UNIVERSIDADE
BEIRA INTERIOR

Role of adenosine on the resistance to temozolomide anti-tumor agent in glioblastoma

Versão final após defesa

Vanda Cristina Simões Nogueira

Dissertação para obtenção do Grau de Mestre em
Bioquímica
(mestrado integrado)

Orientador: Prof. Doutor José Francisco Cascalheira
Co-orientadora: Prof. Doutora Ana Maria Sebastião

Junho de 2020

Acknowledgments

Doze meses passaram desde que cheguei de bagagens a uma nova cidade tão diferente da que me viu crescer, não sabia porém que a aprendizagem num ano seria tanta que traria de volta muito mais do que o que levei. Saí pela primeira vez de casa agarrada a uma oportunidade conseguida pelo meu querido orientador Doutor professor José Francisco Cascalheira.

Ao meu orientador professor Doutor Francisco Cascalheira quero agradecer primeiramente pela oportunidade que me deu e por ter acreditado em mim desde o primeiro momento em que me dirigi a ele. Obrigada por me ter feito saber do meu potencial e acima de tudo por nunca ter duvidado dele, agradeço o apoio durante todo este ano mesmo na fase mais difícil onde eu própria questionei a continuação da tese. Agradeço a compreensão por todos os disparates feitos e o reconhecimento por todas as coisas bem conseguidas. Hoje, que vejo esta fase terminar não me arrependo em nada da minha primeira escolha para este segundo ano de mestrado, o meu orientador.

À minha co-orientadora professora Doutora Ana Sebastião quero agradecer a rápida disponibilidade para me receber no seu laboratório mas acima de tudo a grande ajuda que me ofereceu a nível pessoal durante o mês mais difícil deste ano. Agradeço por não ter duvidado que eu estaria à altura para trabalhar no seu laboratório e pelo sábio conhecimento que partilhou comigo sempre que foi possível.

A um dos motores para a realização da minha tese é difícil arranjar palavras suficientes para agradecer. À Cláudia, obrigada por tudo o que fez por mim e me ajudou a conseguir. Agradeço por nunca ter desistido mesmo após eu cometer erros atrás de erros e tudo dar errado. Agradeço por me ter feito crescer como cientista, como trabalhadora e como pessoa. O otimismo e persistência que aprendi consigo são características que levarei toda a vida e com certeza me ajudarão de futuro.

Ao João, a primeira pessoa a receber-me não só no laboratório mas na nova fase da minha vida. Tenho de agradecer-te por tudo o que me ensinaste e pela paciência com que o fizeste. Juro que pensei que nunca conseguiria fazer um western blot ao fim de te ver tão confiante a fazer mil coisas ao mesmo tempo. Agradeço por teres sido peça fundamental na minha integração no grupo Aseb e por mostrares preocupação pelo meu trabalho do princípio ao fim deste ano. Encontrei um colega mas acima de tudo um amigo e esse foi um dos ganhos desta minha passagem pelo IMM.

Às minhas meninas! O que seria dos meus dias sem vocês? Sem as nossas conversas de desespero ou dos doces partilhados para adocicar os constantes maus resultados. Margarida, Antónia e Daniela agradeço por terem feito parte desta fase comigo, agradeço-vos pelos momentos partilhados. Tenho a certeza que este ano teria sido muito menos risonho sem vocês e se tivesse um grupo para escolher seriam as minhas três companheiras de novo, sem uma sombra de dúvida. A entreajuda e companheirismo que partilhamos nunca vou esquecer e por isso sei que este ano será algo que nos ligará durante a nossa vida.

A todos os Asebs, à Leonor, ao João, ao Diogo, à Carolina, à Joana Ribeiro, à Joana Gomes, à Joana Mateus, à Robertta, à Ana, ao Francisco, ao Miguel, ao Rui, à Sara Tanqueiro, à Sara Paulo, à Beatriz, à Catarina, à Marta, à Nadia, à Rita Belo, à Tatiana, à Maria José, à Sara Xapelli e à Sandra Vaz eu agradeço por toda a simpatia com que me receberam, por todo o apoio sempre que foi necessário, pelas dicas sábias que ajudaram a melhorar o meu trabalho, pela paciência quando foi preciso ensinar o que para vocês já era básico. Foi graças a este maravilhoso grupo que este ano foi proveitoso e que consegui terminar o objetivo inicial. Por isso, a todos vocês o meu muito obrigado.

À Inês Correia, a minha pupila, uma trabalhadora e inteligente jovem que ouviu todos os meus conselhos atentamente e me ajudou eficientemente quando o trabalho não acabava. Eu agradeço. E posso dizer que é com orgulho que fiz parte do teu projeto que tão bem agarraste e defendeste, acredito pelo que conheci de ti que vais ser uma mulher de sucesso com muitos objetivos para realizar impecavelmente como me habituaste.

À Helena Marcelino tenho de agradecer pela disponibilidade em passar os seus conhecimentos e em ajudar sempre que necessário. O meu obrigado por me dar as bases necessárias para eu poder trabalhar num local diferente e com outras metodologias. Foi devido ao seu grande profissionalismo e capacidade de ensinar que cheguei ao IMM sabendo que tinha capacidade para desempenhar as minhas funções.

Porém, como não só de trabalho se fez este ano tenho que lembrar quem me apoiou e esteve comigo incondicionalmente na minha vida pessoal. Às minhas amigas Ana Carolina Proença, Micaela Riscado e Carolina Rosa eu quero agradecer por nunca terem posto a distância que nos separava entre a nossa amizade. Agradeço por me terem apoiado em todos os momentos, por terem ouvido os meus desabafos e desabafado comigo. Agradeço-vos pela quantidade de histórias que partilhamos mesmo estando longe. Não seria possível manter a mesma saúde mental sem vocês ao longo deste ano. A vocês, o meu muito obrigado.

Ao meu namorado, tenho de agradecer por me ter acompanhado de perto durante todo este ano. Por toda a paciência para me ajudar a ultrapassar os momentos menos bons a nível pessoal e profissional. Agradeço por me ter sempre feito sentir em casa mesmo longe dela. Agradeço por me ter feito crescer a nível pessoal e por apoiar o meu crescimento profissional. Obrigado por teres acreditado em mim mesmo quando eu não acreditei e por relativizares as situações ao ponto de serem fáceis de ultrapassar. Este ano não seria possível sem ti.

Para terminar, à minha família tenho de agradecer pelo apoio que me foi dado logo que eu tomei a decisão de ir para muitos kms de distância. Pelo apoio incondicional, por estarem sempre comigo independentemente das decisões que eu tomei. Aos meus pais, por se terem sacrificado para que eu pudesse aproveitar esta oportunidade, por terem aceitado a minha decisão e por demonstrarem sempre querer o melhor para mim. Muito obrigada por me terem dado toda a bagagem necessária para enfrentar os diversos obstáculos da vida. Às minhas tias por me fazerem sentir perto mesmo estando longe, por me ajudarem a desvalorizar o que eu achava serem grandes problemas e por me apoiarem em todas as minhas decisões. Aos meus queridos avós o meu muito obrigado por me fazerem sentir sempre a neta que não estava longe, por mostrarem o seu amor incondicional todos os dias e por todo o suporte que me deram a mim, que estava longe, e aos outros que ficaram e que tanto precisavam deles. Ao meu irmão, agradeço por continuar a ser a minha pessoa independentemente da distância. Por último, aos meus priminhos eu agradeço por todos os momentos divertidos que me proporcionaram, por me fazerem sempre sentir perto e por me fazerem sentir um orgulho para eles. Tenho a certeza que qualquer que seja o rumo da minha vida vocês serão sempre orgulhosos dele e estarão sempre presentes. Acima de tudo sei que a vossa frase será sempre “Desde que te faça feliz…”

Estou segura de que cada dia deste ano foi um pouquinho mais doce por ter cada um de vocês!

Sozinho vais rápido, acompanhado vais longe

Resumo

O Glioblastoma é o tipo de tumor cerebral mais comum e mais agressivo do sistema nervoso central. Constitui 60 a 70% de todos os gliomas. Este tipo de tumores englobam um conjunto de caraterísticas próprias. Apresenta elevada quimiorresitência através da ativação do sistema MRD (sistema de resistência a múltiplas drogas) e aumento da expressão de transportadores ABC que facilitam a expulsão de agentes externos à célula. São encontrados no interior do tecido tumoral vários nichos com subpopulações celulares de células tipo-estaminal de glioblastoma (GSC - "Glioblastoma Stem-like Cells"), que conferem alta capacidade tumorigénica e de quimiorresistência. As GSC encontrarem-se principalmente em zonas de hipoxia e apresentam uma elevada capacidade de autorrenovação, diferenciação celular e propagação. O glioblastoma é designado comumente como um glioma de grau 4 por apresentar características próprias que o diferem de outros tumores cerebrais, como a elevada taxa de proliferação, grande capacidade de invasão, heterogeneidade celular com vários tipos de células e diferentes graus de diferenciação, elevada angiogénese e extensas áreas de necrose. O tempo médio de sobrevivência de um paciente com glioblastoma ronda os 12 meses, sendo que o tratamento padrão passa por recessão cirúrgica, radioterapia e quimioterapia com agentes alquilantes, principalmente. Pesquisas de novas terapias têm surgido tendo como alvo a inibição de proteínas das vias de sinalização que regulam o estado de hipoxia. Por análise de transcriptoma identificaram-se quatro subtipos de glioblastomas de alto grau, sendo eles o pré-neural, neural, clássico e mesenquimal. Cada subtipo apresenta um perfil clínico próprio sendo o mesenquimal o mais agressivo e com mais potencial tumorigénico. Nestes tumores a formação de novos vasos sanguíneos ocorre lentamente, limitando a distribuição de oxigénio e nutrientes nas células. É esta a principal causa das longas áreas de necrose e hipóxia que contribuem para um mau prognóstico da doença. Em condições fisiológicas normais a pressão de oxigénio mantêm-se no intervalo de 20-100 mm Hg, quando as pressões descem abaixo desse intervalo considera-se estado de hipoxia moderado. Se a pressão for inferior a 4 mm Hg é considerado um estado celular de hipoxia severa. Contribuindo para este estado está ainda o facto de o fluxo sanguíneo no interior do tumor não ser contínuo, acontecendo oscilações de estado normal, hipoxico moderado ou hipoxico severo. O estado de hipoxia controla a expressão de vários factores essenciais para a sobrevivência tumoral, instabilidade genómica, invasão, metástases e metabolismo celular. Esta regulação acontece pela activação de factores de transcrição induzidos por Hipóxia (FIH), existindo três tipos de FIHs (1a, 2a e 3a). Com a diminuição dos níveis de oxigénio torna-se possível a ligação dos FIHs aos elementos de resposta a hipóxia (ERHs) iniciando a transcrição de diversos genes. Consoante o tipo

de hipóxia são ativados diferentes tipos de FIHs e a resposta celular é diferente, por exemplo, a ativação do HIF1a tem como alvo enzimas glicolíticas enquanto que o HIF2a atua em proteínas de invasão. O HIF 1a é o que tem o maior nível de expressão no glioblastoma e atua sobre-expressando ectonucleotidases como a CD39 e CD73. Por sua vez, a enzima CD39 contribui para a hidrólise do ATP a AMP e CD73 pela formação de adenosina a partir do AMP. Assim, a sobre-expressão da CD39 e CD73 provoca um aumento de adenosina extracelular. O aumento dos níveis extracelulares de adenosina conduz ao aumento da passagem desta para o interior da célula através dos transportadores de nucleósidos. A adenosina é um nucleósido envolvido na homeostase do metabolismo energético e de ácidos nucleicos e do ciclo de metionina, produz proteção contra insultos neurotóxicos e plasticidade sináptica. A maioris das acções da adenosina são mediadas por activação de receptores de adenosina acoplados a proteína G (A1, A2A, A2B e A3). A concentração deste nucleósido em zonas hipoxicas, esquémicas, tumorais e inflamatórias aumenta cerca de cem vezes relativamente às concentrações fisiológicas. A ação da adenosina mediada pelos receptores em glioblastoma, não é consensual, sendo que em muitos trabalhos é referido um papel anti-apoptotico e noutros, pelo contrário, ações pró-apoptoticas. Novas evidências sugerem que a adenosina também pode atuar por mecanismos intracelulares independentes dos receptores. A adenosina é removida por acção da cinase da adenosina, que catalisa a fosforilação desta a AMP, e pela desaminase da adenosina, qua catalisa a hidrólise da adenosina a Inosina. Intracelularmente a adenosina é formada pela hidrólise do AMP a adenosina catalisada pela 5-nucleotidase. Outra via para a sua formação é mediada pela ação da hidrolase da S-adenosilhomocisteína (SAH) que converte S-adenosilhomocisteína em adenosina e homocisteína. A reacção catalisada pela hidrolase da S-adenosilhomocisteína encontra-se em equilíbrio rápido e, como tal, um aumento de adenosina ou homocisteína conduz ao rápido excesso de S-adenosilhomocisteína. Por sua vez, a S-adenosilhomocisteína é um potente inibidor das transmetilases dependentes de S-adenosilmetionina (SAM), o principal doador de grupos metilo nas células. Os grupos metilo estão envolvidos numa das principais modificações covalentes que ocorrem a nível genético nas células, a metilação. Vários trabalhos têm suportado a hipótese de o aumento intracelular de adenosina poder causar uma hipometilação global do ADN nas células. A diminuição da metilação do DNA é uma das principais características do glioblastoma causando instabilidade genómica e perda de "imprinting", em muitos casos conduzindo à diminuição da expressão de proteínas pró-apoptoticas e por outro lado à sobre-expressão de proteínas que contribuem para a progressão tumoral. A relação entre a adenosina e o metabolismo dos grupos metilo pode contribuir para perceber mecanismos promotores-tumorais neste tipo de tumor.

A metilação do DNA ocorre em locais específicos chamados “ilhas CpG”. No entanto, apesar de se observar uma hipometilação global do DNA no glioblastoma, pode ocorrer hipermetilação local na região promotora de alguns genes específicos. Esta característica dos gliomas confere-lhes a capacidade de bloquear algumas proteínas supressoras de tumor como por exemplo a metiltransferase da O6-metilguanina (MGMT). A MGMT é uma proteína reparadora de DNA cuja sua função é remover grupos alquilo da posição O6 das guaninas sendo que esta é a principal lesão que acontece a nível genético nas células. 60 a 85% dos gliomas apresenta perda local do cromossoma 10 onde está localizado o gene que codifica para a MGMT. Por outro lado, em 45 a 70% dos gliomas o promotor do gene MGMT encontra-se hipermetilado não permitindo a transcrição do gene e expressão da proteína.

O tratamento quimioterapêutico padrão aplicado a glioblastomas baseia-se em agentes alquilantes. Embora este tipo de tratamento já tenha sido imensamente estudado e otimizado ainda apresenta taxas de sucesso demasiado baixas. O temozolomide (TMZ) é um agente quimioterapêutico de primeira linha no tratamento de glioblastoma, o qual apresenta uma taxa de sucesso de apenas 20%. A ação do TMZ baseia-se na adição de grupos metilo nas posições O6 e N7 das guaninas e N3 das adeninas, sendo que a mais agressiva para a célula é a adição na posição O6 da guanina produzindo O6-metilguanina. Este tipo de lesão deveria, em condições normais, acionar o sistema de reparação de mismatch do ADN (MMR). Esta ativação do sistema MMR deveria causar elevada citotoxicidade para a célula e terminar em apoptose. No entanto, em células com expressão de MGMT, o TMZ não consegue exercer qualquer função pois sempre que é adicionado um grupo metilo na posição O6 das guaninas este é imediatamente retirado e a lesão do ADN deixa de existir. Esta é a principal causa de resistência aos tratamentos alquilantes, principalmente porque parecem haver mecanismos que conseguem reativar a expressão de MGMT depois de um tratamento com agentes alquilantes, o que é comumente chamado como resitência adquirida e uma das principais causas da tão pouca taxa de sobrevivência destes pacientes. Por outro lado, mesmo que não exista qualquer expressão da proteína MGMT, se o sistema MMR não estiver funcional também não é acionado qualquer mecanismo que cause citotoxicidade na célula, mais grave ainda é que muitas proteínas que fazem parte deste sistema deixam de estar funcionais depois de um primeiro tratamento com TMZ.

O objetivo deste trabalho consistiu em perceber, por um lado, se a adenosina tem algum efeito nos níveis de expressão de MGMT, uma vez que a possível hipometilação global do ADN causada pelo aumento da concentração de adenosina poderá afetar o nível de metilação do promotor do seu gene. Por outro lado pretende-se estudar se ao

aumentar os níveis de expressão da MGMT, a adenosina diminuirá o efeito citotóxico do temozolomide em glioblastoma. Para tal incubaram-se durante 3 dias linhas celulares de glioblastoma, na presença de um inibidor selectivo da cinase da adenosina, o ABT 702, e na presença ou na ausência de temozolomide. O ABT 702 qual promove o aumento da concentração da adenosina endógena por inibição da sua degradação. Foram estudadas 3 linhas de glioblastoma: U87, SNB19 e U373. Os níveis de expressão de MGMT foi analisado por Wester Blot e a sobrevivência/proliferação celular foi analisada pelo método do CCK-8.

