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Paola Gyuliane Gonçalves

Avaliação da deleção de *MTAP* no desenvolvimento e resposta a terapia e imunoterapia em glioblastomas

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Doutora em Patologia.

Orientador (a): Prof. Dr. Lucas Bidinotto

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Impacto potencial desta pesquisa

Impacto científico esperado: Considerando que os glioblastomas (GBM), tumores cerebrais extremamente agressivos e com prognóstico ruim associado a resistência aos tratamentos convencionais, possuem alta frequência de deleção de *MTAP*, a relevância clínica reside na possibilidade exploratória desta característica na busca de um tratamento mais efetivo. Caracterizamos o fenótipo dos tumores e exploramos tratamentos alternativos baseado na deleção ou não deste gene. Os resultados encontrados apontam para a combinação de duas drogas que podem alterar o crescimento das células neoplásicas, deletadas para *MTAP*, com pouco impacto nas células MTAP positivas, teoricamente não-neoplásicas. Importante, mostramos que a combinação destas drogas pode ser utilizada em tumores cerebrais, pois é capaz de cruzar a barreira hematoencefálica. Por fim, caracterizamos o microambiente dos tumores, baseado na deleção de *MTAP*, e sugerimos uma coorte de pacientes que não seriam elegíveis a imunoterapia, dado as características intrínsecas destes tumores.

Impacto social: No momento, o tratamento padrão para glioblastoma (GBM) utiliza a mesma abordagem a todos os pacientes, e geralmente está associado a alta morbimortalidade. Baseado no conceito de tratamento personalizado, nossos resultados sugerem que futuramente a deleção de MTAP, que ocorre em quase 60% dos pacientes diagnosticados com GBM, pode ser utilizada como um biomarcador de resposta a tratamentos alternativos mais eficazes e na seleção de pacientes para futuros estudos de imunoterapia. Em última instância, isso poderá implicar na sobrevida e/ou qualidade de vida dos pacientes.

Impacto econômico: Nossos estudos sugerem que a expressão de MTAP pode ser utilizada como biomarcador de resposta a terapias alternativas à padrão para o tratamento de glioblastoma (GBM). Esta expressão pode ser conferida de maneira simples e barata, como uma reação de imuno-histoquímica, e, caso os tratamentos sejam mais efetivos, em última instância, esperamos que a utilização deste biomarcador pode impactar economicamente o Sistema de Saúde, já que um tratamento mais efetivo pode levar a melhor resposta, logo, menos gastos com hospitalização, reinternação e cirurgias para tumor residual.

UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Botucatu

ATA DA DEFESA PÚBLICA DA TESE DE DOUTORADO DE PAOLA GYULIANE GONÇALVES, DISCENTE DO PROGRAMA DE PÓS-GRADUAÇÃO EM PATOLOGIA, DA FACULDADE DE MEDICINA - CÂMPUS DE BOTUCATU.

Aos 27 dias do mês de fevereiro do ano de 2025, às 9h, por meio de Videoconferência, realizou-se a defesa de TESE DE DOUTORADO de PAOLA GYULIANE GONÇALVES, intitulada **Avaliação da deleção de MTAP no desenvolvimento e resposta a terapia e imunoterapia em glioblastomas**. A Comissão Examinadora foi constituída pelos seguintes membros: Prof. Dr. LUCAS TADEU BIDINOTTO (Orientador(a) - Participação Virtual) do(a) Faculdade de Ciências da Saúde de Barretos Dr. Paulo Prata, Prof. Dr. GUIDO LENZ (Participação Virtual) do(a) Depto de Biofísica / Universidade Federal do Rio Grande do Sul, Profa. Dra. CAMILA FERREIRA BANNWART CASTRO (Participação Virtual) do(a) Centro Universitário Sudoeste Paulista (UniFSP). Após a exposição pela doutoranda e arguição pelos membros da Comissão Examinadora que participaram do ato, de forma presencial e/ou virtual, a discente recebeu o conceito final: _ _ _ _ APROVADO _ _ _ _ . Nada mais havendo, foi lavrada a presente ata, que após lida e aprovada, foi assinada pelo(a) Presidente(a) da Comissão Examinadora.

Prof. Dr. LUCAS TADEU BIDINOTTO

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Dedico este trabalho a todos os camundongos que deram a vida para este estudo. Obrigado por contribuírem para minha formação e para o conhecimento científico, espero que um dia esse sacrifício seja revertido em melhorias no tratamento do câncer.

Agradecimentos

Este trabalho se iniciou em março de 2020, juntamente com a covid-19 no Brasil. Isso trouxe ainda mais inseguranças e problemas a um doutorado que já não é conhecido por ser fácil. Foi quase um ano e meio afastada do laboratório, assistindo diariamente na TV o pânico que acometia o mundo, e o medo crescia juntamente com o número de mortes. Tenho comigo que ninguém passou imune a esse período, seja pela perda de um ou mais pessoas queridas, ou pelos problemas que a pandemia trouxeram. Eu mesma perdi alguém muito especial para mim, que sei que está orgulhosa de onde cheguei mesmo não estando aqui presencialmente. Então meu agradecimento neste trabalho é um pouco diferente, quero agradecer aos profissionais de saúde que se arriscaram naquele momento, que foram verdadeiros heróis e prestaram um serviço sem igual. A ciência e seus pesquisadores que conseguiram em tempo recorde criar vacinas e imunizar o mundo. As pessoas de serviços essenciais que não puderam parar e se colocaram em risco. Sem essas pessoas e suas contribuições eu não poderia ter dado continuidade a este trabalho, então a minha gratidão é para com elas.

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"I gave my blood, sweat and tears for this"

Taylor Swift – You are on your own kid.

RESUMO

Glioblastoma (GBM) é um tumor cerebral altamente agressivo e letal, caracterizado por crescimento rápido, resistência à terapia e prognóstico ruim. Estudos recentes enfatizaram o papel central da metiltioadenosina fosforilase (MTAP), uma enzima no metabolismo de purina e metionina, em vários tipos de câncer. Entretanto, o impacto do silenciamento do MTAP no GBM ainda não está claro. Neste estudo, investigamos os efeitos do silenciamento de MTAP no crescimento de GBM usando linhagens de células de glioblastoma e um modelo de xenoenxerto. O *knockout* de *MTAP* mediado por CRISPR-Cas9 em células de glioblastoma, implantadas em camundongos imuno deficientes, demonstrou que o silenciamento de *MTAP* atrasou significativamente o crescimento do tumor ($p<0,001$) e alterou a celularidade ($p<0,009$), fibrose ($p<0,006$) e necrose ($p<0,0003$) em xenoenxertos em camundongos imuno deficientes. Mecanicamente, a perda de *MTAP* interrompeu a proliferação ($p<0,001$), a migração ($p<0,001$) e a formação de colônias ($p<0,001$) em estudos *in vitro*. Além disso, o acúmulo de metiltioadenosina (MTA) no microambiente tumoral (TME) promoveu um ambiente imunossupressor e aumentou a angiogênese ($p<0,0001$), o que facilitaria a progressão do tumor. Também exploramos o potencial terapêutico da síntese de purina e dos inibidores de MAT2A no GBM deficiente em MTAP, sugerindo que a 2-Fluoroadenina (2-FA) inibiu a migração celular ($p<0,0001$), a formação de colônias ($p<0,0001$) e a viabilidade do tumor, particularmente em células com *MTAP* deletado. Além disso, na presença de MTA houve letalidade sintética apenas em células $MTAP^{-/-}$, revertendo a citotoxicidade da droga nas células $MTAP^{+/+}$. Entretanto, nenhuma letalidade sintética foi observada com pemetrexede ou AG-270. Essas descobertas destacam o silenciamento de *MTAP* como um potencial alvo terapêutico no GBM e sugerem que direcionar o metabolismo da purina pode oferecer uma estratégia promissora para tumores deficientes em MTAP. Mais estudos são necessários para validar esses resultados in vivo e avaliar a eficácia dos inibidores da via relacionada ao MTA no tratamento do GBM.

Palavras-chave: Glioblastoma, MTAP, deleção, MTA, 2-FA, pemetrexede, AG-270.

ABSTRACT

Glioblastoma (GBM) is a highly aggressive and lethal brain tumor, characterized by rapid growth, resistance to therapy, and poor prognosis. Recent studies have emphasized the role of methylthioadenosine phosphorylase (MTAP), an enzyme in purine and methionine metabolism, in various cancers. However, the impact of *MTAP* silencing in GBM remains unclear. In this study, we investigated the effects of *MTAP* silencing on GBM growth using glioblastoma cell lines and a xenograft model. CRISPR-Cas9-mediated *MTAP* knockout in glioblastoma cells, implanted into immunodeficient mice, demonstrated that *MTAP* silencing significantly delayed tumor growth ($p<0.001$) and altered cellularity ($p<0.009$), fibrosis ($p<0.006$), and necrosis ($p<0.0003$) in immunodeficient mouse xenografts. Mechanistically, *MTAP* loss disrupted proliferation ($p<0.001$), migration ($p<0.001$), and colony formation ($p<0.001$) in *in vitro* studies. Furthermore, Methylthioadenosine (MTA) accumulation within the tumor microenvironment (TME) promoted an immunosuppressive environment and enhanced angiogenesis ($p<0.0001$), which could, in turn, facilitate tumor progression. We also explored the therapeutic potential of purine synthesis and MAT2A inhibitors in *MTAP*-deficient GBM, finding that 2-FA inhibited cell migration ($p<0.0001$), colony formation ($p<0.0001$), and tumor viability, particularly in *MTAP*-deleted cells. Furthermore, in the presence of MTA there was synthetic lethality only in MTAP-/- cells, reversing the cytotoxicity of the drug in MTAP+/+ cells. However, no synthetic lethality was observed with PRMT5 inhibitors or AG-270. These findings highlight *MTAP* silencing as a potential therapeutic target in GBM and suggest that targeting purine metabolism may offer a promising strategy for MTAP-deficient tumors. Further studies are needed to validate these results *in vivo* and assess the efficacy of MTA-related pathway inhibitors in GBM treatment.

Key-words: Glioblastoma, MTAP, deletion, MTA, 2-FA, pemetrexed, AG-270.

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Capítulo I

Revisão da literatura

Gliomas são responsáveis por aproximadamente 70% dos tumores cerebrais (1) e, embora representem menos de 2% dos tumores em geral, são considerados um importante problema de saúde pública, por caracterizarem a segunda maior causa de morte por câncer em pacientes na idade pediátrica e alta morbimortalidade nas demais idades (2). Estes tumores são divididos em astrocíticos, oligodendrogliais e ependimais, de acordo com a sua origem, e são separados em distintos graus de malignidade segundo a Organização Mundial da Saúde (OMS). O Glioblastoma (GBM) corresponde à metade dos casos de glioma (1,2) e pode manifestar-se em qualquer idade. Geralmente afeta adultos, com um pico de incidência entre 45 e 75 anos de idade (3–5). Os GBMs são tumores letais, com uma média de sobrevida de aproximadamente 14 meses, e menos de 10% dos pacientes vivem dois anos após o diagnóstico (5,6). A barreira hematoencefálica e algumas mutações garantem às células alta resistência aos medicamentos de rotina e a sua grande capacidade de infiltração no parênquima cerebral geralmente impede a ressecção cirúrgica completa (4). Além da cirurgia, a terapia padrão foi proposta por Stupp e colaboradores, através de radioterapia com quimioterapia concomitante, a temozolamida (5). Temozolamida é um agente alquilante oral que possui atividade antitumoral comprovada no tratamento de glioblastoma, entretanto, mesmo com o uso da droga, a média de vida dos pacientes é de 14 meses (5). Além disso, déficits cognitivos e repercussões endócrinas acompanham os pacientes durante o tratamento (7), e taxa de cura dos casos de GBM continua muito baixa. Portanto, novos estudos acerca de suas bases moleculares e a melhor compreensão da gênese destes tumores pode levar ao desenvolvimento de novas opções terapêuticas (7,8).

Alterações no número de cópias (CNAs) de pequenas ou grandes porções

do genoma (instabilidade cromossômica) podem levar à amplificação de oncogenes ou deleção de genes supressores tumorais. Nosso grupo de pesquisa perfilou as CNAs mais frequentemente encontradas em gliomas na população Brasileira, tais como ganho do cromossomo (chr) 7, amplificação de *EGFR*, perdas em chr9, chr10, chr13 e deleção homozigótica na região chr9p22.1-p21.3 (9), e estes achados estão de acordo com as alterações descritas na literatura mundial (10).

A deleção em chr9p22.1-21.3, região detentora dos supressores tumorais *CDKN2A* e *CDKN2B,* foi encontrada em aproximadamente 50% dos glioblastomas na população Brasileira (9). Análises *in silico,* usando o The Cancer Genome Atlas (TCGA), também demonstraram frequência de deleção maior que 50% desta região em glioblastomas (11). A deleção de *CDKN2A* passou a ser utilizada na classificação da OMS em 2021 - se um astrocitoma difuso mutante *IDH* exibir deleção homozigótica dos genes *CDKN2A/B*, ele é designado como um glioma de alto grau, mesmo que características histológicas de malignidade, como necrose e proliferação microvascular, estejam ausentes (1). Além de *CDKN2A/B*, análises *in silico* demonstraram outros potenciais genes supressores tumorais presentes nesta região, tais como *PTPLAD2, ELAVL2, MLLT3, KIAA1797*, vários genes da família dos interferons e *MTAP* (11).

O gene *MTAP* codifica a enzima metiltioadenosina fosforilase (MTAP), que é essencial para os processos de reciclagem de adenina e metionina formados durante a degradação dos nucleotídeos. A enzima catalisa o processo de clivagem do subproduto 5'-deoxi-5'-metiltioadenosina (MTA), gerado durante a biossíntese de poliaminas, em adenina e 5-metil-tioribiose-1-fosfato (MTR-1-P) (**Figura 1**) (12). Este servirá de substrato para formar metionina. Adenina será convertida em adenosina monofosfato (AMP) pela enzima adenina fosforribosil transferase (APRT), utilizando o grupo fosforribosil do monossacarídeo fosforribosil pirofosfato (PRPP) e, por fim,

reutilizada nos processos de formação de nucleotídeos (**Figura 1**). Assim, a via de reciclagem se torna essencial para otimizar o gasto de energia intracelular (12).

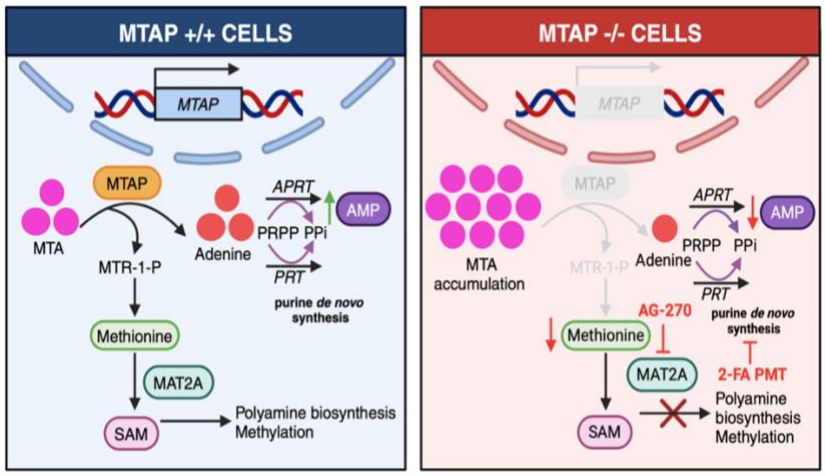


Figura 1 – Esquematização das vias de obtenção de adenina pelas células MTAP +/+ e -/- e os potenciais alvos terapêuticos nas células MTAP -/-.

Nas células MTAP$^{+/+}$, o MTA funciona como agente protetor por ser convertido em adenina pela MTAP. Entretanto, nas células MTAP$^{-/-}$, nenhuma adenina será formada pela conversão de MTA, e a única forma alternativa de obter adenina é pela síntese de purinas *de novo;* entretanto, caso essa via seja inibida, as células MTAP$^{-/-}$ não obterão adenina para suas funções celulares e poderão entrar em apoptose (**Figura 1**) (12).

As drogas 2-Fluoroadenina (2-FA) (**Figura 2**) e Pemetrexede (PMT) (**Figura 2**) são inibidoras de síntese de nucleotídeos a partir de sua conversão em nucleosídeos (PAMP) auxiliada pelas enzimas PRPP e APRT (**Figura 1**). Um estudo demonstrou que 2FA+MTA diminuíram o crescimento de tumores MTAP$^{-/-}$ e as células MTAP$^{+/+}$ não apresentavam diferença do grupo controle (13). Já o AG270 (**Figura 2**), é um inibidor de MAT2A, que converte metionina em SAM (**Figura 1**). O acúmulo de MTA nas células MTAP$^{-/-}$ é pouco estudado, e ainda não se sabe ao certo os efeitos que esse metabólito pode ter no desenvolvimento e prognóstico da neoplasia. Um potencial efeito do

acúmulo de MTA é a ativação alternativa imunossupressora (M2) de macrófagos (14), sugerindo que a perda de MTAP em células GBM pode contribuir para o microambiente tumoral imunossupressor, e que a deleção de MTAP deve ser considerada para caracterizar estados imunológicos de GBM e desenvolver abordagens baseadas em imunoterapia para o tratamento de GBM MTAP$^{-/-}$.

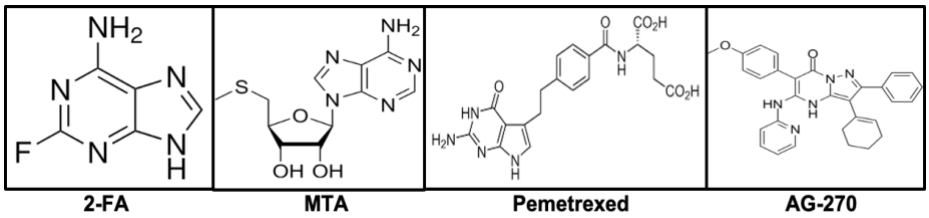


Figura 2 – Estrutura química das drogas utilizadas neste estudo.

Dado que glioblastomas possuem alta frequência de perda do MTAP e prognóstico ruim associado a resistência aos tratamentos convencionais, a relevância clínica deste estudo reside na possibilidade exploratória dessa característica na busca de um tratamento mais efetivo para o paciente. Os resultados deste estudo podem indicar uma importante opção terapêutica que poderá beneficiar os pacientes com GBM que possuem deleção de *MTAP* e sugerir os pacientes com gliomas que responderiam a imunoterapia.

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Role of *MTAP* in human cancers: A review

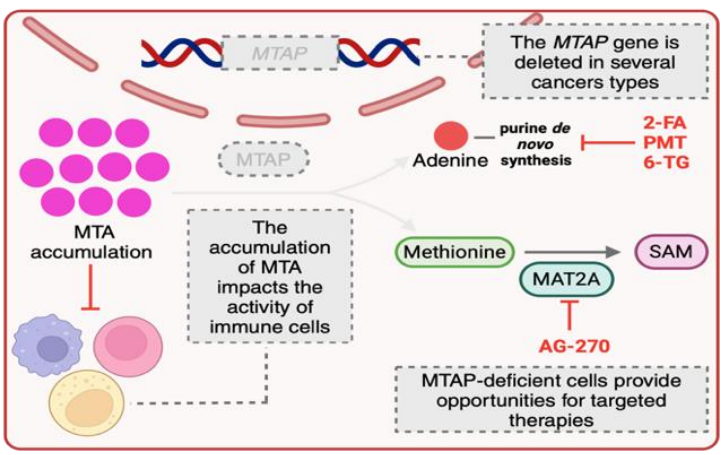
Paola Gyuliane Gonçalves[1,2], Connor Purcell[2], Maximilian Pinho-Schwermann[2], Rui Reis[3,5], Sean Lawler[2], Lucas Tadeu Bidinotto[1,3,4]

1 Department of Pathology, UNESP – Univ. Estadual Paulista, School of Medicine, Botucatu, São Paulo, Brazil
2 Pathology and Laboratory Medicine, Legorreta Cancer Center, Brown University, Providence, RI, USA;
3 Molecular Oncology Research Center, Barretos Cancer Hospital, Barretos, São Paulo, Brazil;
4 Barretos School of Health Sciences, Dr Paulo Prata – FACISB, Barretos, São Paulo, Brazil;
5 Life and Health Sciences Research Institute (ICVS), Medical School, University of Minho, Braga, Portugal;

ABSTRACT

The methylthioadenosine phosphorylase (MTAP) gene plays a crucial role in cellular metabolism, particularly in the salvage pathway of purine nucleotides. *MTAP* deficiency, often resulting from chromosomal deletions, is observed in a variety of cancers, including glioblastoma, pancreatic, lung, and bladder cancers and is associated with a poor prognosis. This genetic alteration leads to an accumulation of 5'-methylthioadenosine (MTA), which in turn disrupts several cellular processes, including the regulation of PRMT5. The loss of *MTAP* has emerged as a potential biomarker for therapeutic targeting, as it confers synthetic lethality to tumors that can be exploited for treatment. From a diagnostic perspective, MTAP status is increasingly being integrated into cancer profiling to help predict prognosis and support treatment decisions. Regarding immunology, the accumulation of MTA and the resultant inhibition of PRMT5 activity can affect immune cell function, influencing T cell activation and tumor immunosurveillance. Understanding the impact of *MTAP* deletion on immune responses offers promising opportunities for combining MTAP-targeted therapies with immunotherapy. Overall, *MTAP* deletion in cancer is a key factor influencing both therapeutic response and immune landscape, and its identification in clinical settings can guide more personalized treatment strategies. In this review, we provide a deeper understanding of MTAP's molecular functions and its role in cellular metabolism and cancer. We also discuss how the loss of MTAP expression could serve as a promising biomarker for guiding treatment decisions.

