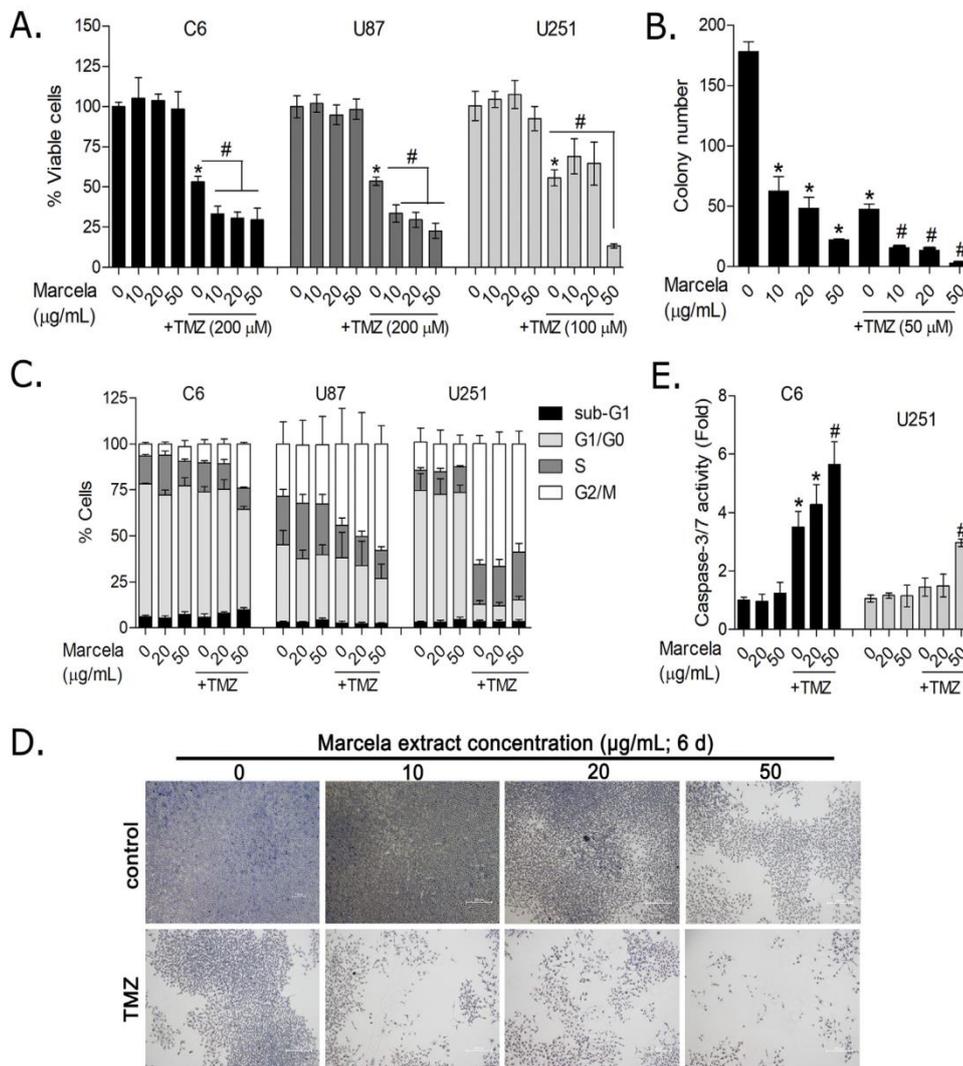
Fig. 2. (A) Cell viability, (B) Clonogenic survival, (C) Cell cycle analysis, (D) Morphological assessment and, (E) Caspase-3/7 activity of glioma cells treated with marcela flavonoid-enriched extracts combined with temozolomide (TMZ). A, C and E experiments were carried out after 72 h treatment. In B and D, U87 cells were treated for 8 and 6 days, respectively. Unless otherwise specified, TMZ concentrations were 200, 200 and 100 μM to C6, U87 and U251 cells, respectively. *Different from control; #different from marcela and TMZ alone at equivalent drug concentrations (n = 3 in triplicate, p < 0.05, ANOVA).

purchased from Sigma–Aldrich (St. Louis, MO, USA) and 3-*O*-methyl-quercetin (M.W. 316.27, purity ≥ 95%) from Extrasynthese (Lyon, France). Achyrobichalcone (M.W. 544.00) fraction was purified by preparative thin-layer chromatography and a crystallization process validated by our collaborators as previously described (Carini et al., 2015).

2.2. Plant material

Inflorescences of *Achyrocline satureioides* (Lam.) DC. (Asteraceae) were acquired from Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas of Universidade Estadual de Campinas/CPQBA-UNICAMP (São Paulo, Brazil, geographical coordinates 22S47'27.46″/47 W6'41.90). Plant samples were collected in April 2013, the flowering period, and inflorescences detached and dried at room temperature for seven days. Afterwards, dried inflorescences were stored at room temperature and protected from light. A voucher specimen was deposited (number 1882428) in the herbarium of Universidade Federal do Rio Grande do Sul (UFRGS).

2.3. Preparation of A. satureioides's hydroethanol extract

The hydroethanol extract of *A. satureioides* was prepared by maceration of dried inflorescences in 80% ethanol (7.5% w/v). The extraction time was eight days, and the extraction mixture was stirred once daily in a semi-industrial extraction tank at room temperature. The mixture was pressed, filtered, and the supernatant was evaporated (Büchi Rotaevaporator R-114). The obtained extract was frozen and dried in a freeze dryer (Edwards Modulyo) and the product was maintained at −20 °C until use.

2.4. Characterization and quantification of flavonoids from extract by HPLC

The dried extract was diluted in methanol and flavonoids present in it were characterized using High Performance Liquid Chromatography (HPLC). Quantification was carried out using two different methods as described in detail by Carini et al. (2013) for achyrobichalcone, and by Bidone et al. (2014) for the others main flavonoids (quercetin, luteolin,
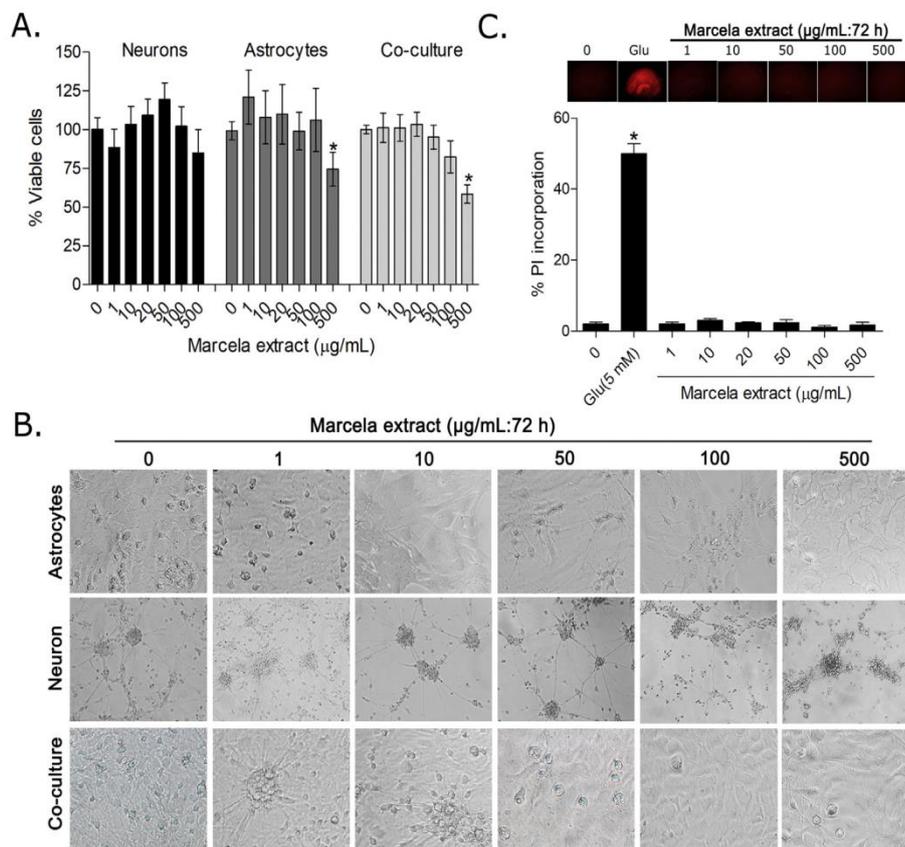
Fig. 3. (A) Cell proliferation by SRB assay and (B) microphotographs (10× magnification) of primary astrocytes, neurons, and their co-culture after 72 h treatment with marcela extracts. (C) Quantification of cell death in organotypic hippocampal cultures evaluated by fluorescence imaging analysis of PI uptake after 72 h treatment marcela extract. Representative microphotographs of hippocampal slices stained with PI are also shown. *Different from untreated controls (n = 3 in quadruplicate, p < 0.05, ANOVA).

and 3-*O*-methyl-quercetin). All samples were filtered using HVLP Millipore membrane (pore diameter 0.45 μm) before HPLC.

2.5. Glioma cells, primary astrocytes, neurons and co-cultures cultivation

Glioma cell lines C6, U87, and U251 were obtained from American Type Culture Collection (ATCC). All cells were cultured in DMEM supplemented with 10% FBS and 1× antibiotic/antimycotic solution (Sigma-Aldrich) at 37 °C in a humidified incubator with 5% CO_2 (Zanotto-Filho et al., 2012). Primary rat astrocytes were prepared from neonate Wistar rats (1–2 days) by mechanical tissue dissociation using calcium-magnesium-free-PBS (CMF-PBS), and were treated after 15 days. Primary rat neurons were isolated from embryonic stage (E18) and used for experiments after one week (Heimfarth et al., 2017). Preparations of astrocyte and neuron primary cultures were approved by the Ethical Committee for Animal Experimentation of Universidade Federal do Rio Grande do Sul (Protocol 27,562).

2.6. Organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures were prepared as described by Stoppini et al. (1991), with modifications (Valentim et al., 2003). Briefly, 400 μm-thick hippocampal slices were prepared from 6- to 8-day-old male Wistar rats using a McIlwain tissue chopper and were separated in ice-cold HBSS (pH 7.2). The slices were placed in culture inserts (Millicell® -CM, 0.4 μm, Millipore®, Bedford, MA, USA), which were transferred to a 6-well culture plate. The medium for slice culture

contained 50% MEM, 25% HBSS, and 25% horse serum, and was changed every three days. Experiments were carried out after 14 days. Quantification of cellular death in organotypic hippocampal cultures was carried out by fluorescence imaging analysis of PI uptake (Noraberg et al., 1999). Briefly, cultures were treated for 72 h with different concentrations of quercetin, 3-*O*-methyl-quercetin, luteolin, achyrobichalcone, and *A. satureioides* extract. Subsequently, 5 μM PI was added to the cultures and incubated for an additional 1 h. Cultures were observed with an inverted microscope (Nikon Eclipse TE300) using a standard rhodamine filter set. Images were captured and then analyzed using Scion Image software. The area of PI fluorescence was determined using the "density slice" option of Scion Image software and compared to the total slice hippocampal area to obtain the percentage of damage (Frozza et al., 2009). The results were analyzed in comparison with a negative control (cells without any treatment) and a positive control (cells treated with 5 mM glutamate for 24 h).

2.7. Cell viability

Sulforhodamine B assay (SRB) measurement of cellular protein biomass was used to estimate cell viability as previously described (Skehan et al., 1990). SRB absorbance was determined spectrophotometrically at 515 nm and results were expressed as percentage of viable cells relative to SRB incorporation in control/untreated cultures (assumed as 100% viable). Glioma cells were plated in 96-well plates (5 × 10³/well) at 70% confluence and treated. Astrocytes were plated in 96-well plates (3 × 10⁴/well) and treated after 14 days. Neurons
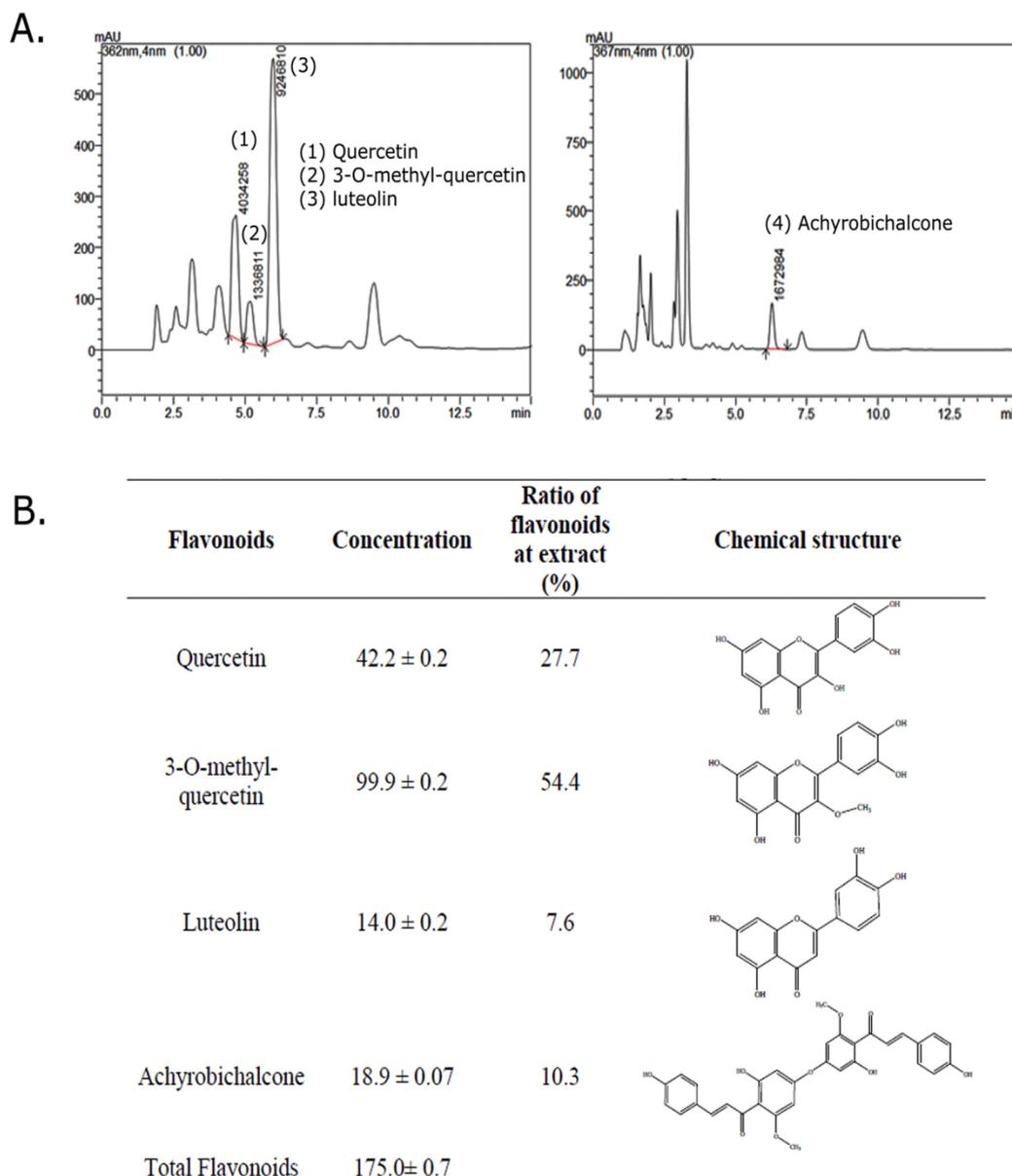
Fig. 4. (A) HPLC profiles and (B) quantification and relative abundance of quercetin, 3-*O*-methyl-quercetin, luteolin, and achyrobichalcone in *A. satureioides* extract.

were plated in 96-well plates (7.5×10^4/well) and treated after 7 days.

2.8. Cell cycle analysis

Glioma cells were plated in 12-well plates (5×10^4/well) to 70% confluence, and were treated with *A. satureioides* extract, TMZ, or isolated flavonoids for 72 h. For cell cycle analysis, the medium and cells were harvested and centrifuged at 1200 rpm for 6 min. Subsequently, the supernatant was discarded, cells were washed with PBS (10 mM), centrifuged, and suspended in 200 µL lysis buffer (Tris-HCl 0.5 mM (pH 7.6); trisodium citrate 3.5 mM; Nonidet-P40 0.1% (v/v); RNAse 100 µg/mL; PI 50 µg/mL). After 15 min, data were collected (10,000 events/sample) using a flow cytometer (BD FACS Calibur flow cytometer, BD Biosciences, CA, USA). FACS analyses were performed in the FLOWJO® software.

2.9. Clonogenic survival assays

Glioma cells (1×10^3) were plated in 24-well plates and treated for 8 days. Then, cells were fixed in paraformaldehyde for 15 min, stained with Giemsa for 20 min, and washed with PBS. Colonies were manually counted, ressuspended in DMSO and spectrophotometrically quantified at 560 and 630 nm.

2.10. Caspase-3/7 activity

Caspase-3 activity was assessed using Caspase-Glo 3/7 assay kit (Promega) following the manufacturer's instructions. The cells were seeded in 96-well plates and assayed after 72 h treatment. Luminescence data were expressed as fold relative to control/untreated cells.

