UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

DEPARTAMENTO DE BIOQUÍMICA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊCIAS BIOLÓGICAS: BIOQUÍMICA

O papel do fator de transcrição STAT3 na quebra da resistência a quimioterápicos em células de glioblastoma

André Simões Pires

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SUMÁRIO

Parte I

RESUMO

Glioblastoma é um tumor cerebral que apresenta uma alta taxa de invasão, sendo que os pacientes portadores do mesmo possuem uma baixa sobrevida. Um dos maiores desafios encontrados é a resistência do tumor ao principal agente quimioterápico utilizado, a temozolomida, além de apresentar um fenótipo de resistência cruzada a outros quimioterápicos tradicionais como a doxorrubicina. Diversos estudos tem mostrado uma ativação contínua do fator de transcrição Transdutor de sinais e ativador de transcrição 3 (STAT3) em glioblastomas resistentes a quimioterapia. Neste trabalho nós examinamos se células de glioma de rato da linhagem C6 que se tornaram resistentes aos quimioterápicos doxorrubicina e/ou temozolamida, apresentam um conteúdo elevado de STAT3 fosforilada (p-STAT3) e se a inibição deste fenômeno pelo inibidor específico de JAK2/STAT3 AG490, poderia sensibilizar novamente estas células a quimioterápicos. Nós observamos que nossas linhagens resistentes apresentaram um IC50 para temozolamida 11 vezes maior do que a linhagem paterna e 33 vezes maior para doxorrubicina em comparação com a linhagem paterna. Além disto, ambas as linhagens resistentes apresentaram um imunoconteúdo elevado de STAT3. A inibição de STAT3 pelo inibidor de JAK2, AG490, apresentou um decréscimo no imunoconteúdo de p-STAT3, uma parada no ciclo celular na fase G2/M e uma consequente ressensibilização das linhagens resistentes aos seus quimioterápicos. Em suma, os dados aqui apresentados nos levam a acreditar que a inibição de STAT3 poderia ser uma interessante terapia adjuvante a ser estudada para a ressensibilização de pacientes com glioblastoma à quimioterapia.

ABSTRACT

Glioblastoma is a brain tumor whit high invasiveness and low survival. One of the main characteristics of the tumor is the resistance to the standard chemotherapy to the alkylating agent temozolomide, and the multidrug resistance to other traditional chemotherapeutics like doxorubicin. Signal Transducer and Activator of Transcription 3 (STAT3) has been shown to be constitutive active in glioblastomas resistant to chemotherapy. In this work we examined if C6 cells that became resistant to temozolomide and/or doxorubicin have increased STAT3 phosphorylation (p-STAT3) and if the inhibition of this phosphorylation could re-sensitize these cells. We observed that our two resistant lineages showed higher IC50 for TMZ and DOX in order of 11 and 33 fold higher respectively, and elevated p-STAT3 immunocontent when compared to their parental C6 lineage. The inhibition of p-STAT3 by AG490 50 μM decreased the p-STAT3 immunocontent, induced a G2/M arrest in cell cycle and re-sensitizes the resistant C6-TR and C6-DR to their chemotherapeutics showing a synergetic effect with both chemotherapeutics used in this work. In summary, data present here suggest that the inhibition of STAT3 could be an interesting adjuvant therapy for glioblastoma cells that became resistant to the traditional chemotherapy.

Lista de Abreviaturas:

BCL2: proteína 2 de linfoma de células B (B-cell lynphoma protein 2)

Bcl-X_L: proteína de isoforma longa relacionada a Bcl2 (Bcl2 related protein long isoform)

C6-DR: linhagem C6 resistente a doxorrubicina

C6-TR: linhagem C6 resistente a temozolomida

CDKs: cinases dependentes de ciclina (cycline dependent kinases)

DOX: doxorrubicina

EGFR: receptor do fator de crescimento endotelial (endothelial growth factor receptor)

ERK: cinase regulada por sinais extracelulares (extracelular regulated cinase)

GBM: glioblastoma multiforme

IL-6: interleucina 6 (interleukin 6)

JAK: cinase Janus ativada (Janus activated cinase)

MGMT: O6-Metilguanina-DNA metiltransferase (O6-Metilguanine-DNA methiltransferase)

NFkb: fator nuclear kappa b (nuclear fator kappa b)

PIAS: proteínas inibidoras de STAT ativada (protein inhibitors of activated STATs)

SOCS: supressores de sinalização por citocinas (signaling of citokines supressor)

STAT3: transdutor de sinais e ativador de transcrição 3 (Signal Transducer and Activator of Transcription)

TMZ: temozolamida

VEGF: fator de crescimento vascular endotelial (vascular endotelial growth fator)

1. INTRODUÇÃO

1.1. Glioblastomas

Glioblastoma multiforme (GBM) é o tumor maligno primário mais frequentemente encontrado em adultos e é classificado como um astrocitoma de grau IV segundo a Organização Mundial da Saúde, classificação dada para tumores particularmente agressivos (Singh et al., 2004). A maior parte dos pacientes diagnosticados com GBM morre no período de 12 a 14 meses a partir do diagnóstico e apenas 5% do total de pacientes diagnosticados consegue sobreviver por mais de 5 anos, porém as terapias utilizadas para prolongar essa sobrevida são extremamente agressivas (CBTRUS., 2011). Durante toda a década passada, uma imensa variedade de tratamentos para GBM vem sendo explorada, porém os sucessos obtidos têm sido relativamente limitados. Esta limitação se deve principalmente aos maiores desafios que são encontrados durante o tratamento, e entre estes desafios nós podemos citar o local onde o tumor é formado e encontrado e principalmente a natureza complexa e heterogênea do mesmo (Kesari, 2011). A maioria dos tratamentos utilizados não consegue alcançar todas as células tumorais e a cirurgia tem se mostrado inadequada, já que o tumor é difuso por natureza e é muito difícil e dispendioso remove-lô completamente sem danificar as regiões sadias do cérebro entorno da massa tumoral (Stummer et al., 2008). O tratamento baseado em quimioterapia também possui sérias limitações, já que muitos quimioterápicos que estão disponíveis não possuem a capacidade de atravessar a barreira hematoencefálica e como consequência disto a disponibilidade do respectivo quimioterápico é altamente prejudicada no local onde o tumor se concentra. Além disto, tem se mostrado que os agentes quimioterápicos comumente utilizados em GBM se acumulam, em baixas concentrações, nos tecidos adjacentes ao tumor e

diversos pacientes tem apresentado GBM resistentes a terapia (Ostermann et al., 2004; Portnow et al., 2009).

Os aspectos genéticos e moleculares da patologia também tem sido alvo frequente de estudo por parte da comunidade científica, o que têm levado a descoberta de novos alvos terapêuticos para o combate ao GBM. Alvos como VEGF (Fator de crescimento vascular endotelial) e EGFR (Receptor do fator de crescimento epidérmico) foram considerados promissores em teste *in vitro*, porém infelizmente os resultados encontrados em estudos pré-clinicos não se mostraram efetivos como era o esperado (Van Meir et al., 2010). Outro alvo que tem sido apontado como promissor no combate a GBM é o transdutor de sinais e ativador de transcrição 3, (STAT3). A ativação contínua da STAT3 tem sido demonstrada como presente em GBM (Abou-Ghazal et al., 2008) e mais recentemente tem sido associada com a resistência que este tumor tem apresentado aos quimioterápicos usualmente utilizados no seu tratamento (Kohsaka et al., 2012; Vlachostergios et all., 2013). Pelos motivos apresentados o fator de transcrição STAT3 parece ser um propício alvo no que se refere ao combate da resistência a quimioterápicos encontrada em GBM e possível re-sensibilização dos pacientes aos mesmos.

1.2. Transdutor de sinais e ativador de transcrição 3 (STAT3)

STAT3 é um dos membros de uma vasta família de fatores de transcrição. O primeiro relato de sua identificação que aparece na literatura data de 1994, em um trabalho que o identifica como um fator ligante de DNA que se liga a um promotor de genes de fase aguda em hepatócitos que foram estimulados com interleucina 6 (IL-6) (Akira el al., 1994). O gene responsável por codificar a síntese de STAT3 se localiza no cromossomo 17q21. O fator de transcrição STAT3 é uma proteína com

peso de 92 quilodaltons e é composta, estruturalmente, de 770 aminoácidos com um domínio N-terminal composto pela estrutura supersecundária conhecida como ‘’Coiled-Coil’’ (duas alfa hélices paralelas), um domínio de ligação ao DNA, um domínio SH2 e um domínio C-terminal responsável pela transativação do fator. No domínio C-terminal é que se encontram os resíduos de tirosina e serina, localizados, respectivamente, nas posições 705 e 727. Quando estes resíduos sofrem fosforilação, STAT3 é ativa e translocada para o núcleo da célula (Aggarwal et al., 2009). Os sinais que disparam a ativação da STAT3 podem ser os mais variáveis possíveis, porém alguns são considerados clássicos por serem observados como ativadores de STAT3 mais usualmente. Como ativadores clássicos nós podemos citar o fator de crescimento epidermico (Cao et al., 1996) e IL-6 (Giordano et al., 1997). A ativação de STAT3 é regulada principalmente por tirosinas cinases, responsáveis por fosforilar a proteína no resíduo tirosina 705. Entre as principais cinases responsáveis pela sua ativação podemos citar a EGFR cinase (Garcia et al., 1997), as cinases Janus ativadas 1 e 2 (JAK) (Tian et al., 1996) e a cinase regulada por sinais extracelulares (ERK) (Megeney et al., 1996). A fosforilação, que ocorre no citosol, leva a dimerização da STAT3, com a subsequente translocação para o núcleo da célula, a ligação do fator de transcrição ao DNA e por fim a transcrição de genes responsáveis por funções de grande importância para a célula como: proliferação, diferenciação e apoptose (Brantley e Benveniste, 2008).