Keywords: MTAP, Cancer, PRMT5, SAM, MAT, MAT2A, Deletion, p16, 9p21.



1. Molecular structure and functions of the *MTAP* gene

Methylthioadenosine phosphorylase (MTAP) is a ubiquitously expressed housekeeping gene that plays a crucial role in biochemical salvage pathways. *MTAP* is located in the 22.1-21.3 region of the short arm of chromosome 9 (1) (**Figure 1**). The loss of this locus has been associated with the development of multiple cancers and linked to poorer clinical outcomes and resistance to immunotherapy. Among the genes present in this locus are the well-characterized tumor suppressors *CDKN2A* (p16) and *CDKN2B* (p15). *CDKN2A* plays a key role in inhibiting CDK4 and CDK6, promoting cell-cycle arrest at the G1 and G2 phases (2). Similarly, p15 controls G1 progression by inhibiting CDK kinase activation. Genetic and epigenetic alterations in *CDKN2A* and *CDKN2B* have been linked to cancer progression, metastasis, recurrence, and poor prognosis in a variety of tumors (2). However, the chr9p22.1-21.3 region also contains more than twenty other less-explored genes which may contribute to cancer development and deserve further investigation, including *MTAP* (**Figure 1**).

Chr9p22.1-21.3 deletion is commonly observed in both solid and hematologic cancers, making it one of the most frequent genetic alterations in human malignancies. Along with *CDKN2A, MTAP* is often co-deleted in certain cancers, particularly in glioblastoma, mesothelioma, pancreatic cancer, and some other solid tumors (1,3). This co-deletion can create a metabolic vulnerability in cancer cells, offering potential therapeutic targets for treatment. MTAP immunohistochemistry (IHC) has shown a significant diagnostic and prognostic value for detecting neoplastic transformation in a wide range of tumors (4).

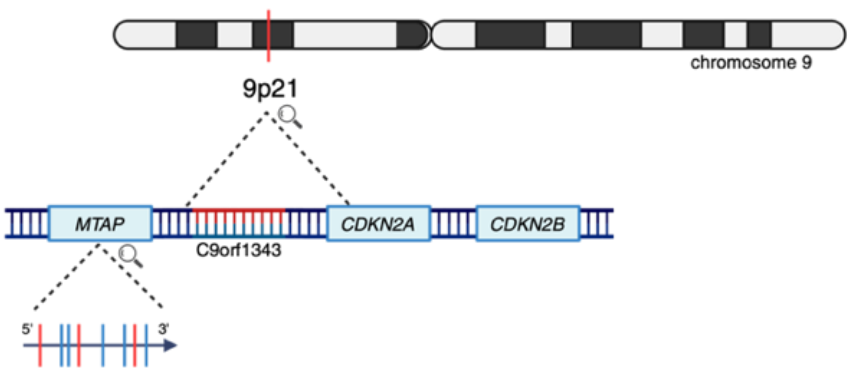


Figure 1 – Molecular structure of the *MTAP* gene and its chromosomal localization. The *MTAP* gene is situated on the short arm of chromosome 9, specifically in the 9p21 region, which also contains other important genes like the tumor suppressors *CDKN2A* and *CDKN2B*. These three genes are close in proximity and are often co-deleted in various types of cancer.

The *MTAP* gene encodes the protein MTAP, an enzyme that plays a key role in the purine salvage pathway, which is essential for recycling nucleotides in cells. Specifically, MTAP catalyzes the cleavage of 5'-deoxy-5'-methylthioadenosine

(MTA), a byproduct of polyamine synthesis, into adenine and methylthioribose-1-phosphate (5) (**Figure 2**). This reaction is crucial for maintaining cellular nucleotide pools and regulating cellular metabolism. *MTAP* is expressed in various tissues, and its activity is vital for cellular homeostasis, especially in rapidly proliferating cells, such as those in cancer or during immune responses. *MTAP*-deficient tumors are often strongly associated with an unfavorable tumor phenotype and linked to a noninflamed tumor microenvironment characterized by PD-L1 expression on tumor cells, lack of PD-L1 expression on immune cells, and a low density of CD8+ lymphocytes (6).

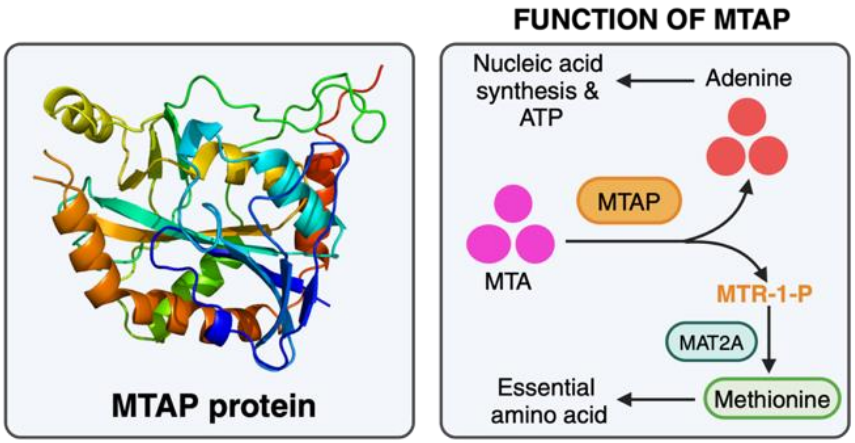


Figure 02 – Function of MTAP. MTAP primarily catalyzes the phosphorolysis of methylthioadenosine (MTA), a byproduct of the polyamine biosynthesis pathway. This reaction results in the production of methionine (an essential amino acid) and adenosine (which can be further utilized in nucleic acid synthesis and ATP).

MTAP is a key enzyme in the methionine salvage pathway, responsible for converting the polyamine synthesis byproduct MTA into methionine. When MTAP is absent, MTA accumulates both intracellularly and extracellularly. Elevated MTA levels inhibit protein arginine methyltransferase 5 (PRMT5), which sensitizes MTAP-deficient cells to inhibition of PRMT5 and methionine adenosyltransferase 2A (MAT2A) (5). MTA accumulation impacts cellular signaling, proliferation, and migration, not only in cancer cells but also in surrounding cells such as stromal fibroblasts and lymphocytes (7). The exact mechanism of action of MTA remains unclear. Notably, MTA has been shown to influence the proliferation of stromal cells and increase their invasiveness (7).

MTAP deficiency is observed in a range of malignancies and is linked to poor survival outcomes in cancer patients. However, the exact mechanisms driving tumor progression following MTAP loss remain unclear. Given its involvement in essential cellular pathways and its potential as a cancer biomarker, MTAP expression and its outcomes is an area of increasing research focus. A deeper understanding of MTAP's molecular functions and its role in cellular metabolism

and cancer could open up new opportunities for diagnostic and therapeutic approaches.

2. *MTAP* status and cancer

MTAP is a critical enzyme in the adenine and methionine salvage pathways, responsible for catalyzing the hydrolysis of MTA. MTA has been suggested to influence key cellular processes, partly by regulating protein methylation. MTAP is widely expressed across various cell types and tissues, and *MTAP* homozygous deletions (HD) occurs in 10-15% of all human cancers, spanning multiple histologies. While *MTAP* deletions have been reported in various tumors (1), in other cancers such as prostate and colon, MTAP may be overexpressed and potentially promote oncogenesis (8–10).

2.1 Bladder urothelial carcinoma

Homozygous 9p21 deletions are frequent in bladder urothelial carcinoma (BLCA), 33% of the patients from TCGA with BLCA had deletion of the locus (11). *MTAP* HD occurs in approximately 30% of BLCA patients and is associated with more aggressive characteristics (12). MTAP loss is associated with poor response to chemotherapy and increased risk to metastasis and cancer progression (12) and is also involved with advanced stage and poor overall survival in BLCA (13,14). For metastatic BLCA, MTAP loss was correlated with higher chance of metastasis in internal organs (14). Importantly, loss of MTAP expression was strongly associated with homozygous *CDKN2A* deletion. MTAP IHC may serve as a surrogate marker for 9p21.3 homozygous deletion, underscoring its clinical significance and potential as both a therapeutic target and predictive biomarker in BLCA (15).

2.2 Brain cancer

Nearly 60% of glioblastoma (GBM) patients exhibit deletions in the 9p21 locus (11), with HD of *MTAP* being one of the most common genetic alterations in GBM. Loss of MTAP leads to significant epigenetic reprogramming, marked by hypomethylation of PROM1/CD133-associated stem cell regulatory pathways. MTAP deficiency promotes the formation of glioma stem-like cells (GSCs), increasing PROM1/CD133 expression and enhancing the tumorigenicity of GBM cells (16). *MTAP* deletion contributes to GBM pathogenesis and is linked to poor prognosis in GBM patients (16). In astrocytoma, negative IDH status and loss of MTAP expression were significantly linked to poorer survival outcomes (17). In addition, patients with low-grade glioma and *MTAP* deletion showed a 7.93-higher chance of death (11). Consequently, since 2021, low-grade gliomas are graded high-grade in the presence of *CDKN2A/B* homozygous deletion (18), and *MTAP* is co-deleted with *CDKN2A/B* in nearly 90% of gliomas (11). However, despite other studies results in gliomas, one study found that reduced MTAP expression was

associated with a better prognostic in GBM (19).

2.3 Breast cancer

The 9p21 locus is often deleted in breast cancer (BC), with *MTAP* being deleted in around 40% of primary BCs (20). Low MTAP expression was significantly associated with poor survival in breast cancer patients (20), whereas MTAP negative metastatic breast cancer was linked to genomic alterations that can affect both targeted therapies and immunotherapies, due to the high-MTA environment typical of these tumors (21). Functional analysis *in vivo* and *in vitro* demonstrated that MTAP could suppress the tumorigenic capabilities of breast cancer cells, including invasion, angiogenesis, tumor growth, and metastasis. In addition, MTAP plays a crucial metastasis-suppressive role by tightly regulating ornithine decarboxylase activity in breast cancer cells (20). MTAP expression was significantly higher in Luminal-A breast tumors compared to triple-negative breast tumors, indicating its reduced expression in more aggressive breast cancer subtypes and suggesting the potential for utilizing *MTAP* status-based approaches in triple-negative breast cancer (22).

2.4 Gastrointestinal Cancers

Gastrointestinal (GI) cancers affect the digestive system and can develop in any part of the gastrointestinal tract, as well as in accessory organs like the liver and pancreas. *MTAP* HD is prevalent in around 9% of GI cancers, being the most prevalent one in pancreatic ductal adenocarcinoma (around 21-30%) and the least in colorectal carcinoma (1.1%) (11,23). In GI tumors, *MTAP* deletion is one of the most common alterations, primarily observed in microsatellite stable tumors with *CDKN2A/B* LOH, particularly in upper gastrointestinal cancers. Other *MTAP* alterations, such as promoter methylation and mutations, have been identified in colorectal cancer, but they are unlikely to result in protein loss or affect drug susceptibility (24).

MTAP is deleted in 19.1% of primary esophageal squamous cell carcinoma (ESCC) tumors. Knockdown of MTAP expression increased invasion and migration of ESCC cells. Conversely, overexpression of MTAP reduced cell invasion and migration. *MTAP* may function as a tumor suppressor gene in ESCC, moreover, *MTAP* deletion contributes to enhanced cell mobility and epithelial-to-mesenchymal transition (EMT) through the GSK3β/Slug/E-cadherin signaling axis (25).

MTAP is deleted in around 30% of pancreatic cancers (11), and its loss is associated with poor prognosis in patients with pancreatic ductal adenocarcinoma (PDAC). Since MTAP expression is typically absent in nonneoplastic pancreas cells, MTAP IHC could be a useful tool to support the diagnosis of PDAC in small biopsy samples (3). *MTAP* deficiency accelerates pancreatic cancer progression through

metabolic reprogramming, offering a potential target for therapeutic intervention (26). 9p21 loss was significantly associated with poor disease-free survival in adenosquamous carcinoma of the pancreas (ASCP) (27).

A significant association was found between MTAP protein expression and tumor progression, as evidenced by an increasing intensity of MTAP staining from normal tissue to carcinoma in colon carcinoma cell lines. Moreover, an association between MTAP activity and interferon (IFN) sensitivity was found, with colon epithelial cells showing minimal response to IFN-gamma, in contrast to the more pronounced response observed in carcinoma colon cell lines (10). MTAP expression was significantly downregulated in three different hepatocellular carcinoma (HCC) cell lines compared to primary human hepatocytes (28). Interestingly, this downregulation was not due to genomic losses or mutations but rather to *MTAP* promoter hypermethylation. Reduced MTAP expression was also confirmed *in vivo* in HCC tissues compared to non-cancerous liver tissue. In MTAP-re-expressing cell clones, invasive potential was markedly decreased, although no significant effects on cell proliferation were observed (28). Additionally, in MTAP-re-expressing cells, treatment with interferon (IFN)-alpha and IFN-gamma resulted in a significantly stronger inhibition of cell proliferation. Furthermore, considering MTAP activity to IFN sensitivity, these results may have clinical implications for developing targeted therapeutic strategies for HCC and colon cancer (10,28). *MTAP* deficiency results in MTA accumulation and disrupts key cellular pathways due to alterations in protein methylation, promoting increased cell proliferation and reducing sensitivity to chemotherapeutic drugs in liver cancer cells (29).

2.5 Lung cancer

MTAP deletion occurs in 13% of non-small cell lung cancer (NSCLC) and is linked to poorer overall survival (30). MTAP loss can lead to different treatment, including immunotherapy options in NSCLC (30). Concurrent loss of both MTAP and p16 predicts poorer outcomes in lung cancer patients (31). Loss of MTAP, along with subtle variations in targeted and immunotherapy options, may influence future combination strategies. MTAP expression serves as an independent prognostic factor and holds greater prognostic value than p16 expression in NSCLC (31). Low MTAP expression may predict a better treatment response but poorer survival outcomes in advanced lung adenocarcinoma (32).

In MTAP-deficient lung cancer cells, reduced sDMA modification inhibits ubiquitination-mediated degradation of vimentin, leading to its stabilization and promoting cell invasion, suggesting a mechanism for metastasis driven by the post-translational regulation of vimentin (33). MTAP and PRMT5 show a negative correlation with vimentin in lung cancer samples, suggesting a mechanism for metastasis driven by the post-translational regulation of vimentin (33).

2.6 Mesothelioma

Mesothelioma is a fatal disease, primarily due to its inherent resistance to existing therapies and the absence of reliable predictive markers and novel molecular targets. *MTAP* HD frequently occurs alongside *CDKN2A* in mesothelioma (11). These alterations are often linked to a worse prognosis and increased resistance to therapies in mesothelioma (34). MTAP loss of expression detected by IHC is a well-accepted surrogate for *CDKN2A* deletion to support a diagnosis in mesotheliomas (3). MTAP IHC is a valuable tool for the diagnosis, prognostication, and potentially stratifying patients for targeted therapies, particularly those involving metabolic vulnerabilities in MTAP-deleted tumors. Given its diagnostic and prognostic significance, MTAP IHC is becoming an increasingly important marker in the clinical management of mesothelioma (35).

2.7 Oral squamous cell carcinoma

Regarding oral squamous cell carcinoma (OSCC), in normal epithelium, MTAP was weakly expressed in the cytoplasm of basal layer cells; however, MTAP was uniformly expressed in the cytoplasm of dysplastic and cancerous cells. Additionally, 38.1% of the cancer cells showed increased nuclear expression of MTAP (36). The concurrent nuclear and cytoplasmic expression of MTAP was associated with poorer differentiation, more aggressive invasion patterns, and features of EMT. Furthermore, this concurrent expression was marginally but significantly associated with worse 5-year relapse-free survival, suggesting that MTAP not only contributes to OSCC oncogenesis but may also promote its aggressiveness by inducing EMT through its role in the methionine salvage pathway (36).

2.8 Prostate Cancer

While *MTAP* deletion is rare in primary prostate tumors, approximately 10% of cases show decreased MTAP mRNA expression, which is associated with poor prognosis (9). Furthermore, this deletion appears to be more prevalent in castrate-resistant prostate cancer compared to primary prostate cancer (9). Additionally, knockdown of MTAP*,* both genetically and pharmacologically, inhibits androgen-sensitive prostate cancer growth *in vivo*. These findings suggest that the methionine salvage pathway plays a crucial role in the homeostatic regulation of metabolite pools in prostate cancer, particularly due to the high flux through the polyamine biosynthetic pathway (8).

2.9 Renal cell carcinoma

Renal cell carcinoma (RCC) is a metabolic disease driven by dysregulated expression of metabolic enzymes (37). Patients with metastatic RCC have a poor prognosis and resistance to existing therapies. Deletions in *MTAP/CDKN2A* are a key

predictor of aggressive tumor progression, poor outcomes, and primary resistance to targeted treatments in RCC (37). However, this deficiency may also suggest potential responsiveness to immune checkpoint blockade. Furthermore, MTAP/CDKN2A loss is linked to sarcomatoid differentiation in RCC, a highly aggressive pathological feature associated with rapid tumor progression and a very poor prognosis (38).

2.10 Others

Accumulation of MTA in *MTAP*-deleted tumors leads to a reduction in protein methylation, disrupting cell signaling and, for instance, activating extracellular signal-regulated kinase (ERK) activity. This alteration promotes a more migratory and invasive phenotype in melanoma cells (7). *MTAP* deletion has been associated with lower overall survival and can be an independent negative prognostic biomarker in Ewing sarcoma (39). *MTAP* deletion was found in 15% of mantle cell lymphoma (MCL) cases. Loss of *MTAP* and its protein expression is linked to poor prognosis in MCL (40).

MTAP deletion was strongly linked to advanced tumor stages and predicted poor outcomes in nasopharyngeal carcinoma (NPC). *MTAP* deletion was associated with an aggressive phenotype and independently predicted worse disease-specific survival and distant metastasis-free survival, highlighting its role in disease progression in NPC. MTAP could serve as an independent prognostic biomarker for NPC, potentially guiding the development of targeted treatments for patients lacking MTAP expression (41).

MTAP deletion occurs in 27.5% to 37.5% of osteosarcoma patients. A study has shown that the absence of MTAP makes osteosarcoma cells more sensitive to methionine restriction via rMETase, offering a promising clinical strategy (42).

3. MTAP as a surrogate biomarker of p16 homozygous deletion

Loss of *MTAP* is frequently associated with homozygous deletion of *CDKN2A,* which codes the tumor suppressor protein p16, (11) since the genes share the same locus (**Figure 1**). Several studies regarding MTAP loss of expression do not report CDKN2A expression status; therefore, some results may be questioned whether the outcomes are due to MTAP or CDKN2A expression status. Further studies using molecular silencing of MTAP may individualize the role of MTAP *per se* in cancer development. Anyway, the frequent co-deletion of these genes is unquestionable (11). Therefore, detecting whether MTAP loss can predict the expression of p16 can provide a useful biomarker in the diagnosis and prognosis of cancers harboring these genetic alterations, mainly due to the limitations of the current detection strategies pf CDKN2A/B deletion (43). In fact, loss of MTAP detected by IHC has been suggested as a surrogate marker for *CDKN2A* HD in various tumors, such as mesothelioma (35) and glioma (44).

Loss of 9p21 is commonly observed in pleural mesotheliomas, where it serves as a well-established diagnostic biomarker. While fluorescence *in situ* hybridization (FISH) has traditionally been the gold standard for detecting 9p21 loss, the loss of MTAP expression by immunohistochemistry IHC is gaining importance as a practical and cost-effective diagnostic surrogate (3). Since 2023, the guidelines for pathologic diagnosis of mesothelioma indicates the use of MTAP and BAP1 immunostains to distinguish benign from malignant mesothelial proliferations (35). MTAP IHC is also a promising surrogate marker for assessing *CDKN2A* status in meningiomas (45).

MTAP loss is emerging as a predictive biomarker in NSCLC since the loss negatively impact the response to immune checkpoint inhibitor (ICI) therapy by silencing the tumor microenvironment and may serve as a predictive marker for novel targeted treatment strategies. Given its growing significance, the incorporation of MTAP IHC into routine diagnostics for NSCLC and other cancers is likely to become standard practice in the near future (46).

CDKN2A HD is a promising biomarker for predicting poor prognosis in IDH-mutant diffuse gliomas (44). The Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy recommends classifying IDH-mutant lower-grade astrocytomas with *CDKN2A/B* HD as grade IV tumors (18). In IDH-mutant astrocytomas and IDH-wildtype glioblastomas, loss of MTAP immunoreactivity could serve as a reliable surrogate for *CDKN2A* HD and provide significant prognostic value, comparable to the direct detection of *CDKN2A* HD (44).

IHC for MTAP loss of expression analyzes could serve as a practical and reliable surrogate biomarker for detecting the absence of functional p16 in tumors, especially when genetic testing for *CDKN2A* HD is not feasible or is limited in clinical practice. MTAP IHC is more cost-effective and easier to perform compared to other methods for detecting p16 deletion. Further, identifying MTAP loss of expression could guide clinical decisions, particularly for MTAP-deficient cancers, and predict sensitivity to emerging therapies and immunotherapies (47–49).

4. The use of *MTAP* deletion as a window for alternative treatments

Due to its metabolic role in cells, *MTAP* deletion has emerged as a potential therapeutic vulnerability in cancer. Cancers with MTAP deletion exhibit a metabolic dependency on alternative pathways, particularly those involving the salvage of methyl donors like S-adenosylmethionine (SAM) and disruption in the purine metabolism pathway, creating a "window" for targeted therapies that exploit these metabolic shifts only and spare normal cells. MTAP presents an opportunity for precision oncology, where the presence or absence of MTAP can help guide treatment decisions, potentially improving the effectiveness of treatments in patients with these specific cancer subtype (**Figure 3**).