Os resultados mostraram que o ABT 702 (15 μM) não afetou a expressão dos níveis de MGMT em nenhuma das linhas. No entanto o ABT 702 mostrou reduzir proliferação celular em células U373. Por outro lado, foi importante avaliar qual a resposta das células ao agente quimioterapêutico TMZ. O TMZ (500 μM) diminuiu a sobrevivência/proliferação celular nas células U373 e SNB19 mas não nas U87. Em condições de concentrações de adenosina aumentada o efeito do TMZ na viabilidade/proliferação celular não sofreu alteração nas células U373, embora fosse observada uma tendência para diminuição do efeito do TMZ na células SNB19. Por último, era importante saber se a diminuição da sobrevivência celular, avaliada pelo ensaio CCK8, produzida por condições de adenosina aumentada, por tratamento com ABT 702, e produzida tratamento com TMZ, envolviam o mesmo mecanismo. Por immunocitoquímica foi possível avaliar que a diminuição celular causada pelo incremento de adenosina não envolve mecanismos de morte celular, enquanto que a diminuição celular após tratamento com TMZ deve-se a mecanismos apoptoticos, tal como descrito na literatura.

Palavras-chave:

Glioblastoma, Adenosina, Temozolomide, O-6-methylguanine-DNA methyltransferase

Abstract

Glioblastoma is a primary brain tumor of glial cells with a high incidence in adults and is one of the most abundant brain cancer pathologies. This type of tumor is associated with high genomic instability caused by global hypomethylation of the DNA, that induces activation of oncogenes, loss of imprinting and increased genomic instability.

Adenosine is a purine nucleoside important to neuroprotection, neuromodulation, synaptic plasticity and immunomodulation. In hypoxic and stress regions in the brain tumors, commonly found in glioblastoma, factors induced by hypoxia (HIF) are activated. These factors modulate the expression of different proteins, particularly the CD39 and CD73. These enzymes are involved in the metabolism of adenosine, increasing the formation of this nucleoside extracellularly. Increased extracellular levels of adenosine lead to increased adenosine uptake into the cell by nucleoside transporters, producing accumulation of intracellular adenosine. Increased intracellular levels of adenosine or homocysteine lead to an increase of S-adenosylhomocysteine (SAH) levels and a decrease of the S-adenosylmethionine (SAM)/SAH ratio, since the reaction of adenosine with homocysteine to give SAH, catalyzed by SAH hydrolase, is near equilibrium. SAH is a competitive inhibitor of SAM-dependent methyltransferases, its accumulation causing global hypomethylation of DNA.

The O-6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair protein whose function is to remove methyl groups from the guanines in DNA. Although, very different mutations can cause the lack of MGMT expression, the hypermethylation of MGMT gene promoter is the main responsible for this.

Early mutations in glioblastoma frequently lead to hypermethylation in the MGMT gene promoter, which causes gene silencing and lack of expression of the MGMT DNA repairing protein. It is known that the expression of this tumor suppressor protein is responsible for the lack of glioblastoma's sensitivity to available first-line treatment, the alkylating drug Temozolomide (TMZ). TMZ is an alkylating agent used in glioblastomas treatment, usually combined with radiotherapy. However, the success of this agent is very low, above 20 % response rat, and the prognosis of the glioblastoma patients around one year. The TMZ effect is influenced by several factors inside the cell, like DNA mismatch repair system, MGMT expression, and base excision repair system. The expected final effect of TMZ is the apoptosis of the glioblastoma cells. Another

important effect is the acquired resistance which happens after the first treatment with TMZ in many patients. In vitro, different mechanisms became active after a first TMZ treatment, like an increase of MGMT expression and mutations in the DNA mismatch repair system proteins.

The aim of this study is to understand if there is a relationship between the increased intracellular adenosine, associated with global hypomethylation of DNA, and the chemotherapeutic resistance to TMZ caused by an increased in MGMT expression and activity.

Three glioblastoma cell lines, U87, U373, and SNB19 were used. Intracellular adenosine levels were increased by incubating cells for 72h with ABT 702 dihydrochloride (ABT, 15μM), an adenosine kinase inhibitor. TMZ (100 and 500 μM) was added for the last 1, 2, 3, 24 and 48 hours of incubation. MGMT expression and localization were visualized by immunocytochemistry and western blot, respectively. Cell viability/proliferation was assessed by CCK8 assay.

The results by western blot analysis showed that increased levels of adenosine do not affect MGMT expression. A decrease in cell viability/proliferation was observed after treatment with ABT in U373 cell line. After immunocytochemistry against active caspase 3, a marker of cell apoptosis, it was possible to see that the decrease of cells number produced by ABT is due to a decrease in cell proliferation and not cell apoptosis.

TMZ (500 μM) was able to decrease the cell viability/proliferation in SNB19 and U373 cell lines. We prove by immunocytochemistry that this effect involves cell death mechanisms mediated by caspase 3 activation, like apoptosis. The combined treatment with TMZ plus an inhibitor of MGMT, we visualized a more accentuated effect on cell survival, but the relative effect of TMZ was not affected because the inhibitor causes its own cytotoxicity.

Increasing levels of adenosine with ABT did not modified the TMZ effect on cell survival in U373 cell line, but in SNB19 cell lines seems to be a recovery of cell survival after combined treatment with ABT and TMZ.

Keywords

Glioblastoma, Adenosine, Temozolomide, O-6-methylguanine-DNA methyltransferase

Index

Chapter 1 1
Introduction 1
1.1 Glioblastoma 1
1.1.1 Glioblastoma Stem-like cells (GSCs) 2
1.1.2 Hypoxic state 3
1.2 ADENOSINE 4
1.2.1 Adenosine in tumor microenvironment (TME) 7
1.3 Methylation in glioblastoma 9
1.4 Temozolomide 11
1.4.1 DNA Mismatch Repair (MMR) 13
1.4.2 Acquired drug resistance 13
Chapter 2 17
Aim of the work 17
Chapter 3 18
Materials and Methods 18
3.1 Materials and cells 18
3.1.1 Cell lines 18
3.2 Methods 19
3.2.1 Cell lines cultures 19
3.2.1.1 Cells freezing, thawing and expansion 19
3.2.1.2 Cell passage 19
3.2.1.3 Cell Counting 19
3.2.2 Drugs and Pharmacology approach 20
3.2.2.1 Propidium iodide 20
3.2.2.2 ABT-702 20
3.2.2.3 Temozolomide 21
3.2.2.4 O6-Benzylguanine 21
3.2.2.5 Cell culture and pharmacological approach 22

3.2.3 Immunocytochemistry 23
3.2.4 Cell viability/proliferation and cell death assays 23
3.2.4.1 Immunocytochemistry with Propidium iodide 23
3.2.4.2 Cell proliferation and viability Assay (CCK8) 24
3.2.5 Western Blot analysis 24
3.2.5.1 Cell lysis and protein quantification 24
3.2.5.2 Western Blot 25
3.2.6 Statistical analysis 26
Chapter 4 27
Results 27
4.1 Expression of MGMT in GBM cell lines 27
4.1.1 Immunostaining 27
4.1.2 Western blot analysis 28
4.1.2.1 Basal expression of MGMT 28
4.2 Increased levels of adenosine do not affect MGMT expression in GBM cell lines 29
4.3 Increased levels of adenosine decrease cell viability/proliferation in glioblastoma cell lines 30
4.3.1 Cell viability 30
4.4 Temozolomide effect in glioblastoma cell lines 31
4.4.1 Immunostaining 31
4.4.2 Cell viability 33
4.5 Adenosine effect in Temozolomide resistance in glioblastoma cell lines 35
4.6 Mechanisms responsible for decreased cell survival in glioblastoma cell lines 37
Chapter 5 40
Discussion, Conclusion and future perspectives 40
5.1 Discussion 40
5.2 Conclusion and Future Perspectives 42
Chapter 6 44
References 44

List of Figures

Figure 1 - Schematic representation of the regulatory mechanism of activated adenosine receptors on apoptosis.

Figure 2 - Adenosine metabolism and signaling in the tumor microenvironment.

Figure 4 - Methyl group metabolism inside the cell.

Figure 5 - DNA repair mechanisms influencing cellular response to temozolomide.

Figure 6 - MGMT and TMZ action.

Figure 7 - Counting procedure. d.

Figure 8 - Chemical structure of iodide propidium.

Figure 9 - Chemical structure of ABT.

Figure 10 - Chemical structure of Temozolomide

Figure 11 - Chemical structure of O6-Benzylguanine.

Figure 12 - Timeline of treatments.

Figure 13 - Evaluation of MGMT location in GBM cell lines.

Figure 14 - Evaluation of MGMT expression levels.

Figure 15 - Evaluation of MGMT basal levels in U87, SNB19 and U373 cell lines.

Figure 16 - Evaluation of MGMT expression in GBM cell lines incubated with the vehicle (CTL) and with ABT 702 (15 μM) for 72h.

Figure 17 - Evaluation of cell viability/proliferation in GBM cell lines incubated with vehicle (CTL) and with ABT 702 (15 μM) for 72h.

Figure 18 - Evaluation of TMZ resistance in GBM cell lines by assessing PI uptake.

Figure 19 - Validation of PI uptake as a cell death marker for GBM cell lines. cope with a 20x objective. 70% ethanol caused a massive cell death in all cell lines.

Figure 20 - Evaluation of TMZ effect in cell viability/proliferation through CCK-8 assay.

Figure 21 - Evaluation of ABT-702 and TMZ treatment in cell survival.

Figure 22 - Evaluation of mechanisms responsible for decreased cell viability/proliferation in U87 and U373 cells.

List of Tables

Table 1 – Description of antibodies used in the assays

List of Abbreviations

ADO	Adenosine
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AMP	Adenosine monophosphate
ADK	Adenosine kinase
BER	Base excision repair
BSA	Bovine serum albumin
DNA	Desoxyribonucleic acid
DNMT	DNA methyltransferase
DMSO	Dimethyl sulfoxide
GBM	Glioblastoma
GSC	Glioblastoma Stem-like Cells
HAF	Hypoxia associated factor
HIF	Hypoxia Induced Factor
Keq	Equilibrium constant
MDR	Multiple drugs resistance system
MGMT	*O*^{6}-alkylguanine DNA alkyl transferase
MSH6	MutS homolog 6
MMR	DNA mismatch repair
MRP1	Multidrug resistance associated protein 1
MTHFR	Methylene tetrahydrofolate reductase
PHD	PHD finger
P-gp	Permeability glycoprotein
PTEN	Phosphatase and tensin homolog
PI	Propidium Iodide
RNA	Ribonucleic acid
RT	Room temperature
SAH	S-adenosilhomocysteine
SAM	S-adenosilmethyonine
TIMPs	Tissue inhibitor of matrix metalloproteinases
TME	Tumor microenvironment
TMZ	Temozolomide

List of scientific communications

Published article

Marcelino H, Nogueira VC, Santos CRA, Quelhas P, Fonseca-Gomes J, Tomás J, Diógenes MJ, Sebastião AM, Cascalheira JF. (2019). Adenosine inhibits human astrocyte proliferation independently of adenosine receptor activation. Journal of Neurochemistry: e14937. doi:10.1111/jnc.14937.

Poster Communications related with this Thesis

1.Vanda C. Nogueira, Cláudia A. Valente, Ana M. Sebastião, José F. Cascalheira. Role of adenosine on the resistance to an anti-tumor agent in glioblastoma cell lines. Meeting of the Portuguese Society for Neuroscience SPN 2019. 31 de May 2019, Lisboa, Portugal

2.Vanda C. Nogueira, Cláudia A. Valente, Ana M. Sebastião, José F. Cascalheira. Role of adenosine on the resistance to an anti-tumor agent in glioblastoma cell lines. XIV Annual CICS-UBI Symposium. 4 july 5 july, 2019, Covilhã, Portugal.

Proceedings Publications

1.Marcelino H, Santos CR, Nogueira VS, Fonseca-Gomes JF, Tomás J, Diógenes MJ, Sebastião AM, Cascalheira JF. 2019. The inhibitory effect of adenosine on human astrocyte proliferation is absent in glioblastoma cell lines. Frontiers in Cellular Neuroscience 13. http://dx.doi.org/10.3389/conf.fncel.2019.01.00004

2.Nogueira VS, Sebastião AM, Cascalheira JF, Valente CA. 2019. Role of adenosine on the resistance to an anti-tumor agent in glioblastoma cell lines. Frontiers in Cellular Neuroscience 13. http://dx.doi.org/10.3389/conf.fncel.2019.01.00009

Chapter 1

Introduction

1.1 Glioblastoma

Gliomas are around 30% of all tumors of the central nervous system and are classified according to the type of cell to which they histological resemble (Bastien et al. 2014).

Glioblastoma (GBM) is the most aggressive primary brain tumor of the central nervous system with the worst prognosis worldwide (Torres et al. 2019). GB is the more common tumor in the glial cells (Spyropolou, 2012). This type of tumor comprises 60%-70% of all gliomas and belongs to grade four subtypes. The time of patient's survival is around 12 months and its higher incidence is in adults (Spyropolou, 2012). This low survival rate is due to the high invasive capacity of GB, which infiltrates healthy brain tissue, leading to a high relapse rate (Torres et al. 2019,).

Glioblastomas can be divided into two groups, primary and secondary. The primary GBs are characterized by not presenting precursor lesions and have a fast and robust development, constituting 90-95% of all GBs (Bastien et al. 2014). Meanwhile, the secondary can accumulate mutations and evolve to a bigger grade of malignancy, with a longer history of disease progression (Fine, 2015).

The standard glioma treatment includes surgical resection followed by radio and chemotherapy (Bastien et. al, 2014).

GB is characterized by a high rate of proliferation, an elevated capacity of invasion, cellular heterogeneity, a high angiogenesis capacity, a long area of necrosis and raised chemoresistance by MDR (multiple drugs resistance system) activation which increases the number of ABC transporters expelling the chemotherapeutic drugs for extracellular space, decreasing their effect (Uribe et al. 2017).

By transcriptome analysis was identified four sub-types of high-grade GBM, pre-neural, neural, classical and mesenchymal, the last one is the most aggressive with bigger growth rates and tumorigenic potential. Each subtype represents a particular clinical profile.

Mutations in isocitrate dehydrogenase (IDH1 and IDH2) are another way to classify these tumors, they occur in most of the high-grade gliomas. These types of mutation divide the classification of the tumors relying on the presence (oligodendroglioma) or absence (astrocytoma) of the co-deletion of chromosome 1p e 19q. The mutations in IDH1 lead to the production of 2-hydroxiglutarate which, in its turn, inhibits the activity of a specific class of histones and DNA demethylases, resulting in DNA hypermethylation of specific genes, which is one of these diseases phenotype.

Other gliomas present mutations in genes which codify regulatory enzymes of chromatin. Mutations in H3F3A inhibit methyltransferases leading in aberrant patterns of DNA methylation (Aoki and Natsume, 2019).

One of the most important characteristics of GB is the global hypomethylation of DNA and could affect around 10 million CpG dinucleotides by haploid genome of the tumor. Experiments where hypomethylation is induced in mice, is enough to initiate the tumorigenesis (Cadieux et al, 2006). The severe hypomethylation of sequences like SAT2 is associated with alterations in the number of chromosomes which suggest that the decrease in methylation could be a determining factor to genetic alterations specific in GB. (Cadieux et al. 2006).

Although the role of genetic mechanisms in the activation of oncogenes and inactivation of tumor suppressors is well documented, very little is known about the epigenetic alterations in GBM.

1.1.1 Glioblastoma Stem-like cells (GSCs)

Inside of this type of tumors exists a subpopulation of stem-like cells which give a raised tumorigenic capacity and chemoresistance. Their capacity to auto-renewal, cell differentiation and propagation are responsible for GB generation and invasiveness (Torres et al. 2019). It is previously described that these cells can differentiate in astrocytes, oligodendrocytes, and neurons with aberrant signals of differentiation. Different aberrant signals culminate in the expression of specific's cellular lines markers together in the same cell. The majority of this type of cells is found in hypoxic zones of tumor. Therefore, the tumoral mass presents different types of cells with several differentiation degrees which end in a bigger problem of resistance because each cell with specific marker is more sensitive to one type of treatment. The stem-like cells activate

different mechanisms of drugs resistance from DNA repair, anti-apoptotic proteins expression and regulators of cell cycle to transporters. Besides all this, in vitro stem-like cells proliferate permanently but in vivo acquire a state of acquiescence and because of that the drugs which the target is components of the metabolism, cannot act. Because the previous studied treatments don´t have the pretend performance, recent studies have bet in new therapies which have as a target the bigger hypoxic regions, the inhibition of proteins and the signaling pathways that are regulated during the hypoxic state (Uribe et al. 2017).