STAT3 também é negativamente regulada por uma ampla série de mecanismos inibitórios, onde podemos citar dois mecanismos como os principais: os supressores de sinalização por citocinas (SOCS) e as proteínas inibidoras de STAT ativada (PIAS). As proteínas denominadas SOCS são uma família de proteínas com 8 membros diferentes identificados até o presente momento (Fuh et al., 2009). O

método de inibição das SOCS é a ligação das mesmas ao domínio SH2 das JAK, inibindo a sua atividade e consequentemente bloqueando a sinalização da mesma, que leva a fosforilação e ativação do fator STAT3 (Fuh er al., 2009). Já em contraste com o método de inibição apresentado pelas SOCS, as PIAS são uma família de genes e fatores de transcrição nucleares. O principal PIAS conhecido que está envolvido na sinalização negativa de STAT3 é o PIAS-3, que se associa com a STAT3 forforilada já no núcleo e consegue bloquear a transcrição dos genes mediada por STAT3 (Chung et al., 1997).

Diversos estudos demonstraram que STAT3 aparece constitutivamente ativa em GBM, tanto em biópsias de pacientes quanto nas linhagens de células. Essa ativação constitutiva foi verificada via comparação do estado de fosforilação da STAT3 de células de GBM com astrócitos de pacientes sadios e tecidos adjacentes ao tumor (Mizoguchi et al., 2006; Rahaman et al., 2002). Além disto, outros estudos já demonstraram que a quantidade de STAT3 fosforilada presente na célula cancerosa se correlaciona diretamente com o grau do tumor, especialmente quando se comparam tumores de graus mais baixos (I e II) com tumores de graus mais altos (III e IV) (Lo et al., 2008). Em uma corte de pacientes que apresentaram GBM, 66% das amostras possuíam STAT3 constitutivamente ativa, já em pacientes com tumores cerebrais de graus I e II somente 27% e 29% das amostras, respectivamente, possuíam esta ativação constitutiva de STAT3 (Lo et al., 2008). Outro estudo demonstrou que 83% dos pacientes analisados com GBM possuíam coloração positiva para p-STAT3 e apesar deste estudo não comparar a fosforilação de STAT3 com o grau do tumor, ele também mostra que a fosforilação da mesma se apresentou aumentada em oito de quatorze pacientes que voltaram a ter ocorrência de GBM depois da quimioterapia com TMZ, sugerindo que STAT3 e a resistência do

tumor a quimioterapia poderiam estar intimamente relacionadas (Kohsaka et al., 2012). Nesse contexto a inibição do fator STAT3 pode ser uma alternativa possível para que se atenue a resistência que o GBM apresenta a quimioterapia, já que pesquisas sugerem que o fator de transcrição não aparenta ser essencial para a manutenção das funções básicas de uma célula sadia, porém a sua ativação aparenta ser indispensável para uma vasta gama de células cancerígenas (Schlessinger e Levy., 2005).

1.3. Quimioterápicos no combate a glioblastomas

Diversos quimioterápicos já foram utilizados no combate a GBM com variadas taxas de sucesso. O principal quimioterápico utilizado no tratamento a este tipo de tumor é o TMZ. TMZ, ou temodal como é comercialmente conhecido, é um agente alquilante que normalmente é administrado por via oral. Após sua absorção, a TMZ é convertida em um cátion metildiazonio, com propriedades alquilantes. Este cátion tem a capacidade de alquilar o DNA levando a uma quebra da dupla fita, e danificando assim a célula cancerosa (Villano, Seery e Bressler, 2009). Porém as dificuldades apresentadas no tratamento com TMZ são advindas, principalmente, da atuação da enzima O6-metilguanina-DNA metiltransferase (MGMT), que remove a metilação encontrada na posição O6 da guanina e com isso se torna a principal indutora de resistência a TMZ presente em GBM (Kokkinakis et al., 2003). Outro quimioterápico que tem despertado o interesse de pesquisadores no tratamento de GBM é a nanocápsula de DOX. DOX é um antibiótico da classe das antraciclinas, que tem sido comumente usado no tratamento de diversos tumores. Porém a forma de nanocápsula lipossômica tem demonstrado uma utilidade clínica limitada e sua utilização tem sido focada, principalmente, no tratamento do câncer de mama e de mieloma múltiplo (Malam, Loizidou e Seifalian, 2009). Além disto, em estudos

passados, as nanocápsulas lipossômicas de DOX se mostraram ineficazes em superarem o fenótipo de resistência a múltiplas drogas apresentado por algumas linhagens de GBM (Hu, Henry-Toulme e Robert, 1995). Em um estudo mais recente, os autores demonstraram que nanopartículas superparamagnéticas de óxido de ferro combinadas com DOX aumentaram a disponibilidade de DOX na região do tumor, diminuíram a viabilidade celular na linhagem de glioma de rato C6 e mais importante, conseguiram contornar o fenótipo de resistência a drogas que foi induzido pela exposição destas células a DOX, tornando a DOX novamente um fármaco de interesse para o estudo de quimioterápicos a serem utilizados no tratamento a GBM (Kievit et al., 2011).

1.4. Inibidores de STAT3

Existem, atualmente, diversas moléculas inibidoras do fator de transcrição STAT3, já que o mesmo tem sido apontado como uma espécie de via central que regula diversas vias de sobrevivência em tumores. Por isso os inibidores têm demonstrado resultados promissores em estudos pré-clinicos, principalmente os que atuam no eixo JAK/STAT3. Um conhecido inibidor é o JSI-124, que demonstrou ter aumentado a taxa de apoptose e diminuído a proliferação em diversas linhagens de GBM (Lo et al., 2008). A inibição de STAT3 advinda de JSI-124 também se mostrou eficaz em sensibilizar essas linhagens a uma vasta gama de quimioterápicos como cisplatina e bis-cloroetilnirourеia (Lo et al., 2008). Outro inibidor que tem sido alvo de estudos é o AZD1480, que demonstrou uma excelente eficácia inibindo o crescimento de GBM tanto em cultura de células quanto em um modelo animal de GBM. Essa eficácia adveio da sua capacidade de inibir a fosforilação de STAT3 pela JAK (McFarland et al., 2011). Por fim, o inibidor específico de JAK2/STAT3 AG490 tem sido avaliado em diversos tipos de tumores, incluindo o GBM. O AG490 reduziu

de forma significante a proliferação celular, migração e capacidade de invasão de diversas linhagens de GBM em cultura de células (Senft et al., 2011). Além disso, o AG490 se demonstrou eficaz na inibição do crescimento da massa tumoral em estudos *in vivo* com pacientes portadores de GBM (Yang et al., 2012) e seguro para ser utilizado já que a inibição de JAK2/STAT3 demonstrou baixa citotoxicidade em células sadias (Rahaman et al., 2002). Estes estudos tem demonstrado que o AG490 pode ser um potencial fármaco no tratamento ao GBM. Apesar de todos dados pré-clinicos já existentes no que tange ao uso de inibidores de STAT3 para o tratamento de GBM, o uso terapêutico destes inibidores continua limitado, e poucos trabalhos demonstram a capacidade destes inibidores em superar a resistência que as células de GBM adquirem a quimioterapia.

1.5. A linhagem C6

A linhagem de GBM C6 foi desenvolvida no final da década de 1960 em um modelo de ratos Wistar, com o intuito de ser utilizada tanto *in vitro* como em modelos animais *in vivo* (Benda et al., 1968). A linhagem possui diversas características histopatológicas que se assemelham as características apresentadas em GBM humano. Entre essas características podemos citar o padrão invasivo da linhagem quando implantada em cérebros de ratos Wistar, que é semelhante ao padrão invasivo de GBM encontrado em seres humanos (Chicoine e Silbergeld, 1995). Em nível celular e histopatológico a linhagem apresenta áreas de necrose, polimorfismo nuclear e altas taxas mitóticas (Auer, Del Maestro e Anderson, 1981), além de apresentar alguns marcadores similares aos apresentados por GBM humano como a proteína S100B (Pfeiffer et al., 1970). A linhagem C6 também possui semelhanças genéticas com GBM humanos, principalmente no gene p16, que se encontra mutado em altas taxas em pacientes com GBM, e também na linhagem C6 (Furnari et al.,

2007). Mais recentemente a linhagem C6 foi utilizada para estudos envolvendo a inibição de STAT3 por compostos naturais, se mostrando também um bom modelo para ser utilizado envolvendo pesquisas com o fator de transcrição citado (Tang et al., 2010). Por todas as semelhanças apresentadas com GBM humano, sua ampla caracterização na literatura e os estudos envolvendo inibição de STAT3, a linhagem C6 foi nosso modelo de escolha para este trabalho.

1.6. Justificativa

Uma vez que STAT3 parece estar super estimulada em pacientes com glioblastoma que apresentam resistência à quimioterapia tradicional (Kohsaka et al., 2012), nossa hipótese é que a inibição deste fator de transcrição pela via JAK2/STAT3 poderia ser uma alternativa para que estes tumores fossem re-ssensibilazados à quimioterapia disponível. Um ponto importante a ser considerado na inibição deste fator em questão é a sua expressão aberrante em glioblastomas quando comparamos com a expressão do mesmo em células sadias, o que, teoricamente, proporcionaria uma possível seletividade do fármaco inibidor utilizado para as células neoplásicas quando comparadas aos tecidos normais (Lo et al., 2008; Mizoguchi et al., 2006). A seletividade é um fator importante quando consideramos os compostos antitumorais, uma vez que isso preveniria os possíveis efeitos adversos aos tecidos sadios. Portanto, essas observações qualificam a inibição do fator de transcrição STAT3 como um possível alvo na ressensibilização de GBM à quimioterapia e um potencial objeto de estudos para que terapias adjuvantes possam ser desenvolvidas.