One promising strategy is to target enzymes like PRMT5, which rely on SAM for methylation reactions. In MTAP-negative cancers, inhibiting PRMT5 or MAT2A

(which produces SAM) can lead to synthetic lethality, selectively killing tumor cells that lack MTAP (50). Another approach for treating *MTAP*-deleted tumors is to target *de novo* purine synthesis, the process by which cells generate purine nucleotides. In normal cells, this pathway is tightly regulated and balanced. However, in MTAP-negative tumors, the inability to properly degrade MTA forces these cells to rely more heavily on *de novo* purine synthesis to meet their metabolic demands, particularly for nucleotide production required for DNA and RNA synthesis (51).

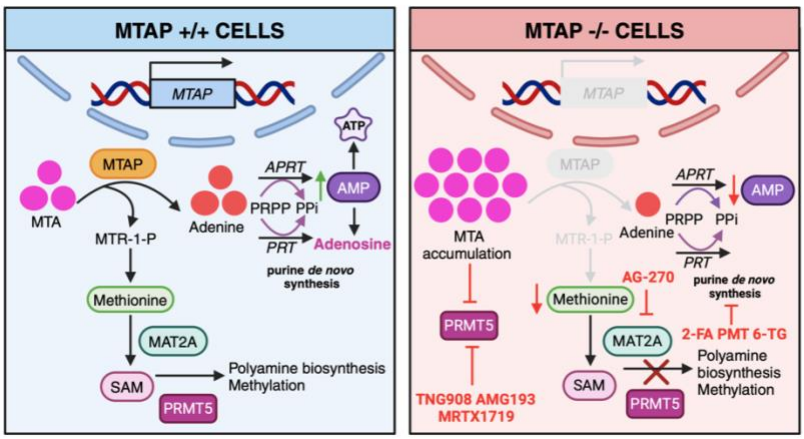


Figure 03 – Differences between MTAP$^{+/+}$ and MTAP$^{-/-}$ cells pathways and the targets therapies that can be used to target specifically MTAP$^{-/-}$ cells. In MTAP$^{+/+}$ cells, MTA acts as a protective agent via conversion to adenine by MTAP. However, in MTAP$^{-/-}$ cells, no adenine will be formed by the conversion of MTA, and the only alternative way to obtain adenine is by de novo purine synthesis; if the de novo pathway is inhibited, MTAP$^{-/-}$ cells will not obtain adenine for their cellular functions and will undergo apoptosis. The drugs 2-Fluoroadenine (2-FA) and Pemetrexed (PMT) are inhibitors of nucleotide synthesis through their conversion into nucleosides (PAMP) aided by the enzymes PRPP and APRT. AG270, on the other hand, is an inhibitor of MAT2A, which converts methionine into SAM. The accumulation of MTA inhibits PRMT5 activity, what leads to an accumulation of SAM.

While these treatments for *MTAP*-deleted tumors show great potential, clinical application is still under investigation. The challenge lies in selectively targeting tumor cells without affecting normal tissues and immune cells, underscoring a need for a deeper understanding of the downstream mediators involved in the treatment effects (50). Also, treatments that rely on MTA accumulation may be affected by the metabolism of MTA by MTAP-expressing stromal cells, potentially reducing the efficacy of these anti-cancer therapies. Furthermore, tumors may develop resistance mechanisms over time, so combination therapies targeting multiple points in the metabolic pathway may be required for sustained efficacy.

4.1 PRMT5

PRMT5, a type 2 arginine methyltransferase, plays a crucial role in regulating

cell growth and survival in cancer. In *MTAP*-deficient cancers, the accumulation of MTA inhibits PRMT5 activity. This inhibition of PRMT5 further sensitizes MTAP-deficient cancer cells to additional PRMT5-targeting therapies, making them more vulnerable to treatment (52). Inhibition of tumor-intrinsic PRMT5 has been shown to enhance antitumor immune responses, suggesting the potential for combining PRMT5 inhibitors (PRMT5i) with cancer immunotherapy; however, global suppression of PRMT5 activity can impair the effector functions of immune cells, potentially limiting the effectiveness of this approach (53).

TNG908 is a clinical-stage PRMT5 inhibitor with an MTA-cooperative binding mechanism, designed to exploit the synthetic lethal interaction between PRMT5 inhibition and *MTAP* deletion. This mechanism enables selective inhibition of PRMT5 in *MTAP*-deleted cancers. Strong preclinical activity in various *MTAP*-deleted xenograft models, along with demonstrated brain penetrance in preclinical studies, supports the potential for clinical development of TNG908 in *MTAP*-deleted solid tumors, including CNS malignancies. TNG908 is currently being evaluated in a Phase I/II clinical trial in patients with *MTAP*-deleted tumors, including GBM (54).

AMG 193 is a CNS-penetrant, MTA-cooperative PRMT5 inhibitor that selectively induces synthetic lethality in *MTAP*-deleted tumor cells. It demonstrated a favorable safety profile, with no clinically significant myelosuppression. Promising antitumor activity was observed across a range of *MTAP*-deleted solid tumors, as evidenced by objective response rates and clearance of circulating tumor DNA (55). In an ongoing Phase 1/2 study, AMG 193 is showing promising clinical activity, including confirmed partial responses in patients with *MTAP*-deleted solid tumors (56,57).

AZ-PRMT5i-1 showed a robust PRMT5-driven *in vivo* efficacy across several MTAP-deficient preclinical cancer models (58). Concomitant administration of exogenous MTA and stable PRMT5i selectively inhibited the growth of *MTAP*-deleted malignant mesothelioma (MM) cells (34).

MRTX1719 is a novel PRMT5 inhibitor that targets the PRMT5-MTA complex. It selectively inhibits PRMT5 activity in the presence of MTA, thereby reducing PRMT5-dependent activity and tumor cell viability. MRTX1719 significantly inhibits the proliferation of *MTAP*-deleted tumors while exerting minimal toxicity on normal cells (53) and effectively reduces activation of the PI3K pathway, a key immune-resistant signaling pathway, suggesting that MRTX1719 has the potential to overcome immune resistance in *MTAP*-deleted tumors (59). Furthermore, MRTX1719 sensitizes MTAP-deficient tumor cells to killing by tumor-reactive T cells (59). When combined with anti-PD-1 therapy, MRTX1719 enhances antitumor activity, resulting in better outcomes in mice bearing MTAP-deficient tumors. (59). Additionally, MRTX1719 demonstrates synergistic anticancer effects when combined with oxaliplatin and gemcitabine in MTAP-deficient cancer cells (60). MRTX1719 demonstrated early clinical signs of activity, including objective

responses in patients with *MTAP*-deleted melanoma, gallbladder adenocarcinoma, mesothelioma, non-small cell lung cancer, and malignant peripheral nerve sheath tumors in a Phase I/II study (61).

4.2 MAT2A

MAT2A is responsible for synthesizing the methyl donor SAM, which is an essential component for methylation reactions in cells. In clinical research, MAT2A inhibitors are being investigated as a treatment for tumors with MTAP deficiency.

AG-270, a potent MAT2A inhibitor, significantly reduces SAM levels in cancer cells and selectively inhibits the proliferation of *MTAP*-deleted cells, both in tissue culture and in xenograft tumor models (62). SCR-7952, a novel MAT2A inhibitor, demonstrates potent and selective antitumor effects on *MTAP*-deleted cancers, both *in vitro* and *in vivo.* Unlike AG-270, SCR-7952 has minimal impact on metabolic enzymes (63). A potential synergistic target for the combination of MAT2A inhibitors is METTL3, which catalyzes the N6-methyladenosine (m6A) modification of mRNA in mammalian cells using SAM as the substrate, and this combination has been shown to impact tumorigenesis. This combination demonstrated synergistic effects both *in vivo* and *in vitro*, resulting in a significant anti-tumor response (64).

Co-deletion of *MTAP* and MAT2A creates a synthetic lethal phenotype by triggering inhibition of PRMT5 (65). MAT2A inhibitors show efficacy in *MTAP*-deleted cancers, but their utility is limited in tumors that express MTAP. Methylthio-DADMe-immucillin-A (MTDIA) is an inhibitor of MTAP that induces a MTAP-deficient phenotype in cells. When combined with AG-270, MTDIA therapy reduced SAM levels and increased MTA in cells, resulting in an inhibition of PRMT5 activity, leading to cell cycle arrest and apoptotic cell death. Unlike direct PRMT5 inhibition, which induces toxicity in normal gut epithelium, the MTDIA and AG-270 combination avoids this issue, making it a safer approach to extend the synthetic lethal strategy to MTAP positive cancers. This offers a potential treatment avenue for a broader patient population, particularly the vast majority (98%) of CRCs that retain MTAP expression (65).

4.3 Pemetrexed

Pemetrexed is a multi-targeted antifolate chemotherapy drug that inhibits several enzymes involved in folate metabolism, primarily thymidylate synthase (TS), dihydrofolate reductase (DHFR) and glycinamide ribonucleotide formyl transferase (GARFT). These enzymes are essential for the synthesis of purines and pyrimidines, which are necessary for DNA and RNA synthesis (66).

In normal cells, MTAP helps convert MTA into MTR-1P and adenine. However, in *MTAP*-deleted tumors, this pathway is disrupted, leading to the accumulation of MTA and a dependence on alternative metabolic routes, particularly in purine metabolism. This creates a metabolic vulnerability that can be exploited and lead

to cell cycle arrest and apoptosis in *MTAP*-negative cancers. The accumulation of MTA in *MTAP*-deficient tumors may further enhance the effect of pemetrexed by disrupting methylation processes and interfering with purine nucleotide synthesis. This can lead to a synthetic lethal effect when combined with pemetrexed, making *MTAP*-deleted tumors more sensitive to the drug (32).

A Phase II trial evaluating pemetrexed in *MTAP*-deleted urothelial carcinoma (UC) found an overall response rate of 43% (14). Additionally, in a retrospective cohort of lung adenocarcinoma, *MTAP* deficiency was also associated with an improved response to pemetrexed (14). A MTAP negative patient with diffuse malignant peritoneal mesothelioma achieved long-term survival of more than 5 years with maintenance therapy using pemetrexed (67). In summary, by targeting the folate pathway and exploiting the synthetic lethality associated with MTAP loss, pemetrexed may offer a promising therapeutic approach for *MTAP*-deleted cancers.

4.4 Inhibitors of *de novo* purine synthesis

Inhibitors of *de novo* purine synthesis have emerged as a promising therapeutic strategy for *MTAP*-deleted tumors, exploiting the metabolic vulnerabilities caused by MTAP loss of expression. Several studies have suggested that inhibiting the *de novo* purine synthesis pathway can selectively target MTAP-deficient tumors (51,68–71). Similarly to pemetrexed, the accumulation of MTA disrupts normal purine metabolism and creates a synthetic lethality vulnerability, where targeting the *de novo* purine synthesis pathway can selectively kill MTAP-deficient cells while sparing normal cells (68).

Inhibitors of *de novo* purine synthesis, such as 6'-thioguanine (6-TG) and 2'-fluoroadenine (2FA), are typically cytotoxic to normal cells. However, pretreatment with MTA can protect normal tissues while still being toxicity to MTAP-deficient cells (71). The rationale behind this approach is that excess MTA can protect normal MTAP-positive cells from the toxicity of purine analogues. MTAP positive cells will catalyze the conversion of MTA to adenine, which then inhibits the incorporation of purine base analogues into nucleotides (51). However, in *MTAP*-deleted tumor cells, no such protection occurs, as adenine cannot be formed. The combination of 2FA and MTA inhibited tumor growth in mouse xenograft models of four different MTAP-deficient tumors (51). The combination of MTA and 6-TG has demonstrated significant therapeutic efficacy, resulting in substantial tumor shrinkage or inhibition of growth in xenograft models of lymphoblastic leukemia and metastatic prostate carcinoma (69). These studies suggest that the combination of MTA and purine analogues could be a promising strategy for treating *MTAP*-deleted tumors (51). Importantly, MTA accumulation affects the microenvironment, leading to a immunologically colder phenotype (48,72), therefore, the addition of MTA in the treatment may be harmful for the patients. However, if the accumulated MTA is be

used by surrounding normal cells, the net effect could be beneficial.

L-Alanosine (ALA), an inhibitor of *de novo* purine synthesis, reduces stemness in MTAP-deficient GBM cells. This reduction in stemness is partly driven by impaired mitochondrial function (70). Additionally, ALA sensitizes GBM cells to temozolomide (TMZ), suggesting that inhibiting purine synthesis could offer therapeutic benefits when combined with the standard care (70). Combining *de novo* purine synthesis inhibitors with other therapies that also target the downstream effects of SAM metabolism, like PRMT5i, could further enhance the efficacy of treatment.

5. Impacts of *MTAP* deletion in immune cells and immunotherapy

Loss of 9p21 is associated with alterations in immune cells and resistance to immunotherapy. While this resistance was once attributed to *CDKN2A/B* loss, studies have pointed to the loss of *MTAP* that drives poor outcomes in immune checkpoint inhibitor (ICI) therapy and reduced tumor-infiltrating lymphocytes (TILs) density (73). *MTAP* deficiency results in the accumulation of MTA, both inside and outside the cell, which profoundly impairs T cell function through the inhibition of PRMT5 and activation of adenosine receptors (ADORA) (73) (**Figure 4**). *MTAP* deficiency alters the tumor immune microenvironment, promoting tumor progression and immune evasion (33). *MTAP* deficiency disrupts both tumor-intrinsic and immune-related pathways, reprogramming cytokine profiles to create a tumor-favorable environment. *MTAP* knockout cells also exhibit a significant increase in the immune checkpoint protein PD-L1, leading to a suppression of T cell-mediated cytotoxicity and triggering markers of T cell exhaustion. MTAP-deficient tumors exhibit reduced immune cell infiltrates, with fewer T lymphocytes and natural killer (NK) cells (33). Treatment with MTA-depleting enzymes reverses this immunosuppressive effect, boosting TIL levels and significantly slowing tumor growth. Further, this approach shows strong synergy with ICI therapy (73).

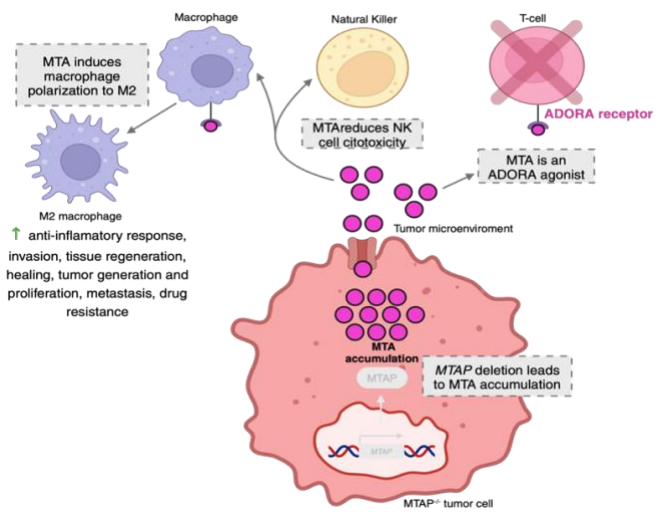


Figure 04 – MTA and immunology. Methylthioadenosine (MTA), the byproduct of

polyamine biosynthesis, plays an interesting and somewhat complex role in immune cells. MTA has been shown to have immunosuppressive properties, especially in the context of inflammatory or autoimmune diseases. One of the key mechanisms is through adenosine receptor signaling. MTA can be converted into adenosine, which binds to adenosine receptors on immune cells, inhibiting immune cell activation and promoting immune tolerance. MTA can also affect macrophages and dendritic cells by inducing a more anti-inflammatory phenotype, which could impair the ability of the immune system to fight infections or tumors. MTA accumulation, particularly in MTAP-deficient tumors, leads to the suppression of immune responses, helping the tumor evade immune surveillance.

The accumulation of MTA also promotes the immunosuppressive alternative activation (M2) of macrophages. This MTA-driven effect on macrophages is independent of IL4/IL3 signaling and is mediated through the ADORA2B receptor (48), which appears to be the specific receptor and signaling pathway for the metabolite MTA in cells (74). MTA also alters the cytokine secretion profile of monocytes and hinders their maturation into dendritic cells (DCs) (72). MTA-treated DCs produced less IL-12 and exhibited an immature phenotype, marked by reduced expression of co-stimulatory molecules CD80, CD83, and CD86, alongside increased expression of monocyte markers CD14 and CD16. As a result, MTA significantly impaired the ability of DCs to stimulate T cells (72). MTA reduced NK cell cytotoxicity against target cells in a dose-dependent manner, while NK cell viability remained unchanged. MTA inhibited NK cell degranulation and cytokine production, both during target cell engagement and upon antibody stimulation. Additionally, MTA interferes with several signaling pathways downstream of the CD16 receptor upon NK cell activation, including the PI3K/AKT/S6, MAPK/ERK, and NF-κB pathways (75). MTA suppresses the proliferation, activation, differentiation, and effector function of antigen-specific T cells without inducing cell death. However, when added to highly activated T cells, MTA exerts cytotoxic effects (76). The Akt pathway, a crucial signaling pathway for T cell activation, is a target of MTA. Additionally, MTA mediates its immunosuppressive effects by disrupting protein methylation in T cells (76). MTA can also inhibit protein sDMAylation (symmetrical demethylation of arginine), and this inhibition may impact the function of FUBP, an enhancer-binding protein (77).

Both MTA and PRMT5 inhibitors impaired T-cell proliferation, viability, and functionality (6). PRMT5 activity plays a crucial role in various cellular processes within human CD8+ T cells that are vital for effective T-cell function. As a result, PRMT5 inhibitors compromise not only tumor cells but also antitumor immune responses. This highlights the need to carefully consider potential side effects on the immune system when developing therapies targeting MTAP-deficient tumors (6).

Conclusions

The homozygous deletion of chr9p22.1-21.3 region, commonly observed in

many cancer types, results in the loss of the *MTAP*, *CDKN2A* and *CDKN2B* genes. MTAP loss is associated with poor clinical outcomes and has been causally linked to unfavorable characteristics such as an immunosuppressive tumor microenvironment. In addition to the phenotypic changes and prognostic differences associated, MTAP loss can serve as a useful biomarker to predict CDKN2A/B deletion, overcoming methodological issues for this detection, proving particularly useful in mesotheliomas and gliomas. Further, MTAP loss of expression provides a therapeutic window which can be used in the development of novel targeted treatments. The multifaceted roles of MTAP loss as a prognostic marker, surrogate for tumor suppressor loss, and indicator of treatment sensitivity underscore its importance to clinical practice and drug development. As research continues to uncover the far-reaching effects of MTAP loss on cellular dependencies and the microenvironment, the importance of MTAP in cancer therapy is sure to expand.

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Objetivos

A presente tese buscou avaliar o papel do gene *MTAP* no desenvolvimento e tratamento de glioblastoma. Para a realização deste estudo, os objetivos específicos foram:

a. Analisar *in silico* a expressão do gene *MTAP* em pacientes com GBM do TCGA, bem como os genes diferencialmente expressos entre pacientes $MTAP^{+/+}$ e $MTAP^{-/-}$;
b. Deletar o gene *MTAP* em linhagens celulares de GBM e realizar estudos *in vivo* e *in vitro*;
c. Verificar se a combinação de 2-FA+MTA possui capacidade de cruzar a barreira hematoencefálica;
d. Verificar o efeito do tratamento 2-FA+MTA como possível terapia alvo para pacientes com deleção de MTAP;
e. Verificar se o MTA pode atenuar a citotoxicidade do 2-FA em células cerebrais normais;
f. Avaliar o papel de MTA na imunologia/imunoterapia;
g. Avaliar o papel do metabólito MTA no desenvolvimento e progressão do GBM.