Fig. 5. (A) Viability of C6, U87, and U251 glioma cell lines after 72 h treatment with differing concentrations of the flavonoids quercetin, 3-*O*-methyl-quercetin, lut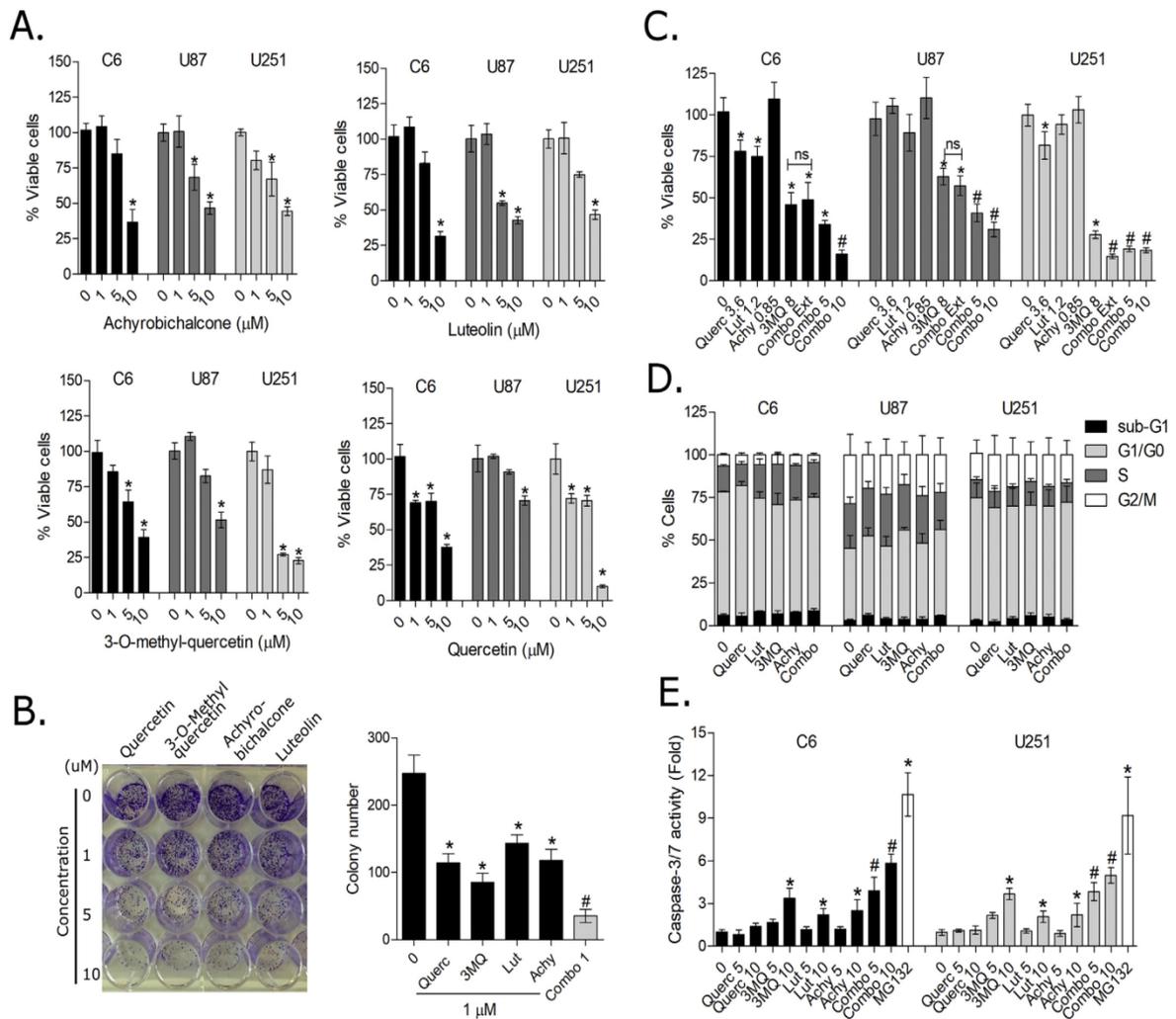eolin and achyrobichalcone as evaluated by SRB assay. (B) Long-term survival of U251 cells after 8 days treatment with flavonoids as evaluated by clonogenic assay. Representative photographs of colonies and quantification of the effect of isolated flavonoids alone or combined ("Combo" column) at 1 μM are shown. (C) Viability of glioma cells treated with isolated flavonoids at concentrations equivalent to those present in the marcela extracts. Flavonoids were incubated alone or combined as equivalent to marcela extract proportions ("Combo ext" column), or combined at 5 and 10 μM each compound. (D) Cell cycle in C6, U87, and U251 cells after 72 h treatment with isolated flavonoids alone (at IC50 levels; 5–10 μM) or combined at 5 μM (Combo). (E) Caspase-3/7 activity in C6 and U251 glioma cells after 72 h treatment with 5 and 10 μM isolated flavonoids or their combination. The proteasome inhibitor MG132 (5 μM) was used as a positive control. Legend: Querc (quercetin); 3MQ (3-*O*-methyl-quercetin); Achy (achyrobichalcone); Lut (luteolin) *Different from control; #different from control and from the respective flavonoid alone at equivalent concentrations (n = 3 in triplicate, $p < 0.05$, ANOVA).

2.11. Cignal finder reporter assay (pathway reporter gene)

Cancer 10-pathway Reporter Arrays from Qiagen provide screening of 10 signal transduction pathways that are critical regulators of cancer (*e.g.* Wnt, Notch, p53/DNA damage, cell cycle/pRB-E2F, TGFβ, NFκB, Myc, HIF-1, MAP/ERK, MAP/JNK). A Cancer 10-pathway Reporter Luciferase Kit (Qiagen) was used to investigate whether any classical cancer-related pathway was modulated by our treatments. The plate was prepared for transfection (0.2 μL P3000 + 0.25 μL lipofectamine in OptiMem medium) and left for 20 min to allow completion of reaction. U251 glioma cells (5 × 10³ cells/well) were seeded in OptiMem containing FBS without antibiotics and, after 24 h transfection, the cells were treated with *A. satureioides* extract (80 μg/mL, IC50 levels), achyrobichalcone (5 μM), 3-*O*-methyl-quercetin (5 μM), or luteolin (5 μM). Luciferase activity was quantified after 24 h using a liquid scintillation counter (Wallac 1409, Perkin–Elmer, Boston, MA, USA).

The change in the activity of each signaling pathway was determined by comparing the normalized luciferase activities (firefly/renilla) of the reporter in treated *versus* untreated wells.

2.12. Statistical analysis

The *in vitro* experiments were repeated at least three times in triplicates/quadruplicates. Results were expressed as mean ± standard deviation (SD) and analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by Tukey post-hoc test (Prism GraphPad® 7.0). Differences were considered significant at $p < 0.05$.
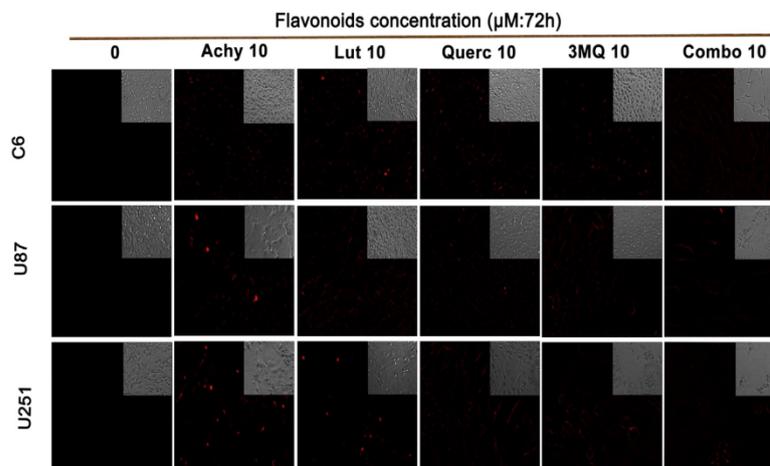
Fig. 6. Representative phase contrast and PI uptake microphotographs of glioma cells treated with flavonoids (10 μM) for 72 h (20× magnification, bar: 100 μm, n = 3 in triplicate). Legend: Querc (quercetin); 3MQ (3-*O*-methyl-quercetin); Achy (achyrobichalcone); Lut (luteolin); Combo (flavonoids combined).

3. Results

3.1. Effect of flavonoid-enriched fraction of A. satureioides extracts on proliferation of glioma cells

By using three different glioma cell lines harboring distinct mutational patterns (*i.e.*, U251$^{p53mut/PTEN\ mut}$, U87 ^{p53wtPTENnull}, and C6 $^{p53wt/PTENmut}$), we observed decreases in cell proliferation of all glioma cell lines at 100 and 500 μg/mL but not at lower concentrations of *A. satureioides* extracts after 72 h incubation (Fig. 1A). In long-term treatment, lower concentrations (10, 20 or 50 μg/mL) inhibited proliferation as evaluated in clonogenic assay (Fig. 1B). These antiproliferative effects were not accompanied by cell cycle arrest (Fig. 1C), even though the cell numbers decreased and cell morphology altered from 100 μg/mL in the three cell lines studied (Fig. 1D). Activation of the cell death pathway increased in C6 cells but not in U251 (the later is more resitant to apoptosis based on our previous data) as evaluated from effector caspase-3/7 activity determination (Fig. 1E). Additional data of *A. satureioides* extract effect in cell migration are given in Online Resource 1, reducing migration from 50 μg/mL (ESM 1).

3.2. Effects of A. satureioides flavonoid-enriched extracts upon TMZ toxicity in gliomas

Treatment with lower and non-cytotoxic concentrations *A. satureioides* flavonoids (at 10, 20 or 50 μg/mL) showed marked cell proliferation inhibitory activity when combined with TMZ (100–200 μM; IC50 72 h), increasing the effect of the chemotherapeutic in C6 and U87 cells whereas U251 cells were not sensitized by 10 and 20 μg/mL extracts in short-term SRB assays (Fig. 2A). Prolonged growth inhibition and additive effect with even lower concentration TMZ (50 μM) were obtained in all concentration in clonogenic assays (Fig. 2B) as well as cell culture morphology was evaluated (Fig. 2D). Cell cycle analysis showed that combination of TMZ with *A. satureioides* extracts (50 μg/mL) resulted in a small reduction of G1 cell population in C6 and U87 cells accompanied by increases in G2/M p, indicating a mitotic arrest at G2 phase. In U251 cells, which lack functional p53 and tend to accumulate in G2/M upon TMZ-induced DNA damage (Zanotto-Filho et al., 2015), TMZ caused G2 arrest, an effect not altered by *A. satureioides* extract (Fig. 2C). Caspase-3/7 activity showed that *A. satureioides* flavonoids combination with TMZ resulted in caspase-3/7 activity in C6 (at 20 and 50 μg/mL) and U251 (50 μg/mL); U87 was not evaluated for this parameter (Fig. 2E). These results suggest that *A. satureioides* flavonoids are more effective, with regard to pharmacological

concentrations used, when administered with TMZ-induced damage.

3.3. Cancer cell selectivity of A. satureioides extracts in central nervous system cell models

Next, we investigated whether *A. satureioides* extracts could affect the viability of non-neoplastic cells of the central nervous system. For this, primary rat astrocytes, neurons, or their co-cultures were treated with *A. satureioides* flavonoids for 72 h (Fig. 3). The extract showed no toxicity to neurons and mild cytotoxic effect was observed in astrocytes and astrocyte:neuron co-cultures (IC$_{50}$ > 500 μg/mL) if compared with its cytotoxic effect in gliomas (IC$_{50}$: 80.0 μg/mL in U251, 180.0 μg/mL in C6, and 220.0 μg/mL in U87). IC$_{50}$ in normal cells were not achieved due to difficulties in obtaining higher concentrations of the extract dissolved in culture medium. It is worthy to note that, at higher concentrations (100–500 μg/mL), proliferation of glioma cells lines was considerably reduced, caspase-3 was activated, and cells exhibited morphological changes typical of cell death (see Fig. 1), whereas astrocytes remained morphologicaly viable (Fig. 3B). This effect upon astrocytes likely occurs due to inhibition of a fraction of cells that keeps proliferative in culture (Dhandapani et al., 2007). In a more complex system consisted of organotypic hippocampal cultures, no cytoxicity was associated with *A. satureioides* extracts at the end of 72 h incubation; high level glutamate-induced cytotoxicity was used as a positive control (Fig. 3C).

3.4. Chemical characterization of A. satureioides extract flavonoids

The flavonoids present in *A. satureioides* extracts were characterized and quantified by HPLC (Fig. 4A). As shown in Fig. 4B, the majoraty flavonoid constituents of the extract were 3-*O*-methyl-quercetin (54.4%), quercetin (27.7%), luteolin (7.6%), which share significant structural similarities. In addition a very unique flavonoid, named achyrobichalcone was detected with approximately 10.3% abundance. These compounds accounted for a total 175 mg/g flavonoids present in our extracts.

3.5. Effect of A. satureioides purified flavonoids upon glioma cells proliferation

Because *A. satureioides* extract affected glioma cell proliferation, the main flavonoids present in the extract (quercetin, 3-*O*-methyl-quercetin, luteolin, and achyrobichalcone) were evaluated individually to determine whether antiglioma activity was due to a single molecule or
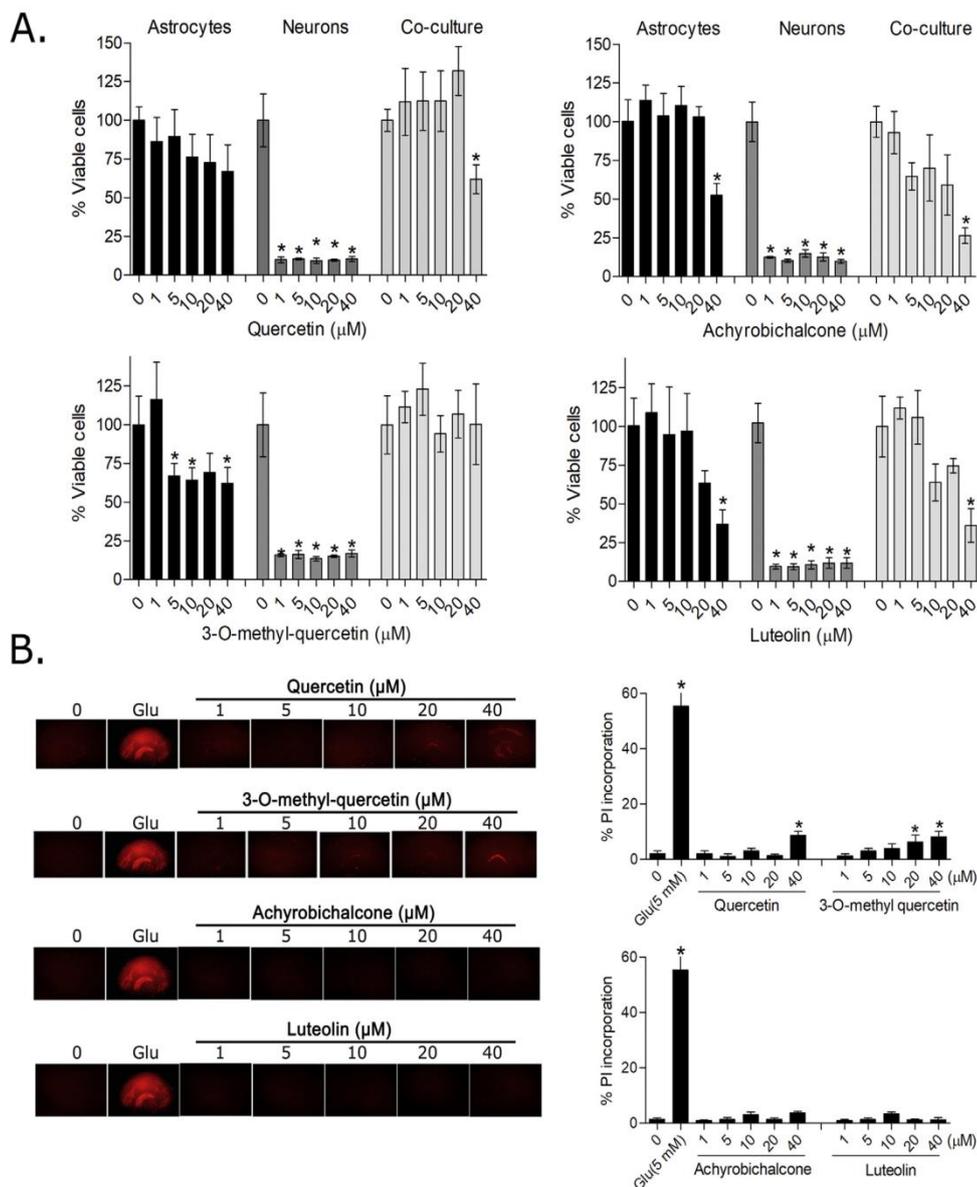
Fig. 7. (A) Cell viability of primary astrocytes, neurons, and co-culture after 72 h treatment with isolated flavonoids. (B) Quantification of cell death in organotypic hippocampal cultures evaluated by fluorescence imaging analysis of PI uptake after 72 h treatment with flavonoids. Representative microphotographs of hippocampal slices stained with PI are also shown. *Different from untreated controls (n = 3 in quadruplicate, p < 0.05, ANOVA).

synergistic. Treatment with the isolated flavonoids at 10 μM or lower concentrations resulted in decreased proliferation of the gliomas cells; IC_{50} values for the differing flavonoids for the three gliomas cells lines were around 5–10 μM (Fig. 5A, Fig. 6 and ESM 3). Clonogenic assays showed antiproliferative effects from 1 μM (Fig. 5B). We also evaluated various combinations of flavonoids at concentratios equivalent to those obtained in *A. satureioides* extracts at 50 μg/mL. We observed that 3-*O*-methyl-quercetin displayed the most significant impact on cell proliferation and its results alone did not differ from combined treatment with the four flavonoids at equivalent extract concentrations ("Combo ext" column) in C6 and U87 but not U251MG, indicating 3-*O*-methyl-quercetin is key for extract antiproliferative effect (Fig. 5C). Furthermore, treatment of glioma cells with combined flavonoids at 5 and 10 μM ("Combo 5 and Combo 10" columns) showed additive effects compared to either compound alone (Fig. 5B right panel and Fig. 5C).

While significant cell cycle changes were not observed (Fig. 5D), caspase-3/7 activity measurement corroborated the activation of apoptotic machinery at higher levels of flavonoids, which is potentiated in combined treatment with the four coumpounds identified herein (Fig. 5E). Additional data of flavonoids effect in cell migration are given in Online Resource 2, in which combined flavonoids inhibited migration (ESM 2).

3.6. Toxicity of isolated flavonoids in normal cell cultures

The effect isolated flavonoids on viability was also evaluated in normal cells. In astrocytes, treatment with flavonoid combinations mostly reduced cell proliferation at higher concentrations whereas neurons were very sensitive to isolated flavonoids, even at lower concentration (1 μM). On the other hand, neuron/astrocyte co-culturing seemed to protect neurons from toxicity (Fig. 7A). With a
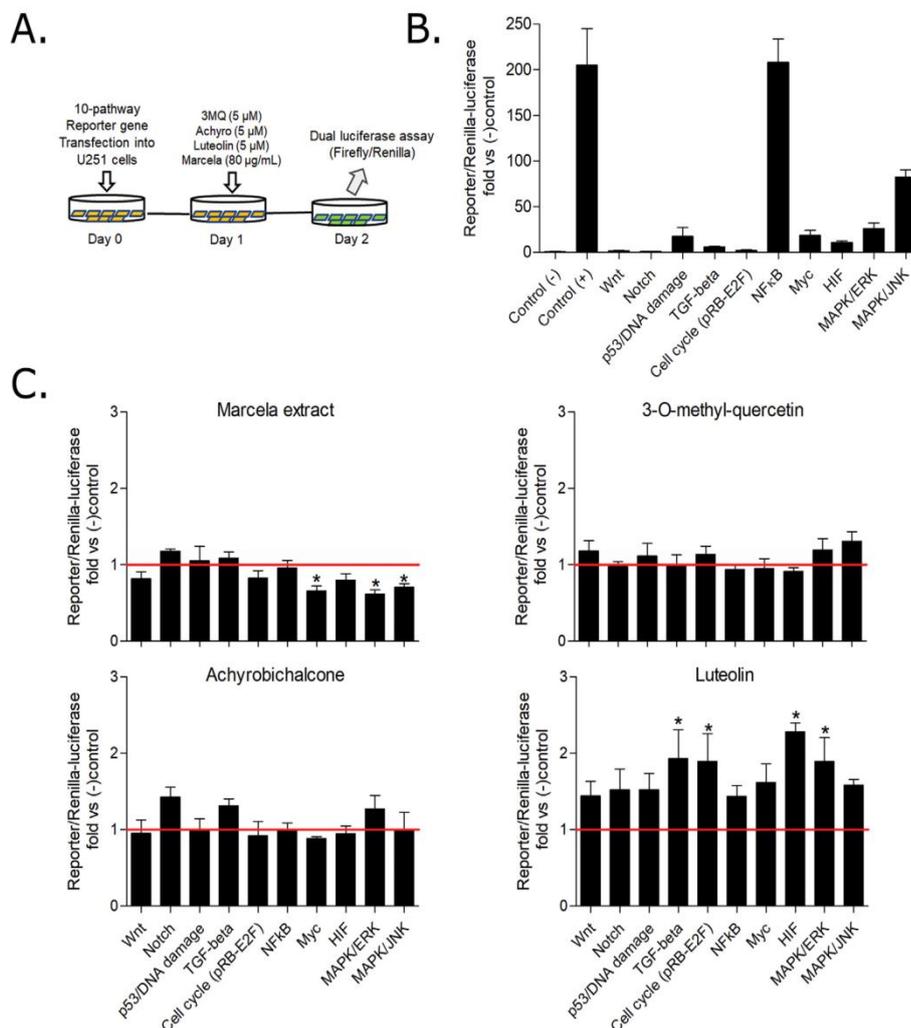
Fig. 8. (A) Schematic representation of reporter gene assays in U251 cells. (B) Relative activation of the 10 cancer related pathways as determined by comparison with positive and negative control constructs in U251 cells after 48 h transfection. (C) Relative activation of cancer signaling pathways after 24 h treatment with isolated flavonoids or marcela extract. Red baseline represents basal pathway activation in untreated cells. *Different from untreated controls (n = 3 in quadruplicate, p < 0.05, ANOVA). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

complimentary approach in organotypic cultures we observed no toxicity in achyrobichalcone and luteolin treatments whereas a low toxicity in hippocampal regions CA1 and dentate gyrus after treatment with high level quercetin and 3-*O*-methyl-quercetin was observed (Fig. 7B).