2. OBJETIVOS

2.1. Objetivo Geral

Tendo em vista os dados apresentados na introdução deste trabalho o mesmo teve como objetivo determinar se a inibição do fator de transcrição STAT3 poderia ser uma alternativa de terapia adjuvante para a ressensibilização de gliomas aos quimioterápicos TMZ e DOX.

2.2. Objetivos Específicos

- Desenvolver uma linhagem de células C6 resistentes a temozolomida (C6-TR); uma linhagem de células C6 resistentes a doxorrubicina (C6-DR) e avaliar os seus respectivos IC50.

- Avaliar o imunoconteudo de p-STAT3 na linhagem C6 paternal e nas resistentes, e verificar se o mesmo se encontra aumentado nas células que apresentam resistência a quimioterápicos.

- Determinar se a inibição de p-STAT3 pelo fármaco AG490 poderia levar a re-ssensibilização das células resistentes aos quimioterápicos utilizados.

Parte II

3. MANUSCRITO: **STAT3 inhibition by AG490 overcomes C6 rat glioblastoma cells induced resistance to temozolomide and doxorubicin**

A ser submetido ao periódico: Toxicology in vitro.

STAT3 inhibition by AG490 overcomes C6 rat glioblastoma cells induced resistance to temozolomide and doxorubicin

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Abbreviations: C6-DR, C6 resistant to doxorrubicin; C6-TR, C6 resistant to temozolomide; CDK, cyclin dependent kinase; DOX, doxorubicin; EGFR, endothelial growth factor receptor; IL-6, interleukin 6; JAK, janus activated kinase; STAT3, Signal Transducer and Activator of Transcription 3; TMZ, temozolomide; VEGF, vascular endothelial growth factor.

ABSTRACT

Glioblastoma is a brain tumor with high invasiveness and low survival. One of the main characteristics of the tumor is the resistance to the standard chemotherapy to the alkylating agent temozolomide and the multidrug resistance to other traditional chemotherapeutics like soxorubicin. Signal Transducer and Activator of Transcription 3 (STAT3) has been shown to be constitutive active in glioblastomas resistant to chemotherapy. In this work we examined if C6 cells that became resistant to temozolomide (C6-TR) and/or doxorubicin (C6-DR) have increased STAT3 phosphorylation (p-STAT3) and if the inhibition of this phenomenom by JAK2/STAT3 inhibitor AG490 could re-sensitize these cells. We observed that our two resistant lineages showed higher IC50 for temozolomide and doxorubicin in order of 11 and 33 fold increases respectively, and elevated immunocontent of p-STAT3 when compared to their parental C6 lineage. The inhibition of p-STAT3 by AG490, a JAK2 inhibitor, decreased p-STAT3 immunocontent, induced G2/M arrest in cell cycle and re-sensitized the resistant C6-TR and C6-DR to chemotherapeutics. In summary, the herein present data suggest that the inhibition of STAT3 could be an interesting adjuvant therapy to treat glioblastoma that became resistant to the traditional chemotherapy.

Keywords: Glioblastoma, resistance, STAT3, AG 490, re-sensitize, C6 cells

1. INTRODUCTION

Glioblastoma (GBM) is the most frequent primary brain tumor and has a classification according to the world health organization as a grade IV tumor. The standard therapy consists of surgical resection, external beam radiation and chemotherapy (Singh et al.,2004). Due to their high invasiveness, tumor heterogeneity, the inability of treatments to effectively reach all tumor cells, the limited drug delivery across the blood-brain barrier and the high likelihood of relapse most of the patients die within two years (Köritzer et al.,2013). The genetics and molecular pathology of GBM has been highly studied, leading to new therapeutic targets like VEGF (Vascular endothelial growth factor) and EGFR (Endothelial growth factor receptor) (Van Meir et al.,2010), but the undergone clinical trials did not confirm the promising results pinpointed from preclinical sets (Van Meir et al., 2010).

Thus far, temozolomide (TMZ), an oral alkylating chemotherapeutic is considered the first line drug in GBM treatment. TMZ is absorbed and converted to an alkylating methyldiazonium cation that damage DNA thereby leading to DNA double strand breaks (Villano et al., 2009). However GBM may mitigate TMZ efficacy by overexpressing the enzime O6-methylguanine- DNA methyltransferase (MGMT), which removes methyl groups from O6 position of guanine leading to resistant phenotypes (Kokkinakis et al., 2003). Another chemotherapeutic that has been used is the liposome encapsulated doxorubicin (DOX), which is an anthracycline antibiotic commonly used in cancer chemotherapy. However, these lipid-encapsulated anthracyclines have limited clinical utility being primarily used in the treatment of breast cancer, AIDS-related Kaposi sarcoma and multiple myeloma (Malam et al., 2009). Noteworthy, in an early study, liposome encapsulated DOX failed to overcome multidrug resistance phenotype in glioma cells (Hu et al., 1995). On the other hand,

novel superparamagnetic iron oxide DOX nanoparticles formulations have been shown to be effective in overcome multidrug resistance in C6 rat glioma cells (Kievit et al., 2011).

Aberrant activation of Signal Transducer and Activator of Transcription (STAT3) has been identified in GBM as well as in a number of others humans cancers (Dasgupta et al., 2009; Yu and Jove.,2004). STAT3 is a member of the Stat family of cytoplasmic transcription factors that are activated by many cytokine and growth factor receptors and downstream substrates (Bromberg and Darnell Jr.,1999). The activation of STAT3 is regulated by phosphorylation of tyrosine 705 by receptor and nonreceptor protein tyrosine kinase, and the main agent that phosphorylate STAT3 is Janus activated kinase (JAK) 1 and 2 (Migone et al.,1995). The phosphorylation of STAT3 in the cytoplasm leads to its dimerization, translocation into the nucleus, and DNA binding; as a result it activates the expression of genes that regulate cell proliferation, differentiation, and apoptosis (Brantley and Benveniste., 2008). Persistent activation of STAT3 is correlate with the malignancy of tumor (Lo et al., 2008) and more recently the constitutive activation of STAT3 was associated with resistance to chemotherapeutics in T98G and U87 GBM cell lines (Kohsaka et al.,2012; Vlachostergios et al., 2013). STAT3 has been considered an potential target for therapeutic intervention in various neoplasms (Yu and Jove, 2009). Its suitability as a therapeutic target is further supported by the fact that STAT3 does not seem to be essential for the survival of untransformed cells, but it is indispensable for many different tumor cells (Schlessinger and Levy., 2005). These evidences showed the importance of STAT3 in the control of survival and proliferation mechanisms of different tumor cells and that its inhibition can lead to promising new adjuvant therapies in GBM.

Based on the aforementioned, the aim of this study is to investigate if C6 rat glioma cells resistant to the chemotherapeutics DOX and/or TMZ have increased phosphorylated STAT3

(p-STAT3), and if the STAT3 pathway inhibition can restore cell sensitivity to chemotherapies.

2. MATERIALS AND METHODS

2.1 Reagents

tyrphostin AG490, doxorubicin-HCl, temozolomide, Hepes, EDTA, trypsin, 3-(4,5-dimethyl)-2,5- diphenyltetrazolium bromide (MTT), Nonidet -P40, spermin tetrahydrochloride, RNAse A from Sigma Chemical Co. (St. Louis, MO, USA). Phospho-Stat3 (Tyr705) and Stat3 antibodies were from Cell Signaling Technology (Boston, MA, USA). Immunoblot reagents were from Bio-Rad Laboratories (Hercules, CA, USA).

2.2 Cell cultures

The rat (C6) malignant GBM cell line was obtained from American Type Culture Collection (Rockville, MD, USA). The cells were grown and maintained in low-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Carlsbad, CA, USA) containing 0.1% Fungizone and 100 U/L gentamicin and supplemented with 10% fetal bovine serum (FBS). Cells were kept at 37°C in a humidified atmosphere with 5% CO2. The C6 control cells were used at low passages to keep the GBM phenotype.

2.3 Drug resistant cell line selection

Rat glioma C6 cells were exposed to different drug concentrations for extended periods to develop the resistance. Dox resistant C6 cells (C6-DR) were developed by exposing C6 cells to increasing doses of DOX (0.235, 0.470, 0.940, 1.88, 3.76, 7.52 μM) for 72 hours, followed by 3-5 days of recovery before exposing to the next concentration. TMZ resistant C6 cells (C6-TR) were exposed to increasing doses of TMZ (62.5, 125, 250, 500, 1000 and 2000 μM) for 72 hours, followed by 4-5 days of recovery before exposing to the next concentration.

2.4 MTT cell viability assay and cell morphology

Dehydrogenase-dependent MTT reduction (MTT assay) was used as an estimate of cell viability (Zanotto-Filho et al., 2010). Cells were plated in 96-well plates (10^4/well) and treated at 50% –60% confluences. The time of incubation in all treatments was 72 hours. At the end of incubation MTT assay was performed. Cell morphology was evaluated by light microscopy (Nikon Eclipse TE 300). The IC50 was calculated and plotted using Graph Prism Software, San Diego, CA, USA.

2.5 Western blotting

Proteins (60 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 10% (wt/vol) acrylamide and 0.275% (wt/vol) bisacryl amide gels and electrotransferred onto nitrocellulose membranes. Membranes were incubated in TBS-T [20 mmol/L Tris – HCl, pH 7.5, 137 mmol/L NaCl, 0.05% (vol/vol) Tween 20] containing 1% (wt/vol) nonfat milk powder for 1 h at room temperature. Subsequently, the membranes were incubated for 12 h with the appropriate primary antibody (dilution range 1:500 –1:1000), rinsed with TBS-T and exposed to horseradish-peroxidase-linked anti-IgG antibodies for 2 h at room temperature. Chemoluminescent bands were detected using X-ray films, and densitometry analyses were performed using Image-J software.