Capítulo II

MTAP silencing inhibits glioblastoma cell proliferation and *in vivo* tumor growth

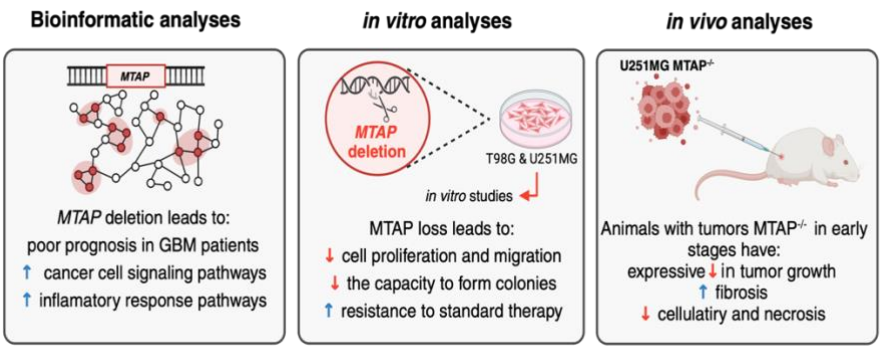
Paola Gyuliane Gonçalves[1,2], Cristiane de Oliveira[1,2], Gabriel Tadeu Heitor Canas[2,3], Leticia Tshikawa Santos[2], Silvia Aparecida Teixeira[2], Renato Silva-Oliveira[2,3], Gustavo Ramos Teixeira[3,4], Sean Lawler[5], Rui Manuel Reis[2,6,7], Lucas Tadeu Bidinotto[1,2,3]*

[1] Department of Pathology, UNESP – Univ. Estadual Paulista, School of Medicine, Botucatu, São Paulo, Brazil
[2]Molecular Oncology Research Center, Barretos Cancer Hospital, Barretos, São Paulo, Brazil;
[3]Barretos School of Health Sciences, Dr Paulo Prata – FACISB, Barretos, São Paulo, Brazil;
[4]Department of Patology, Barretos Cancer Hospital, Barretos, São Paulo, Brazil;
[5] Pathology and Laboratory Medicine, Legeorreta Cancer Center, Brown University, Providence, RI, USA;
[6]Life and Health Sciences Research Institute (ICVS), Medical School, University of Minho, Braga, Portugal;
[7]3ICVS/3B's-PT Government Associate Laboratory, Braga/Guimarães, Portugal;

ABSTRACT

Glioblastoma (GBM) remains one of the most aggressive and lethal brain cancers, characterized by rapid tumor growth, resistance to therapy, and poor prognosis. Recent studies have highlighted the role of methylthioadenosine phosphorylase (MTAP), a key enzyme in purine and methionine metabolism, in various cancers. However, the impact of MTAP silencing in GBM remains poorly understood. In this study, we investigated the effects of MTAP silencing on GBM tumor growth using glioblastoma cells lines and a xenograft model. Using CRISPR-Cas9-mediated MTAP knockout in glioblastoma cells, which were then implanted into immunodeficient mice. Our results demonstrate that MTAP silencing significantly delayed tumor growth compared to controls ($p<0.001$), suggesting that MTAP loss impairs GBM proliferation in early stages. Additionally, histological analysis revealed alterations in cellularity ($p<0.009$), fibrosis ($p<0.006$) and necrosis ($p<0.0003$) in MTAP-silenced xenografts. Mechanistically, MTAP loss induced differences in proliferation ($p<0.001$), migration ($p<0.001$) and colony formation ($p<0.001$). These findings suggest that MTAP silencing disrupts the metabolic and proliferative pathways critical for glioblastoma progression, highlighting MTAP as a potential therapeutic target. Targeting MTAP-deficient tumors via purine synthesis or PRMT5 inhibitors may offer a promising therapeutic strategy. Further studies are warranted to explore the mechanistic basis of these effects and to assess the therapeutic potential of targeting MTAP in GBM treatment.

Key-words: MTAP, deletion, glioblastoma, xenograft.



Introduction

Glioblastomas (GBM) are the most common tumors of the Central Nervous System (CNS) and, despite all advances in molecular studies, the morbimortality remains poor[1]. World Health Organization (WHO) proposed in 2021 that integration of molecular features for classification and grading of some brain tumor types, such as *Isocitrate dehydrogenase (IDH)* mutation, which was able to predict more accurately the clinical behavior of some entities[2].

Molecular studies in GBM have shown deletion of chr9p22.1-21.3 as an event occurring in more than 50% of the patients[3]. This region encompasses several genes, including *CDKN2A/B* (*p16* and *p15*). In fact, studies have pointed to the homozygous deletion (HD) of *CDKN2A/B* as a prognostic marker of gliomas, mainly those which have point mutation in *IDH*[4–6]. However, the variety of methods that can be used for *CDKN2A/B* HD (single-nucleotide polymorphism microarrays, next-generation sequencing, DNA-based methylation techniques and fluorescent *in situ* hybridization) have their own limitations, such as heterogeneous accuracy, discordant results, high costs, which results are difficult to uniformize[7]. Furthermore, there are reports showing discordant results between *CDKN2A* HD and loss of expression[8–10].

Molecular integrated *in silico* studies point to the co-deletion of *CDKN2A/B* and *MTAP* in almost all cases[11] and MTAP protein has been described as with frequent loss of expression in high grade gliomas[12]. Additional studies, therefore, have been proposing the immunohistochemistry for MTAP as a surrogate biomarker for *CDKN2A* HD in both *IDH*-mutant and *IDH*-wild type gliomas[13,14].

Besides, little is known about the effects of *MTAP* deletion in GBM growth. During polyamines synthesis, MTAP cleaves deoxy-5'-methylthioadenosine (MTA) to adenine (which is converted to AMP) and 5-methylthioribose-1-phosphate (which is metabolized to methionine)[15]. Although some studies found that HD in *MTAP* locus was associated with poor survival[11,16], low mRNA expression of *MTAP* was not associated with differential survival, detected by RNA sequencing[11]. However, at protein level, there are contrasting results, with some studies showing that MTAP loss is associated with a poor prognosis in gliomas[17,18], while another showed a better survival of patients with low expression of MTAP[12].

Given the biological role of MTAP and that its role in GBM development and prognosis are poorly understood, we aimed to analyze the differences between MTAP +/+ and -/- tumors, as well the effects of *MTAP* knockout in MTAP silenced GBM cell lines and in the growth of xenografts in NOD scid gamma mouse (NSG) immunodeficient mice.

Methodology

In silico analyses of the MTAP gene in glioblastoma

RSEM-normalized RNA sequencing data from gliomas were acquired from

TCGA and imported into the R platform using the TCGAbiolinks[19] and RTCGAToolbox[20] packages. First, we obtained the copy number alteration (CNA) and paired RNA-Seq data for each patient with glioblastoma, deposited in TCGA. After that, the patients were separated into "deleted" vs "non-deleted" (considering the MTAP gene) according to the CNA, and then the differential expression was calculated with the RNA-Seq data comparing these 2 groups. The differentially expressed genes (DEGs) of normal and deleted MTAP patients were obtained using the eBayes empirical method for differential expression with the LIMMA package[21]. Genes with fold change $\geq$ 2.0 and FDR < 0.05 were considered differentially expressed. The DEGs were subjected to Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, and the terms were considered statistically significant when FDR $\leq$ 0.05. Overall survival was proven using log rank statistical analysis, with significance set at 0.05. The platforms Xena[22], to compile some information data from TCGA, VolcaNoseR[23], to create a volcano plot using the DEGs, and STRING[24], to find MTAP–protein association networks, were also used in this study.

Ethics approval

All experiments were performed according to protocols from the Commission of Ethics in Animal Research (CEUA) from Barretos Cancer Hospital (protocol number 026/2023).

MTAP silencing

MTAP was silenced in U251MG cells using CRISPR/Cas9 methodology according to Menezes *et al*.[12]. In T98G cells the silencing was performed according to the DharmaFECT™ Duo transfection reagent—for siRNA and plasmid co-transfection standard protocol (Dharmacon). To confirm MTAP silencing, the cells were lysed with lysis buffer (50 mM Tris; pH 7.6; 150 mM NaCl; 5 mM EDTA; 1 mM Na3VO4; 10 mM NaF; 10 mM sodium pyrophosphate; 1% NP-40) containing protease inhibitor cocktail (0.01 M EDTA; 1 mM DTT; 1 mM Leupeptin; 1 mM PMSF and 1 µM Aprotinin) and placed on ice for 60 minutes and centrifuged at 13.000 g for 15 minutes. After quantification using Bradford method (Quick Start TM Bradford Protein Assay, Bio-Rad, USA), the total proteins were separated by 12% polyacrylamide gel and transferred onto Trans-Blot Turbo Midi Nitrocellulose Transfer Packs (Bio-Rad, USA). Non-specific proteins block was performed using 5% non-fat milk, and the membrane was further incubated with primary antibodies (MTAP 1:2.000, ProteinTech, USA, Vinculin: 1:4.000, ProteinTech, USA and β actin 1:2.000, Cell Signaling®, USA) sequenced by secondary antibody. Immunodetection was performed using ECL KIT (Amersham Biosciences, Sweden) in ImageQuant LAS 4000 mini (GE Healthcare, USA).

In vitro studies

During the functional assays, described below, the cells were treated with temozolomide (TMZ) (dilution ranging from 1 µM to 1000 µM). For the control of all experiments, DMSO was used at the same dilution as the drug with the highest concentration in the experiment. All experiments are described below.

DAPI phalloidin fluorescence

DAPI and phalloidin fluorescence staining is commonly used to visualize both nuclear DNA and actin cytoskeleton in cells using fluorescence microscopy. T98G MTAP $^{+/+}$ and $^{-/-}$ cells were cultured on chambered coverslip with 8 individual wells (2.000 cells per well) for 48 hours. The cells were fixed after, by adding 4% paraformaldehyde (PFA) in PBS for 10-15 minutes at room temperature. After fixation, the cells were washed 2x with PBS to remove any residual fixative. The cells were permeabilized using 0.1-0.5% Triton X-100 in PBS for 10 minutes. The cells were washed 2x with PBS after permeabilization. The cells were incubated with Alexa Fluor 488-Phalloidin 1:100 dilution for 60 minutes at room temperature in the dark. After, the cells were washed 2x with PBS and incubated in DAPI to a final concentration of 1 µg/mL for 10 minutes at room temperature. The stained cells were imaged using a fluorescence microscope.

Cell proliferation analysis

Cell proliferation was assessed using the BrdU (5-bromo-2-deoxyuridine) assay. A total of 2,000 cells per well were seeded in dark 96-well plates designed for fluorescence measurements. After 24 hours of incubation, cells were treated accordingly, and BrdU labeling was added. Following 24 and 48 hours of treatment, cells were stained with the BrdU detection kit (Roche, cat# 11647229001) following the manufacturer's instructions. The percentage of BrdU-positive cells was quantified using an absorbance reader at 450 nm. The experiment was conducted in triplicate and repeated three times.

Analysis of apoptotic cell death

To evaluate apoptotic cell death, cells were grown in 6-well plates until confluent, at which point the specific treatment was applied, and the cells were incubated for 48 hours with the drugs. Following treatment, cells were stained with SYTOX™ Blue Dead Cell Stain (ThermoScientific, cat# S34857) according to the manufacturer's guidelines. Data acquisition was carried out using a CytoFLEX flow cytometer (Beckman Coulter). The experiment was performed in duplicate and repeated three times.

Cell invasion analysis by transwell

The invasion capacity of cells was assessed using the Biocoat Matrigel

Invasion Chamber (BD Biosciences), which provides a layer of extracellular matrix for cell transmigration. Then, culture medium supplemented with 10% serum, serving as a chemoattractant, was added to the lower compartment of the wells. Cells were trypsinized and seeded at a density of 1.0 x 10^5 in the upper compartment and incubated at 37°C for 24 hours. After incubation, non-invading cells were removed with a swab. Cells that successfully degraded the Matrigel (mimicking the extracellular matrix) and migrated to the lower surface were fixed with 100% methanol and stained with crystal violet. The stained cells were visualized and quantified using a fluorescence microscope (Nikon Eclipse 50i). The experiment was performed in duplicate and repeated twice.

Colony formation assay

For this assay, 4,000 cells T98G MTAP +/+ and -/- were seeded in 6-well plates. After 24 hours of incubation, the treatments (DMSO Control and TMZ at 500 µM) were added to the culture medium. The cells were cultured for 12 days, with media changes every 72 hours. After the 12-day treatment period, the wells were gently washed with dPBS, fixed with 100% methanol, and stained with crystal violet. The plates were then kept at room temperature for 48 hours to dry before being photographed. The experiment was performed in duplicate and repeated twice.

CellTiter-Glo cytotoxicity assay

A total of 2,000 cells per well were seeded in dark 96-well plates designed for fluorescence assays. After an overnight incubation, the appropriate treatments were added. The cells were then incubated for 48 or 72 hours, followed by the addition of the fluorescent reagent CellTiter-Glo® 3D Cell (Promega, cat# G968A), which was incubated for 30 minutes in the dark, before being read using a fluorescence reader. The assay was performed in triplicate and repeated three times. GraphPad PRISM 10 was used to calculate the drug dose-response curve and EC50. The EC50 represents the concentration of agonist that induces a response halfway between the baseline and maximal response.

In vivo studies

In order to characterize the growth of U251MG cells in animals, we performed 2 different experiments. In the first experiment, the total of 16 NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice were distributed in 4 cages (4 animals/cage), maintained under SPF conditions, and received sterile food and water ad libitum. At aging 9 weeks, they were randomized into two groups (n=8/group): G1 received subcutaneously 5x10^6 MTAP$^{+/+}$ cells diluted in 100ul of dPBS and matrigel; G2 received subcutaneously 5x10^6 MTAP$^{-/-}$ cells in the same diluent. This was considered day 0 of the experiment. The animals were weighted, and tumor volume was measured twice a week with a caliper and calculated using the formula: VT =

$(D \times d^2)/2$, where D and d represent the largest and smallest diameters, respectively. At day 45, the animals were euthanized by anesthetics overdose and tumors were collected and fixed in 10% buffered formalin for 48h, followed by inclusion in paraffin blocks. In the second experiment, we performed the same conditions of experiment 1, except for we injected 10^7 cells, and the tumors were allowed to grow longer.

A histopathological characterization of the tumors considering cellularity, necrotic and fibrotic area in both experiments by a blinded expert neuropathologist. For cellularity, the tumors were categorized in low, moderate, or high cellularity. For necrotic area, the tumors were divided in "absent", 0-10% of necrotic area, or >10% of necrotic area. Finally, for fibrotic area, the tumors were classified in "absence of fibrosis", 0-25%, 26%-50%, or >50% of necrotic area.

Statistical analyses

Statistical analyses were conducted using GraphPad PRISM 10 or SPSS Statistics (IBM®) v26. For *in vitro* analyses, one-way ANOVA was applied to compare the means of two or more independent groups to assess whether there were significant differences between population means. For multiple comparisons, two-way ANOVA was used. T-tests were employed for dependent analyses. For *in vivo* studies, results are presented as mean and standard error. Tumor volume was compared between the two groups using t-test, whereas cellularity, necrosis and fibrosis were analyzed using chi-square test. The level of significance adopted was of 5%.

Results

MTAP gene deletion in gliomas is associated with a worse prognosis and cancer signaling pathways

After *in silico* analyses using available data from TCGA, it was found that the *MTAP* gene is deleted in almost 60% of glioblastoma cases, and *CDKN2A* is co-deleted in 90% of the cases, making *MTAP* deletions a surrogate for *CDKN2A* deletions (**Figure 1A**). The gene expression of *MTAP* in the GBM dataset reveals a significant loss of *MTAP* expression, primarily driven by copy number alterations (**Figure 1C**). Additionally, **Figure 1C** highlights the frequent co-deletion of CDKN2A in GBM. When comparing the overall survival of patients according to *MTAP* status, it was noted that patients without *MTAP* gene deletion have a better prognosis when compared to patients with *MTAP* deletion in GBM (**Figure 1D**). In the analysis of differentially expressed genes between $MTAP^{+/+}$ and $^{-/-}$ patients, 607 differentially expressed genes were found, with *CDKN2A/B* being one of the most significant upregulated in $MTAP^{+/+}$ patients, highlighting the relationship between *MTAP* and *CDKN2A*/B (**Figure 1B**), while the most downregulated was the gene *MXRA7*, involved in monocyte-to-macrophage differentiation[25]. STRING showed the MTAP-

protein association network with genes related to adenine biosynthesis process, purine salvage, and positive regulation of NF-κB and chronic inflammatory response (**Figure 1E**). When analyzing the cellular biological processes and signaling pathways related to the differentially expressed genes (**Figure 2**), an enrichment of the Wnt pathway was found, with 34 associated genes. Other cancer-related pathways are also deregulated, such as mTOR and MAPK. Regulation of NF-κB signaling and inflammatory response was also found (**Figure 2**).

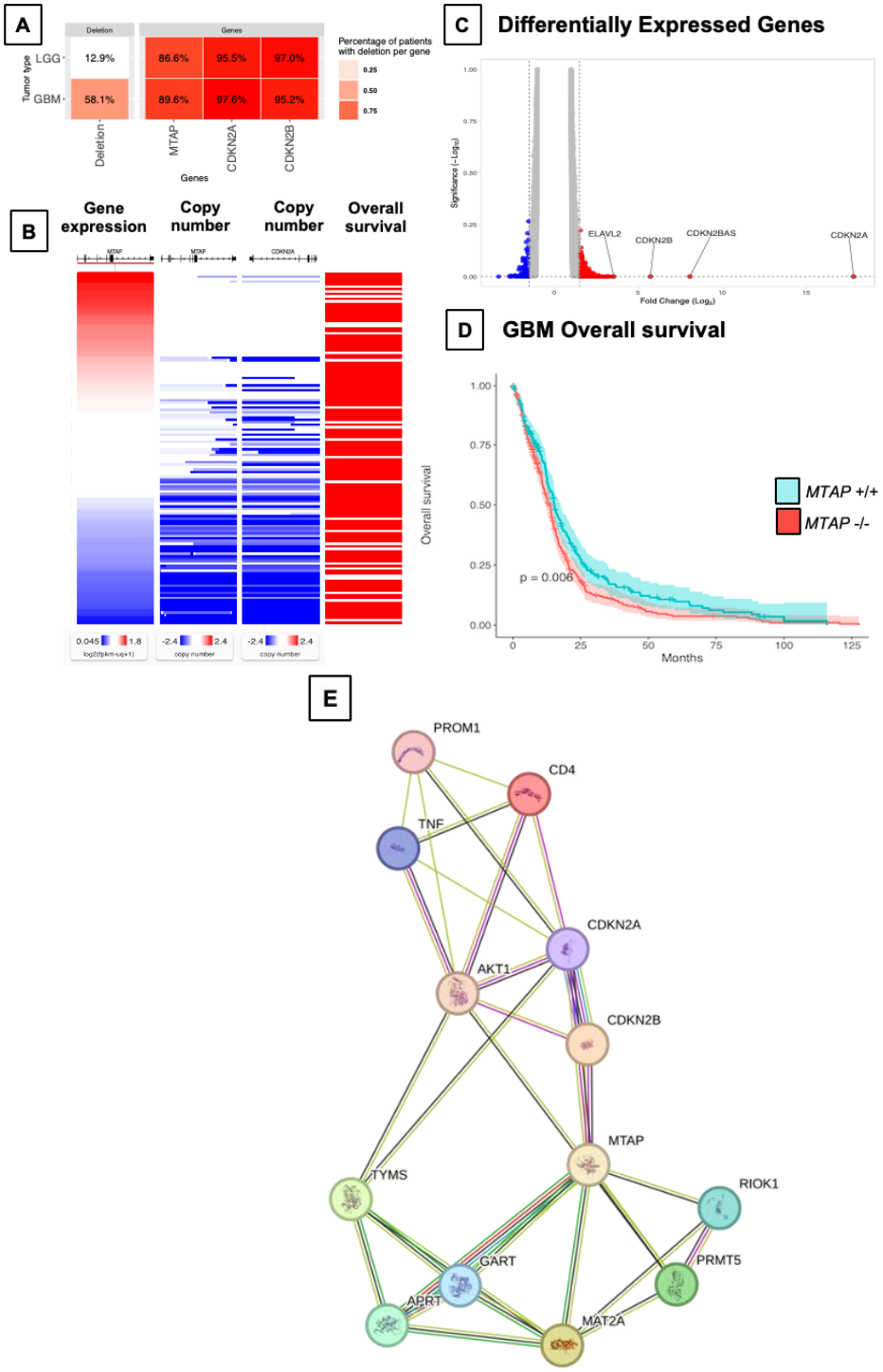


Figure 1 - Percentage of *MTAP* gene deletion and comparison of deletion with *CDKN2A* gene (A), *MTAP* gene expression compared to MTAP/CDKN2A copy number alterations and overall survival (C) in the TCGA GBM cases. Volcano plot showing the differentially expressed genes (B) and differences in overall survival (C) of MTAP$^{+/+}$ and $^{-/-}$ GBM patients from TCGA. MTAP-protein association network using the DEG data (E). *MTAP loss is found in almost 60% of GBM cases, and this loss is associated with poor prognostic in TCGA patients. The DEG*

between MTAP$^{+/+}$ and $^{-/-}$ patients shows a MTAP-protein association network with genes related to adenine biosynthesis process, purine salvage, and positive regulation of NF-κB and chronic inflammatory response.