3.7. Potential mechanisms involved in A. satureioides extract and isolated flavonoids effects in gliomas

Next, U251 human glioma cells were transfected with different pathway reporter constructs for 10 important cancer-related pathways and treated for 24 h with *A. satureioides* extract (80 μg/mL) or isolated flavonoids before assessing luciferase activities (Fig. 8A). Firstly, we determined that the transcription factor NFkappaB followed by JNK and ERK map kinases, MYC and HIF1α are constitutively active in the cell line evaluated (Fig. 8B). While *A. satureioides* extract inhibited JNK, ERK and MYC reporters after 24 h treatment, 3-*O*-methyl-quercetin and achyrobichalcone had no impact upon none of the oncogenic pathways studied. Intriguingly, luteolin increased expression of TGFβ, pRB/E2F, MAPK-ERK and HIF1α (Fig. 8C).

4. Discussion

Flavonoids have been shown to exert a broad spectrum of health-beneficial biological activities, including antiproliferative effect in cancer cells (Tsai et al., 2016). We herein investigated the antiglioma activity of hydroethanol extract of *Achyrocline satureioides* (Marcela), a plant widely consumed in South America, as well as it major constitutents in order to identify potential active or prototypic agents for further drug developement. *A. satureioides* extract showed selectivity in reducing glioma cells proliferation and inducing apoptosis if compared to the normal central nervous system models tested; *i.e.* astrocytes, neurons and co-cultures/organotypic. *A. satureioides* IC50 to glioma cells was ~ 5-fold lower to gliomas compared to normal cells counterparts. When used in combination with TMZ, *A. satureioides* extracts potentiated the effects of TMZ at concentrations lower than those required to cause toxicity alone, indicating that low concentration of extracts contain compounds that affect cell resistance to DNA damage. Clonogenic assays confirmed the long-term effects of low concentrations *A. satureioides* extracts. Of note, U251 glioma cells showed more sensitive to *A. satureioides* extracts (with/out TMZ) even though they showed more resitant to activate caspase-3/7 if compared to C6 and

U87. This different sensitivity profile of gliomas is likely attributed to different mutational patterns among gliomas cell lines which require further investigation.

We next investigated the compounds involved in toxicity of *A. satureioides* extracts. Four flavonoids were isolated and showed antiproliferative and pro-apoptotic effects at an IC50 of ~ 10 µM. Treatment of cells with flavonoid combinations (1 µM in clonogenic and 5 µM in cell viability/caspase-3/7 assays) indicated these compounds may act through additive effects in gliomas. On the other hand, when emulating the combination of flavonoids present in *A. satureioides* extracts (Combo ext. treatments), the toxicity of the quercetin metabolite 3-*O*-methyl-quercetin (3MQ) (Kumar et al., 2016; Okoko and Oruambo, 2009) alone was similar to *A. satureioides* extracts likely because it is the major flavonoid constitutent. The antiglioma effects of quercetin and luteolin have been described in the context of gliomas (Chakrabarti and Ray, 2016; Zamin et al., 2014). For instance, 20 uM luteolin inhibited proliferation and caused apoptosis in U87 and T98G cells (Chakrabarti and Ray, 2016). Quercetin antiglioma effects have been well-described *in vitro*, while *in vivo* data in ortothopic C6 cells implants showed pro-glioma effects (Zamin et al., 2014). In contrast, various studies have shown that quercetin co-delivered with temozolomide led to an improved anticancer effect (Barbarisi et al., 2017; Pozsgai et al., 2013; Sang et al., 2014; Wang et al., 2016). Thus, quercetin data need to be interpreted with cautious. 3MQ and achyrobichalcone effects in gliomas are not reported to the best of our knowledge. Noteworthy, achybichalcone, an exclusive and recently discovered molecule from our plant, induced growth inhibition and caspase activation in the glioma cell lines tested. Although achyrobichalcone is a very novel compound, its effect corroborates with the broad spectrum of biological activities related to flavonoids including anticancer (Chakrabarti and Ray, 2016; Kim et al., 2013; Lamy et al., 2015; Lou et al., 2016; Mielcke et al., 2017; Pan et al., 2015; Sang et al., 2014; Wang et al., 2017; Zamin et al., 2014).

With regard to cancer cells selectivity, treatment with flavonoids alone reduced growth of neurons at low concentrations as low as 1 µM. This effect was not observed in astrocytes, astrocyte:neuron co-cultures and hippocampal slices, probably because astrocytes are well known to protect neurons that are usually more susceptible to the treatment with xenobiotics. In a previous study, Costa et al. (2016) reported the protective effect of astrocytes and microglia upon neuronal progenitor cells, neuronal differentiation, and neuritogenesis induced by flavonoids, which is in agreement with our results. While more toxic to glioma cells, 3MQ also showed more toxicity to hippocampal slices, even though it was small in magnitude and occurred only at concentrations equal or higher 20 µM.

We also investigated the impact of extracts and isolated flavonoids upon some key pathways tipically activated in cancer cells. *A. satureioides* extract was able to reduce MYC and map kinases (ERK and JNK), which showed constitutive activation in the U251 cells. On the other hand, luteolin increased pathways such as TGF-beta, pRB-E2F, HIF and ERK which, intringuily, are frequently related to a more aggressive cancer cell phenotypes by promoting a favorable tumor microenvironment. Quercetin and 3MQ had no effect upon these pathways. These data show that combined effect of *A. satureioides* flavonoids, likely in combination with other compounds present in the extracts, is important to achieve inhibition of cancer cell survival pathways. The influence of polyphenols and flavonoids on gene expression through epigenetic modifications has been reported by Abdul and collaborators (Abdul et al., 2017). Quercetin was able to suppresses human esophageal cancer cell growth by altering DNA methylation, histone acetylation and NF-κB signaling (Zheng et al., 2014). Luteolin inhibited hyperglycemic condition-induced cytokine production in monocytes through epigenetic changes mediated by histone acetylatilases/deacetylases modulation (Kim and Yun, 2017). Moreover, additional pathways not identified herein are likely involved in the effect of individual flavonoids which require further investigation in order to clarify their antiglioma mechanisms.

5. Conclusion

A. satureioides flavonoid-enriched extract showed antiproliferative activity alone as well as in combination with TMZ *in vitro*, potentiating the chemotherapeutic effect of TMZ. The antiglioma activity was also obtained when glioma cells were treated with isolated flavonoids, suggesting that achyrobichalcone, luteolin, 3-*O*-methyl-quercetin, and quercetin have antitumoral activity *per se*. Overall, our results demonstrate that *A. satureioides* extract and its isolated flavonoids are promising for the treatment of glial tumors, although more studies are necessary to explore the molecular mechanisms involved in their antiproliferative effect *in vitro* as well as efficacy *in vivo*.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tiv.2018.04.013.
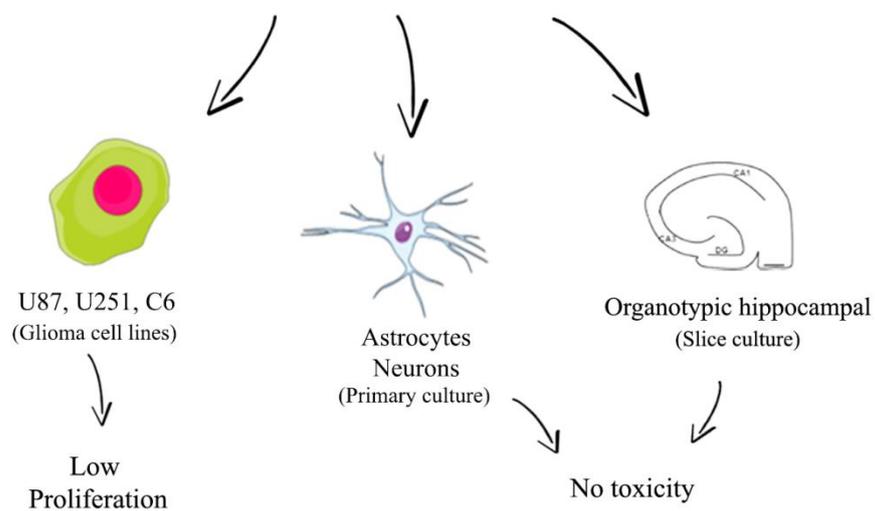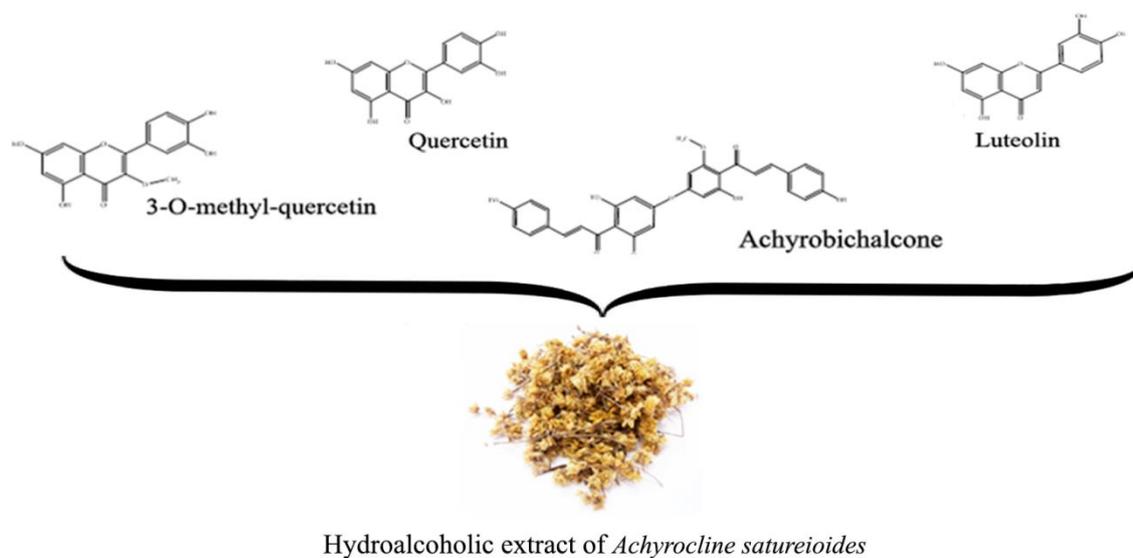
Conflicts of interest

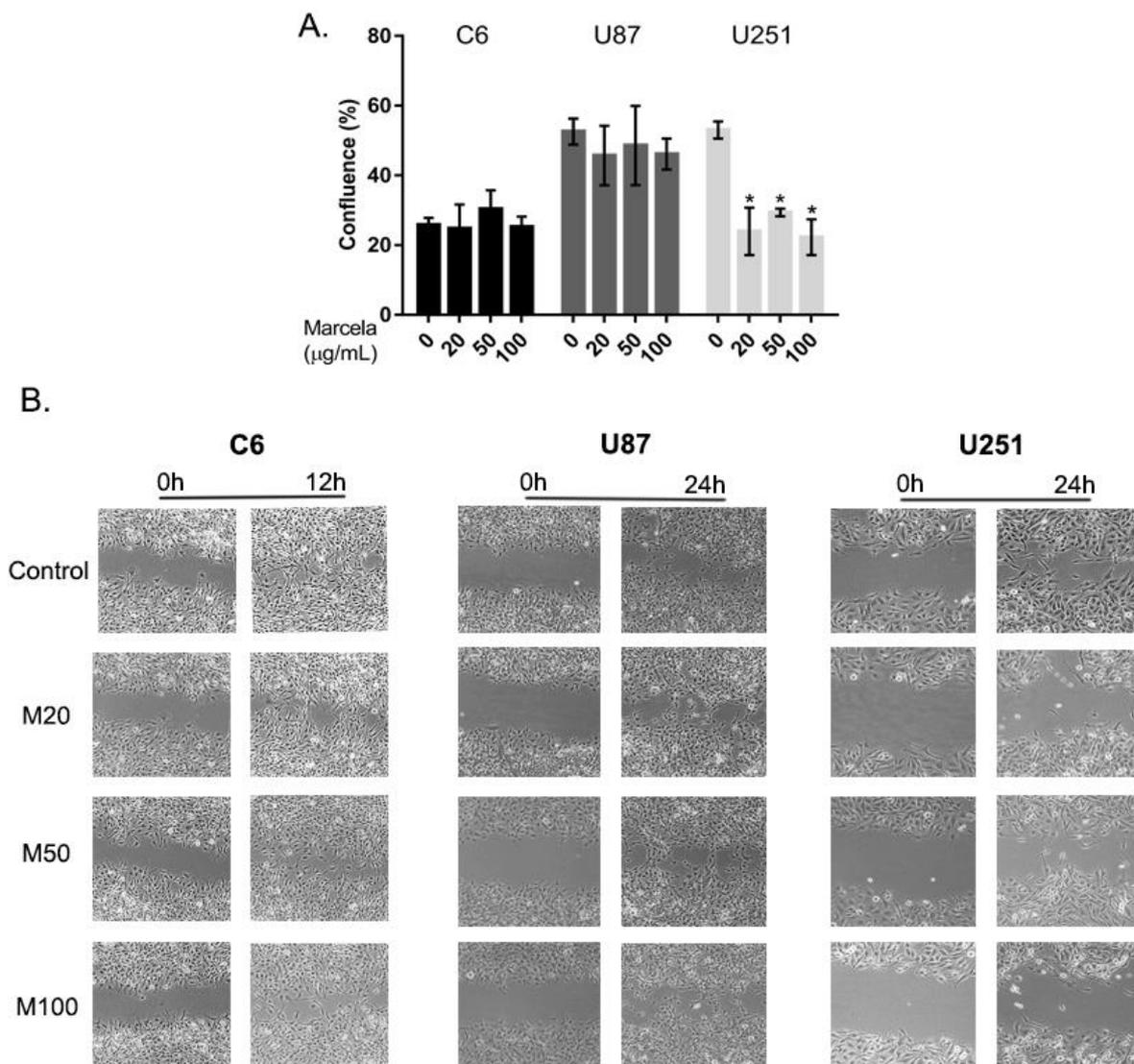The authors declare that there are no conflict of interests.

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3-O-methyl-quercetin

Quercetin

Achyrobichalcone

Luteolin

Hydroalcoholic extract of *Achyrocline satureioides*

U87, U251, C6
(Glioma cell lines)

Astrocytes
Neurons
(Primary culture)

Organotypic hippocampal
(Slice culture)