2.6 Cell cycle analysis

For cell cycle analysis, cells were trypsinized, centrifuged and resuspended in a lysis buffer containing 3.5 mmol/L trisodium citrate, 0.1% vol /vol Nonidet P-40, 0.5 mmol/L Tris-HCl, 1.2 mg/mL spermine tetrahydrochloride, 5 μ g/mL RNAse, 5 mmol/L EDTA and 1μg/mL PI, pH 7.6. Afterward, cells were incubated for at least 10 min on ice for cell lysis, and DNA content was determined by flow cytometry. Ten thousand events were counted per sample.

FACS analyses were performed using the CellQuest Pro Software (BD Biosciences, CA, USA)

2.7 Statistical analysis

The statistical significance was assessed by one-way analysis of variance (ANOVA) followed by Tukey's test. All analysis was performed using the GraphPad Prism (GraphPad Software Inc, San Diego, CA, USA, version 5.0) software. The results are expressed as means ± standard deviation (S.D.) of 3 individual experiments (n=3) unless otherwise indicated; p values were considered significant when $p \leq 0.05$.

3. RESULTS

3.1 Validation of chemoresistance protocol in glioma C6 line

To evaluate if the cell lines becomes resistant to the selected chemotherapeutics we performed the MTT cell viability assay (see methods). C6, C6-TR, C6-DR were treated with crescent doses of their respective chemotherapeutic for 72h. The doses of TMZ used were: 125, 250, 500, 1000 and 2000 uM. The doses of DOX were: 0.470, 0.940, 1.88, 3.76, and 7.52 μM

Both cell lines displayed resistance to their respective chemotherapeutics when compared to the control group. As expected C6-DR showed an IC50 of 12.43 μM which is a 33-fold increase when compared to parental C6, IC50 of 0.376 μM (Figure 1A). C6-TR presented an IC50 of 1710 μM, 11-fold higher than the control group, 151.8 μM (Figure 1B).

At the microscope, all lineages looked morphologically very similar (Figure 1C). The only highlighted difference is that C6-TR have a slightly elongated morphology, slower proliferation, and tended to grow in clusters, instead of spreading out the plate surface.

3.2 p-STAT3/STAT3 immunocontent is higher in resistant cells, and AG490 reduced the p-STAT3 immunocontent

Aiming to determine whether the immunocontent of p-STAT3 is increased in chemoresistant cells, we performe western blot experiment to detect Tyr 705 phosphorylated residue of STAT3. Also, we treated cells with IL-6 10 ng/ml, the classical STAT3 activator for 24 hours as a positive control for STAT3 activation (Zhong et al., 1994; Kohsaka et al., 2012). We performed a C6 full combination of DOX 0.470 μM + TMZ 250 μM + IL-6 10 ng-ml in an attempt to see synergetic effects on STAT3 activation and the concentrations of chemotherapeutics used are to see if the chemotherapeutics are STAT3 activators by

themselves. In fact, all resistant lineages presented an increased immunocontent of p-STAT3 when compared with C6 alone or the positive control (Figure 2). When the resistant lineages were treated with JAK2/STAT3 inhibitor AG490, both showed a significant decrement in p-STAT3 immunocontent, with their immunocontent coming nearly as C6 levels.

3.3 Resistant cells showed multidrug cross-resistance

In order to determine if the acquired resistance to the alkylating agent TMZ could be associated with cross-resistance to the antibiotic DOX (and vice versa), we performed a viability assay with 3 doses of DOX in C6-TR and TMZ in C6-DR lineages to evaluate their aproximate IC50. This approach could reveal a possible multidrug resistance across these chemotherapeutics, since both lineages presents p-STAT3 increased when compared to the C6 parental lineage. C6-TR showed an IC50 for DOX of 1.20 μM (Figure 3), 3,2-fold higher than the IC50 value of 0.376 already estimated for C6 in figure 1. C6-DR presented an IC50 of 673 μM for TMZ, which is 4-fold higher than the previously reported C6 IC50 of 171.8 μM.

3.4 p-STAT3 inhibition re-sensitize resistant cells to their respective chemotherapeutics

Taking into account the increased activation of STAT3 in chemoresistant cell lines, we sought to investigate if inhibiting p-STAT3 activation with the JAK2/STAT3 inhibitor AG490 could re-sensitize the resistant lineages. Then we incubate the cells with their respective treatments and after 72h MTT assay were carried out. AG490 significantly decreased the viability of all 3 cells lineages in a dose dependent manner (Figure 4). However, the level AG490 toxicity was similar across the cell lines, regardless their chemoresistance phenotype. We chose doses of the chemotherapeutics below the IC50 of the resistant cells, to evaluate if AG490 presents a synergetic effect with low doses of the chemotherapeutics. Notewhorty when we combined AG490 treatments with doses below IC50 values of selected drugs in resistant lineages (TMZ

500 and 750 μM; DOX 0.940, 1.88 μM) we clearly observed that STAT3 pathway inhibition could re-sensitize both C6-TR and C6-DR to their respective chemotherapeutic agent (Figure 4).

3.5 AG 490 leads to G2 arrest in cell cycle

Taken into account that STAT3 plays an important role in cell cycle, we decided to analyze the cell cycle distribution by flow cytometry (See Material and Methods). C6, C6-TR and C6-DOX didn't differ in their cell cycles, but surprisingly treatment with AG 490 50 μM caused an arrest in G2 phase in all cell lineages after 72h of treatment (Figure 5), suggesting that arrest in cell cycle could be a mechanism involved in the loss of viability/proliferation when resistant cells are treated with the STAT3 inhibitor AG 490.

4. DISCUSSION

Recently, several studies have shown that the inhibition of STAT3 phosphorylation could lead to an overcome of chemotherapy-induced resistance, in diverse tumor cell lines (Bhagwat et al., 2014; Tang et al., 2010; Wang et al., 2013). Kohsaka et al. (2012) also demonstrated that STAT3 phosphorylation was enhanced in 8 out of 14 samples from patients with recurrent GBM following TMZ treatment compared to matched samples from the same patients before treatment. In fact, our study showed that p-STAT3 is upregulated in two chemotherapeutic-resistant induced C6 lineages, C6-TR which shows resistance to TMZ and C6-DR which shows resistance to DOX.

Our lineages showed a higher IC50 for the respective drugs after the resistance induction protocol, and a cross-resistance was observed, suggesting STAT3 activation as a pleiotropic response to DNA damaging agents. This is in agreement with the current literature, which shows elevated IC50 for similar protocols in the same cell lineage, Kievit et al. (2011), found an IC50 over 20-fold higher in their C6 Dox resistant lineage, while Sun et al. (2012) reached out a 6-fold higher IC50 for TMZ when compared with parental U87 glioma cells. The evidence found in these papers led us to believe that we achieve a resistant lineage in agreement with the current available evidences, confirming the effectiveness of our protocol. Morphologically, we found no difference between C6 and C6-DR which is in agreement with the data found by Kievit et al. (2011), who found virtually none morphological difference in their C6 DOX resistant lineage and their parental surrogates. The tendency to grow in cluster and the slower proliferation that we observed in the C6-TMZ lineage was already observed in lineages of breast cancer resistant to paclitaxel (Chen et al., 2013 Lu et al., 2009), and bufalin in hepatocellular carcinoma (Gu et al., 2014.). Curiously,

STAT3 phosphorylated was found upregulated in paclitaxel-resistant cell lines, thus corroborating with our findings. (Duan et al., 2009).

We investigated if p-STAT3 is upregulated in our C6-TR, or C6-DR, since the immunocontent of p-STAT3 was found increased in other tumor cell lineages resistant to chemotherapy or radiotherapy, like colon cancer (Cross-Knorr et al., 2013), lung cancer (You et al., 2013) and prostate cancer (Liu et all., 2014). Even in GBM p-STAT3 was found highly increased in U87MG and T98G human lineages (Kohsaka et all., 2012). In this present work we used the C6 rat glioma lineage in a protocol of acquired-resistance which reproduce better the development of resistant phenotypes or selection of cells subpopulations in the course of chemotherapy. The C6 cell lineage is an established *in vitro* model with an extensive characterization and genetic analysis (Grobben et al., 2002) and has been used as an *in vivo* model of syngeneic GBM in rats (Zanotto-Filho et al., 2011).

Both resistant lineages presented an increase in p-STAT3 immunocontent when compared to C6 parental lineage, possibly due to an up regulation of upstream regulators. The reason why p-STAT3 is elevated in both drug resistance cell lines is unclear, but we believe that these cells could have developed an autocrine IL-6 secretion that leads the cell to major p-STAT3 activation, constituting a positive feedback whit IL-6 secretion and a resistant phenotype. Although we have not measured IL-6 secretion, this mechanism is already described in adecarcinomas by Grivennikov and Karin.(2008), where they found that cancer cells carrying EGFR mutation were found to produce high amounts of IL-6, which is an activator of JAK, that leads to STAT3 activation, in addition Rahaman et al. (2002) already described that U251 GBM cells are capable of IL-6 autocrine secretion. Since we found that exogenous IL-6 is capable to increase p-STAT3 immunocontent in C6 cells and the continuous exposure to chemotherapies could lead these cells to an EGFR mutation, this

theory could be the explanation for our results. Despite being an acceptable theory, more experiments must be made to really confirm this hypothesis.