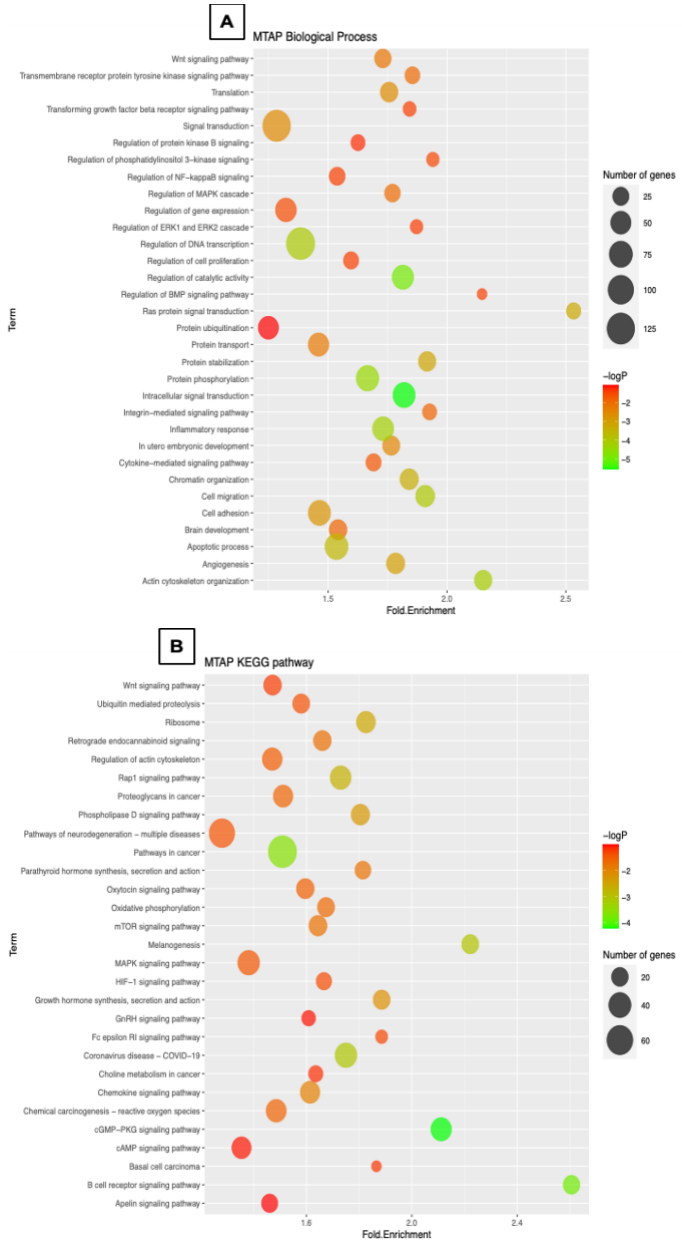


Figure 2 – The biological processes (A) and signaling pathways (B) that the differentially expressed genes between MTAP $^{+/+}$ and $^{-/-}$ patients are related to. *MTAP loss leads to changes in pathways related to cancer cell signaling and inflammatory response.*

MTAP deletion leads to changes in response to standard GBM treatment, while decreases the proliferation and migration of GBM cells

To confirm the MTAP silencing in U251MG cells, we performed western blot, using β-actin and vinculin as internal control. As shown in **Figure 3**, MTAP was

successfully silenced in the cells. Therefore, we used this line as MTAP$^{-/-}$ and the mock cells as control (MTAP$^{+/+}$). These cells were subsequently used in the experiments to characterize the behavior depending on MTAP status. Loss of MTAP results in changes in cell morphology (**Figure 3B and C**), which may be driven by an increase in vimentin expression that leads to a more mesenchymal phenotype. The differences in cell morphology between the cells were confirmed by DAPI phalloidin fluorescence (**Figure 3F and G**).

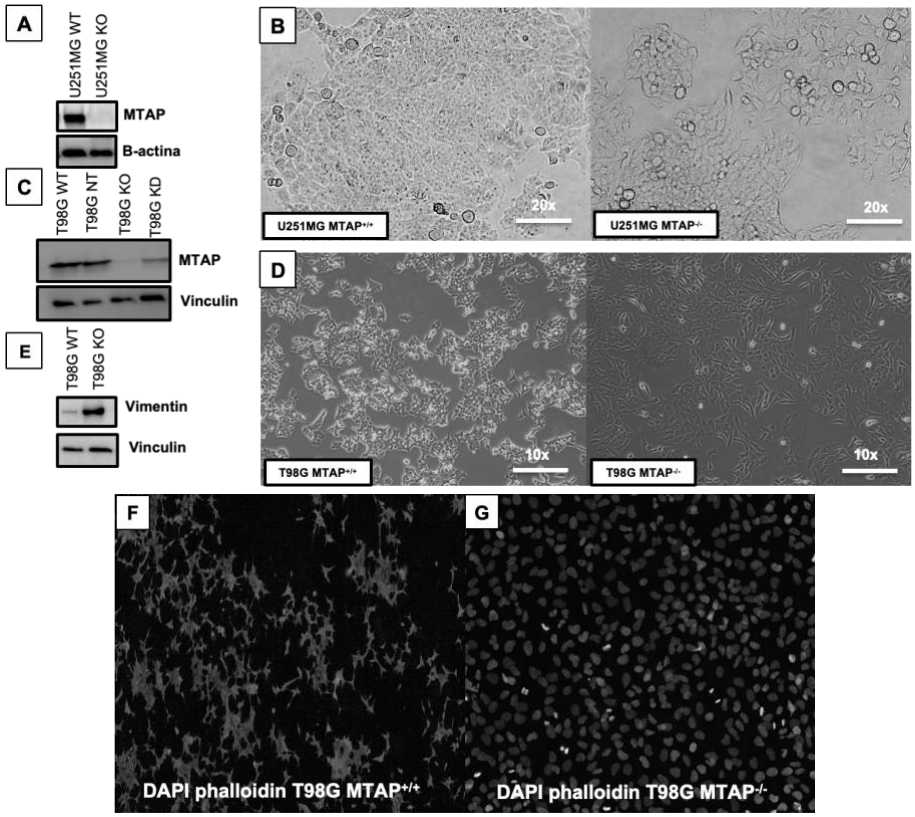


Figure 3 - MTAP protein expression analysis shows silencing in the cell lines U251MG (A) and T98G (C) and the differences of morphology in the U251MG (B) and T98G (D) MTAP $^{+/+}$ and $^{-/-}$ cells. The expression of vimentin in T98G MTAP $^{+/+}$ and $^{-/-}$ cells (E). DAPI phalloidin fluorescence showing the differences in morphology between cells T98G MTAP $^{+/+}$ (F) and $^{-/-}$ (G). *MTAP loss leads to morphological changes in the cells that are related to a more mesenchymal state, with increasing of vimentin expression in cells MTAP$^{-/-}$.*

The loss of MTAP confers an increasing in the resistance of cells to the standard therapy for GBM, the alquilante agent TMZ. The MTAP$^{-/-}$ cells had an increasing of more than 30% in the EC50 when treated with TMZ (**Figure 4**). The U251MG had an increasing from 409 to 638 µM of TMZ (**Figure 4A**), while T98G increased from 329 to 506 µM (**Figure 4B**).

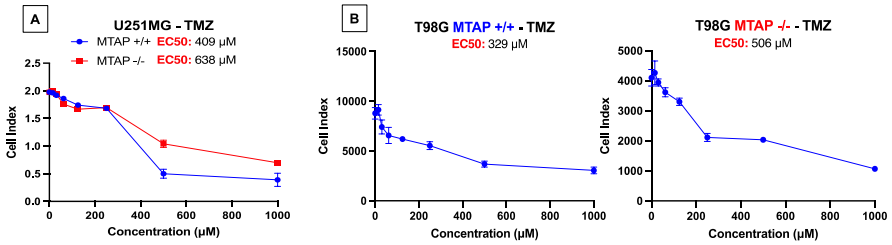
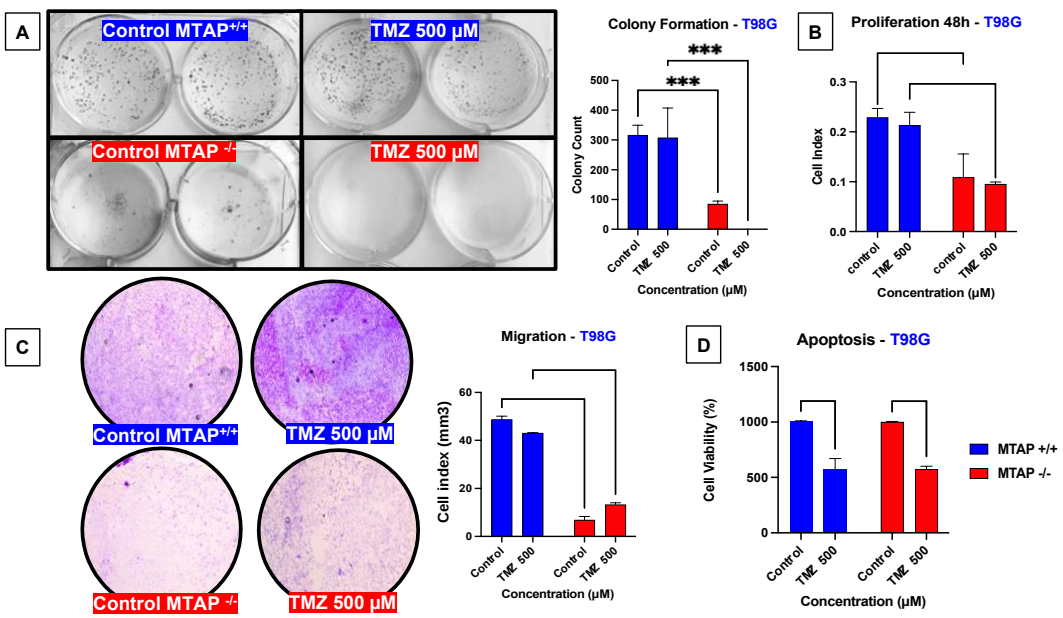


Figure 4 - Dose-response curves showing the cytotoxic effects of the drugs on the MTAP $^{+/+}$ and $^{-/-}$ cell lines U251MG (A) and T98G (B) after 72h of treatment with TMZ, as well as their EC50. *MTAP loss increases the resistance to temozolomide in glioblastoma cell lines.*

T98G MTAP$^{-/-}$ cells showed a reduced ability to form colonies, and treatment with 500 µM TMZ completely eliminated colony formation in the MTAP$^{-/-}$ cells ($p<0.001$) (**Figure 5A**). The proliferation was also affected by MTAP loss ($p<0.001$), although there were found no differences in proliferation after TMZ treatment (**Figure 5B**). In addition, cells MTAP$^{-/-}$ invaded less than MTAP$^{+/+}$ independent of the treatment (**Figure 5C**). Regarding migration, MTAP$^{-/-}$ migrate less than MTAP$^{+/+}$ around 48h ($p<0.01$), however, after this period the cells increase its proliferation rate and gets similar to MTAP$^{+/+}$ cells (**Figure 5E**). In addition, in the presence of 500 µM of TMZ, the cells appear to migrate more. (**Figure 5E**). Evidenced in the EC50 (**Figure 4B**), the T98G cells MTAP$^{-/-}$ need higher concentrations of TMZ to have a cytotoxic effect, the cells entered apoptosis with 500 µM of TMZ treatment ($P<0.01$) (**Figure 5D**).



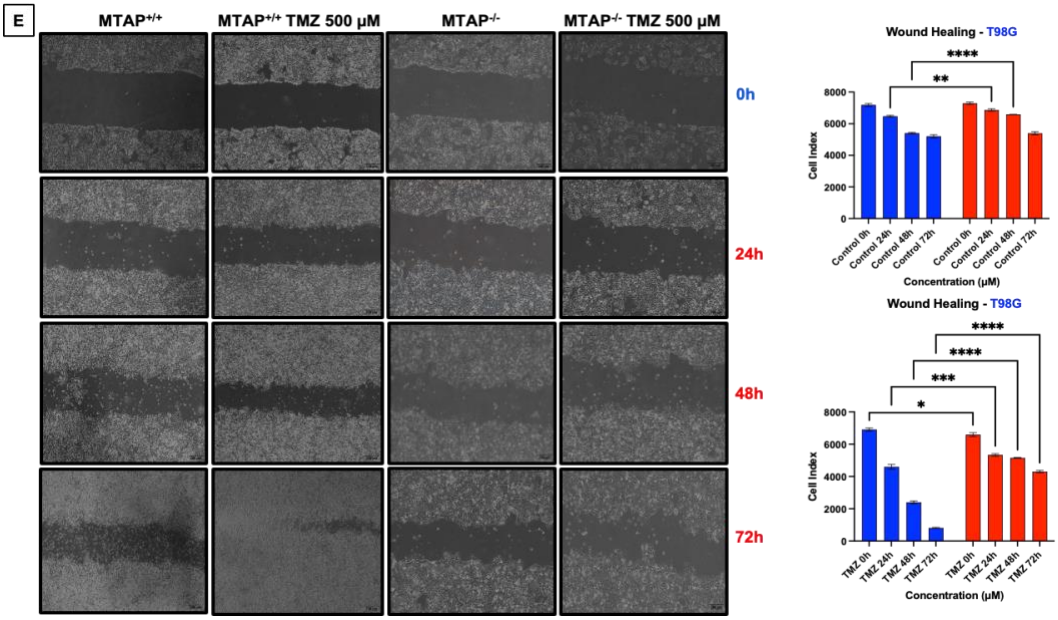


Figure 5 – Differences in the capacity to form colonies (A), proliferate (B), invade (C), enter apoptosis (D) and to migrate (E) between T98G cells MTAP $^{+/+}$ and $^{-/-}$ treated and untreated with TMZ. *The MTAP loss changes the ability of tumor cells to form colonies (p<0,001), proliferate (p<0,001), invade (p<0,001) and migrate (p<0,05-0,0001) in early hours.* *P<0.05 ** P<0.01 ***P<0.001 *** ****P<0.0001

MTAP$^{-/-}$ U251MG cells have lower growth in xenograft model with a distinct histological pattern in early growth

The tumors of the *in vivo* experiment 1 were first palpable approximately after 2 weeks of injection. The tumors MTAP$^{-/-}$ had a lower volume since day 17 (**Figure 6A**, P<0.05). After 5 weeks, the mean volume of the tumors MTAP$^{-/-}$ was strikingly lower than those MTAP$^{+/+}$ (43.0±6.92 mm^3 and 236.9±40.9 mm^3, respectively, P<0.01). The size difference between MTAP $^{+/+}$ and $^{-/-}$ tumors was visibly noticeable (**Figure 6B**). In general, MTAP$^{-/-}$ tumors presented low cellularity (50% of tumors with low cellularity *vs.* 87.5% of the MTAP$^{+/+}$ tumors with high cellularity, P<0.01), no necrosis (100% of MTAP$^{-/-}$ with no necrosis, whereas all tumors MTAP$^{+/+}$ presented necrosis, P<0.001) and high percentage of fibrotic area within the tumor (62.5% of MTAP$^{-/-}$ tumors presented >25% of necrosis, whereas all MTAP$^{+/+}$ tumors presented 0-25% of necrosis, P<0.01) (**Table 1**, **Figure 6C-E**).

Table 1 – Cellularity, Area of Necrosis and Fibrosis of MTAP$^{+/+}$ or MTAP$^{-/-}$ xenografts five weeks post U251MG cells injection.

		MTAP$^{+/+}$ N(%)	MTAP$^{-/-}$ N(%)	P Value
Cellularity	Low	0	4 (50.0%)	**0.009**

	Moderate	1 (12.5%)	3 (37.5%)	
	High	7 (87.5%)	1 (12.5%)	
Necrosis	Absent	0	8 (100%)	**0.0003**
	0-10%	5 (62.5%)	0	
	>10%	3 (37.5%)	0	
Fibrosis	Absent	0	2 (25.0%)	**0.006**
	0-25%	8 (100%)	1 (12.5%)	
	25-50%	0	2 (25.0%)	
	>50%	0	3 (37.5%)	

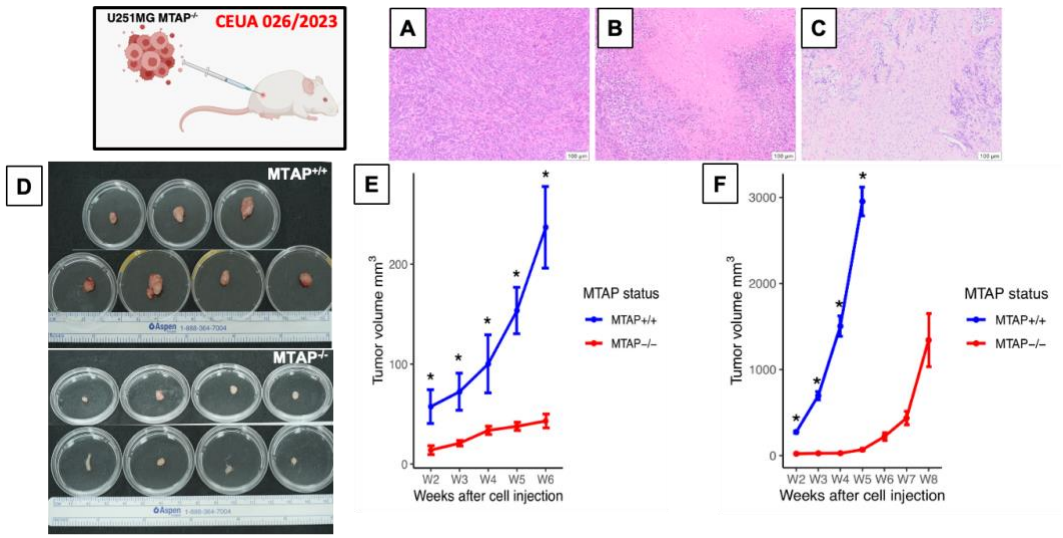


Figure 6 - U251MG xenografts MTAP$^{+/+}$ and MTAP$^{-/-}$ showing high cellularity (A), extensive area of necrosis (B) and extensive area of fibrosis (C). Volume size differences between tumors MTAP $^{+/+}$ and $^{-/-}$ (D). Tumor volume of xenografts considering MTAP status in the first (E) and second experiment (F). *MTAP loss leads to a decrease in the growth of tumors in xenograft in early stages (p<0.001), after a period of plato, the tumors MTAP$^{-/-}$ starts to increase the growth rate (p<0.001), and changes in cellularity (p=0.009), necrosis (p=0.0003) and fibrosis (p=0.006) when compared with tumors MTAP$^{+/+}$.* *P<0.001

MTAP$^{-/-}$ U251MG cells growth progress is resumed after a latent growth period, and the tumors become phenotypically similar

In the second experiment, the tumors were first palpable after 2 weeks of injection. The *MTAP$^{+/+}$* tumors kept a constant growth throughout the experiment, whereas *MTAP$^{-/-}$* presented a latent growth for approximately 5 weeks. At this time, the animals which received *MTAP$^{+/+}$* cells were euthanatized due to the high mean tumor volume 3514.57±90.50mm^3, whereas *MTAP$^{-/-}$* presented a mean of 128.31±22.15mm^3 (P<0.0001). From this point, *MTAP$^{-/-}$* tumors resumed the growth, and were kept for two more weeks, when animals were euthanized, and the tumors had a mean volume of 1341.54±308.58mm^3 (**Figure 6F**). Strikingly,

histopathological analysis showed that both tumor groups presented similar phenotype, with high cellularity, absence of fibrosis, and more than 10% of necrotic area (**Table 2**).

Table 2 – Cellularity, Area of Necrosis and Fibrosis of $MTAP^{+/+}$ or $MTAP^{-/-}$ xenografts at the end of experiment 2.

		$MTAP^{+/+}$ N(%)	$MTAP^{-/-}$ N(%)	P Value
Cellularity	Low	0	0	0.999
	Moderate	0	0	
	High	8 (100%)	8 (100%)	
Necrosis	Absent	0	0	0.999
	0-10%	0	0	
	>10%	8 (100%)	8 (100%)	
Fibrosis	Absent	8 (100%)	8 (100%)	0.999
	0-25%	0	0	
	25-50%	0	0	
	>50%	0	0	

Discussion

Although *MTAP* has been proposed as a surrogate biomarker of p16 homozygous deletion (HD)[13,14], which is present in about 50% of glioblastomas (GBM)[3], little is known in its effect during gliomagenesis. In this study, we found *in silico* enrichment of WNT, mTOR and MAPK pathways in MTAP-deleted patients, and that the silencing of MTAP *in vitro* lead to decreased colony formation capacity and proliferation, as well as limited invasion and increased resistance to TMZ treatment. Importantly, we found *in vivo* that there was a dramatically decrease in early xenografts growth of MTAP-silenced cells, and the growth of these tumors start accelerating after a few weeks.

MTAP is essential in polyamine synthesis, by forming AMP and methionine. However, cells lacking MTAP expression are not able to salvage adenine/methionine from endogenous MTA[26]. We have previously described the frequent deletion of chr9p22.1-21.3, which encompasses MTAP[3], in GBM, and a pan cancer analysis described its loss in several tumor types, such as mesothelioma[27]. In this study, our *in silico* analysis reveals that nearly 60% of GBM TCGA cases exhibit *MTAP* deletion, and this deletion can be used as a surrogate for *CDKN2A* gene deletion, often co-deleted with *MTAP*, which has been used for diagnostic purposes in gliomas since 2021[2]. When comparing overall survival based on *MTAP* status, it was observed that glioblastoma patients from TCGA without *MTAP* gene deletion have a better prognosis than those with *MTAP* deletion, which corroborates another study in GBM[28], and other tumor types[29]. In the analysis of differentially

expressed genes between MTAP $^{+/+}$ and $^{-/-}$ patients, 607 genes were identified. Further examination of the cellular signaling pathways and biological processes revealed Wnt pathway as importantly enriched in this comparison. This pathway is one of the main cellular signaling pathways that regulates a wide variety of biological processes, including differentiation, proliferation, and cell migration. Deregulated activation of the Wnt pathway can result in uncontrolled cell proliferation and evasion of apoptosis[30], important hallmarks of cancer. Other cancer-related pathways are also deregulated, such as mTOR and MAPK. mTOR is an essential protein kinase that plays a central role in the regulation of cell growth, proliferation, survival, and metabolism[31]. Dysregulation of the mTOR pathway is commonly associated with cancer development and progression, as it aids in the elevation of uncontrolled cell growth, resistance to cell death, and increased survival of cancer cells, especially in environments of nutrient stress and hypoxia[31]. The MAPK pathway plays a critical role in the pathogenesis and progression of GBM, promoting cell proliferation, survival, migration, and resistance to therapy[32]. Abnormal activation of the MAPK pathway in GBMs occurs especially through mutations in EGFR (epidermal growth factor receptor) and alterations in Ras and B-Raf. Although targeted therapies aimed at inhibiting MAPK signaling are promising, challenges such as tumor heterogeneity, compensatory signaling, and drug resistance remain significant obstacles[32].