Low
Proliferation

No toxicity

Graphical Abstract

ESM 1 (A) Cell migration of C6, U87, and U251 treated with marcela extract (μg/mL) after 12 h and 24 h by scratch assay. (B) Representative photos (10x magnification) illustrating the effects of treatment. *Different from control (n = 3 in triplicate, $p < 0.05$, ANOVA)
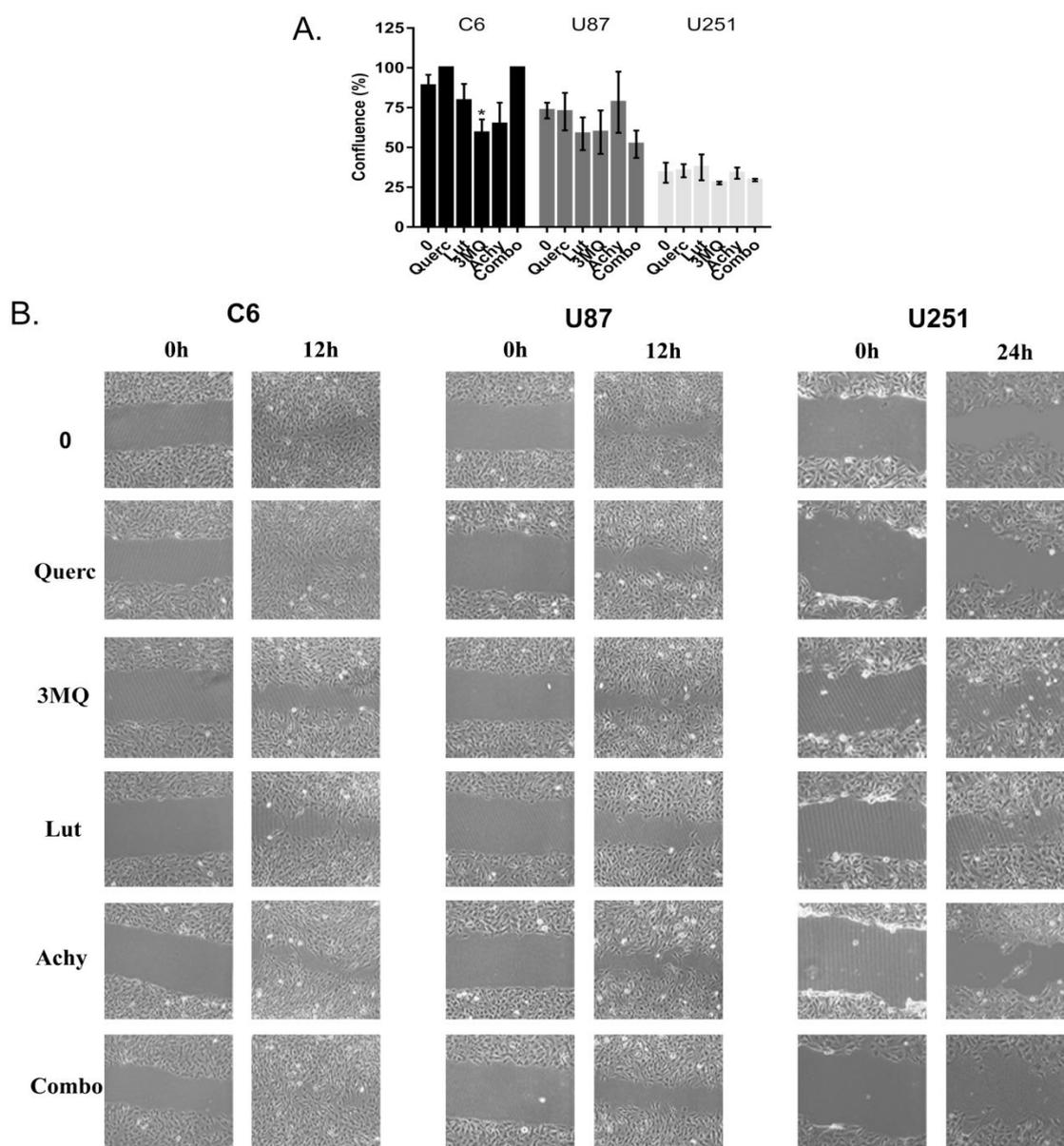
ESM 2 (A) Effect of isolated flavonoids quercetin, 3-O-methyl-quercetin, luteolin, achyrobichalcone (at IC50 levels; 5-10 μM) or combined at 1-5 μM (Combo) in cell migration of C6, U87, and U251 after 12 h and 24 h by scratch assay. (B) Representative photos (10x magnification) illustrating the effects of treatment (n=3). Legend: Querc (quercetin); 3MQ (3-O-methyl-quercetin); Achy (achyrobichalcone); Lut (luteolin). *Different from control (n = 3 in triplicate, $p < 0.05$, ANOVA)
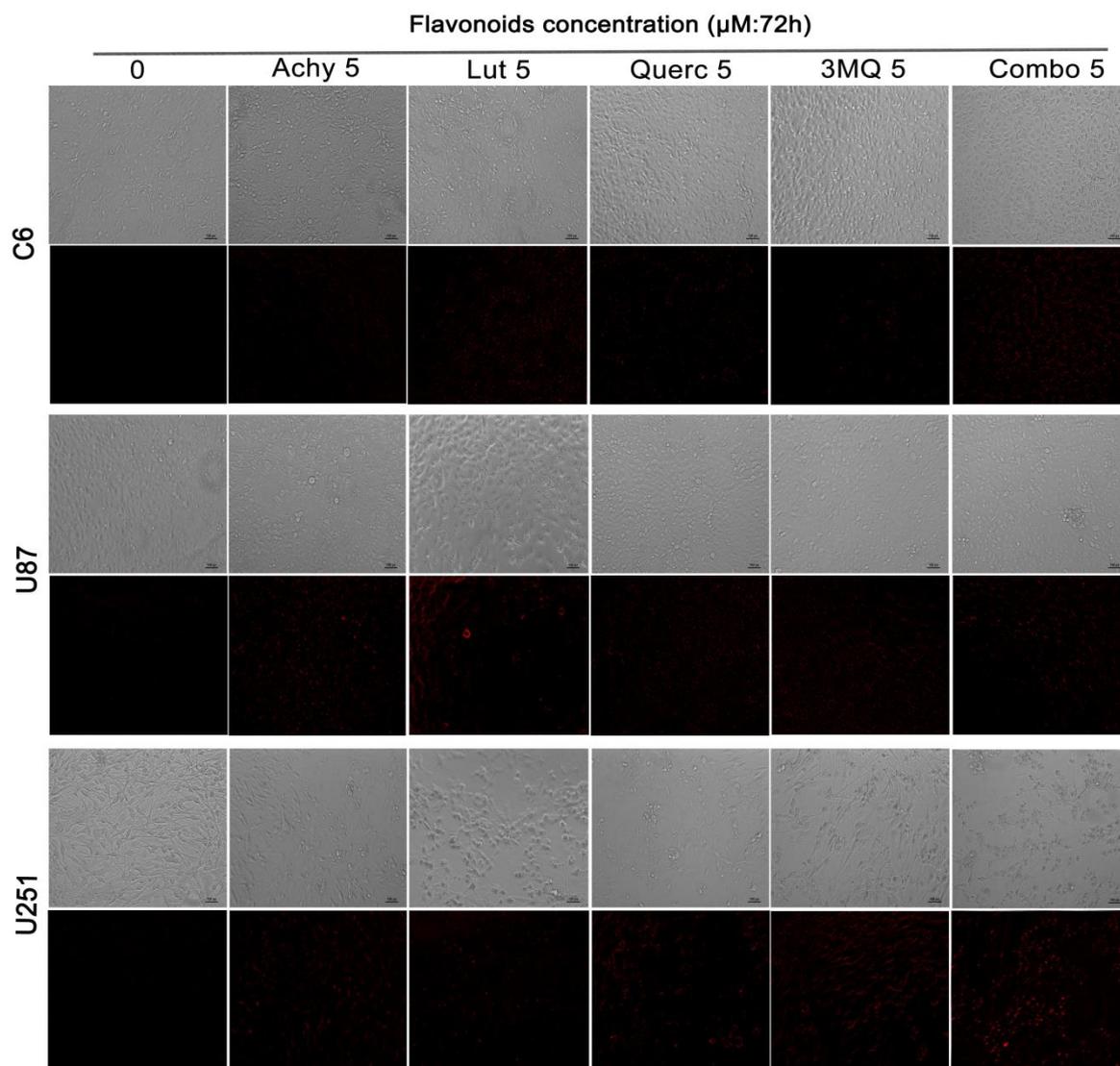
ESM 3 Representative phase contrast and PI uptake microphotographs of glioma cells treated with flavonoids (5μM) after 72 h (20× magnification, bar: 100 μm). Legend: Querc (quercetin); 3MQ (3-O-methyl-quercetin); Achy (achyrobichalcone); Lut (luteolin); Combo (flavonoids combined).

DISCUSSÃO

Vários estudos têm relatado inúmeras atividades biológicas relacionadas aos flavonoides. A planta medicinal *Achyrocline satureioides*, tradicionalmente utilizada pela população da América Latina, apresenta composição rica nos flavonoides quercetina, 3-O-metil-quercetina, luteolina e achyrobichalcona, o que justificaria a diversidade de atividades biológicas associadas a essa planta.

Quercetina tem apresentado atividades anti-aterosclerose, antialérgica, antibacteriana, anticâncer, anti-hipercolesterolêmica, anti-hipertensiva, anti-inflamatória, antiobesidade, antiúlcera, antiviral, neuroprotetora e vasodilatadora (ANAND DAVID; ARULMOLI; PARASURAMAN, 2016). Enquanto 3-O-metil-quercetina é um metabólito da quercetina, geralmente produzido após a ingestão de alimentos ricos em flavonoides, o qual tem um grupo catecol no anel B da quercetina. Alguns estudos indicaram que modificações na sua estrutura química alteram suas atividades biológicas, dentre as quais já foram relatadas ação antioxidante, citoprotetora, imunomodulatória e antiviral (FORMICA; REGELSON, 1995; KUMAR et al., 2016; OKOKO; ORUAMBO, 2009).

Luteolina também tem apresentado atividades anti-aterosclerótica, anticâncer, anti-inflamatória, antioxidante e nefroprotetora (DOMITROVIĆ et al., 2013; LEE et al., 2017; ZHANG et al., 2016). Já a achyrobichalcona, molécula recentemente descoberta nessa planta (HOLZSCHUH et al., 2010), apresenta similaridade estrutural com outras bichalconas com propriedades anticâncer (MDEE; YEBOAH; ABEGAZ, 2003), o que indica uma das potenciais atividades a serem avaliadas. Considerando esse amplo espectro de atividades biológicas dos flavonoides presentes na *A. satureioides*, particularmente contra a proliferação do câncer, nesse estudo nós investigamos a atividade antiglioma do extrato hidroetanólico

dessa planta tanto em modelos de glioma *in vitro* quanto *in vivo*, assim como dos seus flavonoides isolados a fim de identificar a molécula potencialmente ativa.

Considerando a ampla variabilidade genética e morfológica do glioblastoma multiforme, foram utilizadas três linhagens celulares que apresentam diferentes padrões de mutação U251$^{p53mut/PTENmut}$, U87 $^{p53wt/PTENnull}$ e C6 $^{p53wt/PTENmut}$. O extrato hidroetanólico de *A. satureioides* reduziu a proliferação das células de glioma em elevadas concentrações (a partir de 100 µg/mL) após 72 horas de tratamento. Ao serem avaliados os efeitos do tratamento em diferentes modelos de células sadias do sistema nervoso central (astrócitos, neurônios, co-cultura de astrócitos/neurônios e cultura organotípica de fatias do hipocampo) observa-se seletividade do extrato hidroetanólico visto que apresentaram um IC50 aproximadamente cinco vezes maior comparado aos gliomas.

Uma vez que as células sadias do sistema nervoso central não foram afetadas significativamente com o extrato, nós focamos na menor concentração efetiva para as análises. Dessa forma, na curva de sobrevivência em longo prazo, obtida por meio do ensaio clonogênico, observamos que 50 µg/mL do extrato hidroetanólico de *A. satureioides* foi capaz de reduzir consideravelmente a proliferação celular. Além disso, quando utilizado em combinação com temozolomida, o extrato de *A. satureioides* foi capaz de potencializar o efeito do quimioterápico, inclusive em concentrações menores do que aquelas necessárias para ter efeito citotóxico sozinho, indicando assim que baixas concentrações do extrato apresentam compostos que afetam a resistência celular ao dano ao DNA. Deve ser considerado também que a linhagem U251 apresentou maior sensibilidade ao tratamento com o extrato hidroetanólico, inclusive associado à temozolomida, apesar de demonstrar maior resistência à ativação da caspase 3/7 comparada à C6 e U87. Esse diferente perfil de

sensibilidade da linhagem está associada aos diferentes padrões de mutação dessas células que devem ser mais estudadas.

O perfil do ciclo celular é influenciado por muitos fatores como ciclinas, quinases, mutações gênicas, dentre outros reguladores do ciclo celular. Temozolomida apresenta um perfil característico de induzir a parada do ciclo celular na fase G2/M nas linhagens celulares humanas de glioma, entretanto isso não foi observado na linhagem de glioma de rato, provavelmente tal efeito seja relacionado com as diferentes mutações dessas células. Apesar disso, uma significativa alteração do perfil do ciclo celular foi observada na combinação do extrato de *A. satureioides* (50 µg/mL) e temozolomida, em que a combinação aumentou a parada na fase G2/M (C6 e U87), enquanto houve uma pequena tentantiva de reversão na linhagem U251. A parada do ciclo celular em G2/M pela temozolomida é mantida por um longo tempo por influência de p53 e p21$^{\text{Waf1/Cip1}}$, apesar deles não serem tão importantes para iniciar a parada como constatado na fase G1/G0 (HIROSE; BERGER; PIEPER, 2001). Segundo esse mecanismo que ocorre nas células de glioma, o efeito de reversão do extrato de *A. satureioides* em U251 poderia estar relacionado com a característica dessa linhagem celular deficiente em p53, induzindo uma parada transitória de G2/M e, assim, o extrato hidroetanólico poderia induzir uma parada no ponto G1, antes da duplicação do DNA danificado. Seguindo esse mesmo parâmetro, as células C6 e U87 que são linhagens p53 selvagens apresentam uma parada em G2/M mais intensa na combinação do extrato de *A. satureioides* e temozolomida.

Quando avaliados os flavonoides de *A. satureioides* separadamente, eles tiveram um significativo efeito inibindo a proliferação dos gliomas em doses menores que 10 µM. Assim como observado anteriormente com o extrato hidroetanólico, a linhagem U251 apresentou maior sensibilidade ao tratamento com os flavonoides. O tratamento dos gliomas com a

combinação dos flavonoides (1 μM no ensaio clonogênico e 5 μM nos ensaios de viabilidade e da caspase 3/7) indicam que esses compostos podem atuar por meio de efeitos aditivos, potencializando a resposta individualizada dos flavonoides nas células.

A análise da combinação dos flavonoides presentes no extrato de *A. satureioides* (tratamento Combo ext), evidencia toxicidade individual da molécula 3-O-metil-quercetina, a qual tem sido explorada por alguns autores e sua atividade poderia estar correlacionada à sua metilação na posição 3 (KUMAR et al., 2016; OKOKO; ORUAMBO, 2009). Além disso, a toxicidade dessa molécula foi similar ao extrato de *A. satureioides*, o que poderia ser diretamente associado ao fato de 3-O-metil-quercetina ser o flavonoide predominante no extrato. Estudos tem demonstrado atividade antitumoral da luteolina e quercetina no contexto de gliomas (CHAKRABARTI; RAY, 2016; ZAMIN et al., 2014). Luteolina na concentração de 20 μM foi capaz de inibir a proliferação e induzir apoptose nas linhagens de glioma U87 e T98G (CHAKRABARTI; RAY, 2016). O efeito *in vitro* antiglioma da quercetina tem sido bem descrito, entretanto dados em modelo ortotópico de implante de células C6 *in vivo* demonstraram efeito pró-glioma (ZAMIN et al., 2014). Porém, outros estudos evidenciaram que a quercetina co-administrada com a temozolomida potencializa o efeito anticâncer (BARBARISI et al., 2017; POZSGAI et al., 2013; SANG; LI; LAN, 2014; WANG et al., 2016). Considerando esses dados, os efeitos da quercetina devem ser analisados com cautela.

A atividade de 3-O-metil-quercetina e achyrobichalcona em gliomas até o momento não haviam sido relatadas. Em nosso estudo demonstramos que a achyrobichalcona induziu inibição do crescimento e ativação da caspase nas linhagens celulares de glioma avaliadas. Apesar da achyrobichalcona ser uma molécula nova, seu efeito confirma o amplo espectro de atividades biológicas relatadas para os flavonoides, especialmente anticâncer.

Entretanto, quando os flavonoides isolados foram avaliados nas células sadias do sistema nervoso central, concentrações baixas, inclusive 1 µM, reduziram o crescimento dos neurônios. Esse efeito não foi observado nos astrócitos, co-cultura de astrócitos/neurônios e na cultura organotípica das fatias do hipocampo, provavelmente porque os astrócitos são bem conhecidos por atuarem como protetores das células neuronais, as quais costumam ser mais sensíveis a tratamentos com xenobióticos. Esse efeito protetor dos astrócitos e micróglia em relação às células progenitoras neuronais, diferenciação neuronal e neuritogênese induzido pelos flavonoides foi verificado previamente (COSTA et al., 2016), corroborando nossos resultados. Assim como observado no tratamento com os gliomas, 3-O-metil-quercetina foi o flavonóide que demonstrou maior toxicidade para as fatias do hipocampo, apesar de ter sido em menor magnitude e ter ocorrido em concentração igual ou superior a 20 µM.

Em paralelo, nós verificamos o efeito do extrato de *A. satureioides* e dos flavonoides na migração celular dos gliomas. O extrato hidroetanólico diminui a migração celular com maior eficácia em U251 após 24 horas de tratamento, corroborando os dados de maior sensibilidade dessa linhagem nos testes de viabilidade. Ao avaliar os flavonoides isolados, 3-O-metil-quercetina afetou significativamente a migração de C6, apresentando efeitos mais sutis nas outras linhagens celulares de glioma, apesar dos resultados nas demais células não serem estatisticamente significativos, o efeito observado com o extrato de *A. satureioides* poderia ser associado com a predominância desse flavonoide, conforme discutido anteriormente.

Nós também investigamos o impacto do extrato de *A. satureioides* e dos seus flavonoides isolados sobre algumas das principais vias de sinalização tipicamente ativas nas células tumorais. O extrato hidroetanólico foi capaz de reduzir MYC e as MAP quinases (ERK e JNK), as quais aparecem ativas constitutivamente nas células U251. Entretanto, o

flavonóide luteolina (5 µM) aumentou a expressão de TGF-beta, pRB-E2F, HIF-1α e ERK, as quais são vias frequentemente relacionadas com um fenótipo mais agressivo das células do câncer, promovendo um microambiente tumoral mais favorável.

Inúmeros estudos consideram essas vias de sinalização como alvos terapêuticos para o tratamento do câncer uma vez que elas desempenham duplo papel na gênese tumoral. Nos estágios iniciais do tumor, TGF-beta pode ser associado com bom prognóstico, promovendo parada do ciclo celular e apoptose. Entretanto, nos tumores avançados, TGF-beta é relacionado à elevada agressividade e prognóstico ruim (DRABSCH; TEN DIJKE, 2012; PADUA; MASSAGUÉ, 2009). Esse paradoxo da função do TGF-beta no câncer pode ser parcialmente explicado a nível celular, devido à acumulação de alterações genéticas nessa via, resultando na transição da atividade supressora do tumor para promotora tumoral (JAKOWLEW, 2006; NEUZILLET et al., 2015). Além disso, TGF-beta pode desenvolver função pró ou antiangiogênica, de acordo com seu nível de expressão, a qual é uma regulação fundamental nos tumores hipervascularizados como os gliomas (ROY; POIRIER; FORTIN, 2015).

A via pRB/E2F desempenha um papel crítico na regulação do ciclo celular, especialmente na transição da fase G1/S, sendo que as alterações nessa via estão relacionadas com a maioria dos cânceres humanos, como no carcinoma hepatocelular, adenocarcinoma de pulmão, câncer de mama e gliomas, conferindo prognóstico desfavorável (CALIGIURI et al., 2013; CHEN et al., 2016; SHI et al., 2016; YANG et al., 2015). A consequência eventual da desregulação na via pRB/E2F é um acúmulo do fator de transcrição E2F ativo, o qual induz proliferação celular pelo estímulo da expressão de genes essenciais necessários para a replicação do DNA. Portanto, a atividade excessiva de E2F pode danificar as células e resultar em proliferação descontrolada. Além disso, essa via pode apresentar duplo papel, atuando

como um oncogênese assim como sendo capaz de induzir a apoptose (GASRI-PLOTNITSKY et al., 2017; SEVILLE et al., 2005). Outro aspecto interessante é o fato de E2F-1 apresentar diferentes papéis em distintos tipos celulares, sugerindo um efeito tecido específico (GORGOULIS et al., 2002).

O fator indutor de hipóxia (HIF-1α) é um dos fatores de transcrição mais expressos, responsável pela regulação da hipóxia encontrado em glioblastoma multiforme, resultando em extensiva necrose, um marcador histológico do glioma de grau IV (NIGIM et al., 2015). HIF-1α regula a expressão de aproximadamente 60 genes relacionados com a angiogênese, invasão e transição epitélio-mesenquimal, contribuindo para o fenótipo maligno desse câncer. Além disso, regiões necróticas formadas pelo desenvolvimento de vasos anômalos protegem as células tumorais, conferindo resistência à quimioterapia e à radioterapia (HUANG; CHEN; ZHANG, 2016).

A via MAPK consiste em ERK, p-38 e JNK, as quais estão envolvidas em muitos processos celulares. Conforme observado para a maioria das vias relacionadas ao câncer, elas podem desempenhar um papel na oncogênese ou na supressão tumoral. A função da ERK na supressão do tumor é influenciada pela força da sua ativação. Elevado nível de ERK induz supressão tumoral, estimulando a degradação da proteína associada à senescência (DPAS). Quando ocorre a perda da função de supressão do tumor ou das células efetoras de DPAS pode haver a modificação do estado de senescência para a iniciação do câncer. Outra condição é a ativação de reguladores negativos da via ERK, os quais atuam mantendo o estímulo de ativação da via abaixo do limiar necessário e, consequentemente, param a supressão do tumor. A última condição se caracteriza pela ERK hiperativada estimulando mecanismos de *feedback* negativo, reduzindo assim a sinalização mitogênica abaixo do mínimo necessário para a proliferação (DESCHÊNES-SIMARD et al., 2014).