1.1.2 Hypoxic state

Glioblastoma tumor growth does not coincide with an equal increase in nutrients and oxygen for cancer cell maintenance. The growth of this type of tumor is so fast that create areas limited in oxygen and nutrient delivery (Uribe et al. 2017). So, hypoxia represents one of the most curious characteristics in this type of tumor. This state modulates the transcriptional activity of HIFs (factors induced by hypoxia) which in tumoral environment contribute for invasion, proliferation, angiogenesis, drugs resistance, genomic instability, creation of metastasis, survival and metabolism maintenance. The presence of hypoxic niches is associated with a poor prognosis because of the low concentration of O2 in the tumor microenvironment favors invasiveness (Torres et al. 2019). The decrease of oxygen and nutrients flow seems like a contradiction, once that the tumor is characterized by high vascularization, but the key of this point will be de fact of the fast vascularization occurs slowly finishing in aberrant blood vessels and inefficient flow arrival (Uribe et al. 2017).

The normal pressure of oxygen in physiologic conditions is between 20 and 100mm Hg and when this one decreases to 4 to 20 mm Hg the state is called moderate hypoxic but, in several conditions, the values of pressure can be 0,75 to 4 mm Hg. In the brain of a patient with glioblastoma the areas with moderate states of hypoxia are the most common. Is described in literature that the blood flow inside the tumor is not continuous causing oscillations in oxygen pressure and because of that, the glioblastoma is a tumor with cyclic hypoxia in place of chronic hypoxia (Uribe et al. 2017).

Three types of HIFs are known, HIF 1a, HIF 2a and HIF 3a. In normal states the HIF 1a is hydrolyzed on Pro 402 and Pro 654, as consequence of its hydroxylation by HIF prolyl-hydroxylase (PHD), whose activity is oxygen-dependent. These hydrolyzed prolines are recognized by VHL tumor suppressor as part of ECU complexed with E3

ubiquitin ligase, culminating in polyubiquitination and degradation. In the hypoxic state, the PHD activity is inhibited and the HIF affinity to VHL decreases drastically which causes accumulation of HIF 1a. After this, HIF 1a heterodimerizes with HIF 2a and binds to HREs (hypoxia responsive elements) starting the transcription of the target genes.

The 1a factor is mostly associated with a response in acute hypoxia targeting the glycolytic enzymes. On the other hand, the 2a factor acts faster in chronic hypoxia acting in proteins associated with invasion, like MMP. The control of both factors is done by the HAF (hypoxia associated factor) and this activation induces transcriptional activity (Uribe et. al, 2017).

1.2 ADENOSINE

Purines and pyrimidines are fundamental molecules in the cell to cell contact between glial cells and neurons.

Adenosine (ADO) is an endogenous purine nucleoside with small half lifetime found in both intracellular and extracellular spaces (Soleimani et Al. 2018). ADO is considered a neuromodulator instead of a neurotransmitter, since it is not stored in synaptic vesicles or released after membrane depolarization (Ceruti and Abbracchio, 2013).

Its function in several organs is well known, acting in platelet aggregation inhibition; pos-ischemia cardio protection; vasodilatation; mast cells activation; neurotransmission modulation; neuroprotection; immunomodulation; cell death and survival. ADO can be involved in energy hemostasis, metabolism of nucleic acids and in the methionine cycle. Several studies still refer that the regulatory effect of adenosine on cell apoptosis in the tumour microenvironment (TME) depends on the cell line, subtype of adenosine receptor, and the adenosine concentration in tumor niche (Soleimani et al. 2018,). In astrocytoma was shown that several purinergic and adenosinergic pathways control the cell survival and cell death (Ceruti and Abbracchio, 2013).

In physiological conditions, the concentration of adenosine is between 30 and 200 nM but in stressful conditions founded in brain injury, after stroke, in neurogenerative diseases or in hypoxic regions of brain tumors, adenosine can reach micromolar concentrations (Torres et al. 2019).

The increased levels of adenosine are usually detected after the release of ATP followed by its sequential hydrolysis into adenosine by the action of ecto-nucleotidases. The effects mediated by ADO modulate the excitatory effects of ATP in a negative way (Ceruti and Abbracchio, 2013).

The TME is very complex. The immune system interplays with tumor initiation, progression, invasion, and metastasis. Adenosine has a critical role in tumor immune suppression, angiogenesis promotion, proliferation, progression and migration of tumor cells (Soleimani et al. 2018,).

In normal conditions, the main source of adenosine production in the intracellular environment is from 5´-adenosine monophosphate (AMP) hydrolysis catalyzed by 5´-nucleotidases and from S-adenosylhomocysteine hydrolysis catalyzed by S-adenosylhomocysteine hydrolase. The literature describes three routes for intracellular adenosine removal comprising: cleavage by adenosine deaminase into inosine; conversion to AMP by adenosine kinase (ADK) and export to extracellular space by nucleoside bi-directional membrane transporters (Ceruti and Abbracchio, 2013).

The adenosine production in extracellular space by ectonucleotidases includes CD39, which converts extracellular ATP to AMP, followed by adenosine production through ecto-5´nucleotidase activity CD73. Extracellular adenosine also can be produced from alkaline phosphates activity, prostatic acid phosphates pathway and CD38-CD203 pathway. The NAD^+ released by the salvage pathway is hydrolyzed to adenosine by the CD38-CD203 pathway in some tumors including gliomas (Soleimani et a., 2018,). Adenosine levels are maintained in equilibrium by reuptake mechanisms.

Most of the adenosine effects are mediated by G protein coupled receptors (Ars), four types have been being described, A1, A2a, A2b, and A3, which have different functions in apoptosis, as described in figure 1. These receptors are ADO concentration-dependent and their expression change with the levels of oxygen (Soleimani et al. 2018). The A1 and A2a types are activated at low nM values. On the contrary, the A2b and A3 receptors are activated when adenosine reaches micromolar concentrations. These receptors are not only found in the brain, but they are expressed in immune, vascular and endothelial systems (Valdés et Al. 2018). The A2b receptor seems to play a critical role in GB pathogenesis. It's known that A2b has low affinity for adenosine receptors, this fact supports the hypothesis that A2b is activated during high /pathological adenosine concentrations, consequently leading to an increase in the P-gp/ MRP1

expression and drug efflux, culminating in a chemoresistance to Temozolomide (Yan et al. 2019).

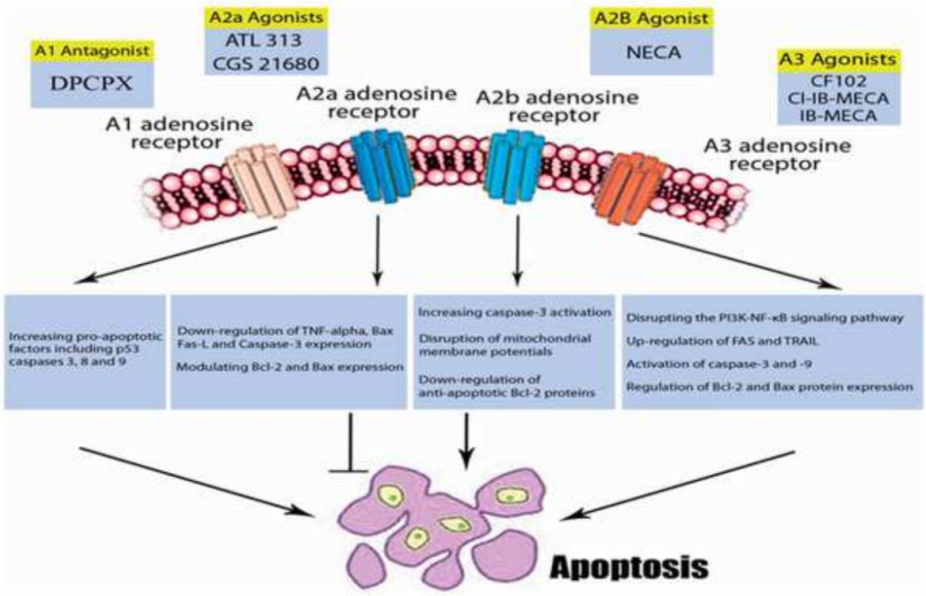


Figure 1 - Schematic representation of the regulatory mechanism of activated adenosine receptors on apoptosis (Adapted from Soleimani et. al, 2018).

Previous studies reported that the stimulation of A1 and A2b culminate in anti-proliferative and pro-apoptotic effects through BAD phosphorylation. However, the role of each receptor when activated in tumor development is still not well clarified because several studies have been published with conflicting results, some studies reporting pro-apoptotic actions while other studies reported anti-apoptotic effects (Soleimani et. al, 2018). Some studies support the idea of inhibitory effects of adenosine on the immune system while other studies report inhibitory effects on metastasis and angiogenesis (Valdés et al. 2018).

1.2.1 Adenosine in tumor microenvironment (TME)

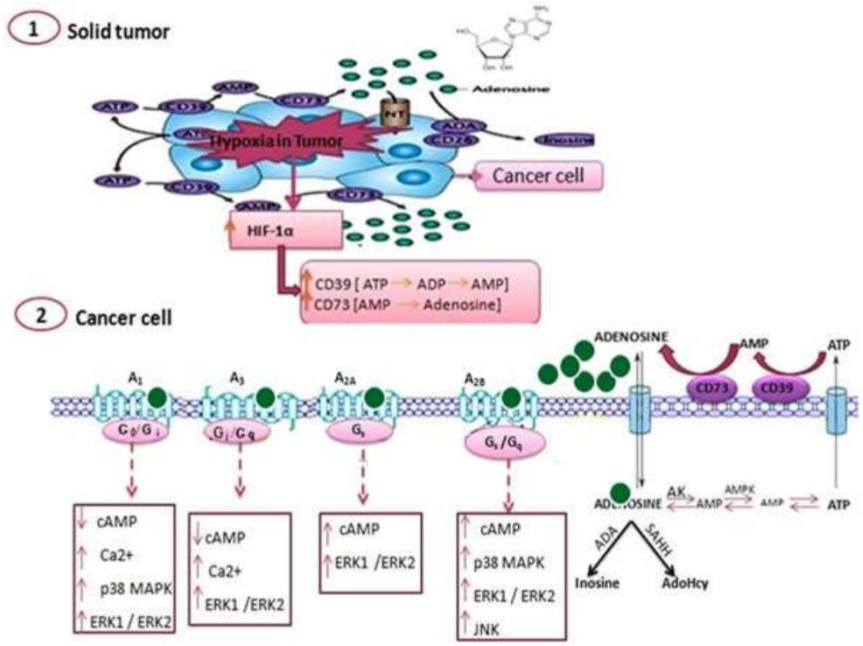


Figure 2 - Adenosine metabolism and signaling in the tumor microenvironment. (a) Hypoxia in solid tumors through HIF-1a (hypoxia-induced factor -1a) upregulates the CD39 and CD73 ectonucleotidases. (b) accumulation of extracellular adenosine in TME activates G-coupling adenosine receptors (A1, A2A, A2B, and A3) on tumor cells (Adapted from Soleimani et al. 2018).

Several studies in the literature reported that in hypoxia, stress, ischemia, inflammation and tumor regions there is an increase of extracellular adenosine, which is thought to be a cellular response to the lower oxygen pressures. The concentration values of this nucleoside in the hypoxic environment can increase 100 times, reaching the 1000-30000 nM interval. This is a consequence of the fact that the HIFs regulate the enzymes involved in adenosine production, increasing CD73 and CD39 expression, as schematized in figure 2. An explanation will be that the promotor of CD73 has a binding location for HIF 1a. On the other hand, it was described that activation of A3 receptors induces an increase in HIF 1a expression and the production of VEGF, thus promoting angiogenesis (Soleimani et al. 2018).

Recent studies showed that increased CD73 expression, with consequently increased adenosine levels, promotes tumor growth and development. In mice with wild type CD73, glioblastomas cells exhibited a high density of blood vessels whereas mice with CD73 knock out (CD73 -), GBM cells had significantly fewer vessels. An increased VEGF expression was observed on GBM in CD73- mice, which promotes endothelial cell

proliferation and glomeruloid vessels formation. CD73 on host epithelial cells regulate extracellular matrix remodeling through MMPs and TIMPs to promote GBM angiogenesis and invasion (Yan et al. 2019).

The increase of extracellular adenosine levels causes a bigger influx of adenosine into the cells a described above. The intracellular increase of adenosine or homocysteine consequently increases S-adenosylhomocysteine (SAH), because the SAH hydrolase catalyzed reaction is near equilibrium. This suggests that in certain conditions of excess of adenosine, the SAH can accumulate with significant cytotoxic effects, as shown in figure 3 (Krendich and Martin, 1977). SAH is a potent inhibitor of reactions catalysed by SAM-dependent methylases, SAM is the universal donor of methyl groups and essential for methylation reactions. The results of several studies show that the intracellular increase of SAH was associated with inhibition of DNA methylation in vivo, resulting in global DNA hypomethylation (Friso et al. 2016).

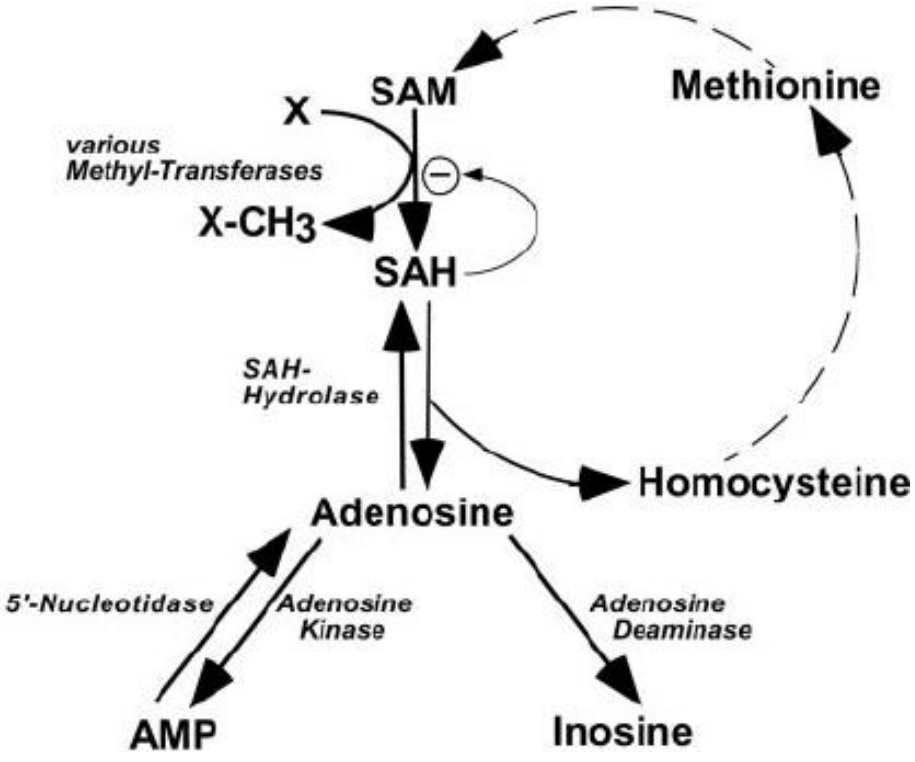


Figure 3 - Pathways of adenosine metabolism. Adenosine is formed either by hydrolysis of AMP or by hydrolysis of SAH, which arises from the action of methyltransferases. Adenosine can be metabolized by ADA or ADK into inosine and AMP, respectively (Adapted from Boison et al., 2002).

1.3 Methylation in glioblastoma

The methylation is a critical step during tumoral growth (Aoki and Natsume, 2019) is also involved in the control of specific genes expression in the tissues, genomic imprinting, and inactivation of X chromosome. This mechanism acts as a protection of exogenous DNA input, like virus DNA (Lafon-Hughes et al. 2008). This covalent modification is regulated by the concerted actions of DNA methyltransferases (DNMTs) and DNA demethylases. DNMTs catalyse the covalent transfer of methyl groups, donated by SAM, to the 5´position of cytosine, forming 5-methylcytosine (Cadieux et al. 2006), this alteration is specific in each species and tissue (Romani et al. 2018).

One of the main characteristics of the tumors is the decrease of global methylation of DNA (hypomethylation) affecting the intergenic regions, repetitive sequences of DNA and bodies of genes including regulatory sequences. The mechanisms associated with malignancy and DNA hypomethylation involve changes in regulatory elements culminating in overexpression of transcriptional genes.

On the other hand, hypermethylation in promoter regions of specific genes, leads to the transcriptional silencing of tumor suppressors and DNA repair genes, which are other phenotypic characteristics of GB. The areas with high density of cytosines and guanines are called dinucleotide cytosine-phosphate-guanine (CpG) islands, in all genome 60 to 70% of all CpG are founded methylated (Lafon-Hughes et al. 2008).

The CpG islands are not only found in local genes promotors but in bodies of genes or regulatory elements like enhancers, silencers or insulators. These elements contain binding locals for transcriptional factors and act by increasing or decreasing the transcription (Cadieux et al. 2006).

Histones are also regulated by methylation and acetylation, which could result in activation or repression of the transcription of several genes. More of one hundred enzymes act in regulating histone modification, forming the modification code of histones which defines the transcriptional activity of the gene. These activities may change, modifying the response to the drugs and benefit the development of the tumor. The new cells that undergo chromatin re-modulation show a transitional drugs resistance which leads to the main problem of this type of tumors which is the quick resistance to the drugs (Romari et al. 2018).