We observed that the increase in STAT3 immunocontent is associated with the resistance that these lineages showed to DOX and/or TMZ, since the cells showed increased p-STAT3, but mainly because they are re-sensitized by treatment with STAT3 inhibitor AG490. These suggests that STAT3 up-regulation is not only correlating with resistance but also playing a key role in this phenomenon. Another interesting result that we found is that C6-TR showed a higher IC50 for DOX, and C6-DR showed a higher IC50 for TMZ when they are compared with parental C6 cells. Recently, Zhang and Wang (2013) showed that vincristine-resistant SGC7901 gastric cancer cell lineage displayed cross-resistance to adryamicin, etoposide, 5-fluorouracil and cisplatin, suggesting a multidrug resistant phenotype (Zhang and Wang., 2013). Agreeing with our data, the cell lineage also presented p-STAT upregulated when compared to parental cells. To best of our knowledge, we found no studies showing that GBM resistant to a determined antitumoral could also be resistant to another chemotherapeutic, even more from different classes and mechanisms of action, as in this case of DOX and TMZ. Since the lineages presented p-STAT3 upregulated and are sensitized by STAT3 inhibition one could assume that p-STAT3 play an important role in GBM multidrug resistance.

The inhibition of p-STAT3 was the main objective of studies aiming to decrease cancer cells viability (Amit-Vanzina et al.,2005; Kim et al., 2007;Nielsen et al., 1997), and STAT3 inhibitors were already tested in GBM cell lines, with great success *in vitro* (Senft et al. 2011; Vlachostergios et al., 2013). Different studies have shown that the inhibition of constitutive activated STAT3 has led GBM cells to decreased proliferation and growth (Michaud-Levesque et al., 2012), apoptosis (Swiatek-Machado et al., 2012) and autophagy (

Siegelin et al., 2010). Our results showed that JAK2/STAT3 inhibitor AG490 reduced the cellular viability of C6, C6-DR and C6-TR, in addition re-sensitized the resistant lineages to doses much lowers to their respective IC50. This result suggest that STAT3 inhibitors are promising for combined therapy in GBM cells that became resistant to the chemotherapy. In addition, there is also interesting to note that, AG490 could reduce cellular viability in cells lines resistant to two different drugs (TMZ and DOX). It suggests that its effectiveness may not be restricted to a specific drug-resistance, if STAT3 activation is proven to be a more general chemoresistant event. Mechanistically, Rahaman et al. (2002) found that U251 GBM cells treated with AG490 50 μM for 72h, inhibited STAT3 activation, with concomitant reduction in steady-state levels of antiapoptotics Bcl-Xl, Bcl-2 and Mcl-1 proteins, and induced apoptosis in the U251 cell lineage, as revealed by Poly (ADP-ribose) polymerase cleavage. Here we add the information that AG490 promotes alteration in the cell cycle progression of chemoresistant gliomas.

STAT3 is a well-known regulator of cell cycle in multiple cell types, especially in cancer cells. STAT3 has been shown as an inductor in the expression of cyclin D1 (Masuda et al.,2012), and a suppressor of p21(Bellindo et al. 1998). Cyclin D1 promotes G1/S transition through activation of cyclin-dependent kinases (CDKs) while p21 mediates cell cycle arrest by inhibiting CDKs and binding to proliferating cell nuclear antigen (PCNA) to inhibit DNA synthesis (Hunter and Pines, 1994). Prior studies demonstrated that constitutively activated STAT3 orchestrates G1/S transition by promoting cyclin D1 gene transcription and down-regulating p21 (Fukada et all.,1998). STAT3 was also described as a down-regulator of TP53 gene expression (Yu and Jove, 2004). We found none significant difference between parental C6 and C6-DR or C6-TR cell cycle distribution, suggesting that chemoresistance is not associated with increased proliferation but potentially with DNA repair or drug detoxification mechanisms. Inhibition of STAT3 with AG490 caused an arrest in G2/M phase of the three

lineages concerned in this work. Taken into account that C6 cells, and other GBMs, exhibit alterations in controllers of G1/G0 arrest cell cycle, as p16 mutations in C6 and p53 mutations in a high percentage of gliomas, it is likely that our cells are arresting in G2/M due to an intrinsic absence of efficient G1/S checkpoints. This G2/M phase arrest was already shown in GBM treated with drugs that inhibit transcription factors relevant for cell survival. Zanotto-Filho et al, 2012 described that the proteasome inhibitor MG-132 caused a G2/M arrest in C6 and U138MG with increased level of $p21^{WAF1}$ at the early steps of apoptosis.

In summary, we demonstrated that C6 cell lines resistant to DOX or TMZ have an increased immunocontent of p-STAT3. Studies using the specific pharmacological inhibitor AG490 confirmed the functional association between STAT3 activity and the resistant phenotypes, our study showed that the treatment with AG490 reduced p-STAT3 protein as well as synergized with DOX and TMZ toward re-sensitization of the resistant lineages to these chemotherapeutics. The inhibition of p-STAT3 also led cells to a cell cycle arrest in the G2/M phase, which can be a possible prelude of apoptosis or mitotic catastrophe. The resistance was not limited to one chemotherapeutic since the two resistant lineages showed a higher IC50 for both drugs when compared to the parental C6. These findings suggest that the inhibition of aberrant p-STAT3 can be an object of study for future therapies aiming to re-sensitize GBM to chemotherapeutics.

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Figure 1

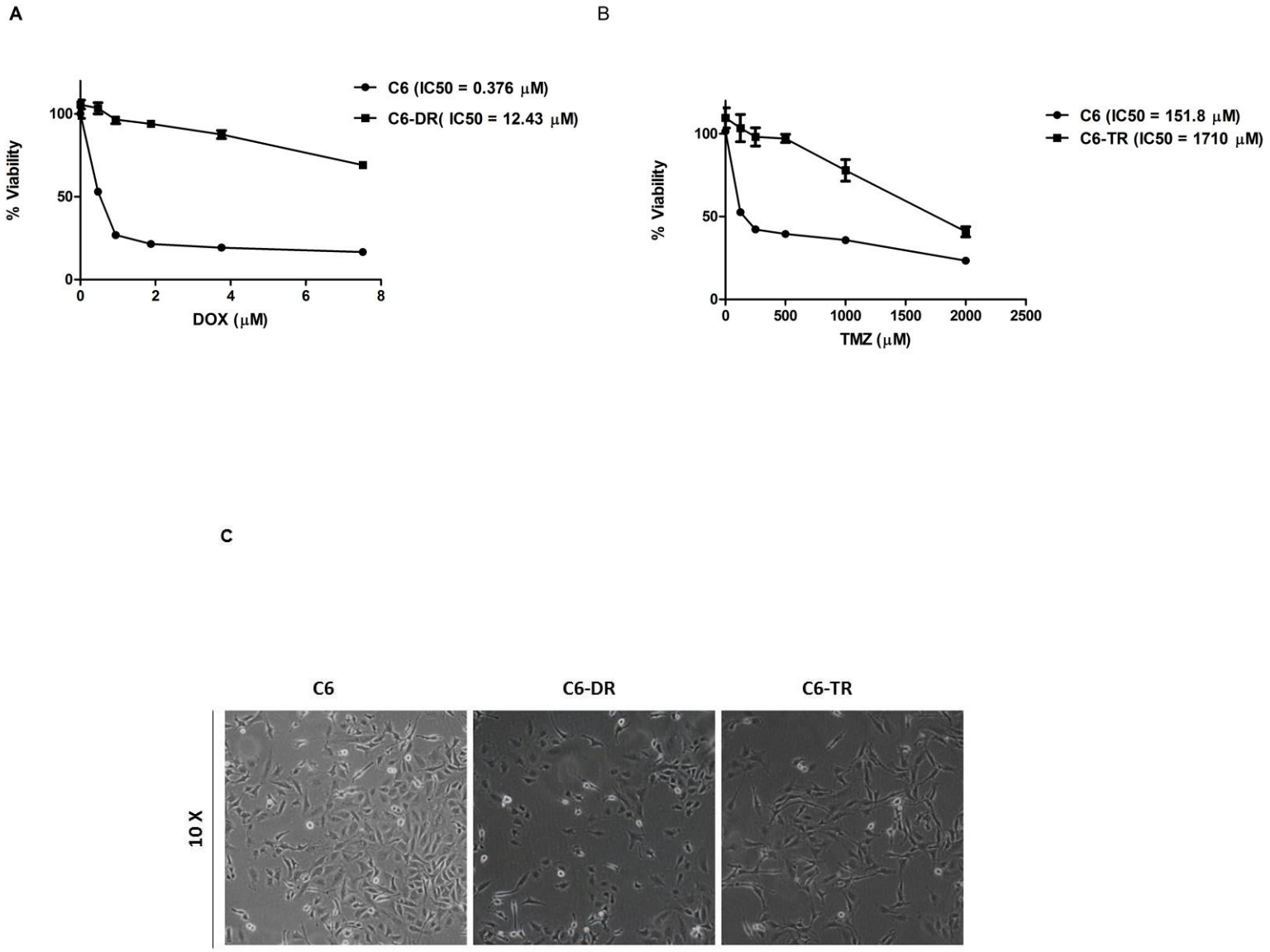

Fig. 1: Comparison between C6 parental and resistant lineages. **A** C6 and C6-DR were treated with the respective concentrations of DOX μM for 72 hours and MTT assay were carried out. **B** C6 and C6-TR were treated with the respective concentrations of TMZ μM and MTT assay were carried out. **C** Cell morphology of all lineages analyzed by phase contrast microscopy (10x magnification), n=3.