De um modo geral, ao longo das análises celulares a luteolina demonstrou ótima redução da proliferação tumoral. No primeiro momento, o aumento da ativação das vias de sinalização TGF-beta, pRB-E2F, HIF-1α e ERK poderia ser relacionado à formação de um microambiente favorável para o crescimento do tumor. Entretanto, conforme descrito essas vias podem desempenhar papel duplo no desenvolvimento do câncer, condição observada também em nossos experimentos, uma vez que esse flavonoide foi tóxico para o glioma em baixas concentrações (5 µM). Os demais flavonoides avaliados não tiveram efeito sobre essas vias de sinalização. Portanto, esses dados mostram que o efeito combinado dos flavonoides em associação com os demais compostos presentes no extrato de *A. satureioides* é importante para inibir as vias de sobrevivência das células de câncer.

Considerando os promissores resultados do extrato de *A. satureioides* nos testes *in vitro* reduzindo a proliferação e a sobrevivência clonogênica das linhagens celulares de glioma, induzindo a apoptose, além de ter potencializado o efeito da temozolomida comparado com a ação do quimioterápico sozinho, fomos investigar o efeito do extrato hidroetanólico no modelo de glioblastoma *in vivo*.

O extrato de *A. satureioides* não apresentou toxicidade sistêmica conforme comprovada pela análise bioquímica do soro (ALT, AST, creatinina e glicose) assim como não foram observadas alterações morfológicas nos tecidos do coração, rim e fígado por meio das análises histológicas. Dano oxidativo foi verificado apenas no rim por meio do ensaio com TBA, indicando peroxidação lipídica com temozolomida, a maior dose do extrato de *A. satureioides* (100 mg/kg), assim como suas combinações. Outro parâmetro avaliado nos grupos de animais tratados foi o peso corporal, o qual não apresentou alteração estatisticamente significativa, sendo considerado um dos indicadores clínicos de severidade de tratamento.

Entretanto, o extrato de *A. satureioides* sozinho não foi capaz de reproduzir os resultados observados *in vitro* e reduzir o volume tumoral no cérebro dos ratos. Além disso, quando o extrato foi administrado junto com temozolomida, ele bloqueou o efeito do quimioterápico. Esse bloqueio pode ser relacionado ao fato dos tratamentos terem sido administrados concomitantemente e desse modo o extrato de *A. satureioides* poderia ter afetado a biodisponibilidade da temozolomida.

Estudos tem avaliado a influência dos flavonoides, especialmente da quercetina, na farmacocinética dos agentes terapêuticos por meio da regulação da fosfo-glicoproteína (P-gp), reduzindo a biodisponibilidade dos fármacos administrados oralmente (BHUTTO et al., 2018; HSIU et al., 2002; LOHNER et al., 2007). Resultados de pesquisas em animais tem comprovado que a P-gp tem um grande impacto na distribuição e absorção tecidual, limitando a absorção celular dos fármacos durante o percurso do sangue até o cérebro (LIN; YAMAZAKI, 2003). Entretanto, é difícil afirmar que essa interação medicamentosa também ocorra no contexto do TMZ, o qual é extensivamente absorvido tanto em humanos quanto em ratos, apresentando uma biodisponibidade oral absoluta de 96-100% (REYDERMAN et al., 2004).

Outro aspecto relevante é a capacidade dos produtos naturais, especialmente dos polifenóis, de inibirem a atividade ou expressão de DNA metiltransferases (DAMMANN et al., 2017). Estudos têm revelado que a quercetina é capaz de modificar a metilação do DNA (ZHENG et al., 2014) e, como bem conhecido, a eficácia do TMZ é potencializada pela metilação do DNA que é mediada pela MGMT (O6-metilguanina-DNA metiltransferase) (BOBOLA et al., 2015). Esses dados ajudam a explicar a perda da atividade quimioterápica observada no nosso tratamento quando administrado simultaneamente com o extrato de *A. satureioides*. Cabe salientar que a quercetina é o segundo composto com maior prevalência na

composição do extrato hidroetanólico de *A. satureioides* e poderia afetar a farmacocinética do TMZ e a metilação do DNA. Além disso, o grupo metila de 3-O-metil-quercetina, flavonoide predominante no extrato de *A. satureioides*, também poderia contribuir para a alteração do status da metilação do DNA.

Esse efeito inesperado do extrato de *A. satureioides* bloqueando a ação do quimioterápico é provavelmente um dos mais importantes no nosso trabalho e deve ser considerado visto que o chá de marcela é popularmente consumido na América Latina, com especial ênfase na região sul do Brasil. Dentre as inúmeras indicações medicinais para o consumo do chá dessa planta destacam-se alívio da dor de cabeça, enjoos e náuseas, os quais são sintomas frequentes em pacientes com glioblastoma assim como resultantes dos tratamentos com quimioterápicos. Assim, de acordo com nossos experimentos o consumo de *A. satureioides*, apesar de não demonstrar toxicidade sistêmica, pode comprometer os efeitos esperados advindos do tratamento com o quimioterápico temozolomida, devendo ser consumido com cautela por pacientes em tratamento.

CONCLUSÕES

- O extrato hidroetanólico de *Achyrocline satureioides*, rico em flavonoides (quercetina, 3-O-metil-quercetina, luteolina e achyrobichalcona), apresentou atividade antiproliferativa *in vitro* nas linhagens de glioma (C6, U87 e U251), assim como quando associado à temozolomida, potencializando o efeito do quimioterápico.

- Esse efeito antiproliferativo foi intensificado quando os flavonoides isolados foram avaliados nas células tumorais, indicando atividade antitumoral da achyrobichalcona, luteolina, 3-O-metil-quercetina e quercetina;

- A combinação dos flavonoides presentes no extrato de *A. satureioides* apresenta um papel importante inibindo vias de sinalização essenciais para o desenvolvimento dos gliomas;

- De um modo geral, o extrato hidroetanólico de *A. satureioides* assim como os seus flavonoides isolados não foram tóxicos para as células sadias do sistema nervoso central (astrócitos, neurônios, co-cultura astrócitos/neurônios e cultura organotípica de fatias do hipocampo) nas mesmas concentrações que afetaram os gliomas;

- Apesar do tratamento com o extrato hidroetanólico de *A. satureioides* não apresentar toxicidade sistêmica, a atividade antitumoral não foi reproduzida no modelo de implantação de glioma *in vivo*;

- O extrato inibiu o efeito do quimioterápico *in vivo*, provavelmente devido à interação do produto natural com o fármaco mediante a administração simultânea. Esse resultado é de grande relevância a fim de conscientizar os pacientes da clínica, visto que o chá de marcela é popularmente consumido na América Latina e pode vir a interferir no tratamento quimioterápico.

PERSPECTIVAS

- Explorar as modificações epigenéticas mediadas pelos flavonoides isolados de *A. satureioides* nos modelos de glioma *in vitro*;

- Avaliar a farmacocinética da absorção da temozolomida quando administrado concomitantemente e em momentos distintos ao extrato hidroetanólico de *A. satureioides*;

- Investigar os efeitos da administração com os flavonoides isolados de *A. satureioides* no modelo de implantação de glioma em camundongos.

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Anexo

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Inflammatory landscape of human brain tumors reveals an NFκB dependent cytokine pathway associated with mesenchymal glioblastoma

CrossMark

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ABSTRACT

The tumor microenvironment is being increasingly recognized as a key factor in cancer aggressiveness. In this study, we characterized the inflammatory gene signatures altered in glioma cell lines and tumor specimens of differing histological and molecular subtypes. The results showed that glioblastoma multiforme (GBM) shows upregulation of a subset of inflammatory genes when compared to astrocytomas and oligodendrogliomas. With molecular subtypes of GBM, the expression of inflammatory genes is heterogeneous, being enriched in mesenchymal and downregulated in Proneural/GCIMP. Other inflammation-associated processes such as tumor-associated macrophage (TAM) signatures are upregulated in mesenchymal, and a subset of 33 mesenchymal-enriched inflammatory and TAM markers showed correlation with poor survival. We found that various GBM tumor-upregulated genes such as *IL6, IL8* and *CCL2* are also actively expressed in glioma cell lines, playing differential and cooperative roles in promoting proliferation, invasion, angiogenesis and macrophage polarization in vitro. These genes can be stimulated by pathways typically altered in GBM, including the EGFR, PDGFR, MEK1/2-ERK1/2, PI3K/Akt and NFκB cascades. Taken together, the results presented herein depict some inflammatory pathways altered in gliomas and highlight potentially relevant targets to therapy improvement.

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Introduction

Despite the numerous advances in the chemotherapeutic protocols used to treat a variety of cancers, glioblastoma (GBM) therapy still rely on the palliative efficacy of combined surgery, radiation and temozolomide. Be that by its particularly protected localization or by its fast growing behavior as well as its inherent ability to acquire chemoresistance, GBM remains one of the most challenging cancers, with a median survival of ~14 months [1,2]. Recent characterization of mutational patterns, copy number alterations and gene expression profiles of human brain tumors led to grouping of GBM into molecular subtypes [1,2]. These multi-omics analyses revealed not only the heterogeneity of GBM but provided avenues for identification of new molecular targets that could benefit specific groups of patients [1,2]. However, these novel possibilities have not been translated into clinical benefits thus far, mostly due to the minimal efficacy observed in clinical trials with inhibitors of GBM deregulated pathways such as EGFR [3], mTOR [4], VEGFR-family-tyrosine-kinases [5,6], Ras-MAPK [7,8] and PI3K/Akt [9,10]. In view of this evading characteristic of GBM, it has been hypothesized that multi-therapies targeting multiple pathways that dictate tumor malignance (such as proliferation, invasion and angiogenesis) or blocking of master regulators of these processes could offer new opportunities [10,11].

There is compelling data that inflammatory crosstalk occurs in the microenvironment of many tumor types, be that by intrinsic production of inflammatory mediators by tumor cells or/and by

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activation of tissue resident and tumor infiltrating peripheral immune cells [12–14]. In addition, tumor microenvironments are inherently complex due to numerous, redundant and complimentary roles of cytokine families of proteins, which function in consonance with matrix metaloproteases, angiogenic factors and cell–cell interaction proteins in order to promote the self-sustained growth of malignant cells as well as to mediate crosstalk between tumors and its non-neoplasic surroundings [12–16]. In this context, it has been shown that the molecular composition of the inflammatory input is capable of orchestrating polarized phenotypes of both cancer and stromal cells thereby dictating tumor heterogeneity and malignance [12–16]. As a consequence of such a complexity, cancer inflammation research is frequently fated to study a limited number of processes at a time, while several co-occurring inflammatory signals are missed.

In this study, we used an integrated system biology standpoint to characterize the inflammatory microenvironment, especially the cytokine component, of glioma cell lines and tumors specimens of different histological and molecular subtypes. We used a curated list of cytokines (e.g., interleukins, chemokines, interferons, tumor necrosis factor and lymphokines) and its receptors as well as other related genes applied to genome-wide gene expression datasets from gliomas cell lines to tumors. Altered gene subsets and their putative transcriptional regulators were identified, and the impacts of modulating some of these inflammatory genes/pathways upon proliferation, invasion, angiogenesis and growth of gliomas as well as macrophage polarization/chemotaxis were studied in vitro.

Materials and methods

Cell lines and treatments

U251MG, U87MG (glioblastomas), MCF-7 (breast cancer; non-invasive control), U937 and THP-1 (monocytic leukemia) cell lines were grown in complete cell culture medium supplemented with 10% FBS plus 1X antibiotic/antimycotic solution (Sigma–Aldrich) at 37 °C in a humidified incubator set at 95% CO_2 and 5% O_2 as recommended by the supplier (ATCC, Rockville, Maryland, USA). When used, pharmacological inhibitors were maintained over the period of treatments, except for conditioned medium preparation as described below. SB225002 (15 μM), UO126 (UO; 15 μM), LY294002 (LY; 20 μM), Stattic (2.5 μM) and temozolomide (200–400 μM) were from Sigma–Aldrich and, unless otherwise specified, incubated at the aforementioned concentrations. Recombinant human IL6, IL8 (Sigma; #I1645), EGF (Invitrogen, #PHG0311) and PDGF-A/B (Invitrogen, #PHG0134) were incubated at 50 ng/mL, 100 ng/mL, 25 ng/mL and 50 ng/mL, respectively.

Neurospheres growth

For neurosphere induction, U251MG and U87 cells were cultured in serum-free neurobasal-A medium supplemented with B27, GlutaMAX-I supplement, 50 ng/mL heparin (Sigma–Aldrich), 20 ng/mL of EGF and 10 ng/mL bFGF (Invitrogen) as described [17]. Cultures were grown until formation of ~100-μm spheres. For passaging, the cells were dissociated with 0.05% trypsin-EDTA (Gibco) for 2 min/37 °C, and re-seeded at 10^5 cells/ml. Neurospheres conditioned medium (CM) was collected and used for angiogenesis assay or ELISA determination of cytokines. For invasion assays, the spheres were dissociated by trypsinization before platting into Boyden chambers (described below).

Conditioned media (CM) preparation

For CM preparation, 12-well-plated cells were incubated with 700 μL serum-free fresh medium for 24 h. This 24 h CM was collected and immediately used for invasion and angiogenesis assays or stored at −20 °C for ELISA. When used, the siRNA sequences were transfected for 24 h to allow gene knockdown, and then a new drug-free medium was replaced and conditioned for 24 h.

Small interference RNA (siRNA)

The siRNAs (30–60 nM) were transfected by reverse protocol using the Lipofectamine RNAiMax reagent (Invitrogen, USA) for 24 h before cell functional assays. Protein knockdown was confirmed by ELISA detection of cytokines in the culture medium or immunoblots against p65-NFκB in whole-cell extracts. Human IL8 (sc-39631), IL6 (sc-39627), CCL2/MCP-1 (sc-43913), CSF1/M-CSF (sc-39393), MMP2 (sc-29398) siRNA sequences were from Santa Cruz Biotechnologies. SignalSilence® NFκB p65 siRNA 1 (#6261) was from Cell Signaling; and Silencer® Select Negative Control#1 (#4390843) siRNA was used as a scrambled control.

ELISA

ELISA quantification of IL6 and IL8 in the culture medium of cells was performed using the Human IL-6 ELISA Kit (RAB0306, Sigma–Aldrich), Human IL-8/CXCL8 ELISA Kit (RAB0319, Sigma–Aldrich) following manufacturer's instructions. Culture medium was diluted up to 50 times as appropriate for each cytokine, and the concentrations were normalized per mL/1×10^6 cells.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Briefly, U937 cells RNA was isolated by Trizol method (from ~10^6 cells), and the cDNA was synthesized by Superscript III Reverse Transcriptase kit (Invitrogen). The IL6, MMP9, IL10, TNF and NOS2 mRNA levels were monitored using SYBR Green primers as follows: IL6 (forward: 5′GGTACATCCTCGACGGCATCT3′; reverse: 5′GTGCCTCTTTGCTGCTTTCAC3′); TNF (forward: 5′CTCTTCTCCTTCCTGATCGTGGCA3′; reverse: 5′GAAAGCATGATCCGGGACGTGGA3′); IL10 (forward:5′GCCTAACATGCTT CGAGATC3′; reverse: 5′CTCATGGCTTTGTAGATGCC3′); MMP9 (forward: 5′GCACGA CGTCTTCCAGTACC3′; reverse: 5′CAGGATGTCATAGGTCACGTAGC3′); NOS2 (forward: 5′ACCTTGTTCAGCTACGCCT3′; reverse: 5′CATTCCCAAATGTGCTTGTC3′); GAPDH (forward: 5′CATCACTGCCACCCAGAAGACTGTGGA3′; reverse: 5′TACTCCTTGGAG GCCATGTAGGCCATG3′). PCR reactions were performed using SYBR® Green PCR Master mix (Invitrogen), and relative mRNA fold-changes (test vs control) were calculated by the $2^{-\Delta\Delta CT}$ method.

ECM invasion and chemotaxis (Boyden chamber assays)

The extracellular matrix (ECM) invasion potential of cancer cells was estimated using the QCMTM 24-Well Cell Invasion Assay Fluorimetric (Millipore). Serum-starved cells were harvested by trypsinization, and 125,000 cells were seeded onto 8 μm pore size inserts coated with ECM in serum-free conditions (top-chamber); 20% FBS was used as a chemoattractant in the bottom chamber. The cells were allowed to migrate for 24–72 h, and invading cells on the bottom side of the insert were quantified by CyQuant GR fluorescent Dye (Ex/Em = 480/520 nm) and compared to cell number curves performed in parallel. In neurospheres invasion assay, the spheres were dissociated with trypsin before seeding. When used, siRNA and pharmacological inhibitors were transfected/treated at the seeding step. For monocyte chemotaxis assay, THP-1 monocyte-like cells were used as previously described [18]. THP-1 cells (125,000/well) were seeded onto top-chambers, and U251MG monolayers were grown in the bottom chamber (with/out a previous 24 h siRNA transfection) in serum-free conditions.

In vitro angiogenesis

The Angiophase kit (MBL Inc.) was used to assess the angiogenic potential of gliomas conditioned media (CM). Kit-provided human endothelial cells were expanded and plated in the presence of gliomas CM for a total of 12 days. The medium was replaced each 4 days. At the end of treatments, the cells were fixed, and the vessel-like structures were immuno-detected with an anti-CD31 antibody and quantified by ELISA. In some experiments, CD31 protein was detected by immunocytochemistry with the substrate BCIP/NBT, which form insoluble precipitates that develop the threadlike structures of anastomosing tubules typical of the capillary bed. Kit-provided VEGF was used as a positive control.

U937 adhesion assay

U937 monocyte-like cells adhesion assay was used as an estimative of the macrophage activating potential of gliomas CM [19,20]. To this, 250 μL of RPMI containing 3×10^5 U937 cells was plated with 250 μL of glioma cells CM (1:1 ratio) in 24-well plates. After 48 h, the U937 conditioned medium was collected (and transferred to U251MG cultures as shown in Fig. 5H assays) and adherent/activated U937 cells were fixed with 4% paraformaldehyde (20 min), washed with PBS and stained with 0.1% Cristal violet (30 min). The cells were washed thrice with PBS and allowed to dry at room temperature. Cristal violet was dissolved in 10% acetic acid and read at 590 nm. The percentage of U937 adhesion was determined as compared to cell number standard curves of cristal violet-stained U937 cells.

Co-cultures of U937 and U251MG

U251MG (1×10^5 cells) and U937 (0.5×10^5 cells) (2:1 ratio) were co-cultured in 12-well plates in DMEM supplemented with 10% FBS plus antibiotics. For assessing glioma proliferation in the presence of U937 cells, co-cultures were kept over a 5 days period. The medium was changed every 24 h to remove unattached/undifferentiated U937 cells. At the end, the cells were fixed, permeabilized, and glioma cells were selectively labeled with an anti-nestin antibody (1:300; AbCam MAB5326 clone 10C2) followed by Alexa 488-conjugated anti-IgG (1:500; 2 h). The cells were PBS-washed and fluorescence was read at Ex/Em 495/520 nm and compared to cell number standard curves following the same immunofluorescence protocol. For in vitro invasion of co-cultures, U251MG cultures (1×10^5 cells) and U937 (0.5×10^5 cells) were co-seeded in the upper chamber of the QCMTM 24-Well Cell Invasion Assay Fluorimetric kit (Millipore) in serum-free medium as described above. The bottom medium consisted of DMEM supplemented with 20% serum as a chemoattractant.