The effect of altered methylation profile was verified in rats and it is enough for initiating the tumorigenesis. The methylation profile of DNA has been associated different grades of astrocytomas (OMS, II,III, IV) and if it is a primary or secondary tumor (Spyropoulou et al. 2013).

The literature data suggest a model by which higher cell proliferation, in a context of inadequate availability of methyl donor by MTHFR deficiency, leads to an increase of proliferative activity in GBM (Cadieux et al. 2006).

Although the role of genetic mechanisms in oncogenes activation and tumors suppressors inactivation in GBM is well documented, very little is known about the epigenetic alterations in this disease. The experimental support for the role of hypomethylation in tumorigenesis by increasing genomic instability and elevating mitotic recombination, is supported from studies of mouse models with hippomorphic alleles for DNA methyltransferase. These mice were developing aggressive lymphomas. Similarly, the global hypomethylation of DNA achieved by a decrease in DNMT1 activity, accelerates the beginning of tumoral formation in a mutant mouse model. Moreover, the removal of DNMT1 in mouse embryonic stem cells increased the rates of mutations, while a double knockout of DNMT1 and DNMT3b in one tumor cellular line results in dramatic hypomethylation and genomic instability visualized by chromosomic translocations. The loss of imprinting generated by the lack of DNMT1 activity in rat's development leads to solid tumors formation (Romari et al. 2018).

As no mutation in DNMT genes was reported in glioblastomas, the decrease of DNMT function and the altered levels of DNA methylation can occur by disturbances in production of universal donor of methyl groups, SAM. SAM is characterized for being the methyl donor group which acts in most of the methylation reactions either of DNA or RNA. For example, diets with methyl deficiency, like reduce methionine or choline, or inadequate sources of coenzymes for methyl group metabolism, like folates or B6 and B12 vitamins caused a decrease in intracellular SAM production leading to genomic hypomethylation (Friso, et al. 2016).

We can see a strict relation between the fact that the tumor found in hypoxic environment, in which intra and extracellular adenosine concentrations are elevated, with the decrease of methylation activity. In section 2, was mentioned that SAH is a powerful inhibitor of SAM-dependent transmethylases. The reaction of SAH hydrolase is reversible and the Keq in the forward direction is very low, 1,4*10-6 M. Because of that, is predictable that the increase of intracellular adenosine levels is followed by an

increase in SAH concentrations and inhibition of SAM-dependent transmethylases. Is important to refer that most cell membranes are impermeable to SAH, so its raise depends mainly to the endogenous rate of production by SAM-dependent transmethylases or by the capacity to metabolize the products of SAH hydrolyze reaction, this process is represented in figure 4.

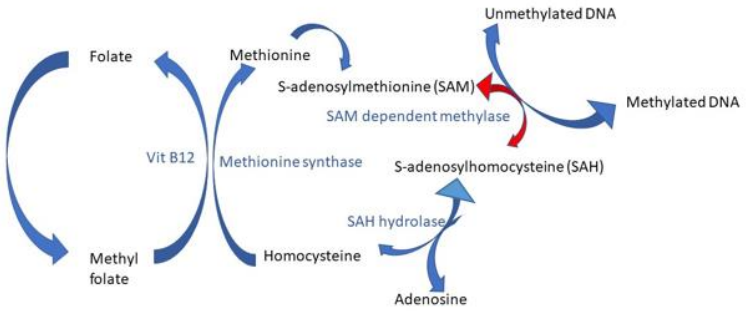


Figure 4 - Methyl group metabolism inside the cell (Hermes et al. 2008).

1.4 Temozolomide

Temozolomide (TMZ) is the chemotherapeutic agent of first line of treatment in glioblastoma. This prodrug is a small lipophilic molecule (194 Da) stable at acidic pH values (Zang et al. 2015). In aggressive treatments this drug is combined with radiotherapy or another chemotherapeutic drug (Jawhari et al. 2016).

The TMZ is rapidly absorbed but is spontaneously decomposed, due to the brain tumor having a more alkaline pH compared with surrounding healthy tissue, to form monomethyl triazene 5-(3-methyltriazen-1-yl)-imidazole-4-carboximide (MTIC). The MTIC reacts with water, liberating 5-aminoimidazole-4-carboxamide (AIC) and a highly reactive cation, methyldiazonium. The mechanism of TMZ action is due to the active species, methyldiazonium cation, which methylates the o6 positions in guanine rich regions and, in smaller levels, the N3 and N7 adenine residues. Thus, TMZ acts like an alkylating agent by adding methyl groups to DNA, producing o6-methylguanines causing misspairs with timine in the next replication cycle. This is the cause of TMZ cytotoxicity, which leads to carcinogenic, mutagenic and toxic lesions (Zang et al. 2015). A mechanism cause G-T imbalance in DNA which activate the DNA MMR (mismatch repair) system. This mechanism only recognizes the mispaired thymine on the daughter strand and remove the timine, this last one is inserted during the next repair synthesis cycle. The cycles lead by MMR can end in very different ways. Normally, the lesions caused by o6-

methylguanine by TMZ action culminate in the stop of cell growth, p53/p21Waf1/Cip1 activation and, lastly, in apoptosis. So, a good response to TMZ requires functional MMR and low levels of MGMT. The most important DNA repair systems impacting the mechanism of action and cytotoxicity of TMZ are MGMT, MMR and Base excision repair (BER), as described in figure 5.

In the cells with decrease MGMT and MMR, these mispair are tolerated and don't activate the repair system, thus are not considered toxic for the cell. As such, the TMZ action is MGMT and MMR dependent but few patients present alterations in MMR system. So, the principal cause why the TMZ doesn´t act like expected seems to be the MGMT activity which repairs directly the mismatches caused by TMZ action.
Although TMZ offers some hope to GB patients, just a 5-year survival rate of only 9,8% is achieved (Zang et al. 2015).

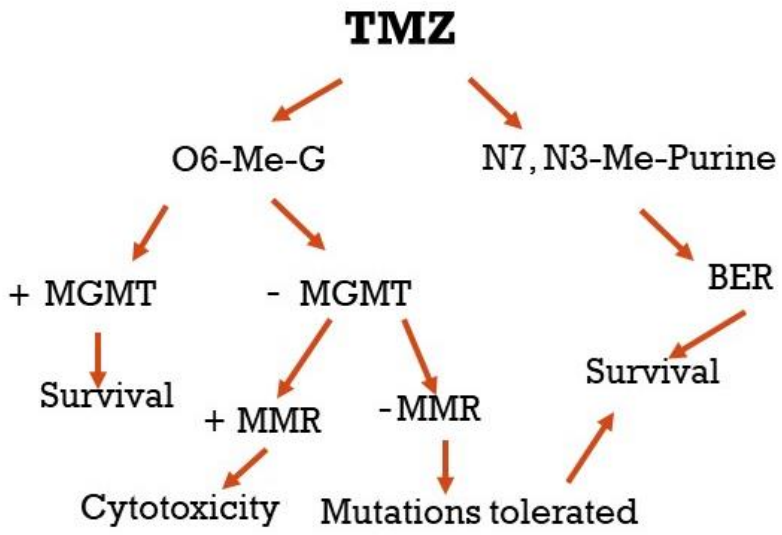


Figure 5 - DNA repair mechanisms influencing cellular response to temozolomide (Zang et al., 2015).

1.4.1 DNA Mismatch Repair (MMR)

The MMR (mismatch repair) system is the mechanism responsible for the recognition and correction of insertion or deletion loops and mispaired bases that may occur during the DNA synthesis. The MSH2, MSH6, MSH2, and MSH3 form two

complexes, MutSa and MutSβ respectively, that binds to mismatch lesions. The complexes recognize different things, the MutSa binds to insertion/deletion mismatch loops of one or two nucleotides and base to base mismatch. So, a loss of MMR causes a higher rate of insertion/deletion mutations in repetitive sequences. The cells with MMR deficient are described to be up to 100-fold less sensitive to methylating agents comparing with the normal cells. In the cells where the 06-MeG-thymine mispairs are not recognized the O6-MeG lesions are tolerated, leading to a surviving with extensive mutagenesis (Zang et al. 2015).

1.4.2 Acquired drug resistance

The acquired resistance comes from a natural selection by the pressure in the chemotherapeutic agent presence. The acquired chemoresistance is a consequence of drug-induced genetic and epigenetic changes in neoplastic cells, inducing and selecting genes which confer a survival advantage. In the beginning, the treatments eliminate the sensitive malignant cells but the drugs-resistant cells keep up with new mechanism, as well as chemo-resistant cancer stem cells which may advance to seed more resistant tumors. As already said, in GB there is a sub-population of stem cells which confer the chemo-resistance to the tumor by different mechanisms. The major contributions to the treatment failure are the multi-drug resistance. Several mechanisms confer to the cells this resistance: decreased cellular drug uptake, increased drug efflux by membrane pumps actively expelling chemotherapeutic agents, intracellular drug inactivation, alteration of drug target by mutations, inactivation or over-expression, enhanced repair of drug-induced DNA damage or suppression of repair resulting in tolerance to DNA lesions and alteration of apoptosis-related genes.

The tumor resistance may be a consequence of the presence of multidrug resistance proteins (MRP1, MRP3, MRP5 and glutathione-S-transferase (GST π)) in the unhealthy tissue. It was described that the expression of multi-drug resistant p-glycoprotein has a negative impact on the TMZ sensitive. Another important aspect is the mutation that gliomas presents in chromosome 10, which inactivates PTEN, causing a constitutively active Akt/PI3K/Mtor/NF-kB signaling leading to naturally apoptosis resistance.

Despite all these factors, the up regulation of MGMT seems to be the principal mechanism of acquired TMZ resistance. A study demonstrated that in untreated tumors the MGMT activity levels round the 37 fmol/mg but in tumors recurrently treated with the TMZ the levels increased to 182 fmol/mg (Zang et al. 2015). This may be a

consequence of the selection of MGMT-expressing cells or induction of MGMT by alkylating agents.

Literature suggests that the intrinsic MMR deficiency is rare in glioblastomas but after treatment, with alkylating agents, mutations appear in MMR proteins leading to development of tolerance to O6-MeG lesions and therefore TMZ resistance regardless of MGMT expression. An example of this is the analysis of MSH6 activity after drugs which reveal a loss of this protein. MSH6 mutations have been found in 26% recurrent GBM cases following alkylating agent chemotherapy. Genomic characterizations of GBM suggest that the initial MGMT promoter methylation in conjugation with alkylating agent treatments leads to a shift in the mutation spectrum affecting mutations at MMR gene loci (Zang et al. 2015).

Previous studies have demonstrated the limited permeability of an intact blood-brain barrier. However, with the presence of tumor cells the BBB becomes heterogeneously disrupted and has been noted as the blood tumor barrier (BTB). The BBB and BTB provide a physical barrier with collaborative cells that inhibit the entry of toxins, including chemotherapy (Jackson et al. 2018)

1.5 O6-methilguanine methyltransferase (MGMT)

The guanine alkylation in O6 position is the more significantly DNA lesion responsible for mutagenic, carcinogenic and cytotoxic properties in all organisms, human included.

The MGMT is a small protein with 22 kDa present in cytoplasm and nucleus. The gene encoding for this protein is localized in 10q26 chromosome and 60-85% of gliomas present a loss of this chromosome (Zang et al. 2015)

The MGMT levels vary widely between different species and tissues. The liver contains the highest level of MGMT activity.

When occurs an alkylation the localization of MGMT change and it becomes more nuclear. The MGMT repairs DNA in a single mechanism of action removing a methyl and alkyl groups of guanines, inhibiting the possible double chain crosslinking in O6G position. The 06-alkyl group is transferred from guanine to the cysteine residue's (cys 145) active site of MGMT in a stoichiometric, auto-inactivating reaction, thereby repairing DNA and inactivating MGMT. This protein binds damaged substrate DNA in the minor groove, the target base is then flipped out of the helix and bound to MGMT, altering the conformation of the DNA binding domain allowing alkylated MGMT to be

detached from DNA and degraded through the ubiquitin/proteasomal system, this mechanism is schematized in figure 6 (Zang et. al, 2015).

Thus, the MGMT acts like a tumor suppressor in the defense against cytotoxicity, mutagenicity, and carcinogenicity of exogenous alkylating agents. So, the cells that express the protein have lower probability of mispairings and mutations. The levels of expression of this protein have normal distribution in the population but in brain tumor patients the levels are very variable. The lower expression is found in glioblastomas. The loss of MGMT activity is mainly associated with the MGMT promoter hypermethylation. The excessive methylation is mediated by 5-methylcytosine methyltransferase and occurs in the cytosines of CpG islands. This modification prevents the binding of the transcription factor and the gene is silenced. This MGMT promotor methylation has been detected in 45-70% high-grade gliomas (Zang et al. 2015).

Nearly 80 % of high-grade secondary gliomas with mutant IDH presents MGMT methylation, suggesting a strong relationship between both mutations. Grade II and III gliomas with IDH mutant, in contrast with other gliomas, carry two copies of chromosome 10. So, MGMT is not completely methylated resulting in resistance to the chemotherapeutic agents (Aoki and Natsume, 2019).

Besides the excess of methylation of MGMT gene promoter which causes the decrease of protein expression, is also known that in glioblastoma the levels of ubiquitinated MGMT are more elevated than the normal, which could explain the lack of protein action by an excessive degradation. This is a determinant factor for the response to the treatment with alkylating agents because de effects of these are contrary to the MGMT action. Moreover, the cells with additional methyl groups present higher radio-sensitivity.

The patients with methylated MGMT have a time of survival around 21.7 months, while the patients with unmethylated MGMT have their time decreased to 12.7 months (Romani, 2018).

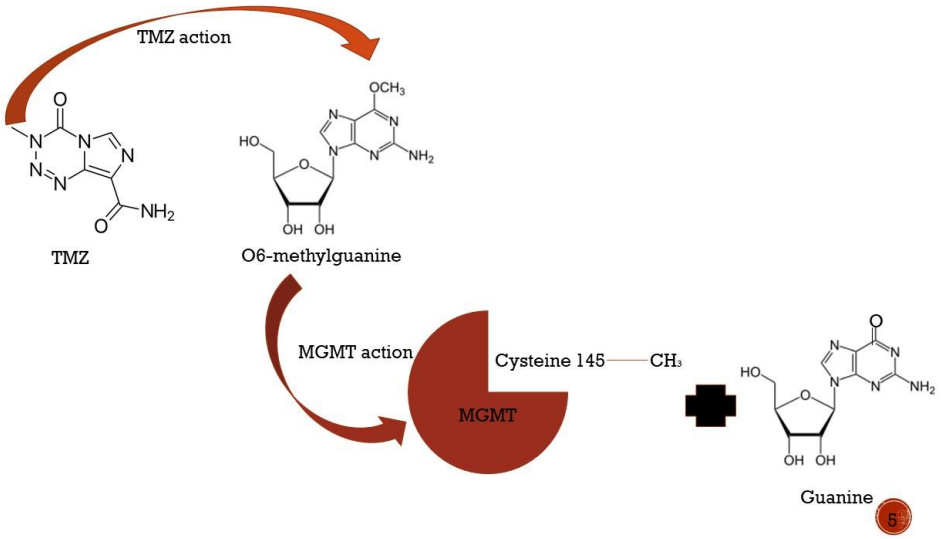


Figure 6 - MGMT and TMZ action. The action of TMZ is the opposite of MGMT. While the alkylating agent adds a methyl group to DNA guanines in O6 positions, the MGMT repair this guanine removing the methyl group. Thus, if the cells have expression and activity of the MGMT, the TMZ can´t do the intended effect and does not present cytotoxicity to the cell.

Chapter 2

Aim of the work

The temozolomide is the main treatment of glioblastoma patients with a low rate of success due to repair proteins activity, like MGMT. In glioblastoma the MGMT gene promoter is usually hypermethylated, reducing MGMT expression and therefore enabling the cytotoxic effect of temozolomide on glioblastoma cells. Increased levels of adenosine can be found in hypoxic regions inside the tumor. On the other hand, increased adenosine concentrations can induce global DNA hypomethylation, by reversal of the near equilibrium SAH hydrolase-catalysed reaction, leading to SAH accumulation and consequent inhibition of SAM-dependent transmethylases. Therefore, increased adenosine levels might increase MGMT expression by decreasing MGMT promoter methylation.

The aim of the present work is:

To explore if adenosine increases MGMT expression.

To understand if increased levels of adenosine affect the response to TMZ in three different human cell lines of glioblastoma.

Chapter 3

Materials and Methods

3.1 Materials and cells

DMEM High Glucose w/ stable L-Glutamine, w/ Sodium Pyruvate medium (Biowest); FBS (Biochrom); DMSO (Sigma); gentamycin (Termofisher); trypsin (Biochrom); ABT-702 (Tocris); TMZ (Sigma-Aldrich); paraformaldehyde (PFA, VWR, Pennsylvania, USA); glycine (Sigma-Aldrich); Triton X-100 (Sigma); HOESCHT (Sigma-Aldrich); protease inhibitors (Sigma); PMSF (Sigma); Bio-Rad DC Protein Assay Kit (Bio-rad); BSA (BSA; NZYtech); Propidium iodide (PI, Sigma); protein molecular weight marker (NZYColour Protein Marker II, NZYtech); PVDF membrane (Bio-Rad); O6-Benzylguanine (Sigma). Stock solutions of ABT-702 and TMZ were prepared in DMSO, while stock solution of O6-benzylguanine was prepared in methanol.