Figure 2

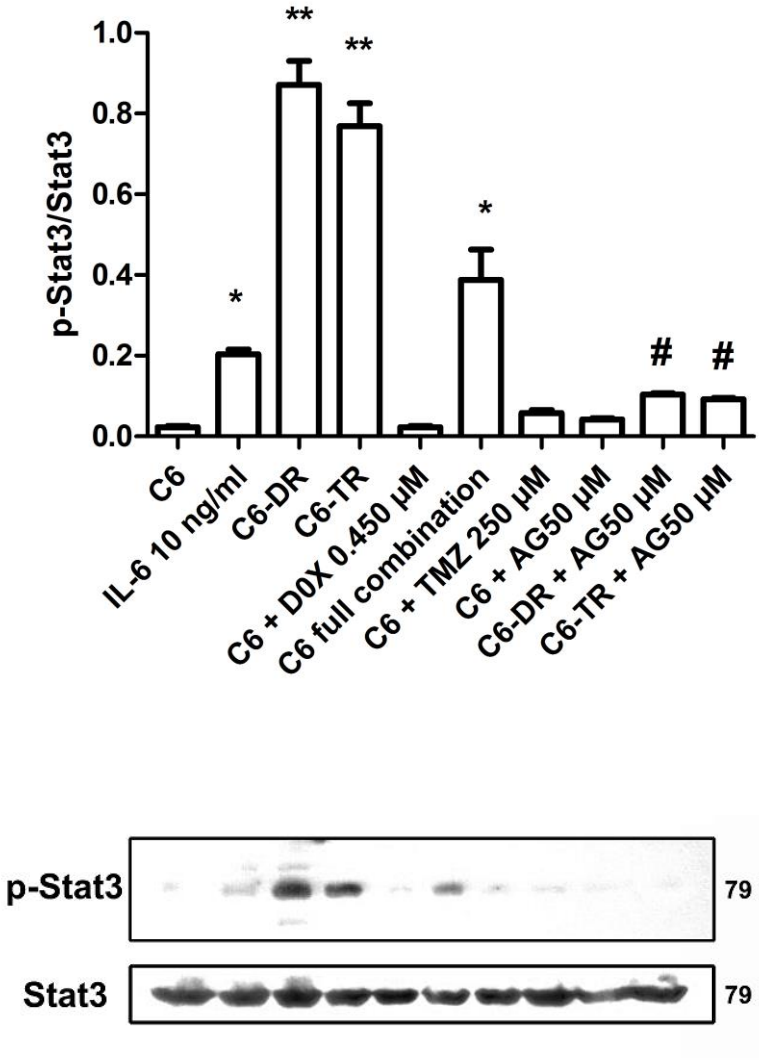

FIG. 2: Immunocontent of STAT3 and p-STAT3 in C6, C6-DR and C6-TR cells. Cells were treated for 72h with the respective treatments and total cellular extract was collected for the analysis. The graph shows the p-STAT3/STAT3 ratio. C6 full combination is C6 + DOX 0.450 µM + TMZ 250 µM + IL-6 10 ng/ml. * Different from C6 ($p<0.05$). ** Different from C6 and IL-6 10 ng/ml ($p<0.05$). # Different from their respective resistant lineage ($p<0.05$), n=3.

Figure 3

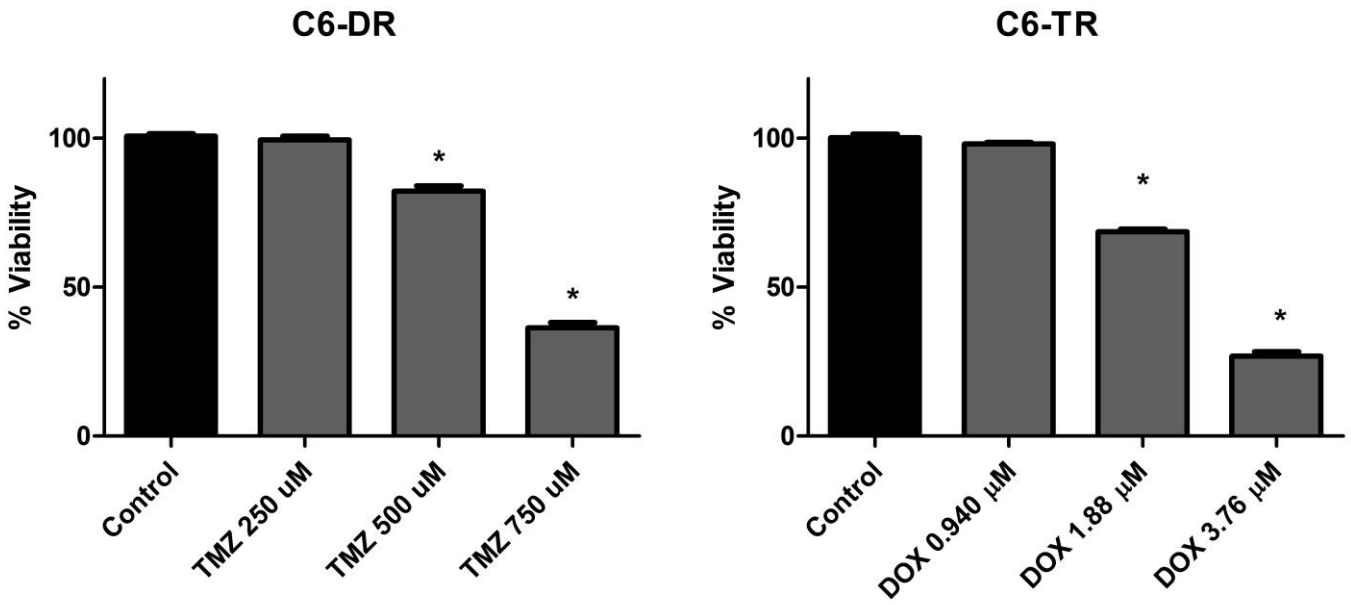

FIG 3: Multidrug resistance in c6-DR and C6-TR. C6-DR and C6-TR were treated with the respective concentrations of DOX µM or TMZ µM for 72 hours and MTT assay were carried out. The IC50 that C6-DR presented for TMZ was 673 µM and C6-TR presented an IC50 of 1.20 µM for DOX. * Different from untreated control ($p<0.05$), n=3.

Figure 4

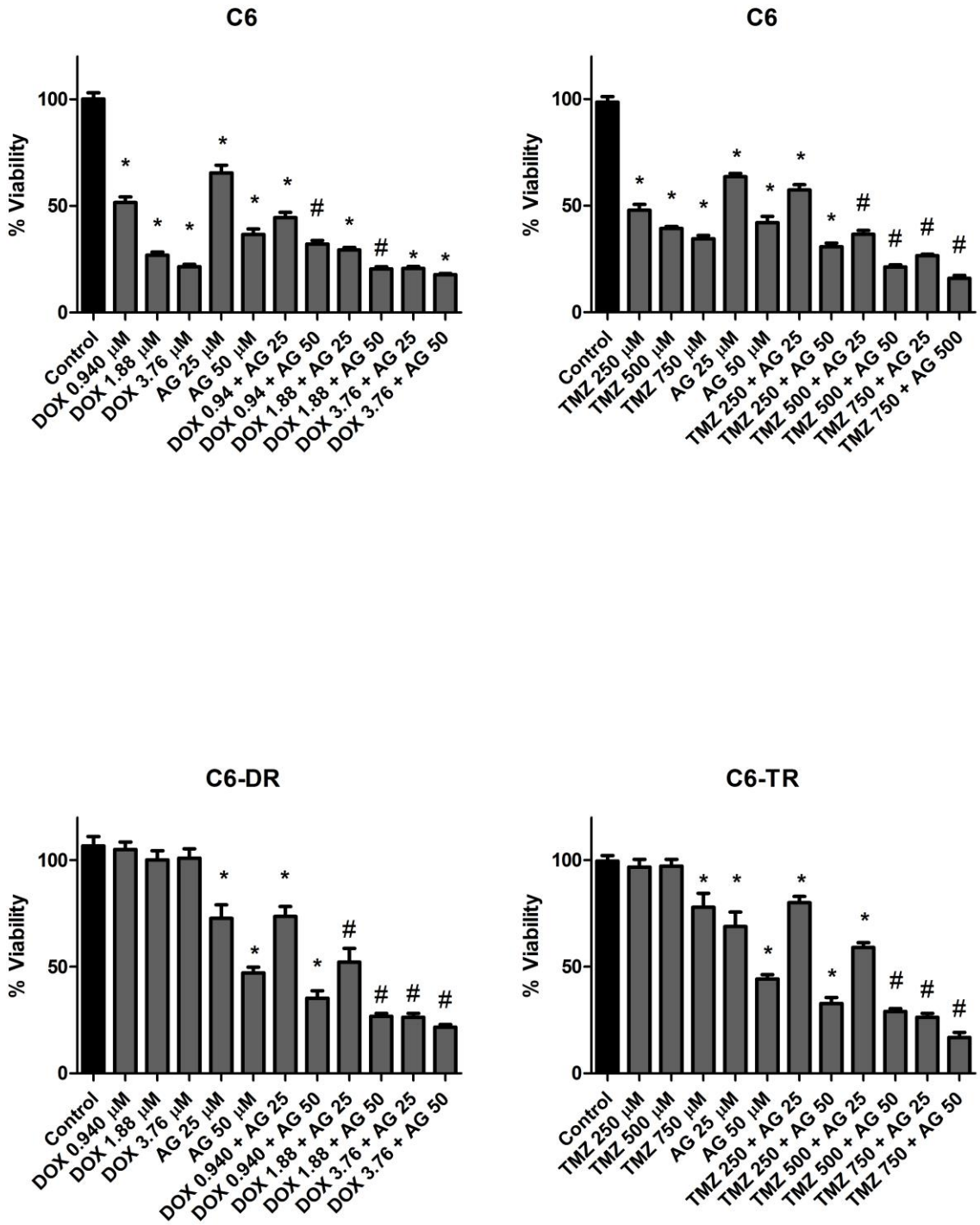

FIG. 4: Treatment with AG490 re-sensitize resistant cells to their chemotherapeutics. MTT assay showing C6, C6-DR and C6-TR treated with DOX, TMZ, AG490 and combinations for 72h. Legends: AG 25: AG490 25 µM; AG50: AG490 50 µM; DOX 0.940: Doxorubicin 0.940 µM; DOX 1.88: Doxorubicin 1.88 µM; DOX 3.76: Doxorubicin 3.76 µM; TMZ 250: Temozolomide 250 µM; TMZ 500: Temozolomide 500 µM; TMZ 750: Temozolomide 750 µM. * Different from the untreated ($p<0.05$). # Different from the monotreatment with the respective dose of chemotherapeutic and the monotreatment with the respective dose of AG490 ($p<0.05$), n=3.