Interestingly, we found *in vitro* that, MTAP silencing reduced colony capacity formation and transwell invasion capacity, which is somewhat conflicting with Hansen et al.[17], that, using an astrocyte model of transformation, found that MTAP loss promoted the growth of glioma stem-like cells (GSCs), which exhibited more potent sphere-forming capabilities. However, both studies found that MTAP loss leads to a higher resistance to temozolomide treatment, what can be explain with the increase of NF-κB in MTAP$^{-/-}$ cells.

NF-κB (Nuclear Factor kappa-light-chain-enhancer of activated B cells) is a protein complex that plays a crucial role in regulating the immune response, inflammation, cell survival, and proliferation[33]. There is a significant correlation between NF-κB expression and MGMT expression in gliomas, with the inhibition of NF-κB activity leading to downregulation of MGMT gene expression and significantly restoration of TMZ chemosensitivity[34]. Hansen *et al.* also found that orthotopic injection of MTAP-depleted cells resulted in more tumorigenic tumors than MTAP-restored cells[17]. There are further reports that explore extensively that, *in vitro*, *MTAP*-deficiency led to increased extracellular MTA[35], a potent inhibitor of PRMT5, which may impair cell viability[36]. This explains in part our early *in vivo* results, that show the dramatically reduced tumor growth in MTAP negative xenografts in immunodeficient mice, with tumors phenotypically different, presenting lower cellularity, necrosis and a larger fibrosis area. In fact, studies show

that MTAP deficiency or dysregulation can influence fibrosis through mechanisms such as altered MTA levels, inflammation, and TGF-β/NFκB signaling. A study identified a link between MTAP and hepatic fibrosis[37], and the loss of the 9p21 locus in mice was associated with bone marrow fibrosis .

Interestingly, when the xenografts were allowed to grow a higher period of time, MTAP-silenced tumors started accelerating the growth, and tumors were phenotypically similar to those MTAP positive. One study found that MTA inhibits melanoma cell proliferation and *in vivo* tumor growth in melanoma[39], similar to our results in GBM. Therefore, we postulate that, during the period of gap in the growth, MTA starts accumulating slowly in the tumor environment[40], but MTAP-expressing stroma is able to metabolize the excessive MTA . However, a longer period of MTA accumulation inclines the microenvironment to a more proliferating and a pro-inflammatory phenotype.

In our study, we found an increasing in the resistance to *MTAP* deletion and response to standard temozolomide treatment. Alternatively, *MTAP*-deleted cells have an altered purine metabolism, which makes them more sensitive to certain treatments, such as purine analogs (e.g., 2-Fluoradenine[41] and Pemetrexed[42]) and inhibitors of the PRMT5 (AG-270[43]). These treatments can exploit the metabolic disruption caused by MTAP loss, leading to increased toxicity in tumor cells while sparing normal cells. Targeting MTAP deletion in GBM represents a novel and promising therapeutic strategy, leveraging the metabolic weaknesses caused by MTAP loss. Further studies must be conducted to test the efficacy of these therapies in *MTAP* deleted GBM cell lines and *in vivo*, and the impact of the MTAP loss of expression and MTA accumulation in the tumor microenvironment.

In conclusion, our study demonstrates that MTAP loss significantly impairs cell proliferation and tumor growth in glioblastoma, highlighting its critical role in maintaining normal cellular function and metabolic balance. The loss of MTAP, commonly observed in glioblastoma, not only disrupts cell signaling pathways related to cancer, but also serves as a key indicator of poor prognosis in GBM TCGA patients. Also, MTAP loss was related to difference in response to the standard treatment and with decreasing in growth both *in vitro* and *in vivo*. These findings suggest that MTAP could be a valuable biomarker for identifying high-risk glioblastoma cases, and its loss may provide a therapeutic window for targeted treatments, such as purine analogs. Ultimately, the identification of MTAP loss as a contributing factor to glioblastoma progression underscores the need for further exploration of its potential as a therapeutic target, with the aim of improving clinical outcomes for patients.

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Inhibitors of purine synthesis and MAT2A as a target therapy to MTAP deleted glioblastoma

Paola Gyuliane Gonçalves[1,2], Connor Purcell[2], Maximilian Pinho-Schwermann[2], Andrea Schmidt[2], Benedito A. Carneiro[2], Wafik S. El-Deiry[2], Rui Manuel Reis[3,4], Sean Lawler[2], Lucas Tadeu Bidinotto[1,3,4]

1 Department of Pathology, UNESP – Univ. Estadual Paulista, School of Medicine, Botucatu, São Paulo, Brazil
2 Pathology and Laboratory Medicine, Legeorreta Cancer Center, Brown University, Providence, RI, USA;
3 Molecular Oncology Research Center, Barretos Cancer Hospital, Barretos, São Paulo, Brazil;
4 Barretos School of Health Sciences, Dr Paulo Prata – FACISB, Barretos, São Paulo, Brazil;
5 Life and Health Sciences Research Institute (ICVS), Medical School, University of Minho, Braga, Portugal;

ABSTRACT

Background: Glioblastoma (GBM) is an aggressive brain tumor associated with poor prognosis and limited therapeutic options. A subset of GBM cases exhibits the loss of Methylthioadenosine Phosphorylase (MTAP), a key enzyme in purine metabolism. MTAP deletion leads to dysregulation of purine salvage and methylation cycles, rendering MTAP-deficient cells highly vulnerable to disruptions in purine metabolism. Recent studies suggest that MTAP loss creates a unique metabolic vulnerability that can be targeted therapeutically. **Objective:** This study explores the potential of purine synthesis and MAT2A inhibitors as targeted therapies for MTAP-deleted glioblastoma. We investigate the role of MTAP in purine metabolism and examine how inhibition of purine biosynthesis and MAT2A could exploit the metabolic defects associated with MTAP loss in GBM. **Methods:** *In vitro* cell viability assays, and tumor growth analyses were conducted to evaluate the therapeutic efficacy of these inhibitors in $MTAP^{+/+}$ and $^{-/-}$ tumor cell line, as well in normal brain cells. **Results:** In $MTAP^{-/-}$, inhibition of purine synthesis by 2-FA resulted in a significant reduction in cell migration ($p<0.0001$), colony formation ($p<0.0001$) and tumor viability, while in the presence of MTA the cytotoxicity of 2-FA to $MTAP^{+/+}$ tumor cells and normal brain cells was reversed. 2-FA and MTA also were able to cross the blood-brain barrier and be delivered and reach the intracerebral tumor. However, we fail to find any synthetic lethality in MTAP-deficient cells using PMT and AG-270. **Conclusion:** Our findings demonstrate that 2-FA represent a promising targeted therapeutic strategy for MTAP-deleted glioblastoma. These results highlight the potential of exploiting MTAP loss as a biomarker for selective therapeutic targeting, offering a novel approach to treat this highly aggressive and treatment-resistant cancer. More studies need to be conducted *in vivo* to confirm our findings.

Key-words: MTAP, GBM, 2-FA, MTA, Pemetrexed, AG-270, TMZ.

Introduction

Glioblastoma (GBM) is the most aggressive and common malignant brain tumor, characterized by rapid proliferation, extensive infiltration into surrounding brain tissue, and resistance to conventional therapies (1). The standard treatment for glioblastoma involves a combination of surgery, radiotherapy, and the use of chemotherapies that can cross the blood-brain barrier (BBB), particularly temozolomide (TMZ) (2). The introduction of tumor treating fields (TTFields) and the potential use of targeted therapies or immunotherapies are expanding the treatment options for this aggressive malignancy (1). However, the prognosis for GBM remains poor, with a median survival of approximately 12-16 months (1) and

ongoing research and clinical trials are crucial for developing more effective therapies that can prolong survival and improve quality of life for patients. Consequently, there is an urgent need for a deeper understanding of the molecular underpinnings of GBM to identify novel therapeutic targets. In particular, recent studies have suggested that the loss of *MTAP*, a gene involved in purine metabolism, may have a critical role in GBM pathogenesis and could offer new avenues for targeted therapies (3).

The *MTAP* gene encodes the enzyme methylthioadenosine phosphorylase (MTAP). MTAP is an important enzyme in the purine metabolism pathway, particularly in the salvage pathway. MTAP is responsible for catalyzing the reversible cleavage of 5'-deoxy-5'-methyladenosine (MTA), a byproduct of the transmethylation reactions, into methylthioribose (MTR) and adenine (**Figure 1**) (4). However, *MTAP* deficiency has been implicated in various types of cancers, where the loss of MTAP activity leads to the accumulation of MTA, which can inhibit key enzymes and further disrupt purine metabolism and cellular methylation. *MTAP* has a deletion frequency greater than 50% among glioblastomas (5,6).

The relationship between MTAP deficiency and cancer has spurred interest in targeting this enzyme as a potential therapeutic strategy. Specifically, purine and MAT2A inhibitors are being explored to exploit the vulnerability caused by *MTAP* loss (3). Inhibition of key enzymes in the *de novo* purine synthesis pathway could lead to a synthetic lethality effect in cells deficient of MTAP (**Figure 1**). Drugs like 2-Fluoroadenine (2-FA) and Pemetrexed (PMT) inhibit nucleotide synthesis by being converted into nucleosides (PAMP) with the help of the enzymes PRPP and APRT. A study showed that treatment with 2-FA and MTA reduced tumor growth in $MTAP^{-/-}$ cells, while $MTAP^{+/+}$ cells showed no significant difference compared to controls (7). MAT2A catalyzes the synthesis of S-adenosylmethionine (SAM) from methionine and ATP (**Figure 1**). SAM is essential for methylation reactions and is particularly critical in rapidly proliferating cells, such as cancers (8). MTAP-deficient cancers cannot salvage methionine via MTAP, making them reliant on MAT2A to produce SAM for survival. MAT2A inhibitors, like AG-270, exploit this dependency by further depleting SAM, exacerbating PRMT5 dysfunction and causing synthetic lethality (9). By inhibiting purine synthesis or MAT2A, therapeutic strategies could specifically target tumors with MTAP loss, sparing normal cells that retain MTAP activity.

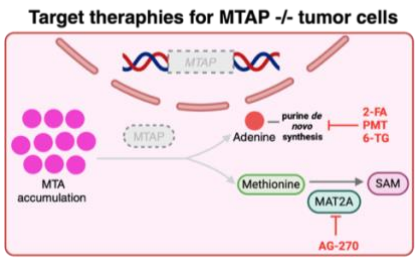


Figure 7 – Schematic of the pathways for obtaining adenine by MTAP+/+ and -/- cells and

potential therapeutic targets. *In MTAP+/+ cells, MTA functions as a protective molecule by being converted into adenine through the action of MTAP. However, in MTAP$^{-/-}$ cells, the conversion of MTA into adenine does not occur, and the only way to acquire adenine is through de novo purine synthesis. If this pathway is blocked, MTAP$^{-/-}$ cells cannot obtain the necessary adenine for their cellular functions and will undergo apoptosis.*

Given the high frequency of *MTAP* deletions in glioblastomas, which are associated with poor prognosis and resistance to standard treatments, we aimed to analyze the drugs 2-FA, PMT and AG-270 as potential target therapies for MTAP-deleted tumors, characteristic present in more than 50% of GBM patients (5,6). By exploring the implications of this molecular alteration, we expect to highlight novel strategies that could improve the clinical outcomes for these patients.

Material and Methods

Characterization of MTAP expression in cell lines

Cell lines obtained from the Brown University cell bank were cultured in specific medium (**Table 1**), in an incubator at 37°C with 5% CO_2 and 60% humidity. After the third passage, the cells were used for *in vitro* experiments. Protein extraction was performed using RIPA lysis buffer (Sigma, cat#20188) according to the manufacturer's protocol with incubation on ice for 1 hour and vortexing every 15 minutes. The extracted proteins were quantified by the BradFord method.

Table 1 – List of cell lines used in this study.

Cell line	Cell line Type	Cell media	Experiments
T98G	Glioblastoma	DMEM 10% SBF	CRISPR, Western blot, *in vitro* analysis
U138MG	Glioblastoma	DMEM 10% SBF	Re-expression, Western blot, *in vitro* analysis
Astrocytes	Normal astrocyte	Astrocyte-specific medium	Spheroids, Western blot & *in vitro* analysis
HCMEC	Brain endothelial cell	Endothelial cell medium	Spheroids, Western blot, *in vitro* analysis & angiogenesis
Pericytes	Normal pericyte	Pericyte-specific medium	Spheroids, Western blot & *in vitro* analysis
BLAD03	Patient-derived organoid	MammoCult™ + components	*in vitro* analysis
HEK-293	Recombinant lentivirus-producing cell	DMEM 10% FBS	Production of lentiviruses to overexpress MTAP

For semi-quantification of MTAP in GBM cell lines, 30 µg of proteins, diluted in 20 µL of 2x Laemmli Sample Buffer (Bio-Rad) and with the amount of H_2O adjusted to a final volume of 20 µL per well were subjected to 12% polyacrylamide gel electrophoresis under denaturing and non-reducing conditions (SDS-PAGE), and transferred to nitrocellulose membrane (Hybond-C™ Extra, Amersham Biosciences)

in the transfer system for 40 minutes. The membrane was then stained with a 0.1% Ponceau S (Sigma) and 5% acetic acid solution. The membrane was then washed and incubated with 5% skimmed milk powder diluted in TBS-T for blocking for 1 hour at room temperature. The membranes were then incubated overnight in the primary antibody at standardized dilutions for each protein (MTAP antibody: 1:2000, ProteinTech cat#11475-1-AP / Vimentin antibody: 1:4000, ProteinTech cat#22031-1-AP). The membranes were then washed and incubated in the corresponding secondary antibody at a dilution of 1:5000 diluted in 5% milk for 1 hour at room temperature. The reaction was revealed by ECL chemiluminescence (GE), detected by the ImageQuant LAS 4000 mini photo documentation system (GE).

Silencing of MTAP by CRISPR/Cas9

The commercial cell lines T98G and U251MG, which normally express MTAP, were selected for silencing using the CRISPR/Cas9 methodology (10). Silencing was performed according to the DharmaFECT™ Duo transfection reagent—for siRNA and plasmid co-transfection protocol (Dharmacon). Cells were plated in a 24-well plate with antibiotic-free DMEM 10% FBS medium with 0.1 mL of cell suspension in each well. The plate was then incubated at 37 °C with 5% CO_2 overnight.

Three siRNAs were used, where each siRNA targets a different region of the gene. The design of the guide RNAs was performed avoiding regions of alternative splicing. Cas-9 and siRNA were diluted in 0% FBS culture medium to a concentration of 25 nM guide RNA per 1 µg/well Cas9. A 60 µg/mL working solution of DharmaFECT Duo transfection reagent was prepared by diluting 12 µL of 1 mg/mL DharmaFECT Duo in 188 µL of serum-free medium. The tubes were incubated for 5 minutes at room temperature.

50 µL of DharmaFECT Duo working solution was added to each sample tube, resulting in a final DharmaFECT Duo concentration of 3 µg/well. No DharmaFECT Duo was added to the non-transfected control, which should contain only serum-free medium. The tubes containing the siRNA and the non-targeting control, with a volume of 100 µL per tube, were incubated for 20 minutes at room temperature. Transfection medium was prepared by adding 400 µL of complete medium without antibiotics to each sample to bring the total volume in each tube to 500 µL. Medium was then removed from the wells of the plate containing cells and replaced with 500 µL of the appropriate transfection medium for each well. Cells were incubated at 37°C in 5% CO_2 for 72 hours.

To select only cells that were transfected with siRNA and plasmid, cell-sorting was performed on a FACS Melody flow cytometer (BD Biosciences) using mCherry fluorescence. After expansion of these cells in culture, protein analysis was performed to confirm silencing by western blot (MTAP antibody: 1:2000,

ProteinTech cat#11475-1-AP), using vinculin and B-actin as endogenous controls (Vinculin antibody: 1:4000, ProteinTech cat#22031-1-AP/ β-actin 1:2000, Cell Signaling® cat#4967, USA).

Functional analyses in cell lines and response to treatments

During the functional assays, described below, the cells were treated with different drugs and concentrations. For the assays using the T98G MTAP $^{+/+}$ and $^{-/-}$ strains, the drugs 2-FA (dilution ranging from 1 µM to 1000 µM), 2-FA (dilution ranging from 1 µM to 1000 µM) combined with 10 µM of MTA), TMZ (dilution ranging from 1 µM to 1000 µM), PMT (dilution ranging from 1 µM to 1000 µM), and AG-270 (dilution ranging from 1 µM to 1000 µM) were used. U251MG MTAP +/+ and -/- cells were treated with 2-FA (dilution ranging from 15 µM to 1000 µM), 2-FA (dilution ranging from 15 µM to 1000 µM) combined with 10 µM MTA, and TMZ (dilution ranging from 15 µM to 1000 µM). DMSO was used as a control in all experiments at the same dilution as the drug with the highest concentration in the experiment.

Cell proliferation analysis

Cell proliferation analysis was performed using the BrdU (5-bromo-2-deoxyuridine) assay. Two thousand cells per well were plated in dark 96-well plates suitable for fluorescence assays. After 24 hours of incubation, the cells were treated specifically and the BrdU label was added. After 24 and/or 48 hours, the cells were stained using the BrdU detection kit (Roche, cat# 11647229001) as recommended by the manufacturer. The percentage of BrdU-positive cells was analyzed using an absorbance reader (450 nm). The assay was performed in triplicate and repeated three times.

Analysis of apoptotic cell death

To assess apoptotic cell death, the cells were cultured in 6-well plates until confluence, when the specific treatment was added, and the cells were incubated for 48 hours. After treatment, the cells were stained with SYTOX™ Blue Dead Cell Stain (ThermoScientific, cat# S34857), according to the manufacturer's recommendations. Data acquisition was performed on a CytoFLEX flow cytometer (Beckman Coulter). The assay was performed in duplicate and repeated three times.

Cell migration analysis by wound healing

Cell migration was assessed using the wound healing technique in 6-well plates. The cells were cultured until reaching approximately 90% confluence. Using a sterile tip, the cells were removed in a line crossing the entire well and the plate was photographed using an inverted microscope coupled to a multimedia system. The corresponding treatments were then added to the culture medium. After 24

and 48 hours in the incubator, the plates were photographed again, and the percentage of space (previously empty) invaded by the cells was assessed using ImageJ software. The assay was performed in duplicate and repeated twice.

Cell invasion analysis by transwell

The matrix Biocoat Matrigel Invasion Chamber (BD Biosciences) was hydrated for 1 hour in serum-free culture medium. Subsequently, the corresponding culture medium supplemented with 10% serum used as a chemoattractant was placed in the lower compartment of the plate wells. The cells were trypsinized and seeded at a density of 1.0×10^5 in the upper compartments of the wells and incubated in an oven at 37°C for 24 hours. After incubation, the cells that did not invade the matrigel were removed with a swab. The invading cells, which were able to degrade the matrigel (analogous to the extracellular matrix) and grow on the lower surface, were fixed with 100% methanol and stained with crystal violet. The cells were visualized and quantified under a fluorescence microscope (Nikon Eclipse 50i). The assay was performed in duplicate and repeated twice.

Colony formation assay

For this assay, 4,000 T98G MTAP $^{+/+}$ and $^{-/-}$ cells were seeded in 6-well plates. After 24-h incubation, the different treatments (DMSO Control, 2-FA 10, 100 and 500 µM, 2FA 10, 100 and 500 µM + MTA 10 µM, PMT 500 µM and TMZ 500 µM) were added to the culture medium. The cells were cultured for 12 days, and the treated media were changed every 72 hours. After 12 days of treatment, the wells were carefully washed with dPBS medium, fixed in 100% methanol and stained with crystal violet. The plates were then kept at room temperature for 48 hours and photographed. The assay was performed in duplicate and repeated twice.

CellTiter-Glo cytotoxicity assay

2,000 cells per well were plated in dark 96-well plates suitable for fluorescence assays. After overnight incubation, the corresponding treatments were added. The cells were then incubated for 48h or 72h, and then the fluorescent reagent CellTiter-Glo® 3D Cell (Promega, cat# G968A) was added, which was incubated for thirty minutes, away from any light source, and read in a fluorescence reader. The assay was performed in triplicate and repeated three times. GraphPad PRISM 10 was used to calculate the drug dose-response curve as well as the EC50.

MTAP re-expression in U138MG cell line

The re-expression MTAP plasmid from Genecopeia was insert in a lentivirus using the Hi-Fi PCR (NEB, USA). After, the lentiviruses were transduced to U138MG cell line for 48h, and the cells were selected with 25ng of puromycin for 96h. The MTAP re-expression was confirmed using western blot.

IncuCyte

For these analyses, tumor cells were cultured at 37˚C (5% CO_2, humidified atmosphere) and after the third passage the cells were plated in 96-well plates with 2,000 cells per well. The cells were incubated overnight, and then the respective treatment was added. The plates were then taken to the IncuCyte instrument, and the imaging parameters were set to phase imaging type, with capture intervals every 6 hours for 3-4 days. After the image capture time, the IncuCyte software was used to analyze the images evaluating the growth rate and confluence parameters. The assay was performed in triplicate and repeated three times.

Blood-brain barrier spheroid formation and fluorescence analysis

Astrocytes, pericytes and human brain endothelial cells (HCMEC) were cultured in specific culture media. After the third passage, the cells were plated at 1.500 cells per well in 96-well plates with low attachment and culture medium for spheroid formation. The cells were placed in the incubator for 48 hours to allow spheroid formation, and then drug treatment was added (DMSO control, MTA 100 µM, 2-FA 100 µM, 2FA+MTA 100 µM, PMT 100 µM, AG270 100 µM and TMZ 100 µM). The spheroids remained in the treatment medium for 72h and were then stained with Dextran for 3h, fixed in formalin 10% for 10 minutes and then their fluorescence was analyzed under a confocal microscope. After that, the fluorescence of the cells was measured using the Image J program, where after analysis the corrected total cellular fluorescence (CTCF) was calculated using the following formula: CTCF = Integrated Density - (Area of the selected cell X Average fluorescence of the background readings). Twenty-four spheroids were made per treatment, and the assay was repeated twice.