3.1.1 Cell lines

Three different immortalized cell lines of glioblastoma were acquired from ATCC:

-U373 (ATCC® HTB17TM), an adherent immortalized human cell line of glioblastoma grade III, astrocytoma, from a 61 years old male brain with epithelial morphology;

-U87 (ATCC® HTB14TM), an adherent immortalized human cell line of glioblastoma from a male brain with epithelial morphology;

-SNB19 (ATCC® CRL2219TM), an adherent immortalized human cell line of glioblastoma, from a 47 years old male brain with fibroblast morphology.

3.2 Methods

3.2.1 Cell lines cultures

3.2.1.1 Cells freezing, thawing and expansion

Cells were kept in -80ºC in cryopreservation vials with $5*10^5$ cells/ml per vial in DMEM medium composed of DMEM High Glucose w/ stable L-Glutamine and sodium pyruvate, supplemented with 40% of FBS and 10% of DMSO to prevent the formation of crystals.

For thawing, the vials were slowly unfrozen and seeded in a flask with DMEM High Glucose w/ stable L-Glutamine and sodium pyruvate medium supplemented with 10% of FBS and 0,05% of gentamycin, referred as DMEM complete medium in the following sections.

3.2.1.2 Cell passage

When cells reached 90% confluency, cell passage was performed to ensure cell viability without stress. Cells were washed twice with phosphate buffered saline (PBS) and detached by adding 1 ml of trypsin-EDTA solution (0.25% in PBS) for 5 min. Trypsinization was stopped with 1 ml of DMEM complete medium. The cells were resuspended gently and centrifuged for 5 minutes at 1000 rpm. The supernatant was discarded. The pellet was re-suspended in 1 ml of fresh DMEM complete medium.

3.2.1.3 Cell Counting

For the different assays, a specific platting density of cells was necessary for each cell line. Since the cell lines under study had different growth rates, the initial platting density was adjusted to each cell line, so that all cell lines reached the same cell density at the end of the incubation period. For counting, 20 µl of cells suspension was added to 20 µl of trypan blue solution. The mix was well homogenized and 30 ul was placed in a Neubauer chamber (Fig. 7). Four different fields were counted and averaged. Finally, the total number of cells were calculated through:

Figure 7 - Counting procedure. Image of a Neubauer chamber (A) and representation of the grid (B). Four fields counted were counted and averaged.

3.2.2 Drugs and Pharmacology approach

3.2.2.1 Propidium iodide

Propidium iodide (PI) is a popular red-fluorescent nuclear and chromosome counterstain represented in figure 8. Since propidium iodide is not permeant to live cells, it is also commonly used to detect dead cells in a population (Dumitriu et. al, 2001).

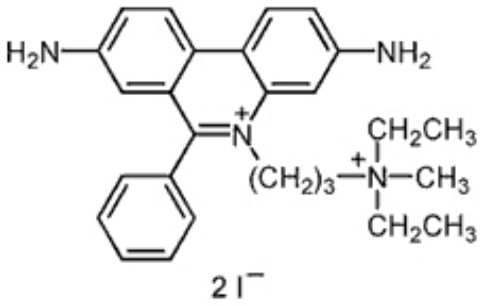


Figure 8 - Chemical structure of iodide propidium.

3.2.2.2 ABT-702

ABT is a potent non-nucleoside adenosine kinase inhibitor (IC = 1.7 nM), selective over other sites of adenosine interaction represented in figure 9 (A1, A2A and A3 receptors, adenosine transporter and adenosine deaminase) (Jarvis et. al, 2000).

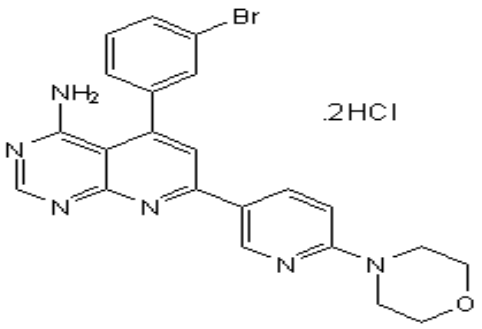


Figure 9 - Chemical structure of ABT.

3.2.2.3 Temozolomide

Temozolomide (TMZ) is a DNA methylating agent and drug resistance-modifying agent with anti-tumoral and anti-angiogenic actions represented in figure 10.

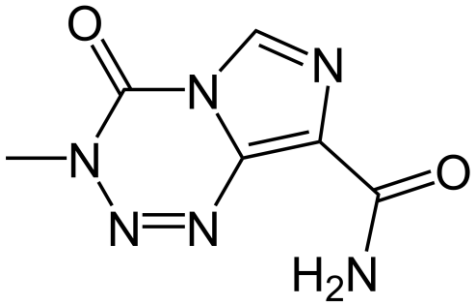


Figure 10 - Chemical structure of Temozolomide

3.2.2.4 O6-Benzylguanine

O(6)-benzylguanine (O6-BG) is an antineoplastic agent that binds the DNA repair enzyme O(6)-alkylguanine DNA alkyltransferase (MGMT), resulting in inhibition of MGMT-mediated DNA repair, represented in figure 11. It is widely used in various DNA repair mechanism studies and potentiates the effects of other chemotherapeutic agents that damage DNA (Bobustuc et. al, 2012).

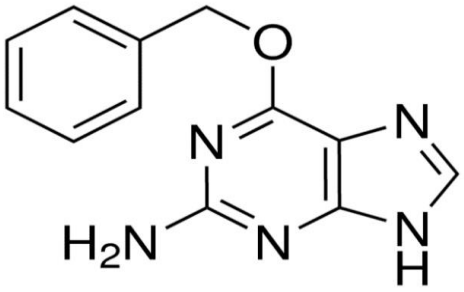


Figure 11 - Chemical structure of O6-Benzylguanine.

3.2.2.5 Cell culture and pharmacological approach

GBM cell lines U87, SNB19 and U373 were plated at 13000, 4800 and 3600 cell/cm2, respectively, and grown at 37ºC, in a humidified atmosphere of 5% CO2 and 95% atmospheric air, in DMEM complete medium. Unless otherwise stated, the above conditions were used in all studies.

When testing the effect of ABT 702, two days after plating cells were treated for another 3 days with ABT 702 (15 µM). For TMZ, two concentrations were tested, 100 and 500 µM. TMZ was applied to the cells for 1, 2, 3, 24 and 48 hours. The effects of O6-BG (50 µM) was tested for 96 hours. Whenever testing the effect of drug(s), a parallel control assay was performed, where the same volume of vehicle was applied to the well. At the end of the incubation period, to finish the stimulus, the cells were washed twice in PBS. The timeline of the treatments is represented in Figure 12.

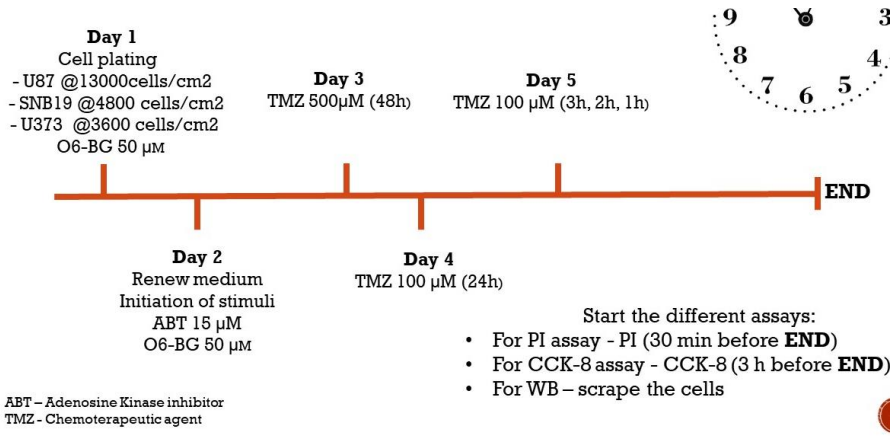


Figure 12 - Timeline of treatments.

3.2.3 Immunocytochemistry

For immunocytochemistry (ICC) GBM were cultured in 24-wells plates with one glass coverslip inserted in each well. Cells were washed twice with PBS and fixed with 4% PFA for 15 minutes at RT. PFA residues were washed with freshly prepared 0,1M glycine in PBS for 10 minutes. The cells were then permeabilized with 0,5% Triton X-100 in PBS for 10 minutes and blocked with 10% FBS in PBS for 1 hour at RT. Cells were incubated overnight at 4ºC with 20 µl of primary antibodies, diluted in blocking solution. In the next day cells were washed in PBS-T (PBS with 0,05% Tween-20) and incubated for 1 hour in 20 µl of secondary antibodies diluted in blocking solution. After this step is mandatory to protect the coverslips from light since secondary antibodies are coupled to fluorophores. For the last washes, the coverslips were placed in a new 24-wells plate and incubated for 5 minutes at RT with 50 µl drop of the nuclear marker HOESCHT (1:100 dilution in PBS from a 2mM stock). Cover-slips were mounted in glass covers with a small drop (4µl) of Mowiol (2,4g Polyvinylalcohol 4-88, 600 mM glycerol, 200mM tris, pH 8.0, in Milli-Q water) mounting medium. Fluorescence images were visualized under a wide field fluorescence microscope (Axiovert 200, Zeiss, Germany).

3.2.4 Cell viability/proliferation and cell death assays

3.2.4.1 Immunocytochemistry with Propidium iodide

The cells were treated for 30 minutes with a PI solution at 7 µM. After the treatment, the cells were washed twice with PBS and fixed with 4% PFA for 15 minutes at RT. The residues of PFA were washed with freshly prepared 0,1M glycine in PBS and cells were permeabilized with 0,5% Triton X-100 in PBS for 10 minutes. Nuclei were stained with Hoechst, as explained in the previous section, and coverslips were mounted in covers over a small 4 µl drop of Mowiol mounting medium. Lastly, the immunostaining was visualized under a wide field fluorescence microscope (Axiovert 200, Zeiss, Germany). The number of Hoechst stained nuclei and PI-positive cells were counted in 5 fields per coverslips per condition.

3.2.4.2 Cell proliferation and viability Assay (CCK8)

CCK-8 assay was used to measure the cell viability/proliferation. Cell counting kit-8 (Dojindo Molecular Technologies, Inc) allows very convenient assays by using highly water-soluble tetrazolium salt. WST-8[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)2H-tetrazolium, monosodium salt] produces water-soluble formazan dye upon reduction by cell dehydrogenases in the presence of an electron mediator.

Cells were platted at the right density for each cell line, in a 96-wells plate (100 µl/well) and incubated 72 hours with test drugs. At the end of incubation, 10 µl of CCK-8 solution were added to each well. After 2.5 hours of incubation absorbance was measured at 450 nm using a micro-plate reader. The absorbance of a well without cells (blank) was subtracted to the total absorbance and results were expressed as fold increase of the control.

3.2.5 Western Blot analysis

3.2.5.1 Cell lysis and protein quantification

For western blotting analysis, cells were cultured in 6-wells plates. At the end of the experiment cells were washed twice with PBS, 100 µl of complete lysis buffer (containing 50mM Tris pH 8.0 (EDM Milipore), 1mM Ethylenediamine tetraacetic acid (EDTA, Sigma), 150mM NaCl (EDM Milipore), 1% Nonyl phenoxlpoylethanol (NP-40, Fluka Biochemika) and 10% glycerol (Sigma)) and supplemented with protease inhibitors and 1mM phenylmethylsulfonyl fluoride (PMSF, Sigma), to prevent protein degradation by proteases released in the cell disruption process. Cells were detached from the wells with a cell scraper. The cell lysate was placed in a cryotube and immediately frozen in liquid nitrogen. The cell lysates were kept at -80°C until further analysis. For protein quantification, the cell lysate was thawed, and the cells were further dissociated with a needle doing up and down movements for 5-6 times, followed by 15 minutes agitation at 4°C and centrifugation at 13000 rpm for 10 minutes at the same temperature. The supernatant was collected in a new micro-tube.

Total protein was quantified using the Bio-Rad DC Protein Assay Kit (Bio-rad), which is a colorimetric assay for protein concentration in detergent solubilized samples. For quantification, 10µl of BSA (0.05-1mg/ml) protein standards and samples were added in duplicate in a 96 well flat bottom plate. After that 25 µl of reagent A' (reagent A and S in 100:2 proportion) followed by 200µl of reagent B were added. The plate was

covered and gently agitated to mix all reagents. After 15 min, absorbance was read at 750 nm in a microplate reader (Infinite M200).

3.2.5.2 Western Blot

The western blot technique allows the relative quantification of proteins and was used to quantify MGMT protein expression and cleaved caspase-3.

60 µg of total protein lysates in 100µl of the final volume, were mixed with 7,5 uM of 6x loading buffer (12% sodium dodecyl sulfate-SDS, 0,015% bromophenol blue, 36% glycerol, 720 nM dithiothreitol, 420 mM Tris, pH 6.8). The samples and protein size marker (Protein Marker II, NZYtech) were resolved on 12% SDS-PAGE gel, at 80 volts, until marker starts to separate and then at 120 volts during approximately 1.5 hour. SDS-PAGE-separated proteins were electrotransferred to PVDF membranes using a semi-dry transfer system at a current of 350 Amp for 1.5h. Membranes were stained with Ponceau S solution to evaluate protein transference efficacy. After this step, the membrane was washed with TBS-T (200mM Tris/HCl pH 7.6, 1.5M NaCl and 0.1% Tween-20 (Sigma-Aldrich)) for three times. Membranes were blocked 1 hour in 3% BSA diluted in TBS-T at RT. Following blocking, membranes were probed overnight at 4ºC with the primary antibodies diluted in TBS-T with 3% BSA on a rotating shaker. Finally, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies diluted in 3% BSA in TBS-T for 1 hour at RT. Before and after incubation the membranes were washed three times in TBS-T.

The ECL (enhanced chemiluminescence) detection (Western Lightning Plus-ECL, PerkinElmer) was used to visualize immunoreactivity. In ECL detection, the luminol is oxidized by HRP in the presence of H_2O_2 and an enhancer, producing 3-aminophthalate that emits light. The emitted light was detected by exposing the membrane to the Chemidoc MP Imaging System (Bio-rad). The time of exposure depends on the protein analyzed. The images were acquired in ImageLab software. The integrated intensity of each band was calculated using computer-assisted densitometry analysis with ImageJ (NIH) software 1.44b. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control.

The antibodies used in the immunocytochemistry and western blotting are listed in Table 1.

Table 1 - Description of antibodies used in the assays.

Antibody		Company	Dilution for ICC	Dilution for WB
Primary	Mouse α-MGMT (23.2)	Novus Biologicals	1:250 1:500	1:500
	Mouse α-GAPDH	Thermo Fisher Scientific, Invitrogen	----------	1:1000
	Rabbit α-GFAP	EMO Milipore Cor. USA	1:500	----------
	Rabbit α-cleaved caspase-3	Cell Signaling TECHNOLOGY	1:200	1:500
Secondary	α-mouse IgG (H+L)-HRP conjugate	Biorad	----------	1:10000
	α-rabbit IgG (H+L) conjugate	Biorad	----------	1:10000
	Donkey α-mouse coupled to Alexa Fluor 568	Invitrogen	1:250	----------
	Donkey α-rabbit coupled to Alexa Fluor 488	Invitrogen	1:250	----------

3.2.6 Statistical analysis

Statistical analysis was carried out with GraphPad Prism (version 6.0). Data were compared and expressed as mean ± SEM of at least 3 independent experiments. Comparisons of means between two groups were performed using Student´s t-test, and between three or more groups by one-way ANOVA followed by Tukey H or LSD post hoc tests. Results were considered statistically significant when $p < 0.05$.

Chapter 4

Results

4.1 Expression of MGMT in GBM cell lines

4.1.1 Immunostaining

After platting the cells of GBM cell lines in 24-wells plates, and incubating for 72 hours in the presence of vehicle (CTL, control) or the ADK inhibitor ABT 702 (15 μM), immunocytochemistry was performed in order to evaluate MGMT expression and localization. The GFAP expression was also assessed in the three cell lines of glioblastoma. As shown in Fig. 13, it is possible to observe the expression of MGMT protein and GFAP and both control ABT 702 treated cells.