Figure 5

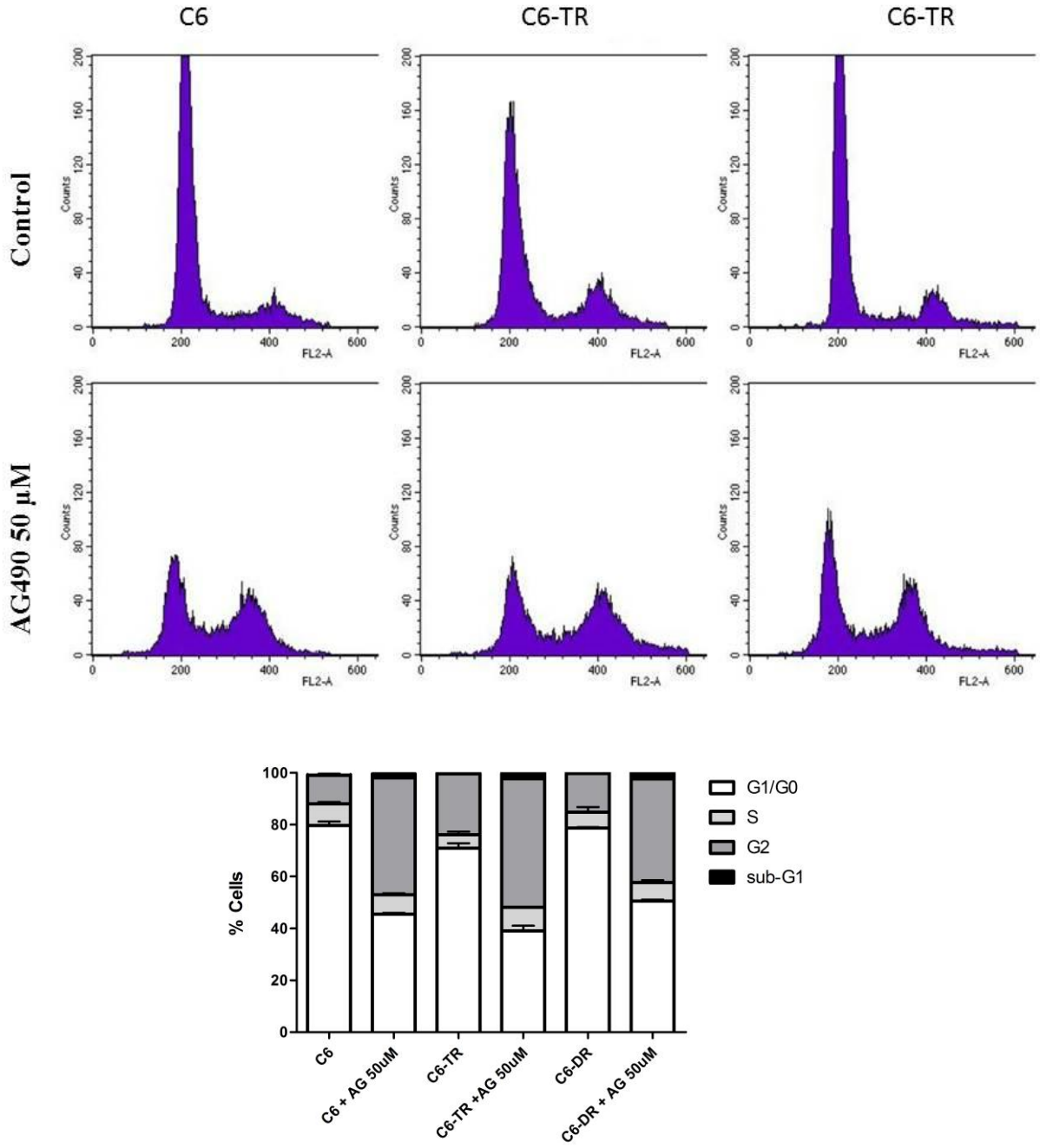

Fig. 5: AG490 induce G2/M cell cycle arrest. Flow cytometry for determination of cell cycle distribution in untreated and treated with AG490 50 µM cells for 72 h. n=3.

Parte III

4. DISCUSSÃO

Durante os últimos anos, uma ampla gama de estudos tem demonstrado que a inibição do fator de transcrição STAT3 poderia levar a uma quebra da resistência a quimioterapicos presente em muitos pacientes, sendo que os resultados positivos têm sido observados em diversas linhagens (Bhagwat et al., 2014; Tang et al., 2010; Wang et al., 2013). Também já foi demonstrado em um estudo realizado com pacientes portadores de GBM que obtiveram uma recorrência deste GBM após a quimioterapia com TMZ, que em 8 das 14 amostras analizadas o conteúdo de p-STAT3 estava elevado quando comparado com o de pacientes com GBM que não passaram pela quimioterapia (Kohsaka et al., 2012). O nosso trabalho demonstrou que o imunoconteudo de p-STAT3 se apresentou elevado em duas linhagens que induzimos resistência à quimioterapia, a linhagem C6-DR que apresentou resistência a DOX e a linhagem C6-TR que apresentou resistência a TMZ.

Ambas as linhagens apresentaram um IC50 elevado, quando comparado com a linhagem C6 paterna, para seus respectivos fármacos após o nosso protocolo de indução de resistência. Nossos dados concordam com o que está disponível na literatura atualmente, podendo citar um estudo com a mesma linhagem que utilizamos onde o autor encontrou um IC50 para Dox mais de vinte vezes maior do que o IC50 apresentado pela linhagem C6 paterna (Kievit et al., 2011). O IC50 que nossa linhagem apresentou é cerca de trinta e três vezes maior do que o da linhagem paterna. Já a nossa linhagem resistente a TMZ apresentou um IC50 onze vezes maior que a linhagem controle, que também vai de encontro com os dados de outro autor que se utilizou da linhagem de GBM humano U87, desenvolveu o seu protocolo de resistência e encontrou um IC50 seis vezes maior quando comparado

com a linhagem paterna (Sun et al., 2012). Morfologicamente, nós não encontramos nenhuma diferença notável entre as linhagens C6 e C6-DR, concordando novamente com Kievit et al. (2011). Porém na nossa linhagem C6-TR nós observamos uma tendência de crescimento em ‘’ilhas’’, e uma proliferação significantemente mais lenta quando comparada com a da linhagem C6. Essas características já foram previamente observadas por outros autores em diferentes linhagens, como linhagens de câncer de mama resistentes a paclitaxel (Chen et al., 2013; Lu et al., 2009); e linhagens de hepatoma resistentes a bufalina (Gu et al., 2014). Curiosamente, algumas linhagens resistentes a paclitaxel também apresentaram uma maior fosforilação de STAT3 (Duan et al., 2009).

Depois de estabelecida a resistência, nosso próximo objetivo foi investigar se o imunoconteudo de p-STAT3 estaria elevado nas nossas linhagens C6-DR e C6-TR, já que outros trabalhos foram capazes de demonstrar que p-STAT3 se apresenta elevado em diversas linhagens de células tumorais resistentes à quimioterapia ou à radioterapia, como linhagens de câncer de cólon (Cross-Knorr et al., 2013), câncer de pulmão (You et al., 2013) e câncer de próstata (Liu et al., 2014). Até mesmo em GBM já foi encontrado um aumento do conteúdo de STAT3 nas linhagens de humanos U87MG e T98G (Kohsaka et al., 2012). Nossas duas linhagens resistentes apresentaram um imunoconteudo elevado de p-STAT3 quando comparadas com a linhagem paterna. Esse dado sugere que o aumento do imunoconteudo de p-STAT3 pode estar relacionado com a resistência a DOX ou TMZ que estas células apresentaram. O imunoconteudo destas linhagens é maior inclusive do que o imunoconteudo apresentado pela C6 tratada com IL-6 exógeno, que é um conhecido ativador de STAT3 (Zhong et al., 1994). O porquê deste imunoconteúdo estar elevado nestas duas linhagens ainda não está claro, porém

nós acreditamos que possa ser devido a um aumento do mecanismo secretor de IL-6 destas células, que levou consequentemente a uma maior ativação de STAT3, e que por sequencia criou um feedback positivo com a secreção de IL-6 e leva a um fenótipo de resistência destas células. Este mecanismo já foi previamente descrito por Grivennikov e Karin em 2008, onde os autores mostraram que células de tumores que possuiam mutações em EGFR produziam maiores quantidades de IL-6, que é um ativador de JAK, levando assim a uma maior ativação de STAT3. Além disto, Rahaman já demonstrou em 2002 que algumas linhagens de GBM possuem uma secreção natural de IL-6. Já que demonstramos no nosso trabalho que IL-6 exógena é capaz de aumentar o imunoconteudo de p-STAT3 da linhagem C6 paterna, a contínua exposição das células aos quimioterápicos durante o protocolo de resistência pode ter levado a uma mutação de EGFR, explicando assim o nosso resultado. Apesar de ser uma teoria plausível, por enquanto ela é só uma especulação e teremos que realizar estudos adicionais para avaliar se as nossas linhagens resistentes possuem uma maior secreção de IL-6, para que assim possamos confirmar esta hipótese que levantamos.