Cell-substrate electrical impedance detection (ECIS) assay

ECIS is a technique that measures cellular resistance and blood-brain barrier function in real time. For this assay, 200 µl of type I collagen was first plated on a special reading plate of the machine, and after 1 hour of incubation, 20,000 HCMEC endothelial cells were added per well. The plate was then placed on the machine inside the incubator, and the software was set to multifrequency 8W10E+ - frequency 4/16/64KH2, with reading intervals every 600s. The culture medium was changed every 24 hours, and after a reading plateau of approximately 48h, the cells were treated with their respective drugs for 5 days. After the readings, the resistance values (ohm) were used to identify the drugs capable of crossing the barrier. The assay was performed in duplicate and repeated twice.

Statistical Analysis

Statistical analyses were performed using the GraphPad PRISM 10 statistical program. One-way ANOVA ("analysis of variance") was used to compare the means

of two or more independent groups to determine whether there is statistical evidence that the associated population means are significantly different, and two-way ANOVA for multiple comparisons. T-tests were used for dependent analyses. A statistical significance of 5% was considered.

Results

***MTAP* deletion leads to changes in tumor cells and can be used as a therapeutic target in MTAP$^{-/-}$ tumors**

First, the *MTAP* gene was silenced in the T98G cell line using the CRISPR/Cas9 technique (**Figure 2A**). In addition, MTAP expression was restored in U138MG cells (**Figure 2B**) using a re-expression plasmid (**Figure 2C**).

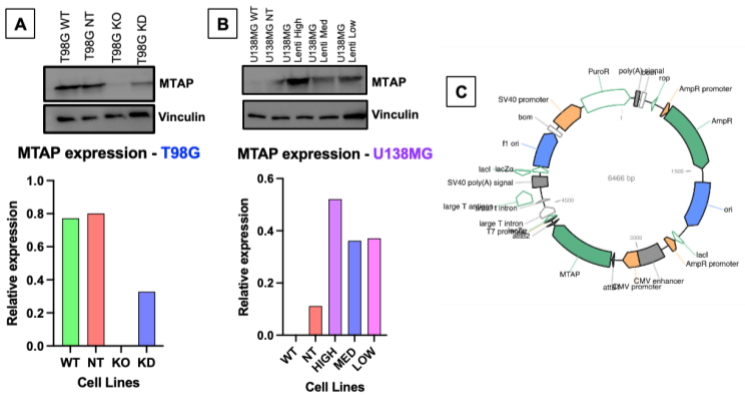


Figure 8 – MTAP protein expression analysis shows silencing in the cell lines T98G (A) and re-expression in U138MG (B). The MTAP re-expression plasmid representation used in the study (C). "WT" represents wild type cells, which no transfection; "NT" represents "no targeting" cells, which were subjected do transfection, but with no targeted guides; "KO" represents Knock-Out cells and “KD” knock-down.

In the cytotoxicity analyses of the drugs in T98G MTAP $^{+/+}$ and $^{-/-}$ cells, it was observed that treatment with 2-FA was extremely cytotoxic for both cells, regardless of the concentration, with the EC50 of MTAP $^{+/+}$ cells being 12 µM and MTAP $^{-/-}$ of 7 µM (**Figure 3A**); however, the addition of MTA to the treatment decreased the cytotoxic effect of the drugs in MTAP $^{+/+}$ cells, the EC50 of 12.5 µM increasing to 136.11 µM, while maintaining the cytotoxic effects in MTAP $^{-/-}$ cells, with an EC50 <10 µM for both (**Figure 3A-B**). Treatment with AG-270 was more cytotoxic to MTAP $^{+/+}$ cells, and only at high concentrations had an effect on MTAP $^{-/-}$ cells (EC50 62 µM vs 845 µM, **Figure 3C**). For TMZ, the cells MTAP$^{-/-}$ showed an increase in the resistance to the treatment compared to MTAP$^{+/+}$ (EC50 329 µM vs 506 µM, **Figure 3D**), whereas treatment with PMT was not cytotoxic to either cell line at concentrations lower than 1000 µM (**Figure 3E**).

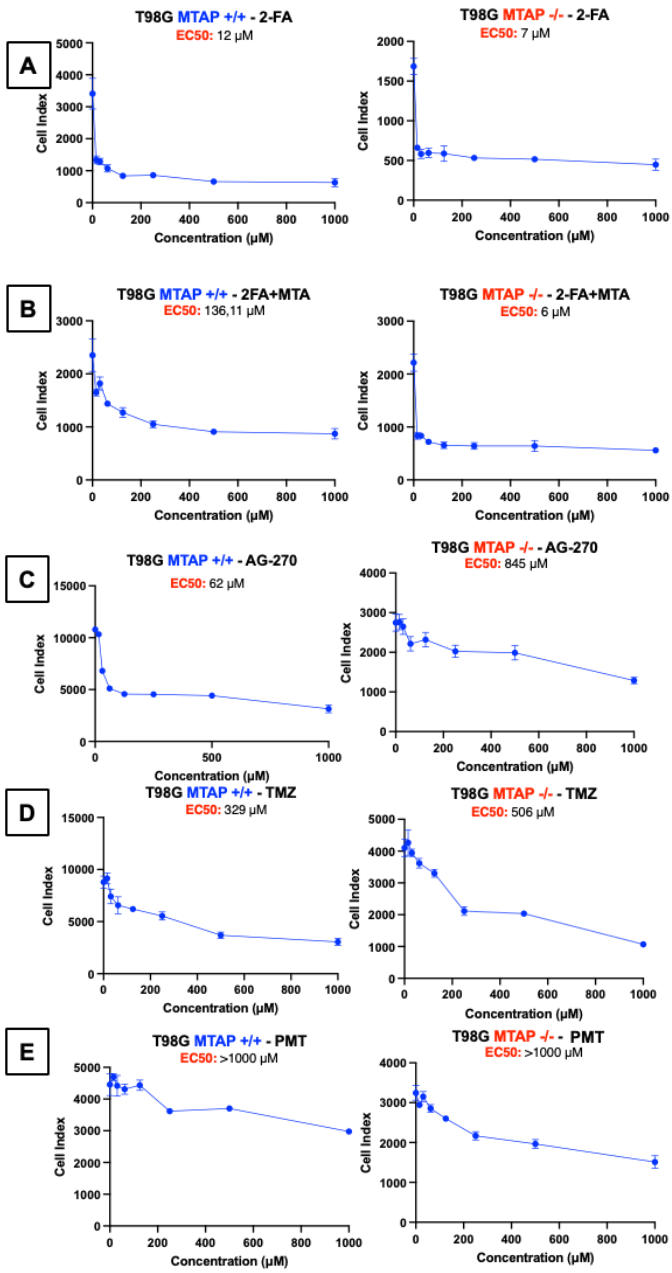
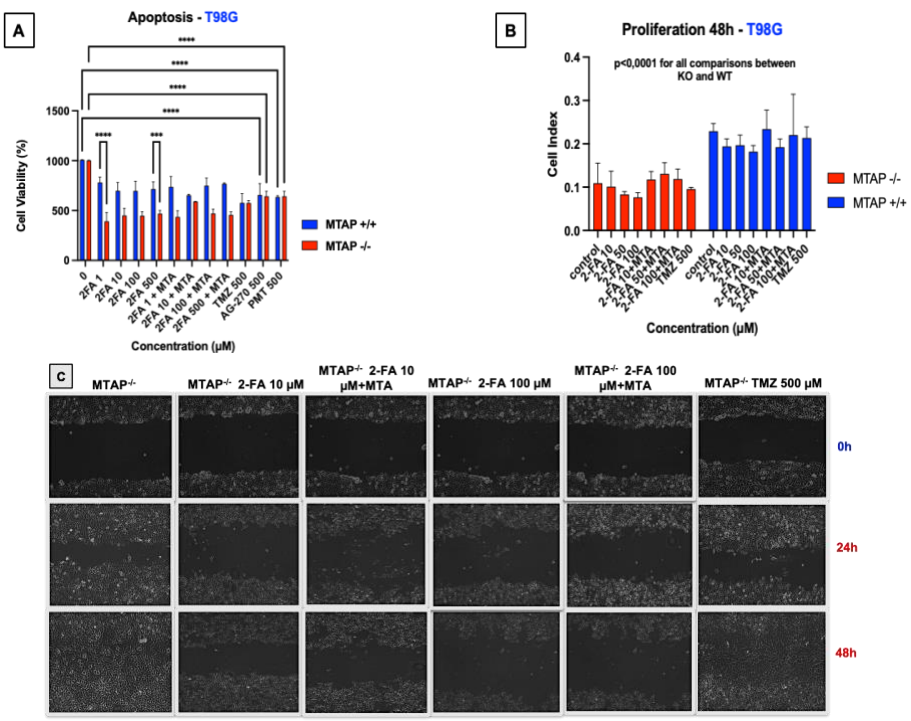


Figure 9 – Dose-response curves showing the cytotoxic effects of the drugs on T98G MTAP $^{+/+}$ and $^{-/-}$ cell lines after 72h of treatment, as well as their EC50. *Treatment with 2-FA was extremely cytotoxic for both cells, regardless of the concentration, with the EC50 of MTAP $^{+/+}$ cells; however, the addition of MTA to the treatment decreased the cytotoxic effect of the drugs in MTAP $^{+/+}$ cells, while maintaining the cytotoxic effects in MTAP $^{-/-}$ cells. Treatment with AG-270 was more cytotoxic to MTAP $^{+/+}$ cells, and only at high concentrations had an effect on MTAP $^{-/-}$ cells. The same was true for TMZ, whereas treatment with PMT was not cytotoxic to either cell line at concentrations lower than 1000 µM.*

To further explore the cytotoxicity findings, apoptosis analysis was performed on T98G MTAP $^{+/+}$ and $^{-/-}$ cells. Treatment with 2-FA did not lead to apoptosis in MTAP $^{+/+}$ cells regardless of drug concentration, while MTAP$^{-/-}$ cells

underwent apoptosis regardless of concentration, and even the addition of MTA did not affect the treatment (p<0.0001) (**Figure 4A**). These results confirm the findings of cytotoxic analysis, that treatment with 2-FA is cytotoxic and induces apoptosis in MTAP$^{-/-}$ cells. The proliferation analysis by BrdU shows that T98G MTAP$^{-/-}$ cells have an extremely significant lower proliferation (p<0.0001) when compared to MTAP$^{+/+}$ cells, regardless of the treatment used, and that treatments with 2-FA, 2-FA+MTA and TMZ 500 µM had no direct effects on cell proliferation (**Figure 4B**). Cells MTAP$^{-/-}$ migrated more in the control and in the treatment with TMZ, while the cells treated with 2-FA and 2-FA+MTA migrated less independent of the drug concentration (p<0.0001) (**Figure 4C**). In the colony formation assay, it was possible to observe that MTAP silencing decreased the ability of T98G cells to form colonies, although silencing increased the sensitivity of the cells to the drug TMZ (p<0.0001) (**Figure 4D-E**). Treatments with 2-FA, 2-FA+MTA and PMT inhibited colony formation in both cell lines, regardless of the concentration (**Figure 4D-E**). The treatment with 2-FA and 2-FA+MTA significantly reduced the capacity of invasion of the cells MTAP $^{+/+}$ and $^{-/-}$ (p<0,0001) (**Figure 4F**). The results of proliferation and apoptosis indicate that the use of 2-FA as a potential targeted therapy for patients with MTAP$^{-/-}$ glioblastoma is plausible, since it has a cytotoxic effect on MTAP-deleted cells, in addition to inducing apoptosis in the cells, and with the presence of MTA (which is present in the microenvironment of MTAP$^{-/-}$ tumors) it is not cytotoxic to MTAP$^{+/+}$ cells. Furthermore, 2-FA can decrease invasion in both cell lines, while the treatment has the ability to inhibit colony formation and migration in tumor cells MTAP$^{-/-}$.



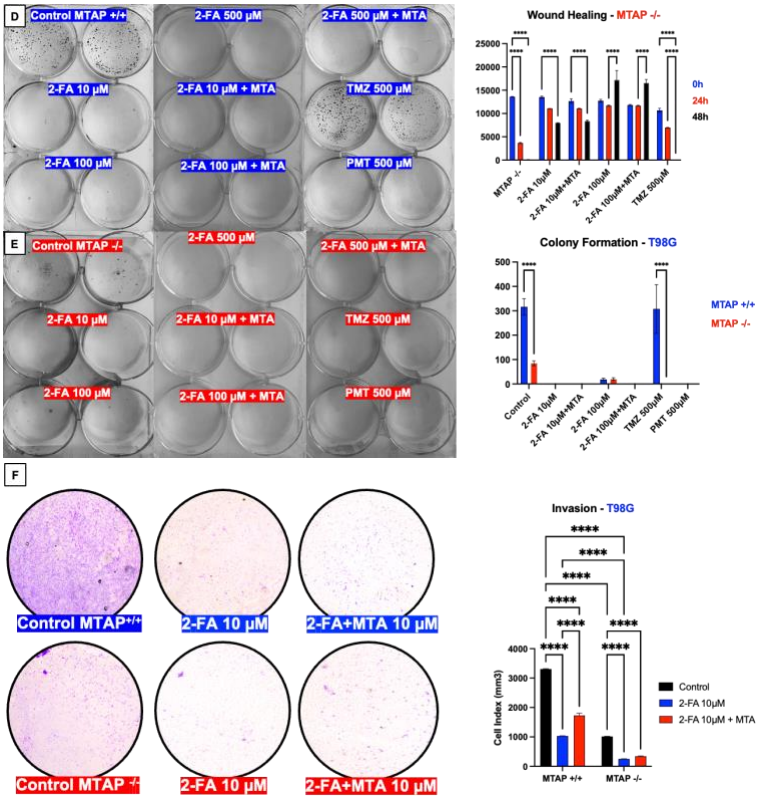


Figure 10 – Results showing cells in apoptosis after 48h of treatment with the drugs (A). Results of proliferation assay in T98G MTAP$^{+/+}$ and MTAP$^{-/-}$ cells after 48h of drug treatment (B). Wound healing assay showing migration and proliferation of treated HCMEC cells after 24h (C). Colony formation in T98G MTAP$^{+/+}$ (D) and MTAP$^{-/-}$ (E) cells after 12 days of drug treatment. Transwell assay showing the invasion capacity of cells MTAP +/+ and -/- untreated and treated (F). *The results from proliferation and colony formation assays suggest that 2-FA could be a viable targeted therapy for patients with MTAP-/- glioblastoma. It demonstrates cytotoxic effects specifically on MTAP-/- cells, induces apoptosis in these cells, and, in the presence of MTA (which is found in the microenvironment of MTAP-/- tumors), it does not affect MTAP+/+ cells. Additionally, 2-FA inhibits colony formation and migration in MTAP-/- tumor cells.*

The IncuCyte shows the proliferation of the cells over time. In T98G MTAP $^{+/+}$, the treatment with 2-FA was cytotoxic, however the addition of MTA protects the cells from the cytotoxicity, while TMZ started to act in the cells after 80h (**Figure 5A**); while in T98G MTAP $^{-/-}$ cells, it was observed that treatment with 2-FA was extremely cytotoxic regardless of MTA addition and again the TMZ resistance in MTAP$^{-/-}$ cells was showed (**Figure 5A**). The same results were found in U138MG MTAP $^{+/+}$, $^{-/-}$ and ^{Low}, with 2-FA being cytotoxic for both cell lines, but only MTAP+/+ benefits from MTA addition (**Figure 5B**). MTAP ^{Low} also showed an increase in the resistance to TMZ (**Figure 5B**). MTAP $^{-/-}$ bladder cancer organoid from a patient showed that the 2-FA is cytotoxic for other MTAP$^{-/-}$ cells, not only GBM cell lines (**Figure 5C**).

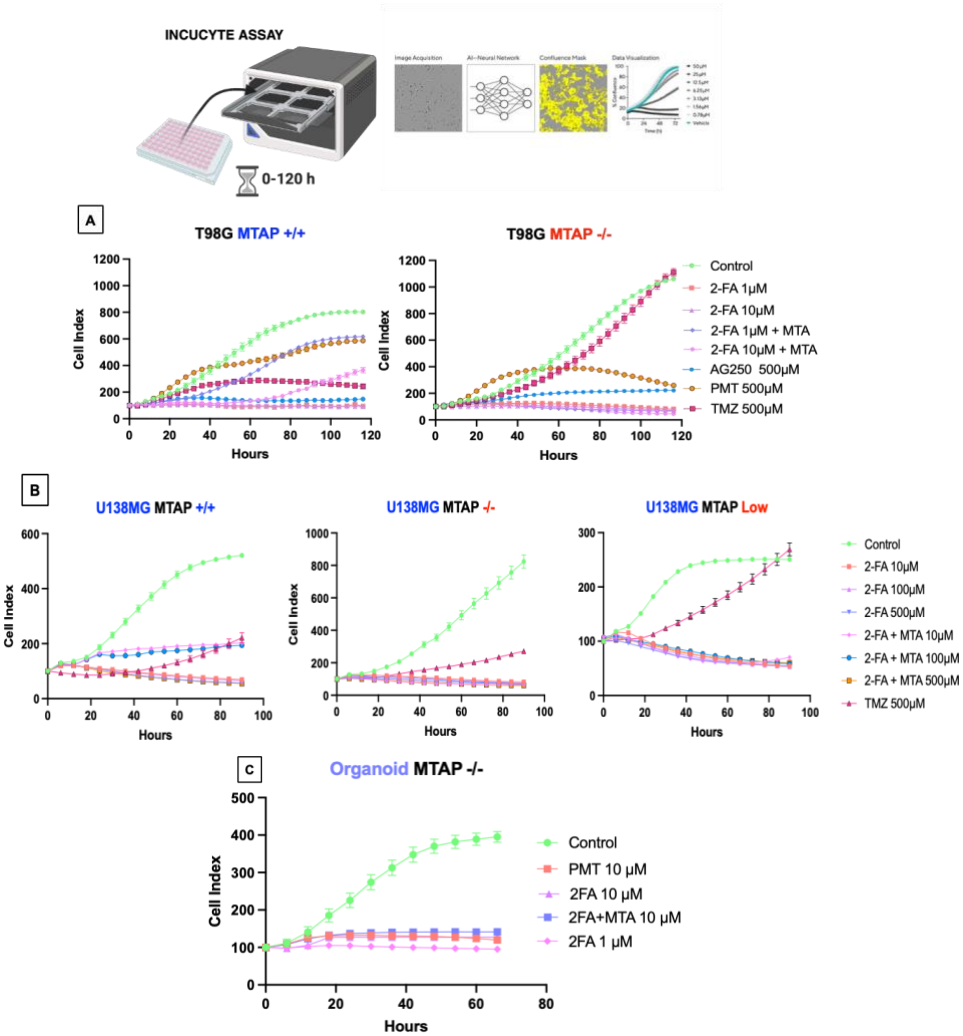


Figure 5 – Proliferation curves showing the cytotoxic effects of the drugs on T98G MTAP $^{+/+}$ and $^{-/-}$ (A), U138MG MTAP $^{+/+}$,$^{-/-}$ and ^{low} (B) and BLAD03 *MTAP* $^{-/-}$ (C) cell lines over time untreated and treated. *Treatment with 2-FA was highly cytotoxic to all cell lines; however, the addition of MTA reduced the cytotoxic effects in T98G and U138MG MTAP +/+ cells, while maintaining the cytotoxicity in MTAP -/- cells. Additionally, 2-FA was cytotoxic to other cell types beyond GBM, as demonstrated by its effects on the **MTAP-/- bladder organoid cell line.*

MTAP is expressed in normal brain cells and MTA helps to attenuate the cytotoxicity of 2-FA in normal cells

The MTAP protein is expressed in normal brain cells, such as astrocytes, pericytes and endothelial cells (**Figure 6A**). To choose an ideal targeted treatment, it is essential that the drugs do not cause damage to non-neoplastic cells, but only to tumor cells. Therefore, we sought to evaluate the cytotoxic effects of the drugs in normal brain cells, as well as their effects on apoptosis. The 2-FA, despite being cytotoxic to normal cells, mainly astrocytes with EC50 of 18.54 µM (**Figure 6C**), its cytotoxicity was extremely attenuated with the addition of MTA in the treatment,

the EC50 increasing for a concentration greater than 1000 µM (**Figure 6D**). The pericytes have a lower expression of MTAP compared to the other normal cells (Figure A), and that can explain why the addition of MTA did not impact as much the cytotoxicity of 2-FA in those cells (**Figure 6D**). For AG-270 drugs were not cytotoxic to cells at doses lower than 1000 µM, regardless of the cell type (**Figure 6E**), while PMT was cytotoxic in astrocytes and pericytes even at low concentrations, with EC50 of 12 and 16 µM respectively (**Figure 6F**). The TMZ drug was cytotoxic only at high concentrations for all cell types (**Figure 6G**). For when the percentage of cells that underwent apoptosis after treatment with the drugs was assessed, it was noted that treatment with 2-FA induced apoptosis in normal cells, but MTA reversed this effect in all cell types analyzed (**Figure 6B**). Together, these results reinforce the use of 2-FA as a potential targeted therapy for patients with MTAP$^{-/-}$ glioblastoma, since the cytotoxic and apoptosis-inducing effects on normal cells are attenuated with the presence of MTA.