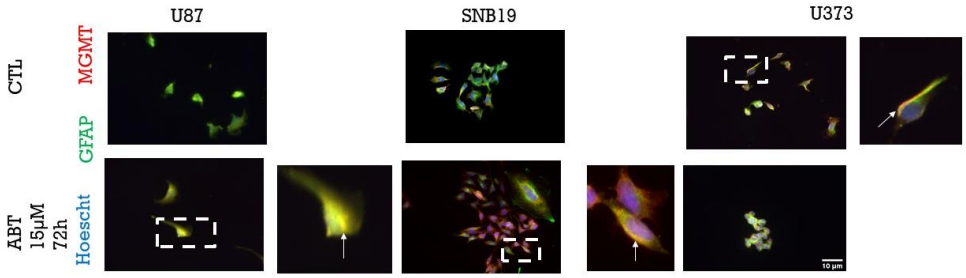


Figure 13 - Evaluation of MGMT location in GBM cell lines. Representative images of the perinuclear location of MGMT in the cells by immunocytochemistry. The cells were seeded and grown for 72h in the absence (CTL) or in the presence of ABT 702 (15 μM). Images of GFAP-stained GBM cells (green), MGMT (red) and Hoechst-stained nuclei (blue) were acquired on a widefield microscope with a 20x objective. Magnified images of the dashed areas are shown. Scale bar, 10 μm.

4.1.2 Western blot analysis

4.1.2.1 Basal expression of MGMT

The levels of MGMT, upon incubation with the vehicle (CTL) and with the ADK inhibitor ABT 702 (15 μM), were quantified in all cell lines by western blotting.

The immunoreactive bands of MGMT (25 kDa) and GAPDH (37 kDa) are represented in Fig. 14. GAPDH was used as the loading control. For each cell line, the integrated intensity obtained for MGMT band was normalized for the GAPDH band intensity.

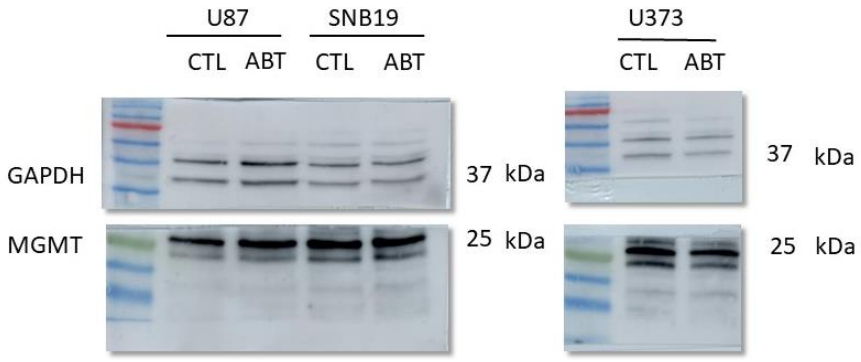


Figure 14 – Evaluation of MGMT expression levels. Representative immunoreactive bands of MGMT (22 kDa) and GAPDH (37 kDa) in U87, SNB19 and U373 cell lines incubated with the vehicle (CTL) and with ABT 702 (15 μM).

The basal levels of MGMT were quantified in U87, SNB19 and U373 cell lines. The results showed that SNB19 cell line depicts a higher level of MGMT, compared to U87 and U373 cell lines. U87 and U373 have a similar MGMT expression (Fig. 15).

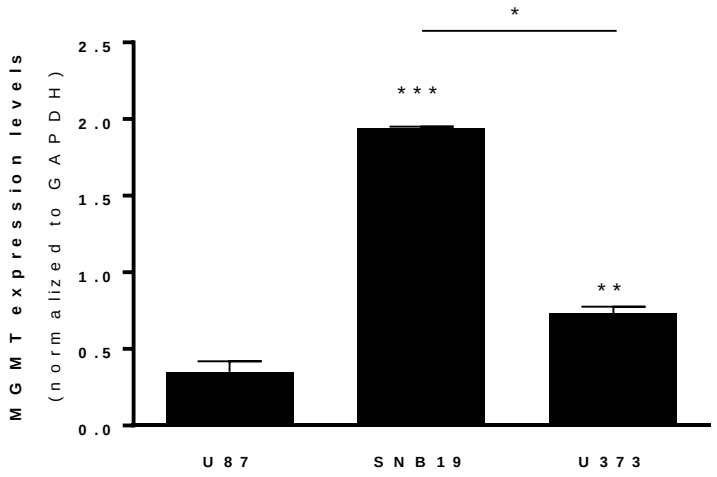


Figure 15 – Evaluation of MGMT basal levels in U87, SNB19 and U373 cell lines. Quantification of basal levels of MGMT in GBM cell lines normalized to the reference protein GAPDH. All values are mean±SEM. N=3, *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA followed by Tukey pos-hoc test. Statistical tests were performed in comparison to U87 cell line, except if otherwise indicated by the connecting lines above the bars.

4.2 Increased levels of adenosine do not affect MGMT expression in GBM cell lines

The cells were incubated for 72h the cells with ABT-702, an inhibitor of adenosine kinase which increases endogenous adenosine concentration by decreasing its catabolism (Boison, 2013). Therefore, western blotting was used to assess if increased adenosine levels, promoted by 72h of ABT-702 incubation, affected MGMT expression in GBM cell lines under study.

As can be observed in Fig. 14, no visible differences in MGMT expression is observed between the control conditions and ABT-702-treated GBM cells. This observation was confirmed through quantification of integrated intensity of immunoreactive bands using ImageJ software (Fig. 16).

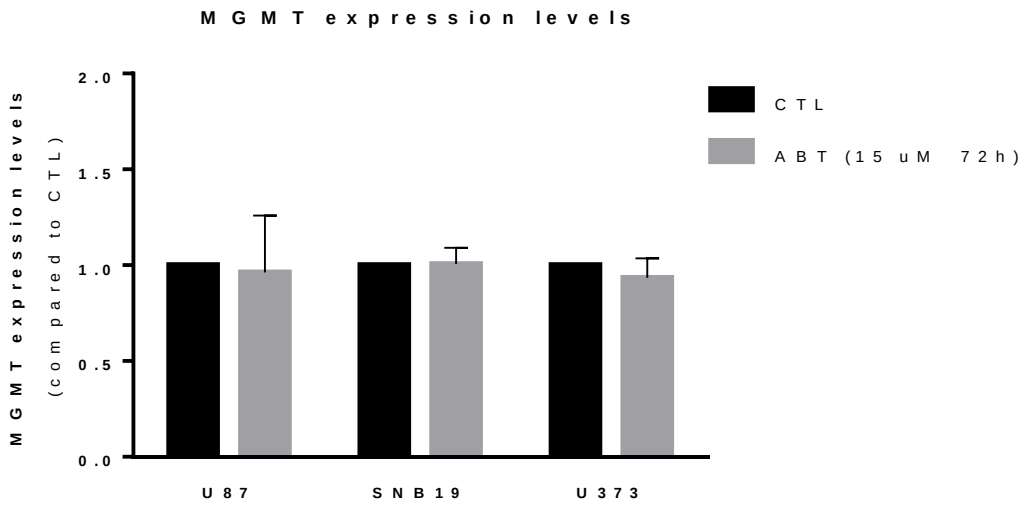


Figure 16 – Evaluation of MGMT expression in GBM cell lines incubated with the vehicle (CTL) and with ABT 702 (15 μM) for 72h. All values are mean±SEM. N=3, one-way ANOVA followed by Tukey pos-hoc test. Statistical tests were performed in comparison to CTL condition of each cell line.

4.3 Increased levels of adenosine decrease cell viability/proliferation in glioblastoma cell lines

4.3.1 Cell viability

The cells, platted in 96-wells plate, were treated with ABT-702 (15 μM) for 72 hours and a CCK-8 assay was performed to measure the cell viability/proliferation. The data was the average of six independent assays. ABT-702 showed a tendency for decreasing cell viability/proliferation in SNB19 cell line and promoted a statistically significant decrease in U373 cell lines ($p<0.05$) compared to control. In U87 cells, ABT did not modify viability/proliferation (Fig. 17).

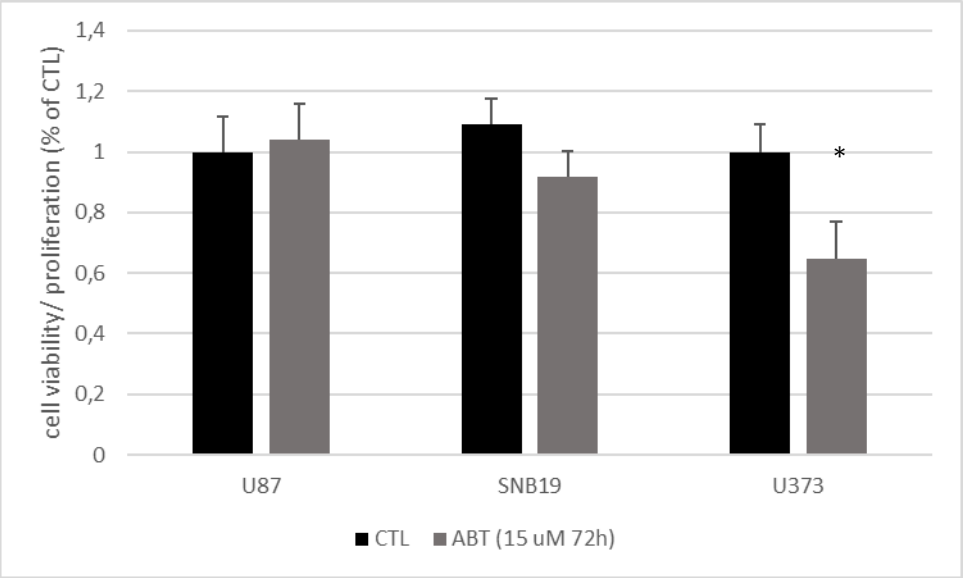


Figure 17 – Evaluation of cell viability/proliferation in GBM cell lines incubated with vehicle (CTL) and with ABT 702 (15 μM) for 72h. Data is expressed as percentage of CTL. All values are mean±SEM. N=6, *p<0.05, one-way ANOVA followed by Tukey pos-hoc test. Statistical tests were performed in comparison to CTL condition of each cell line.

4.4 Temozolomide effect in glioblastoma cell lines

4.4.1 Immunostaining

To assess cell death induced by TMZ, cells were treated with PI (7 μM), a fluorescent DNA intercalating agent not membrane-permeable, which only enters the cells when the cell membranes are damaged. After the incubation, immunocytochemistry was performed (as described in section 2.3), and the cells were visualized in a wide-field fluorescence microscope at 493nm excitation/636 nm emission.

Contrary to expectations no PI-positive cells were visualized with TMZ (100μM) treatment during 1, 2, 3 and 24 hours (Fig. 18).

Other concentrations of PI (15 μM, 25 μM, and 50 μM) and cell densities were tested, but the results obtained were essentially the same.

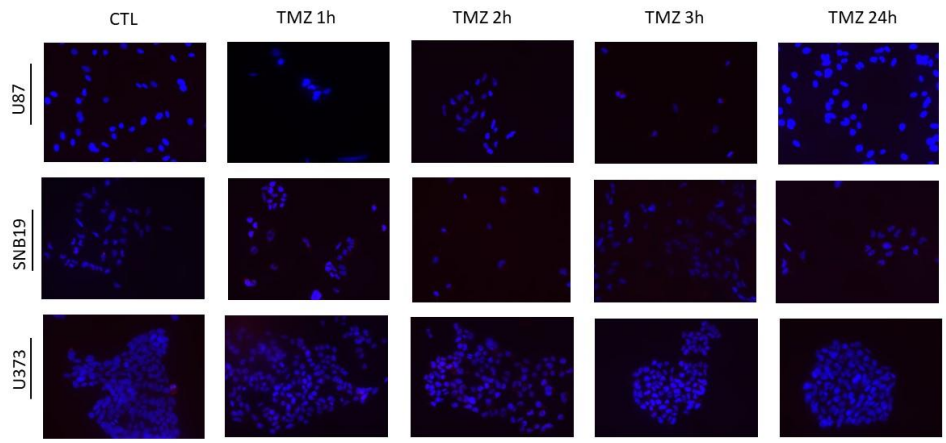


Figure 18 – Evaluation of TMZ resistance in GBM cell lines by assessing PI uptake. GBM were treated with TMZ at 100μM for 1, 2, 3 and 24 hours. No PI-positive cells were observed in GBM cells in any condition.

To validate the PI uptake assay a positive test was performed. GBM cells were submitted to ethanol 70% and the protocol used to assess TMZ cytotoxicity was performed. Cells were visualized under a wide-field fluorescence microscope. As expected, 70% ethanol caused a massive cell death in all GBM cell lines (Fig. 19), thus validating this assay to assess cell viability.

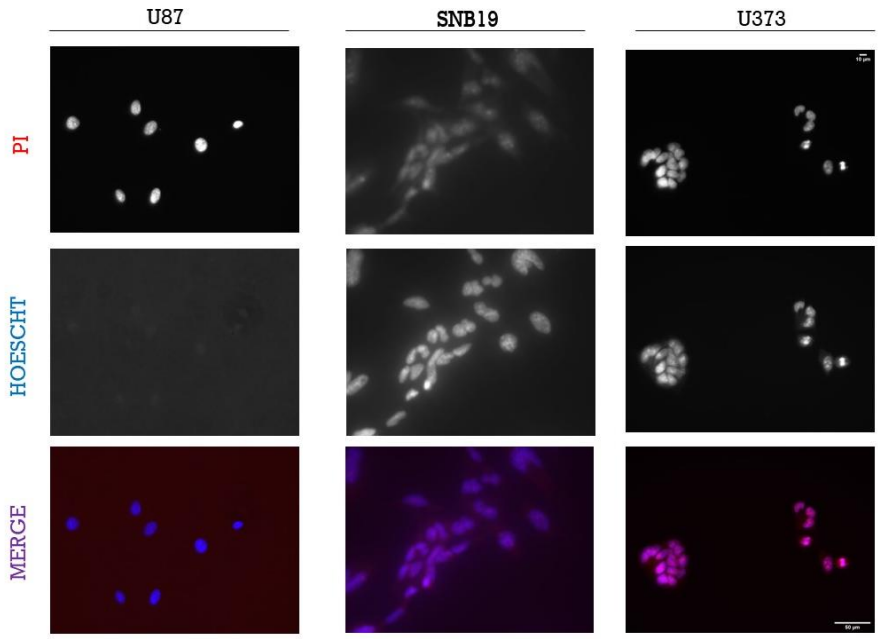


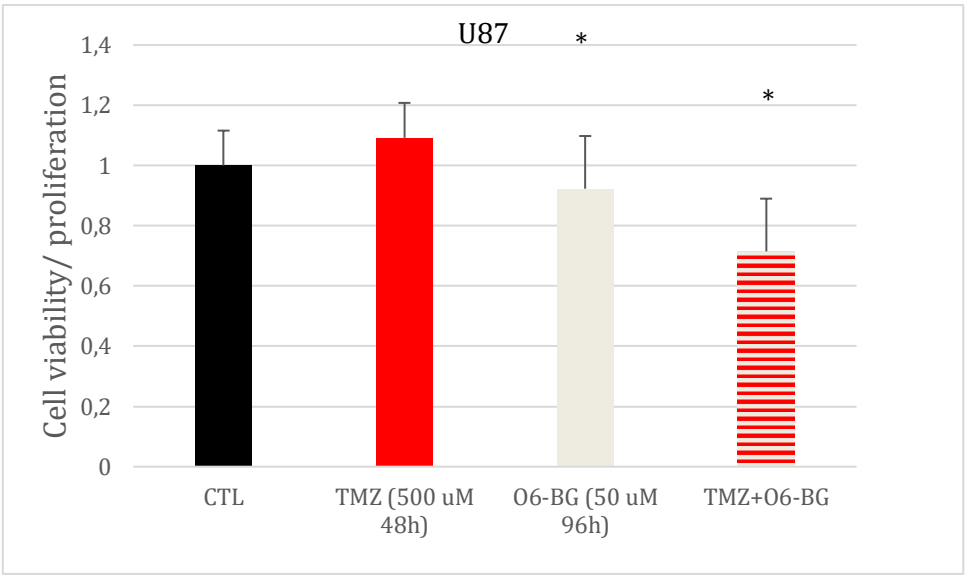
Figure 19 - Validation of PI uptake as a cell death marker for GBM cell lines. Representative images of the PI uptake assay for U87, SNB19 and U87 cells after incubation with ethanol 70%. Images were obtained under a widefield fluorescence microscope with a 20x objective. 70% ethanol caused a massive cell death in all cell lines.