Um resultado que particularmente despertou nosso interesse foi o resultado de viabilidade celular, que mostrou que a linhagem C6-TR obteve um maior IC50 para DOX e a linhagem C6-DR obteve um maior IC50 para TMZ quando comparadas com a linhagem C6 paterna. Esse resultado se assemelha a outro estudo, onde os autores induzem resistência à vincristina na linhagem de câncer gástrico SGC7901, e observaram que a sua linhagem resistente, além de exibir a resistência a vincristina que já seria esperada também exibiu resistência a adriamicina, 5-fluoracil e cisplatina (Zhang e Wang., 2013). Curiosamente, a linhagem resistente dos autores também apresentou um imunoconteudo elevado de

p-STAT3 quando comparada com a linhagem controle. Não encontramos nenhum estudo que demonstrasse que linhagens de GBM aonde fosse induzida a resistência a um quimioterápico específico também apresentassem resistência a outros quimioterápicos a que a linhagem não tenha sido previamenta exposta. O nosso trabalho demonstra que ambas as linhagens resistentes apresentam resistência aos dois quimioterápicos utilizados, mesmo não sendo previamente expostas a um deles. Como estas linhagens apresentaram um aumento no conteúdo de p-STAT3, nossos dados sugerem que STAT3 deve possuir um papel importante no fenótipo de resistência a múltiplas drogas, muitas vezes observado em GBM.

A inibição de STAT3 tem sido o alvo de muitos estudos envolvendo a busca por redução na viabilidade de tumores (Amit-Vanzana et al., 2005; Kim et al., 2007; Nielsen et al., 1997), e os inibidores farmacológicos de STAT3 têm sido usados em linhages de GBM com uma taxa considerável de sucesso (Senft et al., 2011; Vlachostergios et al., 2013). Diferentes estudos já demonstraram que a inibição de STAT3 que está constitutivamente ativa em GBM leva a um decréscimo na proliferação e crescimento destas células (Michaud-Levesque, Bousquet-Gagnon e Béliveau, 2012), indução de apoptose (Swiatek-Machado et al., 2012) e autofagia (Siegelin et al., 2010). O nosso estudo demonstrou que o inibidor farmacológico de JAK2/STAT3 AG490 reduziu a viabilidade celular das linhagens C6, C6-TR e C6-DR bem como ressensibilizou as linhagens resistentes à quimioterapia ao seu quimioterápico em doses significativamente menores do que as doses apresentadas por seus IC50. Este resultado nos sugere fortemente que a inibição de p-STAT3 pode ser uma terapia auxiliar que deve ser avaliada em pacientes que possuem GBM resistentes a quimioterapia, já que o AG490 conseguiu reduzir a viabilidade de duas linhagens resistentes a diferentes quimioterápicos, demonstrando que além de

ser efetivo, o AG490 não tem seu efeito restrito a apenas um tipo de resistência. Rahaman et al, já demonstrou, em 2002, que células de GBM humanos U251 tratadas com AG490 na dose de 50 μM por 72h, que foi o mesmo tratamento que utilizamos, tiveram a ativação de STAT3 significantemente reduzida, além de apresentarem uma concomitante redução no conteúdo das proteínas antiapoptóticas Bcl-Xl, Bcl-1 e Mcl-1. Isto levou estas células a entrarem em apoptose, que foi observado através do conteúdo da enzima poli ADP-ribose polimerase na forma clivada. Apesar do nosso trabalho não apresentar nenhum experimento que possa confirmar a morte celular, ou possíveis mecanismos da mesma, a perda da viabilidade celular e o efeito sinérgico que AG490 na dose de 50 μM apresentou quando administrado em conjunto com TMZ e/ou DOX, nos leva a acreditar que a inibição da ativação de STAT3, via JAK2, é eficiente na ressensibilização a quimioterapia na linhagem C6, e deve ser explorada em estudos futuros para comprovar a sua efetividade como terapia adjuvante em pacientes com GBM.

O fator de transcrição STAT3 também tem sido associado como um importante regulador do ciclo celular, em diversos tipos de células e especialmente em células tumorais. Sua função como indutor da expressão da proteína ciclina D1, por exemplo, contribui em muito para o seu papel de regulador do ciclo celular de células cancerígenas (Masuda et al., 2002). A regulação negativa de p21 por STAT3 também é um evento de suma importância no ciclo celular (Bellindo et al., 1998). A ciclina D1 tem o papel de promover a continuidade do ciclo celular através da transição da fase G1/S para G2 através da ativação de cinases dependentes de ciclina (CDKs). Já a função de p21 é promover uma parada no ciclo celular caso haja algum dano significativo a DNA detectado na célula, através da inibição das CDKs e ligação do mesmo ao antígeno nuclear de proliferação celular (PCNA),

inibindo assim a síntese de DNA (Hunter e Pines., 1994). Como já foi citado anteriormente, estudos demonstram que a ativação constitutiva de STAT3 é responsável por promover a continuidade do ciclo celular, promovendo a transição da fase G1/S devido ao aumento da transcrição do gene responsável pela expressão de ciclina D1 e também inibindo a expressão de p21, fazendo com que o ciclo celular não possa ser interrompido (Fukada et al., 1998). Isso faz com que células que possuem o DNA mutado, como células tumorais, possam continuar proliferando. Também já foi descrito que a ativação contínua de STAT3 pode regular negativamente a expressão do gene TP53 (Yu e Jove., 2004) e se considerarmos que p53 é um conhecido, e amplamente encontrado em diversos tipos celulares, inibidor de proliferação e indutor de apoptose (Yu e Jove., 2004), o fator de transcrição STAT3 novamente aparece como uma peça chave da regulação do ciclo célular em células tumorais. Considerando todas estas evidências apresentadas, nós decidimos investigar se haveria alguma diferença no ciclo celular das três linhagens e se o tratamento com AG490 poderia alterar o ciclo celular destas linhagens de alguma forma.

Não identificamos nenhuma diferença significativa entre o ciclo celular das três linhagens utilizadas, porém observamos que o tratamento com AG490 na dose de 50 μM se mostrou capaz de induzir uma parada no ciclo na fase G2/M em todas as linhagens utilizadas neste estudo. Esta parada no ciclo em G2/M já foi demonstrada em outros trabalhos envolvendo GBM e inibidores de fatores de transcrição, inclusive por colegas de laboratório. No trabalho realizado por Zanotto-Filho et al. em 2012, foi demostrado que o inibidor de proteassomo MG-132, utilizado para inibir a ativação do fator de transcrição nuclear kappa b (NF-Kb) também causou uma parada no ciclo na fase G2/M das linhagens C6 e U138MG.

Além da parada relatada, o inibidor foi capaz de aumentar o imunoconteudo de $p21^{WAF}$ que é característica dos estágios iniciais de uma célula que entra em apoptose. Nós encontramos esta mesma parada em G2/M, e a perda de viabilidade das células submetidas ao tratamento com AG490, sugere que este mesmo mecanismo pode estar ocorrendo nas linhagens resistentes a quimioterápicos.

5. CONCLUSÃO

5.1. Conclusões Gerais

Conseguimos demostrar ao longo deste estudo que linhagens de GBM C6 portadoras de resistência aos quimioterápicos TMZ e DOX possuem um elevado imunoconteudo de p-STAT3 quando comparado com a linhagem paterna. A resistência que estas linhagens apresentaram não foi somente a um tipo de quimioterápico, demonstrado uma resistência múltipla. Demonstramos também que o fármaco inibidor de STAT3 AG490 foi capaz de diminuir este imunoconteudo de p-STAT3 e ressensibilizar ambas as linhagens à quimioterapia. A inibição de STAT3 também levou a uma parada do ciclo celular na fase G2/M, que pode ser um prelúdio de apoptose. Portando nossos dados sugerem que células de GBM resistentes à quimioterapia possuem STAT3 superexpressa e que a inibição deste fator pode ser uma terapia adjuvante a ser explorada em pacientes resistêntes à quimioterapia.

5.2. Conclusões Específicas

- Obtivemos sucesso na criação de linhagens de células C6 resistentes tanto a DOX quanto a TMZ, que pode ser evidenciado pelos elevados IC50 encontrados nestas duas linhagens.

- O imunoconteúdo de p-STAT3 se encontrou significativamente elevado em ambas as linhagens resistentes quando comparadas com a linhagem C6 paterna, demonstrando uma relação entre resistência a quimioterapia e ativação de STAT3.

- A inibição de STAT3 pelo fármaco AG490 se mostrou efetiva na ressensibilização das linhagens de GBM aos quimioterápicos utilizados neste estudo.

6. PERSPECTIVAS

Como perspectivas do estudo, temos a intenção de avaliar o conteúdo de STAT3 em linhagens de GBM humano, além da eficácia de outros inibidores farmacológicos de STAT3 para a ressenssibilização a quimioterápicos. Também podem ser apontadas como perspectivas a caracterização dos tipos de morte celular que estão ocorrendo nestas células, bem como a quantificação de secreção de IL-6 pelas mesmas. Além disso, desenvolver um modelo *in vivo* de implante das nossas células C6 resistentes C6-TR e C6-DR para que possamos avaliar a eficácia da inibição de STAT3 *in vivo* e a influência do ambiente fisiológico do tumor na eficácia do nosso tratamento.

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