Cell viability and cell cycle analysis

MTT (3-(4,5-dimethyl)-2,5-diphenyl tetrazolium) reduction by cellular dehydrogenases, cell counting and cell cycle analyses were performed as previously described [21].

Gene expression dataset analysis

Inflammatory and tumor associated macrophage (TAM) gene sets

A set of 249 genes comprising the main cytokine groups such as interleukins (IL genes), chemokines (CXC, CC and CXC3 subfamilies), interferon, TNF, Toll-like receptor families and their respective receptors as well as other mediators such as hematopoietins was derived from HUGO gene families (www.genenames.org/cgi-bin/genefamilies/) and KEGG database (hsa04060: Cytokine–cytokine receptor interaction pathway) as detailed in Table S1 and Fig. S1. Gene markers associated with tumor-associated macrophages/microglia (TAM) were curated from [14,22–25] as annotated in Table S1. TAM markers included the surface markers *CD14, FCGR3A* (CD16), *ADGRE2* (CD312), *CD68, HLA-DRA* (MHC-II), *ITGAM, CD200R1, PTPRC* (CD45), *CHI3L1* (YKL-40) and *CSF1R*, the M2 macrophage-related genes *CD163, MSR1, MRC1, CD209, IL10, CLEC10A, CLEC7A* and *CXCR4*, and the M1 markers *TNF, IL12A, IL12B, CD80, CD86* and *NOS2*.

Datasets

Gene expression profiling of gliomas cell lines in vitro was evaluated using the E-MTAB-2706 dataset, which comprises 27 glioma cell lines evaluated by RNA sequencing (from ArrayExpress). For gene expression analysis among glioma histological subtypes, the GSE68848 (Rembrandt cohort; n = 580 samples), GSE16011 (n = 284 samples) and GSE4290 (n = 180 samples) Affymetrix U133Plus 2.0 microarray datasets were downloaded from GEO and independently evaluated. Only the samples annotated as "astrocytoma", "oligodendroglioma" and "GBM" were analyzed. For analysis across GBM molecular subtypes [1], the TCGA microarray dataset (Affymetrix HT-HG-U133A genechip; Cell 2013 dataset) comprising 520 previously annotated samples (".CEL" files of 144 classical/Class, 155 mesenchymal/Mes, 83 neural/Neu, 99 proneural/Pro and 39 GCIMP/GC) was downloaded from TCGA Data Portal (https://tcga-data.nci.nih.gov/tcga/). The GSE65363, GSE1128, GSE49162 and GSE334432 microarray datasets (from GEO Omnibus) were used to evaluate the impact of modulating NFκB (by means of NFKB1 shRNA or IκB-SR overexpression), PDGF and EGF receptors activation; MEK1/2-ERK1/2 and PI3K/Akt (by means of UO126 and LY294002 treatment, respectively) upon inflammatory genes expressions in glioma cell lines.

Data processing

Microarray data were imported and the process of summarizing gene expression for each probe set constituted of (i) Background correction for adjusting for hybridization effects, (ii) Normalization by Affymetrix Microarray Suite 5 (Mas5.0), and (iii) Summarization by selecting the most dispersed rows to obtain a single value for each gene. These operations were carried out in the Bioconductor packages affy and hgu133plus2.db. Significance analysis of microarrays (SAM) (FDR < 0.05) was applied to determine features differentially expressed across the disease subtypes, and only entities with at least ±2.0 fold-change in any subtype comparison were considered as differentially expressed genes (DEG). With the GSE68848, GSE16011 and GSE4290 datasets, genes with differential expression in at least 2 out of 3 cohorts were assigned as DEG. With the TCGA samples, genes with +2-fold-change as compared to at least 3 other subtypes were considered as enriched with a given subtype. In RNA sequencing of glioma cells (E-MTAB2706), genes with median Reads Per Kilobase per Million mapped reads (RPKM) >1.5 across the entire cell panel were considered as significantly represented/expressed in vitro. For heatmap representation, normalized expressions of DEG were log2 transformed, Z-scores were calculated and hierarchical clustering was performed using Euclidean Distance and Ward's linkage as dissimilarity measures and clustering method, respectively. Principal component analysis (PCA) of DEG across gliomas of all histology was performed using the R-environment statistical and visualization package available in the Metaboanalyst 3.0 [26].

Pathway enrichment analysis (PEA)

PEA was estimated using the Pathway Annotation Tool available in Enrichr (http://amp.pharm.mssm.edu/Enrichr/) and DAVID bioinformatics (https://david.ncifcrf.gov/). Disease-related pathway terms were excluded from the output list.

Transcription factor enrichments (TFE)

TFE was performed using the ChIP Enrichment Analysis (ChEA, 2015 release) tool available in Enrichr (http://amp.pharm.mssm.edu/Enrichr/enrich) [27]. From an input list of DEG, the ChEA program computes over-representation of transcription factor targets based on shared overlapping targets and binding site proximity from the ChIP-X database.

Pathway signature expression

From our curated list of inflammatory and TAM markers afore described, we firstly pooled all genes with differential expression (DEG) in at least one glioma subtype comparison (i.e., among histological or molecular subtypes) and assigned them into their respective pathway (inflammatory or TAM). Genes with unaltered expression in any comparison were removed. Thus, with a given process, pathway signatures consisted of the same gene modules. Pathway Signature Expression index was obtained by plotting the calculated median Z-score of each DEG as a single group per subtype, therefore providing a comparative expression of inflammatory and TAM pathways across disease subtypes (see detailed in Fig. S1B). Kruskal–Wallis followed by Dunn's were used for statistical comparisons at a p < 0.05.

Patient survival analysis

We used the SurvExpress tool [28] in order to assess the association of altered expression of inflammatory genes with glioma patient's survival. SurvExpress uses the prognostic index (PI), also known as the risk score, to generate risk groups. PI is known as the linear component of the Cox model $PI = \beta 1x1 + \beta 2x2 + \ldots + \beta pxp$, where xi is the expression value and the βi can obtained from the Cox fitting. Patients were split into 4 risk groups (low-, low/medium-, medium-, medium/high-and high risk), and Overall Survival was selected as clinical endpoint. The initial screening included the individual risk assessment of all inflammatory and macrophage markers (total of 267 genes) as described in Table S1. The screening was performed in two of the largest cohorts with the database (TCGA GBM n = 538 GBM; LGG/GBM TCGA n = 660 (512 Low-grade glioma/LGG + 148 GBM); access: 11/2016) using the following parameters: i) "Brain" (21); ii) Database: cohort; iii) "Maximum variance" probe for genes with multiple probes; iv) "original (quantile normalized)" values of gene expression; v) "Maximize risk groups" for risk group stratification. Only genes showing differential expression between high-risk versus low-risk groups in both cohorts were considered as associated with survival, and were selected for gene signature analysis. Kaplan–Meier plots, log-rank test of differences between risk groups, and hazard-ratio (HR; estimated by fitting a CoxPH using risk group as covariate) were calculated [28].

Results

Characterization of inflammatory genes expressed in glioma cell lines in vitro

We initially evaluated RNA sequencing data of a panel of 27 glioma cell lines aiming to identify the inflammatory genes constitutively expressed in glioma cells growing in vitro (Fig. 1A–B). We found that 70 of 249 (28%) genes show constitutive expression. Majority of them codify interleukins, TNF and interferon receptor subunits, indicating that glioma cells may undergo immune modulation by cytokines present in the microenvironment (Fig. 1A–B). With regard to ligands, classical cytokines such as *MIF, IL8, IL6, CCL2* (MCP-1 protein) *and CSF1* (colony stimulating factor-1) as well as other less studied in the context of gliomas (*IL11, IL32, MDK* (midbrain midkine), *LIF* (leukemia-inhibitory factor), *CKLF* (chemokine-like factor) and *CLCF1*) showed high levels of expression across the cell panel, indicating that glioma cell lines may modify the tumor microenvironment through production of these soluble mediators (Fig. 1A–B). Interestingly, classical genes such as *TNF and IL10* did not show consistent expression across this set of glioma cell lines (data not shown).

Inflammatory gene expressions are enriched in GBM and are overrepresented in the mesenchymal subtype

We next set out to determine if the inflammatory alterations observed in cell lines transcriptomes are also observable in human gliomas of differing subtypes. By independently evaluating microarray datasets of gliomas of all histology, we obtained 32 DEG (30 up- and 2 down-regulated) in GBM when compared to less aggressive subtypes; i.e. astrocytoma/Ast or oligodendroglioma/ODG (Fig. 2A). Expression of the Inflammatory Pathway Signature is higher in GBM followed by astrocytoma and then a lower level in ODG (Fig. 2B). In addition, principal component analysis (PCA) of the inflammatory gene expressions across histological subtypes showed that a fraction of GBM overlapped with astrocytoma and ODG specimens (Fig. 2C). Based on this evidence, we sought to test whether such an intra-GBM subtype heterogeneity could be associated with the distinct molecular profiling of
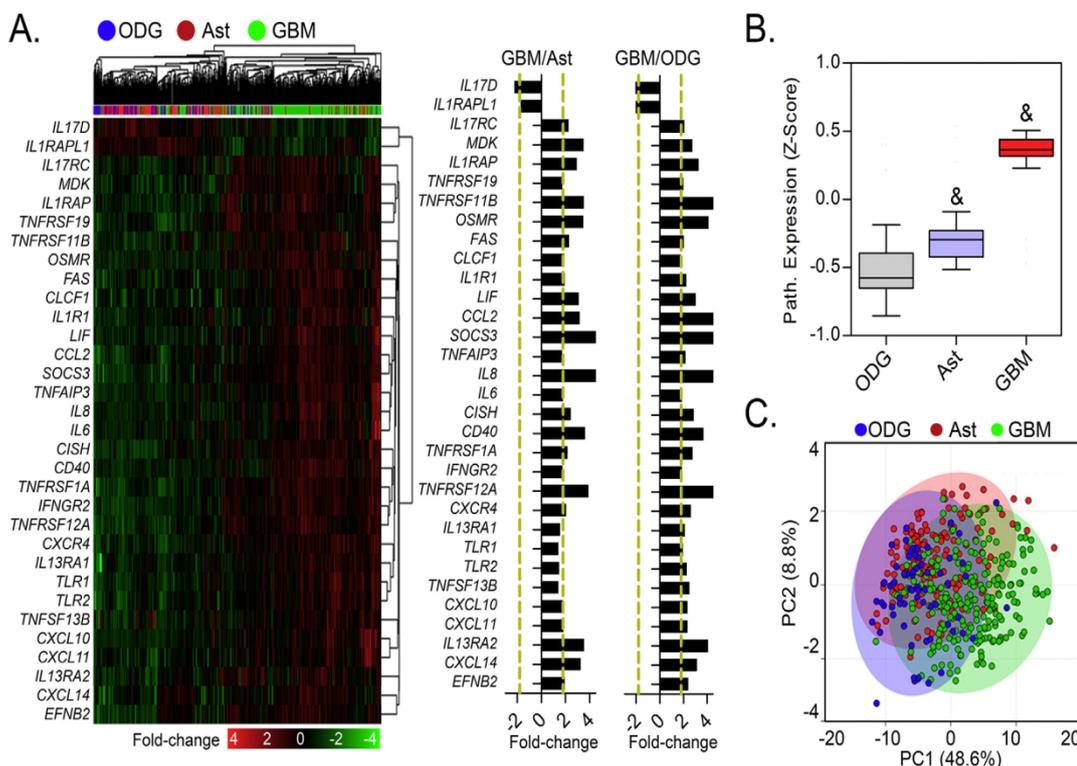
Fig. 1. Inflammatory gene expression profiling of glioma cell lines. (A) Heatmap representation of the inflammatory genes expressed in a panel of glioma cell lines (E-MTAB-2706 RNA sequencing dataset; gene expression cut-off: median RPKM > 1.5). Gene expressions are plotted as Log2 of the RPKM value. (B) Box-plot representation of the most expressed inflammatory ligands and receptors across glioma cells in the E-MTAB-2706 dataset. Each box comprises the distribution of mRNA RPKM values for a given gene across the entire cell panel (Whiskers: 5–95 percentile).

GBM as previously characterized in TCGA studies [1]. By grouping GBM into molecular subtypes, we observed that mesenchymal (Mes) tumors showed upregulation of 60 transcripts compared to other subtypes (Fig. 3A; also see Fig. S2). This cumulated in an upregulated Inflammatory Pathway Signature with Mes tumors followed by classical/neural, proneural and then a lower expression with the CpG island methylator phenotype, GCIMP (Fig. 3B; Fig. S2). PEA analysis confirmed that genes belonging to "Cytokines", "chemokines" and "JAK-STAT" pathways are enriched with the Mes subtype when compared to all-GBM (Fig. 3C; detailed in Table S2). Complimentary, TFE analysis suggested that the NFκB member p65 (*RELA gene*) and STAT3 are putative regulators of the inflammatory gene repertoire of GBM, especially the Mes-enriched gene set (Fig. 3D; Table S2). Overlapping analysis showed that 12 genes, including the ligands *IL8, CCL2, IL6, OSMR,* and *LIF,* are expressed in glioma cell lines in vitro as well as showed upregulation in all-GBM and Mes (Fig. 3E). Noteworthy, 36 genes were enriched with Mes (especially CCL and CXCL family members) but showed neither differential expression when all-GBM were analyzed as a unique phenotype nor constitutive expression in glioma cell lines (Fig. 3E). Additional genes (namely *CXCR4, CXCL10, CXCL14* and *EFNB2*) were found upregulated in all-GBM/Mes but showed low/no expression in majority of the glioma cell lines in vitro. Taken together, these data indicate that the inflammatory microenvironment gains complexity (i.e. enriches) when comparing in vitro, low- to high-grade gliomas, and the Mes subtype of GBM.

Inflammatory mediators cooperate to different malignance processes

We then went on to determine whether and how some glioma cells derived mediators may contribute to different malignance phenotypes. In the assays presented hereafter, we addressed the role of IL8, IL6, CCL2 and CSF1 in different aspects of glioma biology, given that these cytokines stood out as highly enriched in gene expression analyses of cell lines, GBM and Mes tumors. We initially set out that, in keeping with RNA sequencing of cells (Fig. 1), protein levels of IL8 and IL6 were readily detectable in the culture medium of U251MG and U87MG gliomas (Fig. 4A). By siRNA knockdown of these cytokines (validated in Fig. 4A–B) we were able to determine that: i) IL6 depletion, and IL8 with minor magnitude, reduces the long-term proliferative rate of glioma cells (Fig. 4C); ii) glioma cells show a highly invasive activity in Boyden chambers, which is inhibited by IL6 knockdown or the positive control MMP2 siRNA whereas IL8 knockdown or the CXCR2/IL8-receptor antagonist SB225002 showed a minor effect (Fig. 4D); iii) CM from glioma cells stimulated angiogenesis when incubated with endothelial cell cultures and, in contrast to observed in the aforementioned assays, U251MG CM depleted of IL8, but not of IL6, as well as addition of SB225002 to U251MG CM attenuated angiogenesis in vitro (Fig. 4E). We also noted that glioma cells growing as neurospheres – which display enhanced oncogenic potential [17] – showed a greater production of IL8 and IL6 (Fig. 4A–B), invasion (Fig. 4D) and angiogenic potential (Fig. 4E) than their respective parental

Fig. 2. Inflammatory gene expression profiling across histological subtypes of gliomas. (A) Heatmap representation of the 32 genes differentially expressed among Astrocytoma (Ast), Oligodendroglioma (ODG) and GBM specimens and fold-ratio of DEG in GBM/Ast and GBM/ODG (Rembrandt dataset). (B) Pathway Signature expression of the 32-gene module across gliomas of all histology (Whiskers: 5–95 percentile). (C) Principal component analysis of inflammatory gene expressions in gliomas of all histology. &different from all other groups; (Kruskal–Wallis, post-hoc Dunns; p < 0.05).

adherent cultures; although it has been reported that IL6 and IL8 are not directly associated with neurosphere formation [17]. Based on these results, it is possible to delineate an autocrine role of IL6 in cell invasion whereas IL8 seems more committed to paracrine stimulation of angiogenesis. Less promising, IL6 and IL8 depletion promoted additive, but not synergistic, decreases in cell viability when combined with TMZ/temozolomide (Fig. 4F–G). Until 72 h incubation, IL8/IL6 knockdown caused neither evidence of apoptosis in Annexin-V/PI assay (data not shown) nor sub-G1 formation in cell cycle analysis (Fig. 4G). Thus, the decrease in cell numbers (Fig. 4C) and the increase in G1 phase of cell cycle (Fig. 4G) could be result of an antiproliferative not-cytotoxic effect of IL8/IL6 depletion; agreeing with previous data with IL8 capture antibody and SB225002 [29]. Noteworthy, although the basal expression of IL6 receptors (IL6R and IL6ST) shown in Fig. 1 data may help to explain the autocrine effects of IL6, the E-MTAB-2706 RNA-sequencing as well as RNAse protection assays [16,30] have not detected *CXCR1* and *CXCR2* (IL8 receptors) mRNA expression in various glioma cell lines. However, IL8 is capable of binding glioma cells, but this seems to involve IL8 interactions with glycosaminoglycans [16].

GBM secretoma promotes macrophage chemotaxis and polarization

Peripheral immune cells are a significant component of the GBM tumor mass [14,24]. There is cumulating evidence that GBM cells crosstalk with tumor-associated macrophages (TAM) and the resulting secretoma milieu is capable of promoting tumor aggressiveness [14]. We wanted to characterize these mechanisms in our model, and we found that, corroborating with Fig. 3 data, Mes GBM showed the highest expression of TAM markers followed by neural,

classical and lower levels with proneural and GCIMP (Fig. 5A and Fig. S2). This TAM signature included genes classically related to M2-like macrophages (such as *IL10, CD163, MSR1 and MRC1*; detailed in Fig. S2) as well as general macrophage surface markers (*CD14, FCGR3A, ADGRE2, HLA-DRA*; Fig. S2); typical M1 markers including *TNF, IL12, TLR4* and *NOS2* were not differentially expressed among molecular subtypes (data not shown). It is key to note that glioma TAM show phenotypic differences from M1/M2 cells obtained in in vitro models of macrophage differentiation [reviewed in [14,22–24]]. However, if TAM markers are enriched in GBM tumors, it is expected that glioma cells are capable of secreting a repertoire of immune chemoattractant and modulatory soluble factors which we wanted to identify, at least in part. Firstly, RNA sequencing of glioma cells showed that they express none of the well-known in vitro macrophage polarizing factors such as TNF-α and Interferon-gamma (*IFNG*) for M1, or IL4 and IL13 for classical M2 (Fig. 5B). On the other hand, some inducers of glioma TAM (as described by [14]) such as TGF-beta (*TGFB1, TGFB2, TGFB3*), Colony Stimulating Factors (*CSF1, CSF2 and CSF3*), *CCL2*/MCP-1, *HGF* and *VEGFA* are constitutively expressed at high levels in majority of the glioma cell lines (Fig. 5B).