4.4.2 Cell viability

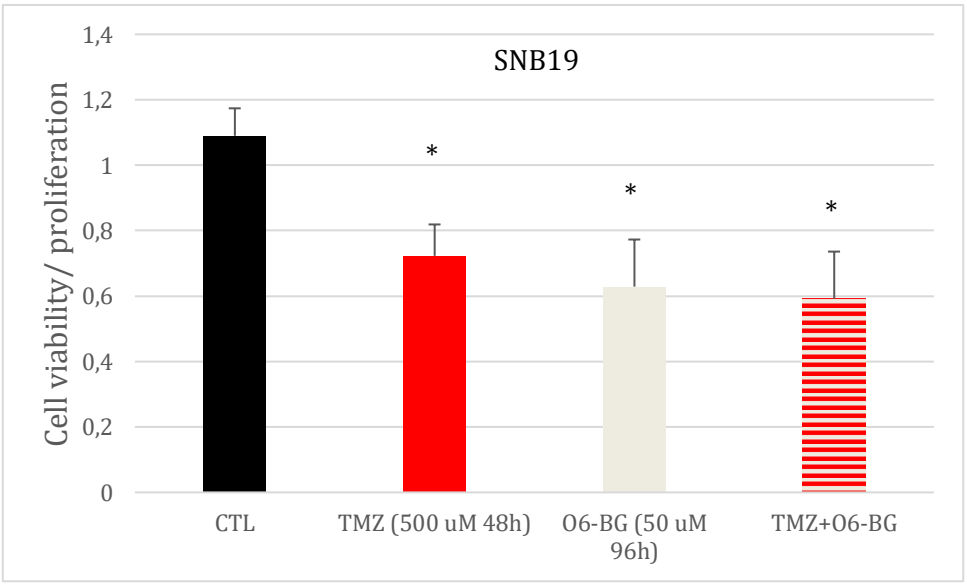
Since it was not possible to observe TMZ-mediated cytotoxic effects using PI uptake assay, other methods were examined to assess TMZ effect on cell viability and proliferation. Other TMZ concentrations and incubation times were also tested.

We start by using the CCK8 viability assay after treatment with TMZ at 500 μM for 48 hours. The results show a decrease in cell viability/proliferation in U373 and SNB19, but not in U87, cells (Fig. 20). The treatment with the inhibitor of MGMT (O6-BG) was performed for 96 hours at 50 μM, with the inhibitor being renewed after the first 24 hours. The O6-BG causes a decrease in cell survival in all cell lines. The presence of O6-BG does not affect significantly ($p>0.05$; ANOVA followed by LSD post hoc test) the effect of TMZ in any of the cell lines.

A


U87
Cell viability/ proliferation
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
*
CTL
TMZ (500 uM 48h)
O6-BG (50 uM 96h)
TMZ+O6-BG

B


SNB19
Cell viability/ proliferation
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
*
*
CTL
TMZ (500 uM 48h)
O6-BG (50 uM 96h)
TMZ+O6-BG

C

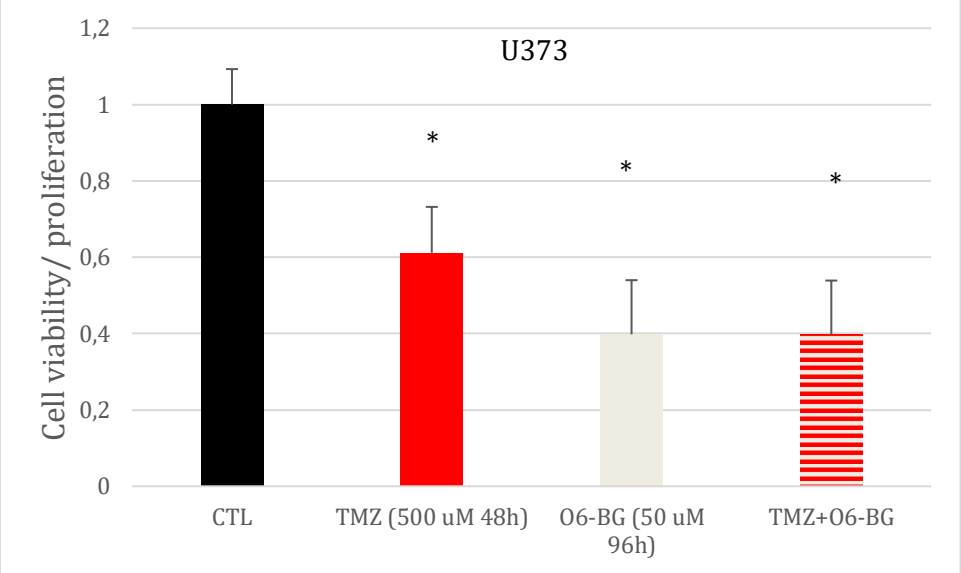
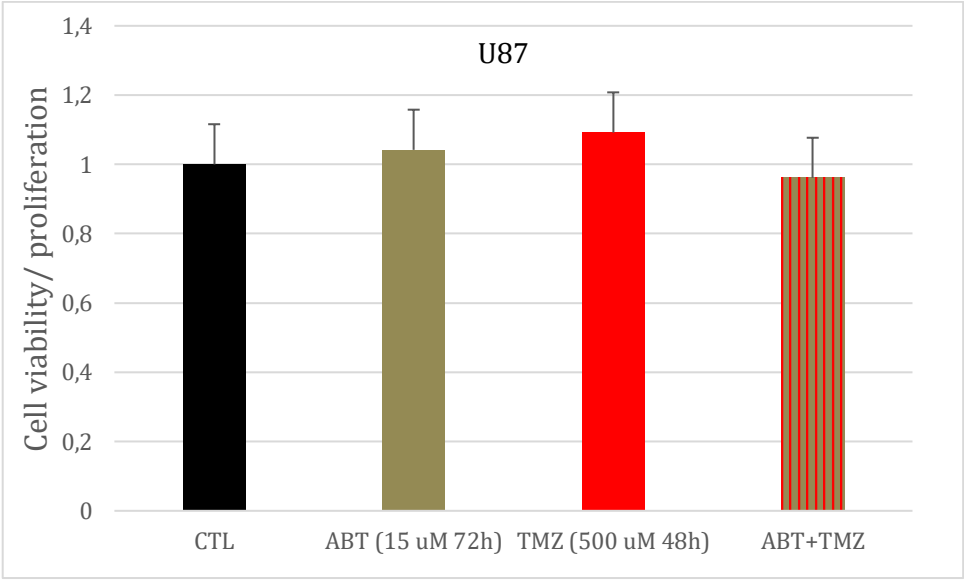


Figure 20 - Evaluation of TMZ effect in cell viability/proliferation through CCK-8 assay. U87 (A), SNB19 (B) and U373 (C) cells were incubated in the presence of vehicle (control) or TMZ (500 µM) and in the presence or in the absence of O6-BG (50 µM). Data is expressed as percentage of vehicle. All values are mean±SEM. n=3, *p<0.05, one-way ANOVA followed by LSD post hoc test. Statistical tests were performed in comparison to CTL.

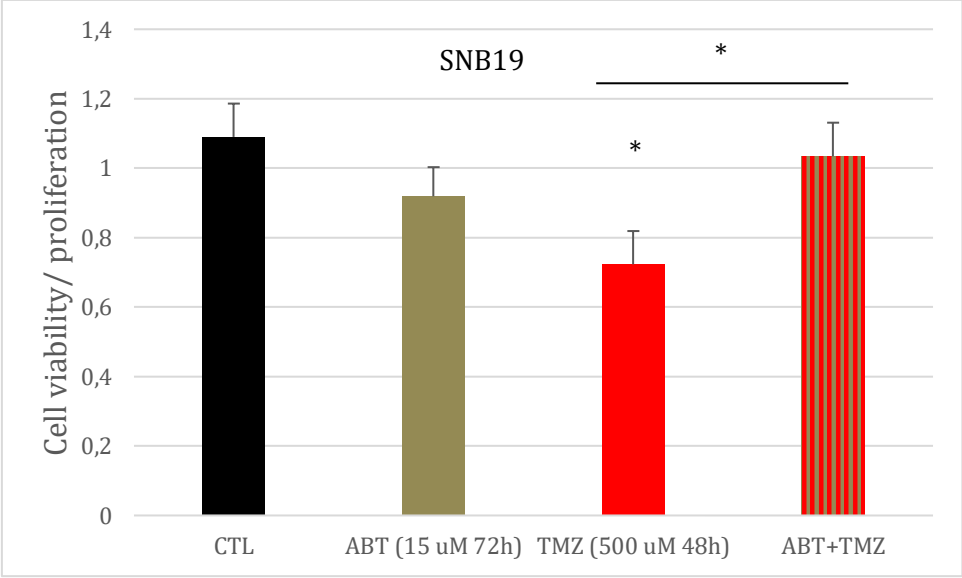
4.5 Adenosine effect in Temozolomide resistance in glioblastoma cell lines

To evaluate if increased levels of adenosine affected TMZ effect on cell viability/proliferation, assessed by CCK8 assay, cells were incubated with TMZ (500 µM) in the absence or presence of ABT 702 (15 µM). As can be observed in Fig. 20, the results are not consensual in the three GBM cell lines. In U87 cells, no effect of TMZ or ABT 702, either alone or in combination, was observed. ABT 702 alone only significantly decreased cell viability/proliferation in U373 cell lines, while TMZ was able to decrease cell viability/proliferation in U373 and SNB19 cell lines. The presence of ABT 702 did not prevent the effect of TMZ in U373 cells. However, in SNB19 cell lines ABT-702 treatment reverted the decrease of cell viability/proliferation caused by the TMZ treatment (Fig. 21).

A


U87
Cell viability/ proliferation
1,4
1,2
1
0,8
0,6
0,4
0,2
0
CTL
ABT (15 uM 72h)
TMZ (500 uM 48h)
ABT+TMZ

B


SNB19
*
*
Cell viability/ proliferation
1,4
1,2
1
0,8
0,6
0,4
0,2
0
CTL
ABT (15 uM 72h)
TMZ (500 uM 48h)
ABT+TMZ

C

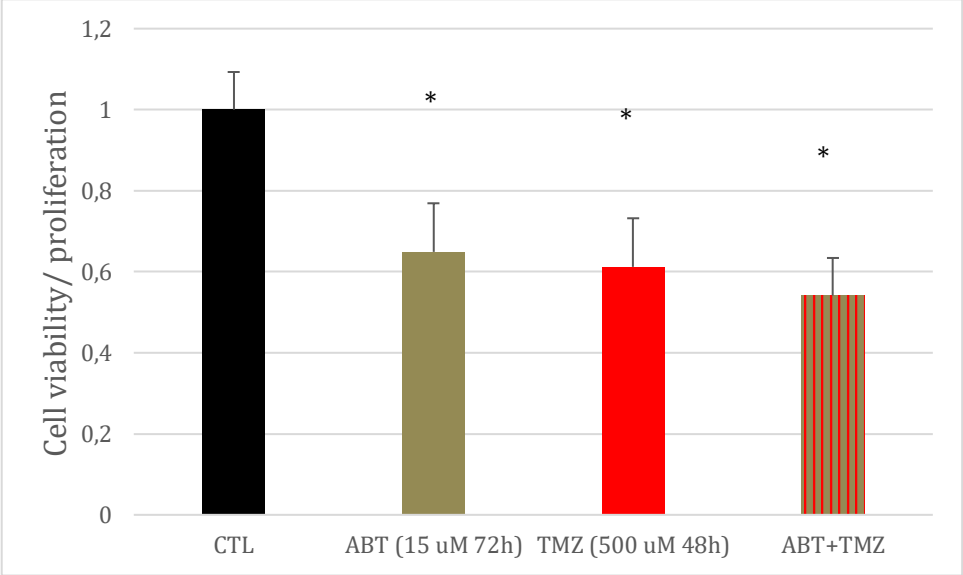


Figure 21 - Evaluation of ABT-702 and TMZ treatment in cell survival. Graph of cell survival (Figure 19. Evaluation of ABT-702 and TMZ treatment in cell viability/proliferation. U87 (A), SNB19 (B) and U373 (C) cells were incubated with vehicle (CTL) or TMZ (500 µM) and in the presence or absence of ABT 702 (15 µM). Data is expressed as percentage of CTL. All values are mean±SEM. n=3-6, *p<0.05, one-way ANOVA followed by LSD post hoc test. Statistical tests were performed in comparison to CTL, except if otherwise indicated by the connecting line above the bars.

4.6 Mechanisms responsible for decreased cell survival in glioblastoma cell lines

The results showed that increased levels of adenosine and TMZ decreased cell viability/proliferation in some of the cell lines under study. However, the mechanisms responsible for that decrease are not made clear because the absorbance read in CCK8 assay is proportional to the number of cells in the wells. So, the decrease in cell viability/proliferation can be due to an increase in cell death or to a decrease in cell proliferation.

To explore the mechanisms responsible for the decreased cell viability/proliferation, an immunocytochemistry assay was performed. Nuclei were stained with Hoechst and an antibody against active caspase-3 was used to identify cell death by apoptosis, since the activation of caspase-3 is the effector caspase for apoptosis (Fig. 22).

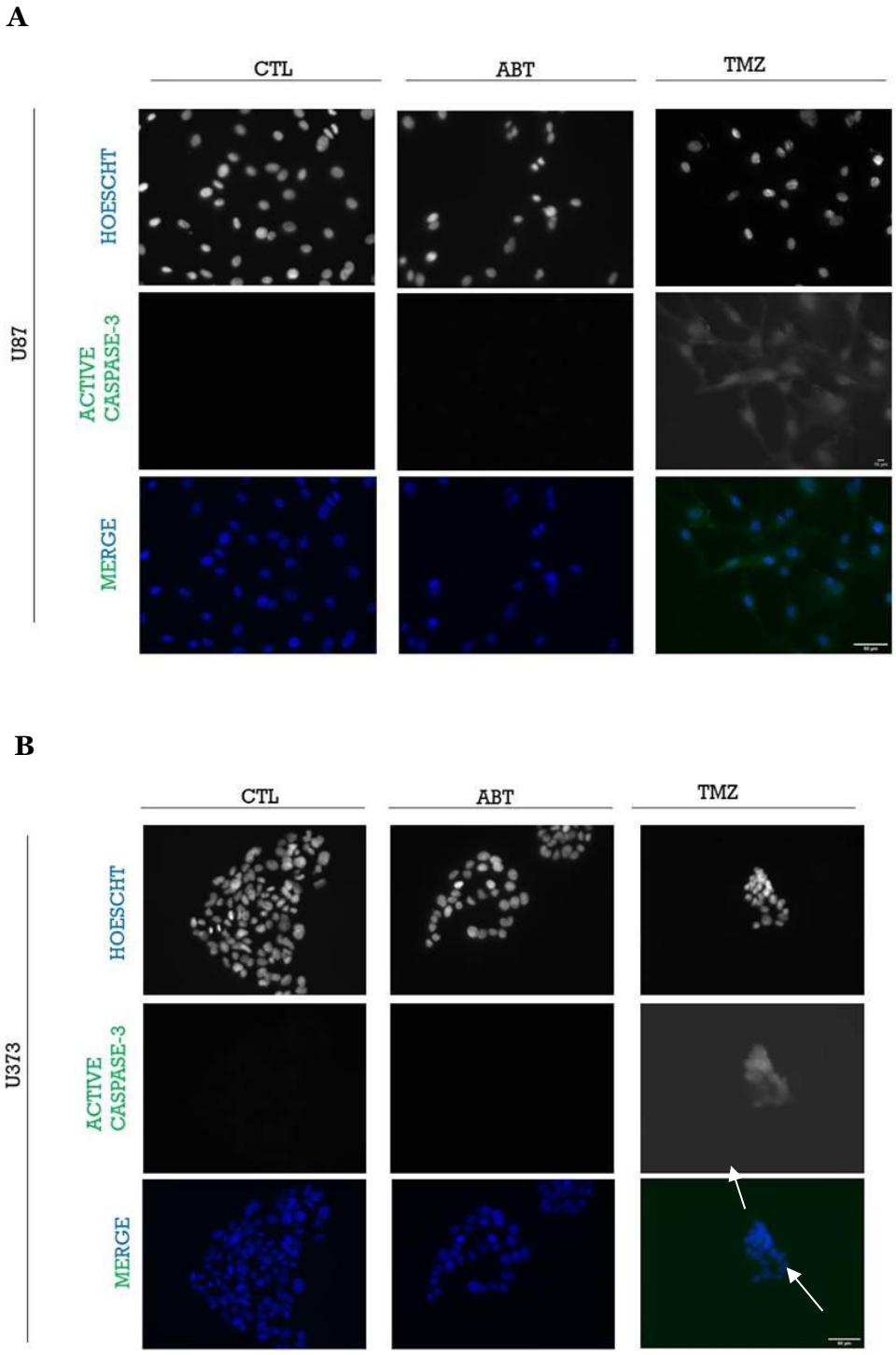


Figure 22 - Evaluation of mechanisms responsible for decreased cell viability/proliferation in U87 and U373 cells. Cells were incubated with vehicle (control), TMZ (500 µM) or ABT-702 (15 µM). Cells were grown for 120h. When present, ABT-702 was added after 48h while TMZ was added after 72h of incubation. Representative images of Hoechst stained nuclei and caspase-3 in U87 (A) and U373 (B) cell lines. Arrows indicate caspase-3-positive cells.

After treatment with ABT 702, no caspase-3 staining can be observed in U87 or U373 cell lines (Fig. 21). Since ABT 702 decreased cell viability/proliferation assessed by CCK8 assay (Fig. 21) in U373 cells, the results suggest that the effect of ABT 702 in U373 cells reflects a decrease in proliferation without affecting cell death.

Contrastingly, TMZ promotes caspase-3 activation in both U87 (Fig. 21A) and U373 (Fig. 21B) cells. Curiously, TMZ did not affect cell viability/proliferation assessed by the CCK8 assay in U87 cells (Fig. 18 and 19).