By using the U937 model, we found that exposure of U937 cells to gliomas CM promoted U937 cell attachment as an early step of macrophage activation process in vitro (Fig. 5C–D). Glioma cells CM also upregulated the M2-related anti-inflammatory cytokine IL10 (~6-fold), IL6 (~3.5-fold) and the pro-invasive metaloprotease MMP9 (~2.8-fold) [32] in U937 cells (Fig. 5E). In contrast, the M1-related genes *TNF* and *NOS2* were not altered (Fig. 5E), agreeing with the lack of TNF-α production in TAM isolated from murine gliomas [31]. When CSF1, and IL6 in a minor extent, were depleted from gliomas CM by siRNA, U937 adhesion was inhibited
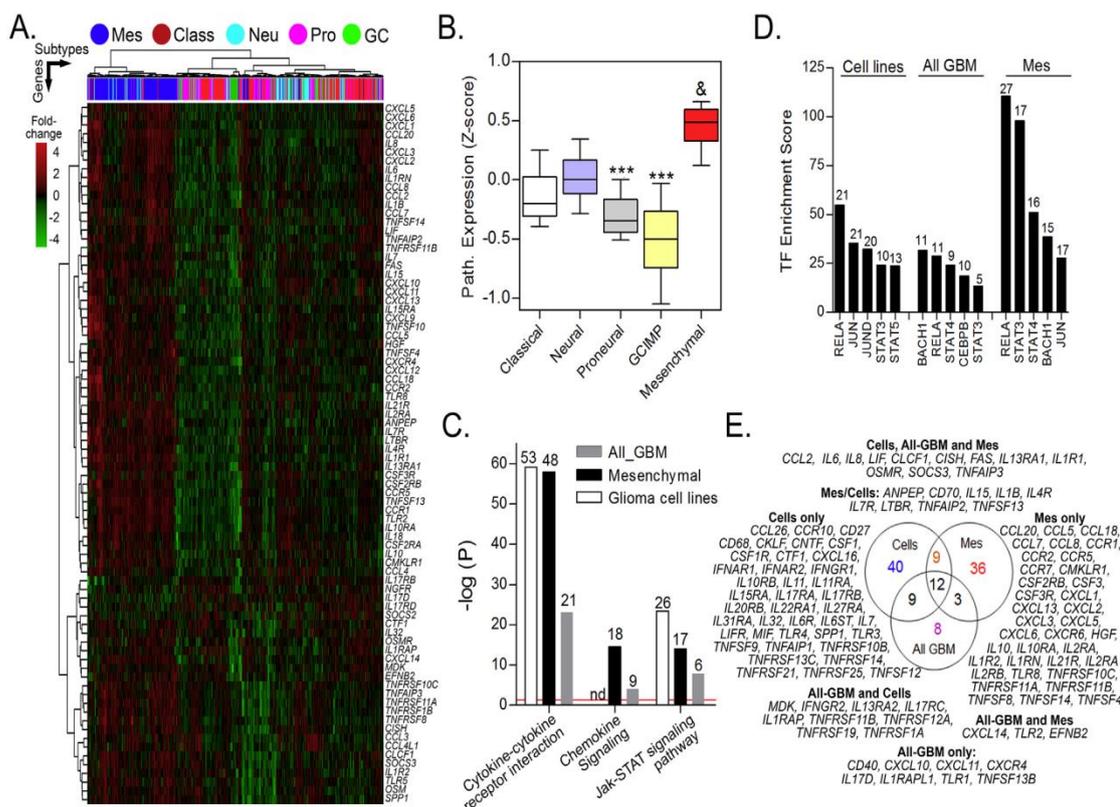
Fig. 3. Inflammatory genes expression across molecular subtypes of gliomas. (A) Heatmap representation and (B) Pathway Signature expression of genes differentially expressed (Top 80 DEG between at least 2-subtypes) across molecular subtypes of GBM derived from the TCGA database. Representative box-plots of some DEG are available in Fig. S2. (C) PEA showing the processes associated with "cell lines expressed", "all-GBM upregulated" (from GBM versus ODG and Ast) and "Mes-enriched" inflammatory genes. (D) Transcription Factor Enrichment (TFE; CheA2015) analysis for prediction of TF regulating the inflammatory genes expressed in glioma cell lines (E-MTAB-2706 dataset), upregulated in GBM (Rembrandt datasets) and enriched with Mes subtype (TCGA dataset). Putative targets of each TF are detailed in Table S2. With C and D, the number of genes detected with each pathway/TF is annotated on the top of graph columns. (E) Venn diagrams showing gene overlaps among i) glioma cells expressed; ii) GBM tumors upregulated and iii) Mes-enriched genes. &different from all other groups; ***Different from classical, Mes and neural subtypes (Kruskal–Wallis, post-hoc Dunns; $p < 0.05$); nd: not-detected.

(Fig. 5C–D); IL8 or MMP2 knockdown had no impact upon this parameter (Fig. 5C). Corroborating, recombinant IL6, but not IL8, treatment of U937 cells promoted cell adhesion (Fig. 5C, right bars).

In a different approach, co-culturing of U251MG with U937 cells prompted adhesion of U937 to U251MG monolayers (Fig. 5F). We also observed that either co-culturing of U251MG with U937 (Fig. 5G) or incubating U251MG cells with U937 CM prepared post-stimulation with gliomas CM (Fig. 5H) also stimulated glioma cells proliferation. This suggests that U937 cells polarized by gliomas CM may secrete soluble mediators that stimulate proliferation of gliomas. Co-culturing also triggered invasion of U251MG through the ECM (Fig. 5I). With the monocyte chemotaxis model, U251MG CM stimulated transwell migration THP-1 cells towards the U251MG compartment (Fig. 5J), an effect significantly inhibited when CCL2 production was depleted in U251MG; IL8/IL6 knockdown effect was minor (Fig. 5J). Note that *CCL2* is the unique CCL family member highly expressed in glioma cells in vitro (Fig. 1) whereas its receptors *CCR2* and *CCR1* were undetectable in the panel of RNA sequenced gliomas (E-MTAB-2706 dataset; data not shown). It suggests that CCL2 is likely evolved to exert paracrine functions in biology of gliomas.

Inflammatory and TAM genes associated with poor survival are upregulated in mesenchymal and downregulated in proneural/GCIMP subtypes

We next asked whether inflammatory genes are associated with altered survival rates in gliomas. In this intent, we grouped patients

by risk/survival categories (low to high-risk) and observed that only 33 out of 267 entities (Inflammatory + TAM markers) showed significantly upregulated expression in the high-risk group as evaluated in two datasets (Fig. 6A–B). These genes included interleukins (*IL6, IL8, IL1B*), CCL/CXCL family members (*CCL2, CCL20, CXCL10, CXCL11, CXCL14*), receptors (*IL10RB, CXCR4, IL13RA1, IL13RA2, OSMR and CCR1*) and others such as *MDK, LIF* and *FAS* as well as TAM markers (*CD14, CD163, TLRs and CHI3L1*) (Fig. 6B). Upregulation of this 33-gene set, rather than individual evaluation of each gene as a single factor (data not show), was associated with a median survival of ~4.7 and 23 months in the high- and low-risk groups, respectively (Fig. 6A). Pathway Signatures analysis showed that expression of high-risk related genes is higher in the Mes subtype, followed by Neural and Classical, and a significantly lower index was found in proneural and its derived subtype [33] GCIMP (Fig. 6C). In keeping with our results, *CCL2, IL6, CXCL10, IL10RB, IL13RA2, OSMR* and *CHI3L1* were already noted as related to poor prognosis in other GBM studies [34–38].

GBM dysregulated pathways promote inflammatory gene expression: involvement of NFκB-dependent STAT3 activation

We next sought to test whether some classical GBM altered mechanisms could participate in the induction of inflammatory genes. In this intent, we analyzed microarray datasets of glioma cells exposed to modulators of classical GBM dysregulated pathways such as EGF, PDGF, MEK/ERK1/2, PI3K/Akt and NFκB as available in public repositories (Fig. 7A). It was interesting to note
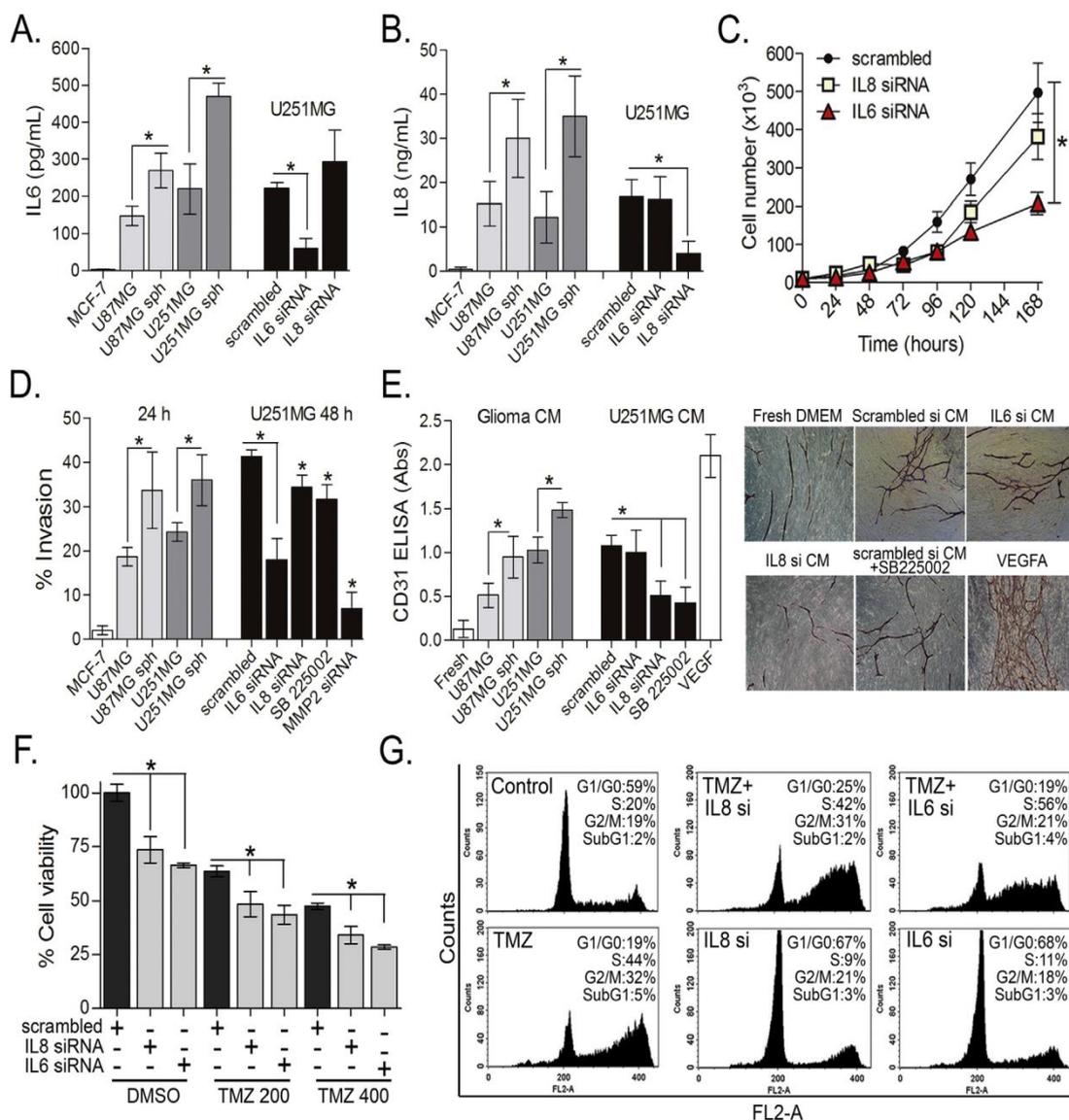
Fig. 4. Inflammatory genes cooperate to drive malignance in gliomas. (A–B) ELISA quantification of IL6 and IL8 proteins in the CM of glioma and GSC-enriched neurospheres; validation of siRNA efficiency in U251MG is also shown. (C) Impact of IL6 and IL8 depletion by siRNA on proliferation (cell counting) of U251MG cells. (D) Transwell assays showing the invasiveness potential of parental gliomas cells and its derived GSC-enriched cultures (seeded post neurospheres digestion), and the effect of IL6, IL8, MMP2 knockdown or SB225002 on U251MG invasion. MCF-7 cells were used as non-invasive controls. (E) In vitro angiogenesis assays showing the effect of CM from parental or GSC-enriched cell lines on CD31 expression in microvessel-like structures. The "fresh" group denotes non-conditioned DMEM controls. The effect CM prepared from IL6 and IL8 knocked down U251MG cells is also shown. The "SB225002" group represents the U251MG CM added of SB225002 at 15 μM. Microphotographs of CD31-stained vessel-like structures are also shown. (F) MTT cell viability and (G) Cell cycle analysis showing the effect of IL6 and IL8 depletion by siRNA on TMZ (200 μM) toxicity in U251MG cells (72 h exposure). *denotes differences at indicated comparisons (One-way ANOVA, post-hoc Tukey, $p < 0.05$; $n = 3$ in triplicate).

that PEA from an input list comprising all DEG genes (i.e. both inflammatory + unrelated) in each condition resulted in a significant enrichment in inflammation associated pathway terms (i.e., "cytokine", "chemokine" containing terms; "NOD-like receptor signaling among others) as well as JAK-STAT and MAPKs (Fig. 7A and S3 and Table S2). For instance, key genes whose function was herein studied such as IL6, IL8 and CCL2 displayed induction in EGF and PDGF-treated cells and downregulation in cells following exposure to ERK1/2 and PI3K inhibitors (UO126 and LY294002, respectively) (Fig. S3 and Table S2). Expression of the IKB super-repressor (IκB-SR) in TNF-treated glioma cells (GSE49162), or downregulation of the NFκB family member NFKB1 (GSE65363 dataset) resulted in downregulation of *IL8, IL6, CSF3, PTGS2, CCL2, LIF, CXCL2, CXCL3, IL1B and CXCL14* (Fig. S3) and their related

pathways (Fig. 7A and Table S2). Corroborating the TFE results in Fig. 3, NFκB-p65 (RELA), followed by STAT3 and NUCKS1 (Nuclear Casein Kinase and CDK substrate 1), stood out as putative transcriptional regulators of EGF, PDGF, MEK-ERK1/2 and PI3K regulated genes in these models (Fig. 7A and Table S2).

With U251MG cell lines, we first confirmed that STAT3 and NFκB are constitutively active (i.e. show nuclear localized distribution) and are capable of activating their DNA binding consensus sequences as determined by immunofluorescence and reporter gene assays, respectively (Fig. S3); and agreeing with [38–42]. Basal activation/phosphorylation of ERK1/2 and PI3K/Akt are described in our previous work with U251MG [21] and [1,11]. In keeping with microarray prediction, we used ELISA assays to confirm that IL8 and IL6 production decreased by MEK1/2-ERK1/2 and PI3K inhibitors
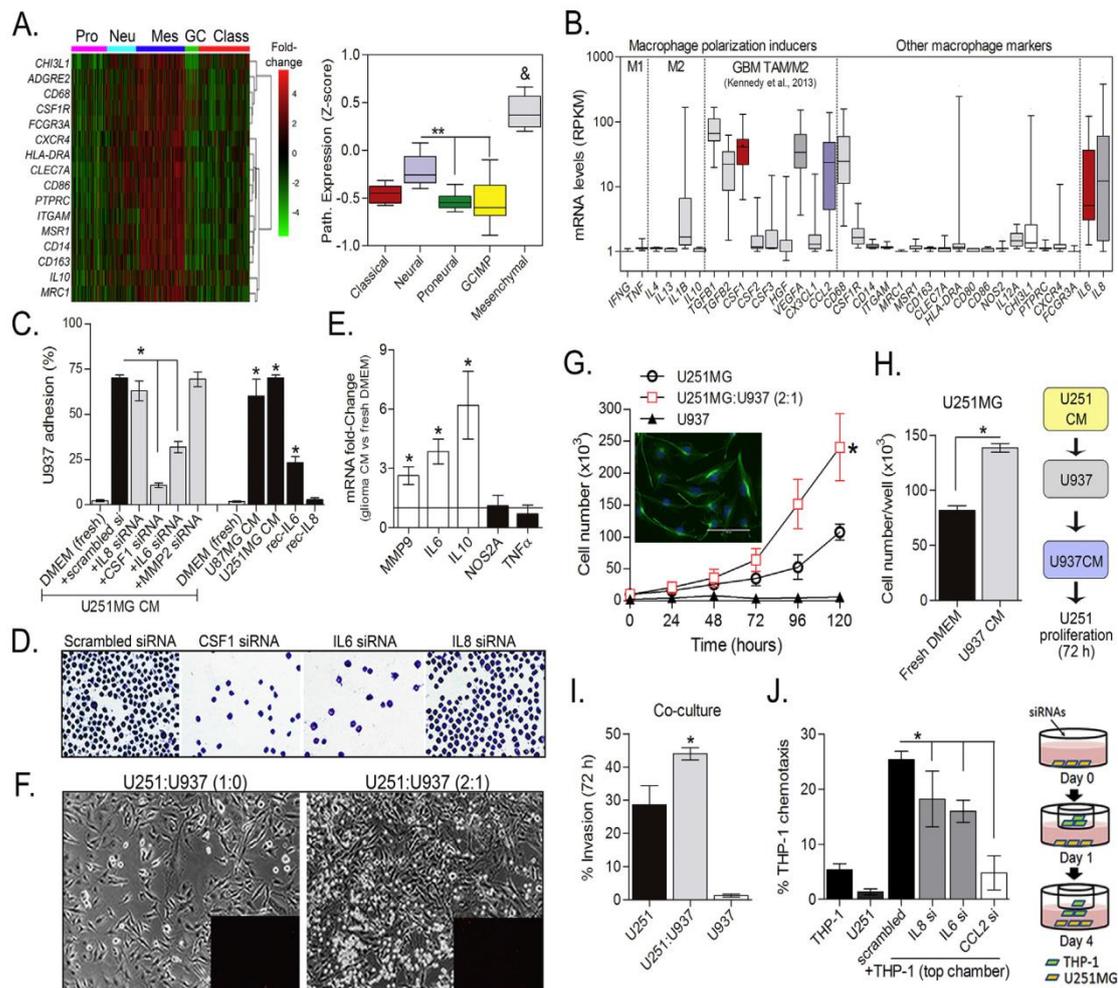
Fig. 5. TAM phenotypes across glioma subtypes, and macrophage modulation by GBM secretoma. (A) Heatmap representation and Pathway Signature expression graphs showing the relative expression of TAM markers in glioma of different histological or molecular subtypes. See Fig. 2 legend for datasets information. Also see Fig. S2 for gene expression plots of TAM markers across subtypes. (B) Basal mRNA expression (RPKM levels) of known macrophage polarization inducers and macrophage surface markers in the panel of glioma cells lines from the E-MTAB-2706 dataset. Each box comprises mRNA RPKM values of each cell line sequenced with the panel plotted as a single group. (C–D) U937 adhesion quantification (C) and representative microphotographs of adhered U937 (D) showing the effect of U251 and U87MG CM as well as CM prepared from scrambled, IL8, IL6 or CSF1 depleted (by siRNA) U251MG. The U937 cells were exposed to glioma CM for 48 h prior to adhesion assessment. (E) qRT-PCR showing mRNA fold-changes (CM-treated versus fresh DMEM-treated control) of *IL10, IL6, MMP9, TNF* and *NOS2* genes in U937 exposed to U251MG CM for 48 h. (F) Representative phase contrast microphotographs (100×) and Propidium iodide/PI staining for cell death control (insert) after 3 days of U251MG:U937 co-culturing. (G) Glioma cells proliferation rate in U251MG:U937 co-cultures as determined by detection of Nestin-positive cells (see Methods). A representative Nestin (green) immunofluorescence with DAPI is also shown. (H) Cell counting assays showing proliferation of U251MG glioma cells incubated with CM from U937 cells activated by gliomas CM. U937 cells were exposed to U251MG CM for 48 h to allow activation into macrophages-like phenotypes, and then incubated with a new fresh medium for an additional 24 h. This U937 CM was incubated with U251MG cells and cell numbers were counted after 3 days. (I) Transwell invasion assays of U251MG alone, U937 alone or U251MG:U937 co-cultures after 72 h of incubation. (J) The effect of U251MG glioma cells CM (with/out cytokine knockdown by siRNA) on chemotaxis of THP-1 cells as determined by adapted Boyden chamber assay. Legends: si (siRNA). In vitro: *Different from cells incubated with fresh RPMI/DMEM or at indicated comparisons (One-way ANOVA, post-hoc Tukey; p < 0.05; n = 3 in quadruplicate). &Different from all other groups (Kruskal−Wallis/Dunn's, p < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(UO and LY, respectively) and NFκB inhibition (p65 knockdown) as well as increased following recombinant EGF and PDGF treatment (Fig. 7B, Fig. S3 for validation of NFκB/STAT3 inhibition). We also knocked down mutant p53[R273H] by siRNA in U251MG and found no impact upon IL8 and IL6 production, indicating that described mutant p53 gain-of-function is not related to cytokine production in these cells (data not shown). On the other hand, the STAT3 inhibitor Stattic led to a ~1.5-fold increase in IL8 levels, agreeing with [43]; IL6 was not altered at the concentrations used (Fig. 7B). In addition, inhibition of NFκB or STAT3 alone decreased cell invasion (Fig. 7C) and caused long-term toxicity in U251MG cells (Fig. 7D); these effects were more pronounced with combined inhibition of STAT3 and NFκB (Fig. 7C–D). This probably occurs because NFκB-mediated IL6 production may activate STAT3 activity via IL6 receptor activation in gliomas [40]. In fact, incubation of U251MG

cells with the IKK inhibitor BAY117082 (BAY), and p65 and IL6 depletion by siRNAs, promoted partial inhibition of STAT3 reporter gene activity, indicating that STAT3 is downstream, at least in part, of the NFκB-IL6 axis (Fig. S3). MEK/ERK1/2 and PI3K inhibitors also showed anti-invasion activity (Fig. 7C) as well as inhibited glioma cells growth − although their cell viability impact was less robust than obtained with NFκB and STAT3 inhibition (Fig. 7D).

Discussion

Inflammation has been implicated in the initiation and progression of a variety of cancers [12−14]. In this study, we characterized the inflammatory gene sets altered in glioma cell lines and tumors of different molecular and histological subtypes, some of its upstream regulators, as well as identified poor survival associated
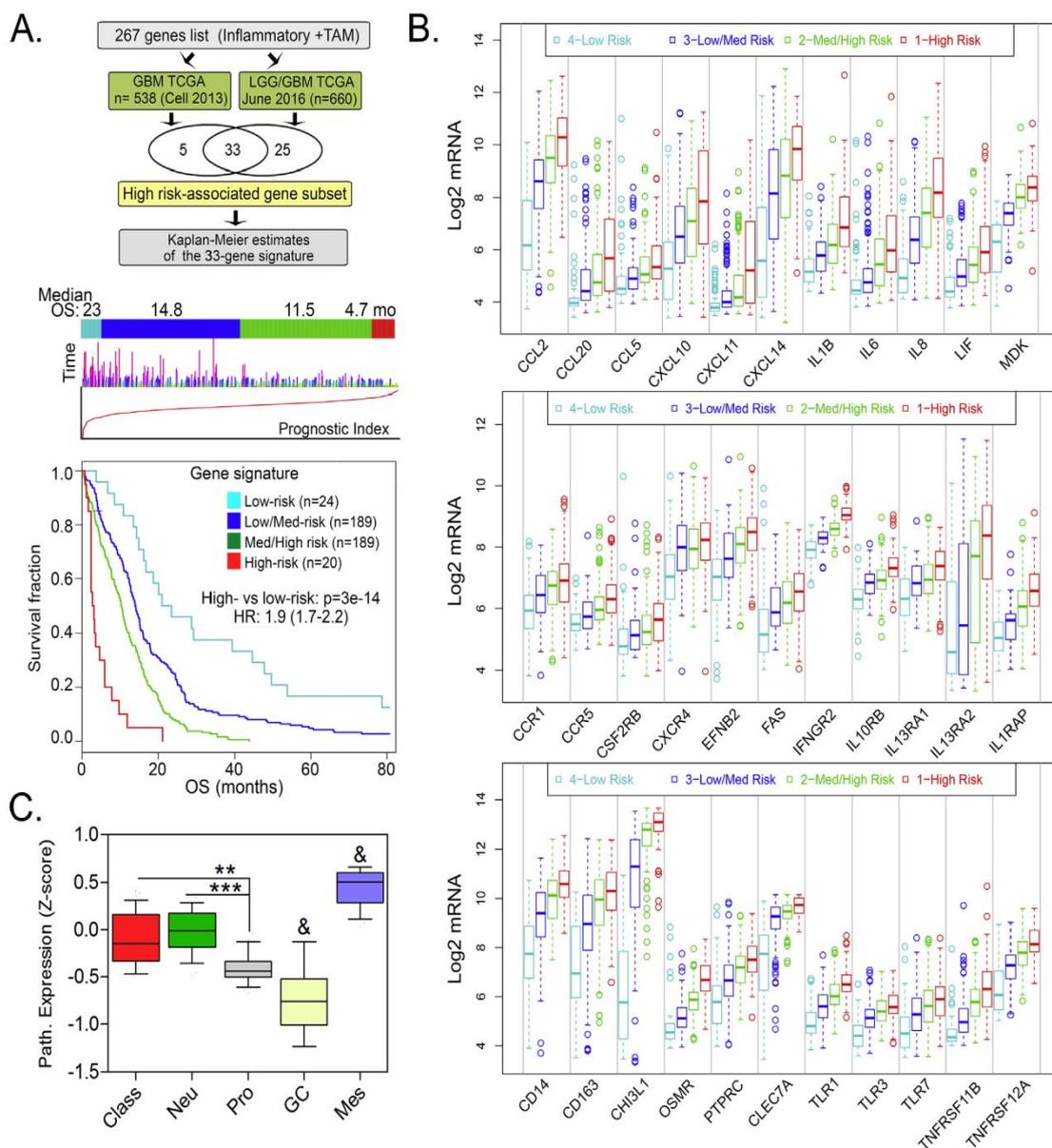
Fig. 6. Inflammatory and TAM markers associated with poor survival of GBM. (A) Patients from the "GBM TCGA" and "LGG/GBM TCGA" cohorts (see methods) were independently evaluated for the risk associated with upregulation of inflammatory and TAM genes (total: 267 genes) using the SurvExpress tool. From this initial screening, only 33 genes with differential expression in high vs low-risk groups overlapped between the 2 cohorts. These genes were used to build Kaplan−Meier plots of the 33-gene signature as shown. (B) Relative expression of the 33 risk-associated genes across risk groups as determined by SurvExpress ("GBM TCGA" dataset; Endpoint: death). All genes showed statistical significance at a p < 0.001 (One-way ANOVA, Tukey) as compared to low-risk group gene expression. (C) Pathway Signature Expression plot showing the relative expression of the 33 high-risk associated genes across molecular subtypes of GBM ("GBM TCGA" dataset). &different from all other groups (p < 0.05); asterisks denote differences at indicated comparisons (**p < 0.01; ***p < 0.001; Kruskal−Wallis, post-hoc Dunn's.

genes. We believe that the herein presented data can be not only useful to understand the differences among GBM subtypes and the biological aspects underpinning their inflammatory phenotype, but also to highlight genes and master regulators of this network which can be tested as potential targets. We found that many of the GBM tumor overexpressed genes are also constitutively expressed in glioma cell lines, indicating that part of the inflammatory machinery is intrinsic of glioma cells. Moreover, the inflammatory gene signatures displayed gains of expression and gene member enrichments from low-grade related (Astrocytoma and ODG) to GBM. Even with GBM, we observed some level of heterogeneity,

with Mes tumors showing upregulation of both cytokines and TAM signatures if compared to other subtypes. Many of these Mes upregulated genes were lost (i.e. not detected as DEG) when all GBM subtypes were evaluated as a single group, and many of them showed significant correlation with survival. Conversely, inflammatory signatures including those associated with high-risk/short survival showed downregulated expression in GCIMP samples, the less aggressive GBM subtype [33]. Correlation methods developed by Leiserson et al. [44], in consonance with TCGA studies [1], identified that EGFR, NF1 and PDGFRA are mutually exclusive genetic alterations of GBM which have strong association with GBM
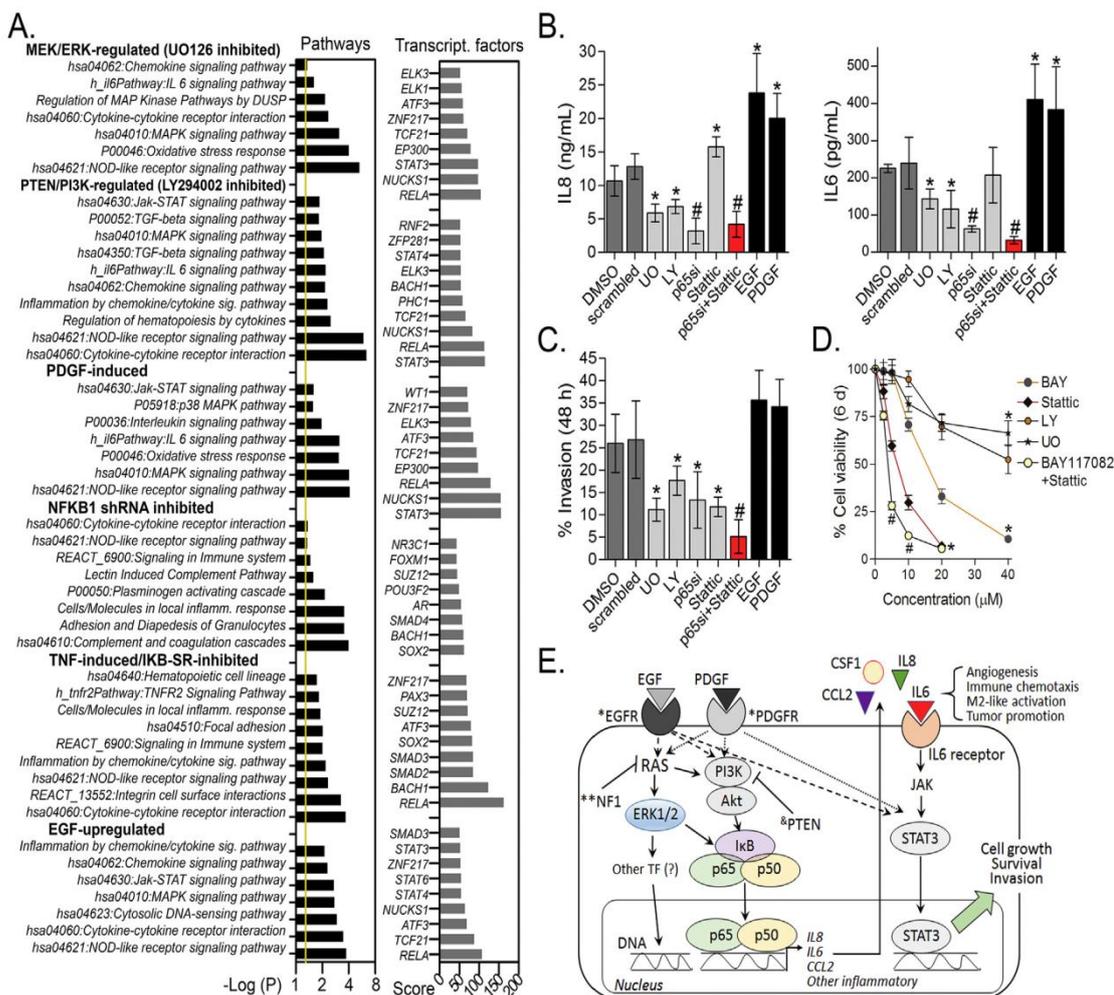
Fig. 7. **NFκB as a regulator of GBM secretoma production and STAT3 activation**. (A) PEA and TFE of genes upregulated by PDGF, EGF, MEK/ERK1/2 (UO126-inhibited genes), PTEN/PI3K/Akt (LY294002-inhibited genes) and NFκB (by means of both NFKB1 shRNA-inhibited and TNF-induced IKB repressor/IKB-SR-inhibited genes) as assessed by microarray. Datasets were downloaded and analyzed as described in Materials and methods; also see Fig. S3 and Table S2 for additional details. (B) ELISA assays showing the effect of MEK/ERK1/2, PI3K/Akt, STAT3 inhibitors (UO126, LY294002 and Stattic, respectively), NFκB-p65 knockdown (siRNA), and recombinant PDGF and EGF upon IL8 and IL6 levels in the culture medium of U251MG at 24 h post-incubation. (C) Invasion assays showing the effect of the above described inhibitors after a 48 h treatment in U251MG cells. (D) MTT assays showing the effect of the IKK/NFκB inhibitor (BAY117082), UO126, LY294002 and Stattic in U251MG treated for 6 d. *Different from vehicle/untreated; # different from either drug alone (One-way ANOVA, Tukey; p < 0.05). (E) Working model showing some of the most common genetic alterations (EGFR, PTEN, NF1 and PDGFR) of GBM and their crosstalk with ERK1/2, PI3K/Akt, NFκB and STAT3 as based on the herein presented data and references. Key: *mutated and/or amplified; **mutated; &mutated and/or deleted.

molecular subtypes: EGFR amplification is associated with Classical, PDGFRA amplification with Proneural, and NF1 mutation with Mes. In addition the PI3K negative regulator PTEN is frequently deleted or mutated in GBM [1,2]. NF1 mutation and PTEN mutation/deletion lead to RAS/MEK1/2/ERK1/2 and PI3K/Akt cascades activation, respectively, which have been described as upstream modulators of NFκB in GBM [45–47]. In addition, ligand or mutation-induced activation of EGFR and PDGFR pathways causes activation of ERK1/2, PI3K/Akt, NFκB and STAT3 in gliomas [47–49]. Taken together with Fig. 7 data, it suggests that different GBM subtypes carry non synonymous alterations capable of redundantly sustain activation of same intracellular cascades thereby promoting cytokine production by different means. Even though it may explain why GBM are more inflammatory if compared to astrocytoma and ODG, the mechanisms underpining the inflammatory enrichment observed with Mes remains undefined.

Our in vitro assays showed that glioma cells take advantage of their diversified secretoma components to orchestrate different malignant phenotypes. While some genes prompted cell growth and invasion via autocrine mechanisms (i.e. IL6), others were more

committed to angiogenesis (IL8) and macrophage chemotaxis/M2-like/TAM activation (CCL2 and CSF1) in a paracrine manner. This paracrine mechanism correlates with the lack/low expression of IL8 [15,16,30] and CCL2 receptors (CCR2) in glioma cell lines, agreeing with [50]. Zhang et al. also showed that IL8 and CCL2 are highly expressed in glioma cells and co-culturing of gliomas with microglia led to a CCL2-dependent IL6 production by microglial cells, which promoted GBM invasiveness; an effect emulated by directly incubating recombinant IL6 with glioma cells [50]. It is also key to note that there are intrinsic fluctuations in the mRNA expression of inflammatory genes across the cell lines panel shown in Fig. 1, suggesting that the same molecular heterogeneity observed in human GBM tumors/subtypes may also exist in the cell lines used in vitro.

There is consistent evidence that IL8 [16,29,51], IL6 [15,52,53], FAS [53,54], CSF1 [55,56], midkine/MDK [57], CCL20 [58], OSM/OSMR [38] and other genes identified herein are potentially relevant as individual targets for drug development. On the other hand, TFE analysis indicated that NFκB - and STAT3 with a lesser number of targets – are putative transcriptional regulators of the

inflammatory repertoire of GBM, being especially enriched in Mes. STAT3 activation was mediated in part via an NFκB-dependent IL6 autocrine loop (agreeing with [40]), and STAT3 inhibitor emulated the anti-invasive and growth inhibitory effects of IL6 knockdown. In addition to IL6 [42], the Oncostatin-M receptor OSMR – which was found enriched with Mes and correlated with poor survival (Figs. 2–3) - may also contribute to STAT3 activation and Mes differentiation in GBM [38]. Corroborating, Carro et al. identified STAT3 and CEBPB as two master regulators of the Mes transformation of GBM [59]. In the NFκB side, proneural glioma stem cells treated with TNF-α undergo differentiation to Mes phenotype in an NFκB-dependent/IκB-SR-regulated manner via downstream activation of STAT3, CEBPB and TAZ leading to radiation resistance [60]. Thus, while it is not novel that blocking NFκB and STAT3 promotes growth inhibition and anti-invasive effects in glioma cell lines [41,61–67], this augmented NFκB/IL6/STAT3 axis associated with Mes subtype may be relevant to targeted therapies.

With our experiments, rebound inductions of NFκB and IL8 were observed with Stattic treatment alone (Fig. S3). Activated STAT3 may occupy IL8 promoter to directly repress transcription, particularly in PTEN deficient gliomas [43]. In addition, STAT3 is also capable of sequestering NFκB within the cytoplasm, thereby inhibiting IL8-mediated tumor progression in KRAS mutant lung cancers [68]. Hence, NFκB-dependent pro-inflammatory activation may be an off-target effect of STAT3 inhibition. Our results showed that combined NFκB and STAT3 inhibitors overcame this off-target effect of STAT3 inhibitor alone, maintaining low IL6 and IL8 levels and enhancing anti-invasive and cytotoxic effects if compared to single agents. An elegant study from McFarland et al. verified that combined NFκB and STAT3 inhibition, (with Withaferin A and AZD1480, respectively) was more efficient than single agent treatment in vitro and in vivo models of gliomas [40]. In addition, mice brain implanted gliomas show increased NFκB activity, and depletion of IKK2 or expression of IκB-SR prolonged animal survival [67]. Despite these positive in vitro and pre-clinical evidences, it is important to take into account that the balance between the role of NFκB/STAT3 in immune system and their pro-tumoral function will dictate the antitumor efficacy when translating NFκB/STAT3 inhibitors into clinical settings. Based on the aforementioned, our future studies will test the relevance of the herein identified genesets and processes as therapeutic targets in preclinical models of glioma.

Acknowledgements

Conflicts of interest

The authors declare that no conflict of interest exists.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.canlet.2016.12.015.

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