Chapter 5

Discussion, Conclusion and future perspectives

5.1 Discussion

Glioblastoma is the main tumour of central nervous system with poor prognosis. The common treatment of this disease includes surgical resection, radio, and chemotherapy with alkylating agents being the Temozolomide the most used. The temozolomide presents a low rate of success in the patient's treatment, only increasing the survival of the patients in about 12 months. The action of TMZ depends on several factors: the MMR system and the MGMT protein are the determinants in the final effect of TMZ. MGMT expression is the main responsible for the lack of TMZ effect. This protein antagonizes the effect of alkylating agents repairing the DNA lesions and promoting cell survival. The expression and activity of MGMT are associated with the higher chemoresistance and the lor time of patient's survival. With the aim of characterization of MGMT expression in the three GBM cell lines, the basal expression levels were quantified by western blot analysis. The major level of MGMT expression was founded in SNB 19 cell lines while the other two lines have near equal and lower levels. The MGMT becomes activated when occurs a possible DNA lesion and their location change to facilitate the action. After visualization the immunocytochemistry images was possible to observe a well-defined perinuclear localization, which is important because when the MGMT is inactive it surrounds the nucleus but when MGMT becomes active it acquires a nuclear localization to performer its function. In the present study, we found that, when endogenous adenosine is increased by inhibiting is phosphorylation with an ADK inhibitor, MGMT appears to translocate to the nucleus in the SNB19 cell line. Curiously, in this cell line, the presence of the same ADK inhibitor decreased the inhibitory effect of TMZ on cell viability/proliferation.

Another important characteristic of Glioblastoma is the long areas of necrosis and hypoxia when the vascular vessels are aberrant due to the fast and disorganized vascularization. These regions are featured by the expression of specific proteins and factors which promote cellular resistance in these conditions, namely the HIFs. The HIF modulates the action of different enzymes like CD39 and CD73 which, in its turn, modulate the adenosine metabolism increasing their expression levels in these areas.

Adenosine is a nucleoside responsible for many actions of maintenance and cell contact between neurons and glial cells. The adenosine can modulate the tumoral growth and the cellular response to the drugs. The expression and activation of A2b receptor by high concentrations of adenosine can be responsible for the increased chemoresistance of GB to the alkylating agents (Yan et. al, 2019). Other mechanisms may be behind this modulation. Since adenosine is capable to cause a decrease in global methylation by receptor-independent intracellular mechanisms emerged the hypothesis that this mechanism might decrease methylation in MGMT promoter and reestablish its expression levels. After analysis by western blot was possible to observed that increased levels of adenosine did not modulate the MGMT expression in the studied GBM cell lines.

The effects of TMZ and of increased adenosine levels on cell viability/proliferation was also studied in three GBM cell lines. The results showed that in general, the TMZ induces cytotoxicity except in U87 cell lines. When a MGMT inhibitor was present, it caused a decrease in cell viability/proliferation by itself but did not affect the effect of TMZ on cell viability/proliferation.

Although increased adenosine levels do not affect MGMT expression in the present work, other mechanisms can be capable to increase the chemoresistance of GB cells. As previously said the expression and activation of A2b receptors to high concentrations of adenosine can be responsible for the increased chemoresistance of GB to the alkylating agents (Yan et. al, 2019). Curiously, in SNB19 cell lines, results showed an increased resistance to the chemotherapeutic agent temozolomide when levels of adenosine were increased. This is an important find since these cell lines present the highest basal level of MGMT expression, though the MGMT expression levels were not modulated to the increased adenosine the relation between these compounds should be further studied, namely the modulation of MGMT activity and nuclear translocation by increased adenosine levels.

The results demonstrate that increased levels of adenosine decrease cell survival in the SNB19 and U373 cell lines as previously demonstrated in astrocytes by our group. After analysis of the mechanisms responsible to the decreased cell viability/proliferation was possible to observe that after increasing adenosine levels by treatment with ABT-702, there was no activation of mechanisms initiators of cell death, like activation of caspase 3, indicative of cell death mediated by apoptosis. Contrastingly, the inhibitory effect of TMZ on cell viability/proliferation, seems to involve activation of apoptosis and cell death mechanisms, which are in accordance with what has been described in the literature (Zang et. al, 2015).

5.2 Conclusion and Future Perspectives

Glioblastoma is the main type of tumors of the central nervous system. The median time of survival of GB patients is around one year. The treatment consists of surgical recession, radiotherapy, and chemotherapeutic agents. The main chemotherapeutic drug used is Temozolomide, an alkylating agent whose function is to damage to DNA, adding methyl groups in guanines. The tumors resistance to chemotherapeutic agents is well known and documented. Although, the mechanisms responsible for this resistance are not fully clarified yet. The resistance mediated by MGMT activation was already studied but there is not the only mechanism. Several proteins are responsible for tumors resistance to the drugs and the acquired resistance aggravates this effect (Zang et. al, 2015). MGMT is a repair protein which is function is to remove methyl groups for the O6 positions in guanines. So, TMZ and MGMT act in opposite directions. In most of the glioblastomas, the gene of MGMT is silenced and there is no expression of this protein. However, the microenvironment of tumor is very complex, and it is possible that this protein is not the only one involved in the resistance to this chemotherapeutic agent, TMZ.

The importance of increased levels of adenosine, founded in hypoxic regions of tumors, in the cell response to TMZ action was not clear, different results were obtained in the different cell lines. However, cells with different phenotypes and mutations respond differently to the environment and to chemotherapeutic agents and this is the main problem of the treatment of glioblastomas.

Adenosine plays an important role in the brain, particularly in neuromodulation, immunomodulation and in synaptic plasticity. The adenosine activity mediated by adenosine receptors is the subject of many studies. However, the role of intracellular mechanisms of action of adenosine not mediated by receptors has also been described, particular in situations of increased adenosine concentrations such as hypoxia. Recent research suggests that intracellular adenosine plays a role in methylation of DNA and cellular proliferation. Inside the cell, the adenosine and homocysteine are in fast equilibrium reaction with SAH which means that if the levels of adenosine or homocysteine increase, the levels of SAH follow the others. SAH which is an inhibitor of SAM-dependent transmethylases. The SAM, in its turn, is the main methyl donor in the cells and plays an important role in DNA methylation. Previous results obtained by our group, showed that increased levels of adenosine in human astrocytes caused a decrease in cell proliferation without activation of cell death mechanisms, which was independent of receptor activation and probably involving reversal of SAH hydrolase-catalysed reaction with consequent accumulation of SAH and inhibition of transmethylases .

Similarly, in this project was observed a decrease in cell proliferation in U373 cell lines when adenosine concentration was increased by inhibition its degradation, with no activation of caspase 3, a marker of cell death by apoptosis. The adenosine role in brain tumors or neurodegenerative diseases might be a key for the prognosis and the treatment of these diseases and deserves further investigation.

Chapter 6

References

Ali-Osman F., Friedman H., Srivenugopal K., Yuan X. 1995 “Ubiquitination-Dependent Proteolysis of O6-Methylguanine-DNA Methyltransferase in Human and Murine Tumor Cells following Inactivation with O6-Benzylguanine or 1,3-Bis(2-chloroethyl)-1-nitrosourea)”. Clinical Breast Cancer

Aoki K. and Natsume A. 2019. “Overview of DNA methylathion in adult diffuse gliomas”. Brain Tumor Pathol

Berger M., Hirose Y., Pieper R. 2001. “p53 Effects Both the Duration of G2/M Arrest and the Fate of Temozolomide-treated Human Glioblastoma Cells”. Cancer Research

Binabaj M., Bahrami A., ShahidSales A., Joodi M., Mashhad M., Hassanian S., Anvari K., Avan A. 2017. “The prognostic value of MGMT promoter methylation in glioblastoma: A meta-analysis of clinical trials”. J Cell Physiol

Bobustuc G., Smith J., Maddipatla S., Jeudy S., Limaye S., Isley B., Caparas M., Constantino M., Shah N., Baker C., Srivenugopal K., Baidas S., Konduri S. 2012. “MGMT inhibition restores ERα functional sensitivity to antiestrogen therapy”. Mol Medicine

Bobustuc GC, Kassam AB, Rovin RA, Jeudy S, Smith JS, Isley B, Singh M, Paranjpe A, Srivenugopal KS, Konduri SD. 2018. “MGMT inhibition in ER positive breast cancer leads to CDC2, TOP2A, AURKB, CDC20, KIF20A, Cyclin A2, Cyclin B2, Cyclin D1, ERα and Survivin inhibition and enhances response to temozolomide”. Oncotarget

Brandes A., Franceshi E., Paccapelo A., Tallini G., Biase D., Ghimeton C., Danieli D., Zunarelli E., Lanza G., Silini E., Sturiale C., VolpinL., Servadi F., Talacchi A., Fioravanti A., Foschini M., Stefania, Pession A., Ermanii M. 2017. “Role of MGMT Methylation Status at Time of Diagnosis and Recurrence for Patients with Glioblastoma: Clinical Implications”. The Oncologist

Cadieux B., Ching T., VandenBerg S., Costello JF. 2006. “Genome-wide Hypomethylation in Human Glioblastomas Associated with Specific copy Number Alteration, Methylenetetrahydrofolate Reductase Allele Status, and Increased Proliferation”. Cancer research

Ceruti S. and Abbrachio M. 2013. “Adenosine Signaling in glioma cells” Glioma Signaling

Ciechomska I., Marciniak M., Jackl J. and Kaminska B. 2018. “Pre-treatment or Post-treatment of Human Glioma Cells with BIX01294, the Inhibitor of Histone Methyltransferase G9a, Sensitizes Cells to Temozolomide”. Front. Pharmacol

Costello J., Futsched B., Tanoil K., Graunkes D., and Pieper R. 1994. “Methylation in the Promoter and Body of the 06-Methylguanine DNA Methyltransferase (MGMT) Gene Correlates with MGMT Expression in Human Glioma Cells”. Biological Chemistry

Cui D. and Xu X. 2018. “DNA Methyltransferases, DNA Methylation, and Age-Associated Cognitive Function”. International Journal of molecular Sciences

Dumitriu IE., Mohr W., Kolowos W., Kern P., Kalden JR., Herrmann M. 2001. “(5,6-carboxyfluorescein diacetate succinimidyl ester-labeled apoptotic and necrotic as well as detergent-treated cells can be traced in composite cell samples”. Anal Biochem

Fine H., McNeill K., Bastien J. 2015. “Molecular Characterizations of Glioblastoma, Targeted Therapy and Clinical Results to Date”. Cancer

Friso S., Udali S., Santis S., Choi S. 2016. “One-carbon metabolism and epigenetics”. Molecular Aspects of Medicine

Head R., Fay M., Cosgrove L., Fungf K., Rundle-Thiele D., Martin J. 2017. “Persistence of DNA adducts, hypermutation and acquisition of cellular resistance to alkylating agents in glioblastoma”. CANCER BIOLOGY & THERAPY

Hegi M., Diserens A., GorliaT., Hamou M., Tribolet N., Weller M., Kros J., Hainfellner J., Mason W., Mariani L., Bromberg J., Hau P., Mirimanoff R., Cairncross G., Janzer R., Stupp R. 2005. “MGMT Gene Silencing and Benefit from Temozolomide in Glioblastoma“. The new england journal of medicine

Hermes M., Geisler H., Osswald H., Riehle R., Kloor D. 2008. “Alterations in S-adenosylhomocysteine metabolism decrease O6-methylguanine DNA methyltransferase gene expression without affecting promotor methylation”. Biochemical Pharmacology

He W., Liu W., Yang S., and Yuan F. 2015. “Chemotherapeutic effect of Tamoxifen on Temozolomide-Resistant Gliomas”. Anticancer Drugs

Ingrosso D., Perna A. 2009. “Epigenetics in hyperhomocysteinemia states. A special focus on uremia”. Biochimica et Biophysica Acta

Jackson S., Weingart J., Nduom E., Harfi T., George R., McAreavey D., Ye X., Anders N., Peer C., Figg D., Gilbert M., Rudex M. and Grossman S. 2018. “The effect of an adenosine A2A agonist on intra-tumoral concentrations of temozolomide in patients with recurrent glioblastoma”. Fluids and Barriers of the CNS

Jawhari S., Ratinaud M. and Verdier M. 2016. “Glioblastoma, hypoxia and autophagy: a survival-prone ‘ménage-à-trois’”. Official journal of the Cell Death Differentiation Association

Kaina B. and Christmann M. 2019. “DNA repair in personalized brain cancer therapy with temozolomide and nitrosoureas”. DNA Repair

Knedich N. and Martin D. 1977. “Role of S-adenosylhomocysteine in adenosine mediated toxicity in cultured Mouse T lymphoma cells”. Cell

Kuo C., Liu J., Shiah H., Ma L. and Chang J. 2007. “Tamoxifen accelerates proteasomal degradation of O6-methylguanine DNA methyltransferase in human cancer cells”. Journal of Cancer

Lafon-Hughes L., Tomaso M., Méndez-Acuña L., López W. 2008. “Chromatin-remodelling mechanisms in cancer”. Mutation Research

Martin d, Kredich N. 1997. “Role of S-Adenosylhomocysteine in Adenosine-Mediated Toxicity in Cultured Mouse T Lymphoma Cells”. Cell

Neonatal hepatic steatosis by disruption of the adenosine kinase gene - Scientific Figure on ResearchGate. Available from: https://www.researchgate.net/figure/Pathways-of-adenosine-metabolism-Adenosine-is-formed-either-by-hydrolysis-of-AMP-or-by_fig1_11374143 [accessed 18 Jul, 2019]

Romani M., Pistillo M. and Banelli B. 2018. “Epigenetic Targeting of Glioblastoma”. Frontiers in Oncology

Silber J., Mueller B., Ewers T., and Berger M. 1993. “Comparison of O6-Methylguanine-DNA Methyltransferase Activity in Brain Tumors and Adjacent Normal Brain”. Cancer Research

Soleimani A, Bahreyni A, Roshan MK, Soltani A, Ryzhikov M, Shafiee M, Soukhtanloo M, Jaafari MR, Mashkani B, Hassanian SM. 2018. “Therapeutic potency of pharmacological adenosine receptors agonist/antagonist on cancer cell apoptosis in tumor microenvironment, current status, and perspectives”. Journal of Cellular Physiology

Spyropoulou A., Papavassiliou A., Piperi C., Adamopoulos C. 2013. “Deregulated Chromatin Remodeling in the Pathobiology of Brain Tumors”. NeuroMolecular Medicine

Synowitz M., Glass R., Färber K., Markovic D., Kronenberg G., Herrmann K., Schnermann J., Nolte C., Rooijen N., Kiwit J. and Kettenmann H. 2006. “A1 Adenosine Receptors in Microglia Control Glioblastoma-Host Interaction”. Cancer Research

Torres A., Erices J., Sanchez F., Ehrenfeld P., Turchi L., Virolle T., Uribe D., Niechi I., Spichiger C., Rocha J., Ramirez M., Salazar-Onfray F., Martín R., Quezada C. 2019. “Extracellular adenosine promotes cell migration/invasion of glioblastoma Stem-Like Cells through A3 Adenosine Receptor activation under hypoxia”. Cancer Letters

Uribe D, Torres Á, Rocha JD, Niechi I, Oyarzún C, Sobrevia L, San Martín R, Quezada C. 2017. “Multidrug resistance in glioblastoma stem-like cells: Role of the hypoxic microenvironment and adenosine signalling”. Molecular Aspects of Medicine

Váldes F., Luna V., Arévalo B., Brown N. and GutiérrezM. 2018. “Adenosine: Synthetic Methods of Its Derivates and Antitumor Activity”. Mini Rev Med Chem

Wainfan E. and Poirier L. 1992. “Methyl Groups in Carcinogenesis: Effects on DNA Methylation and Gene expression”. Cancer research

Wiewrodt K., Nagel G., DreimCuller N., Hundsberger T., Perneczky A. and Kaina B. 2018. “MGMT in primary and recurrent human glioblastomas after radiation and chemotherapy and comparison with p53 status and clinical outcome”. Int. J. Cancer

Williams-Karnesky R., Sandau U., Lusardi T., Lytle N., Farrell J., Pritchard E., Kaplan D., and Boison D. 2014. “Epigenetic changes induced by adenosine augmentation therapy prevent epileptogenesis”. The Journal of Clinical Investigation

Wong S., Zhang X., Zhuang J., Chan H., Li C. and Leung G. 2012. “MicroRNA-21 Inhibition Enhances In VitroChemosensitivity of Temozolomide-resistant Glioblastoma Cells”. ANTICANCER RESEARCH

Yan A., Joachims M., Thompson L., Miller A., Canoli P. and Bynoe M. 2019. CD73 promotes glioblastoma pathogenesis and enhances its chemoresistance via A2b adenosine receptor signaling”. JNeurosci

Zang J., Stevens M. and Bradshaw T. 2012. “Temozolomide: Mechanisms of Action, Repair and Resistance”. Current molecular pharmacology

Zhang J., Stevens M., Laughton C., Madhusudan S., Bradshaw T. 2010. “Acquired Resistance to Temozolomide in Glioma Cell Lines: Molecular Mechanisms and Potential Translational Applications”. Oncology