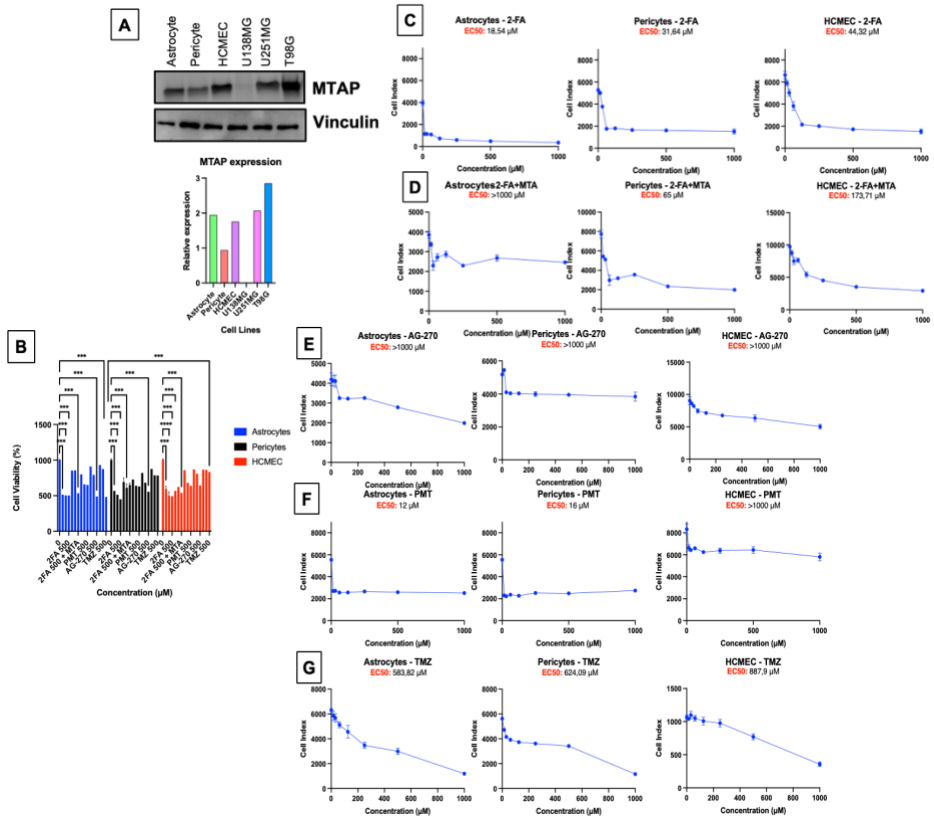


Figure 6 – Analysis of MTAP protein expression in normal brain cell lines and in glioblastoma cell lines (A). Results showing the cells undergoing apoptosis after 72 hours of treatment (B). Results showing the cytotoxic effects of the drugs on normal brain cells after 72 hours of treatment (C-G). *These results support the potential of 2-FA as a targeted therapy for patients with MTAP-/- glioblastoma, as the cytotoxic and apoptosis-inducing effects on normal cells are reduced in the presence of MTA.*

The combination of 2-FA+MTA is a viable treatment for glioblastoma since both drugs are able to cross the blood-brain barrier

Another fundamentally important point in choosing therapies for glioblastomas is choosing drugs that have the ability to cross the blood-brain barrier (BBB) and reach the tumor. Fluorescence analysis shows whether the drug was able to permeate the barrier and enter the spheroids. As expected, it was found that temozolomide was able to cross the BBB (**Figure 7A-B**). It is already known that PMT does not cross the BBB (11), but we found that the inhibitor AG-270 also appears not to penetrate the barrier (**Figure 7A-B**). Furthermore, MTA and 2-FA were able to penetrate the spheroids, as well as the combination of the two drugs (**Figure 7A-B**), indicating that the treatment is viable for central nervous system tumors, since the drugs can be delivered into the tumors. The ECIS assay confirmed our findings, where MTA, 2-FA and the combination were able to break the cellular resistance of BBB in a more efficient way even than TMZ (**Figure 7C**). However, 2-FA alone and AG-270 were cytotoxic for the cells (**Figure 7C**).

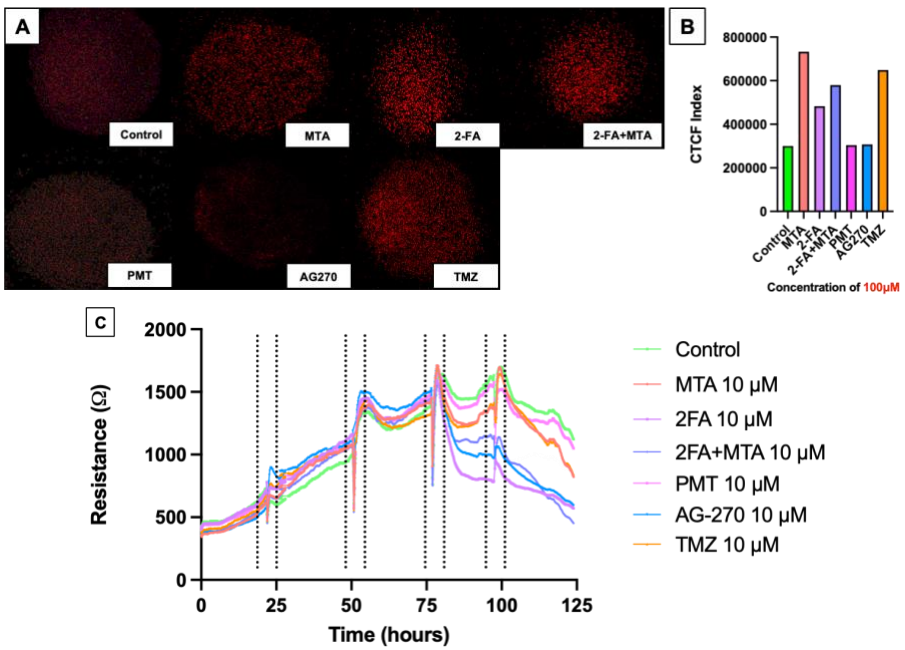


Figure 7 – Confocal microscope analysis of Dextran fluorescence in spheroids of the blood-brain barrier (A) and the corrected total cellular fluorescence calculation rate (B). Real time measurement of electrical resistance across endothelial monolayer with different treatments (C). *MTA and 2-FA are capable of crossing the blood-brain barrier and reaching glioblastoma tumors.*

Discussion

Glioblastoma is one of the most aggressive and treatment-resistant brain tumors, characterized by its rapid growth, invasiveness, and poor prognosis (1). A significant challenge in GBM therapy lies in the tumor's ability to evade

conventional treatments, often due to the BBB and immune evasion (1). MTAP loss is a common alteration in a subset of GBMs and is associated with an aggressive tumor phenotype (3). In tumors with MTAP deletion, purine synthesis becomes critically dependent on the alternative salvage pathways, purine synthesis inhibitors can target this metabolic vulnerability in MTAP-deleted tumors by blocking key enzymes involved in purine metabolism. In this study, we found that 2-FA, a purine synthesis antagonist, is highly cytotoxic to cells in general, and the presence of MTA attenuates its cytotoxicity in MTAP $^{+/+}$ cells, which, could be an important strategy to target specifically MTAP$^{-/-}$ cells. 2-FA also inhibits migration and colony formation in the tumor cells.

Tang *et al.* (7) used human fibrosarcoma cell lines and NIH 3T3 MTAP $^{+/+}$ and $^{-/-}$ mouse cells in their study and found that 2-FA had a cytotoxic effect on the cells after 48 hours of exposure and that the addition of 10 μM MTA did not affect the cytotoxicity in MTAP$^{-/-}$ cells, but drastically reduced the cytotoxicity in MTAP$^{+/+}$ cells (7). Besides the promising observations made by the authors, the further observations were focused on *in vivo* assays, and there was a lack of comparison with the standard TMZ treatment (7). Consistent with the literature (12), we observed that MTAP loss confers increased resistance to TMZ treatment, which appears to be linked to elevated NF-κB levels in MTAP$^{-/-}$ cells. Inhibition of NF-κB activity resulted in the downregulation of MGMT gene expression, leading to a significant restoration of TMZ chemosensitivity (13).

In the literature, other drugs related to purine synthesis to treat MTAP$^{-/-}$ cells have been studied. One of the most widely studied is pemetrexed, especially for urothelial cancer, since it is already approved by the FDA (14). However, for glioblastomas, the use of pemetrexed is still little explored, since there are reports that it does not cross the blood-brain barrier (15). Here, we report that, in fact, PMT is not able to cross BBB and did not show any effect on GBM tumor cells besides inhibit the colony formation.

The BBB is a protective structure formed by specialized endothelial cells that line the blood vessels in the brain and hinder the passage of many substances, including medications, to the central nervous system (CNS). This barrier is essential for protecting the brain against toxins and pathogens, but it can also hinder the treatment of conditions such as cancer (16). In our findings, MTA, 2-FA, and the combination had the ability to cross the BBB. Taken together, besides crossing BBB, which is an important point to drug delivery for brain tumor, the combination of 2-FA and MTA would be effective against tumor cells, since more than 50% of GBM patients present deletion in MTAP (5,6), and MTA could protect non-tumor tissue from the cytotoxicity, since they are MTAP $^{+/+}$.

Current research on MAT2A inhibitors is in its early stages, and preclinical studies have shown promise in selectively targeting MTAP-deficient tumors without affecting normal tissues . However, in this study we found that AG-270 was more

cytotoxic to normal brain cells than tumor cells. New studies have to performed to check the use of the drugs combined in MTAP-deficient cancer cells, and studies *in vivo* are also necessary.

Conclusion

Purine synthesis antagonist 2-FA represent a promising therapeutic strategy for targeting MTAP-deleted glioblastomas. 2-FA, in the presence of the metabolite MTA, could offer selective targeting of MTAP-deficient tumors, potentially improving treatment efficacy while minimizing off-target effects of MTA in tumor cells. While *in vitro* data is promising, further research and *in vivo* studies are needed to assess the safety, efficacy, and potential for combining 2-FA with existing therapies. If successful, 2-FA+MTA could become a novel and effective therapeutic approach for a subset of almost 60% of GBM patients that have the deletion of *MTAP*.

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MTA accumulation leads to microenvironmental changes in glioblastoma

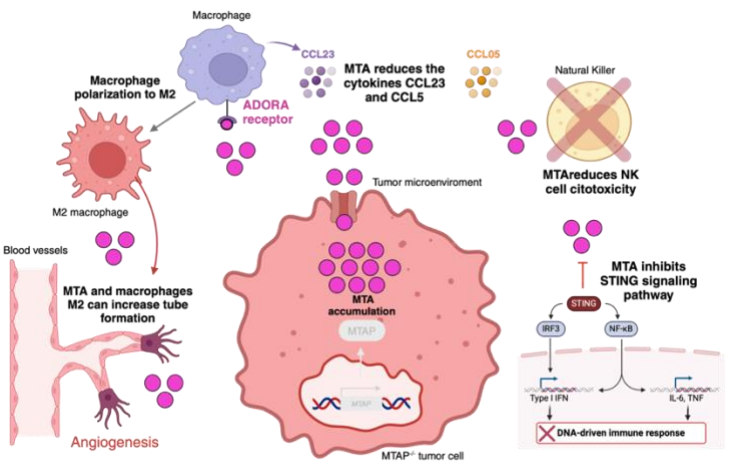
Paola Gyuliane Gonçalves[1,2], Andrea Schmidt[2], Noe Mercado[2], Maximilian Pinho-Schwermann[2], Rui Manuel Reis[3,5], Lucas Tadeu Bidinotto[1,3,4], Sean Lawler[2]

1 Department of Pathology, UNESP – Univ. Estadual Paulista, School of Medicine, Botucatu, São Paulo, Brazil
2 Pathology and Laboratory Medicine, Legeorreta Cancer Center, Brown University, Providence, RI, USA;
3 Molecular Oncology Research Center, Barretos Cancer Hospital, Barretos, São Paulo, Brazil;
4 Barretos School of Health Sciences, Dr. Paulo Prata – FACISB, Barretos, São Paulo, Brazil;
5 Life and Health Sciences Research Institute (ICVS), Medical School, University of Minho, Braga, Portugal;

ABSTRACT

Methylthioadenosine (MTA) is a metabolite derived from the polyamine biosynthetic pathway, and its accumulation within the tumor microenvironment (TME) has recently garnered attention for its potential role in modulating cancer progression. In glioblastoma (GBM), a highly aggressive and treatment-resistant brain tumor, MTA accumulation is associated with significant microenvironmental changes that influence tumor growth and immune evasion. This study aims to explore how MTA affects key components of the GBM TME, including immune cells and angiogenesis. We highlight the impact of MTA on immune cell polarization, particularly the promotion of an immunosuppressive environment that allows for tumor immune evasion. Furthermore, MTA accumulation is influencing endothelial cells and macrophages to promote angiogenesis, which supports the rapid growth and spread of GBM. Overall, MTA accumulation plays a pivotal role in shaping the GBM microenvironment, and targeting MTA-related pathways may offer new therapeutic opportunities for disrupting GBM growth and improving patient outcomes.

Key-words: MTA, glioblastoma, macrophages, angiogenesis, microenvironment.



Introduction

MTA (5'-methylthioadenosine) is a byproduct of polyamine synthesis and plays a crucial role in cellular metabolism, particularly in the regulation of purine metabolism and the modulation of the tumor microenvironment (TME). The TME is a dynamic and complex ecosystem surrounding tumor cells, consisting of stromal cells, immune cells, extracellular matrix components, and blood vessels[1]. This

environment significantly influences tumor progression, immune evasion, metastasis, and response to therapy in glioblastoma (GBM)[1]. MTA contributes to several key aspects of the TME, including immune cell modulation and tumor cell proliferation[2–5]. By affecting the balance of pro- and anti-inflammatory signals, MTA can promote or suppress immune responses, which impacts tumor growth and metastasis[2–5].

MTAP (methylthioadenosine phosphorylase) is an enzyme involved in the breakdown of MTA, a byproduct of the polyamine biosynthesis pathway. The loss of MTAP leads to the accumulation of MTA in the cells and TME. This accumulation has been linked to various cellular changes, including alterations in immune cell function, metabolism, and tumor microenvironment dynamics.

Angiogenesis is the formation process of new blood vessels. Angiogenesis is a critical process in both normal physiology and disease, particularly in cancer, where the growth of new blood vessels is essential for tumor growth and metastasis[6]. Angiogenesis can be affected by TME, mainly with the increasing in the expression of angiogenic factors and inflammatory cytokines, along with the presence of hypoxia[7]. MTA has yet not been implicated in modulating angiogenesis. However, immune cells and angiogenesis are closely intertwined, with immune cells often promoting or inhibiting angiogenic processes in response to signals from the TME[8]. Understanding the interplay between MTA, immune cells, and angiogenesis could offer new therapeutic opportunities for targeting both immune modulation and vascularization in cancer treatment.

Glioblastoma (GBM) is a highly aggressive and malignant primary brain tumor that originates from glial cells, specifically astrocytes. It is characterized by rapid growth, extensive invasion into surrounding brain tissue, and a poor prognosis[9]. In cancers like GBM, where MTAP loss is common[10,11] the buildup of MTA can influence immune cell functions, such as T cell activation[4] and macrophage polarization[12], which are vital for both tumor progression and immune evasion. Therefore, understanding the impact of MTAP loss and MTA accumulation is critical for exploring new therapeutic approaches aimed at targeting these metabolic alterations in GBM. Therefore, the aim of this study was to elucidate the role of MTA in GBM TME, mainly in angiogenesis modulation and immune cells.

Material and methods

For this study, the brain endothelial cells HCMEC and T98G MTAP $^{+/+}$ and $^{-/-}$ were used. The cells were treated with MTA (ranging from 1 pM to 500 µM), Adenosine (ranging from 10 to 500 µM) and VEGF (vascular endothelial growth factor) (at a concentration of 100 ng). DMSO, at the same dilution as the highest drug concentration used in each experiment, served as the control. Detailed descriptions of all experiments are provided below.

Cell migration analysis by wound healing

Cells were cultured in 6-well plates until they reached approximately 90% confluence. A sterile pipette tip was used to create a straight line across the well, removing cells along the path. The plate was then photographed using an inverted microscope with a multimedia system. After adding the corresponding treatments to the culture medium, the plates were incubated for 24 and 48 hours. The plates were photographed again, and the percentage of the initially empty space invaded by cells was analyzed using ImageJ software. The assay was performed in duplicate and repeated twice.

Cell migration analysis by transwell

The transwell (BD Biosciences) was hydrated for 1 hour with serum-free culture medium. Next, the corresponding culture medium supplemented with 10% serum, acting as a chemoattractant, was added to the lower compartment of the wells. Cells were trypsinized, seeded at a density of 1.0×10^5 cells in the upper compartment, and incubated at 37°C for 24 hours. After incubation, non-invading cells on the upper surface of the matrigel were removed with a swab. Cells that had invaded and degraded the matrigel (mimicking the extracellular matrix) and migrated to the lower surface were fixed with 100% methanol and stained with crystal violet. The cells were then visualized and quantified under a fluorescence microscope (Nikon Eclipse 50i). The assay was performed in duplicate and repeated twice.

Cell culture in hypoxia condition

The cells were cultured in a hypoxia chamber (Coy Laboratory Products) with oxygen set to 1%, 30% humidity, 5% CO_2 and at 37°C. The levels of O_2 and CO_2 were monitored. After 48h under hypoxic conditions, the cells were removed, and the RNA was extracted using the Qiagen extraction kit.

RT-PCR

For total RNA extraction, the RNeasy mini kit from Qiagen was used. After trypsinization of the cells, the cells were centrifuged at 13,000 RPM for 15 minutes at 4°C, where the supernatant was discarded. Using the RNeasy mini kit, the samples were first lysed and then homogenized. Ethanol was added to the lysate to provide optimal binding conditions, and the lysate was then transferred to the RNeasy silica membrane, where the RNA bound to the column and all contaminants were efficiently washed away. Residual amounts of remaining DNA were removed using an on-column DNase treatment. The pure and concentrated RNA was eluted in 40 µL of water. RNA concentrations were measured on the NanoDrop (Thermo Scientific). 500ng of total RNA were treated with the Turbo DNase Free kit (Thermo Scientific), the purified RNA was synthesized into cDNA using the Superscript III

reverse transcriptase kit (Thermo Scientific), according to the manufacturer's protocol. Three probes were designed for the ADORA2A, ADORA2B and HPRT1 genes to analyze the mRNA expression of each gene using the Taqman technique (Thermo Scientific) for qRT-PCR. QuantStudio 6 (Thermo Scientific) was used to perform the qRT-PCR and analyze the results.

ELISA for quantification of cytokines

Cytokine quantification in the culture supernatant was carried out using enzyme-linked immunosorbent assay (ELISA) from R&D Systems (Minneapolis, USA). The analytes measured were CCL5, CLL23 and VEGF, following the manufacturer's protocol. Absorbance values for both the samples and standard curve were read at 450 nm using a spectrophotometer (µQuant, Biotek Instruments Inc). The concentrations of the analytes were determined based on the linear regression of the standard curve.

Endothelial cell tube formation assay – angiogenesis

Brain endothelial cells (HCMEC) were cultured at 37˚C (5% CO2, humidified atmosphere) with special culture medium for endothelial cells (ScienCell, cat#1021). First, 50 µL of growth factor-reduced matrigel was added to 96-wells. After 1 hour in the incubator, 15,000 cells were added per well along with the corresponding treatments (DMSO control, MTA 1pg:500µM, Adenosine 10µM:500µM, VEGF 500ng) in triplicate. After 24 hours of incubation, pictures were taken using a microscope with a built-in camera. The number of tubes formed was counted using AngioTools in ImageJ (18). The assay was performed in triplicate and repeated three times.

Antibody-dependent cell-mediated cytotoxicity (ADCC) assay

The ADCC assay was performed to evaluate the cytotoxicity mediated by natural killer cells (NKs), Dinutuximab, MTA and 2FA in T98Gs MTAP +/+ and -/- cells. Tumor cells were plated and incubated overnight in a 96-well culture plate at 37°C. ADCC was initiated by the addition of NK92 M1 cells as effector cells (1:8 as the effector: target (E:T) ratio) and the respective drugs (DMSO control, NK cell-only control, IgG antibody control, MTA, Dinutuximab and 2-FA+MTA) at 100 µM. The plate was incubated for 48h at 37°C (5% CO2, humidified atmosphere). After 48h, the medium with the treatment and NK cells was removed, 100 µL of CellTiter Glo was added per well and incubated for 30 minutes, and then the plate was read to evaluate cytotoxicity in tumor cells. The assay was performed in triplicate and repeated three times.

Transformation of monocytes into macrophages and polarization assay

TPH-1 monocytes were cultured in their own medium. After the third

passage, TPH-1 cells were plated in 6-well plates, with 100,000 cells per well. 1 µL of Phorbol 12-myristate 13-acetate (PMA) was added to every 5 mL of medium to induce the transformation of monocytes into macrophages. After 24 h of incubation, macrophages were incubated with 20 ng/mL of IFN-γ and 10 pg/mL of LPS to induce polarization in M1, and 20 ng/mL of IL-13 and IL-4 for M2. Some macrophages were also treated with different concentrations of MTA (dilution ranging from 10 µM to 500 µM) to analyze its effects on macrophage polarization. After 48h of treatment, protein extraction was performed from the cells using RIPA buffer and expression analyses of the proteins CD38 (1:2000, ProteinTech cat#84070-5-RR), EGR2 (1:1000, ProteinTech cat#13491-1-AP), Arg-1 (1:4000, ProteinTech cat#16001-1-AP), IRF5 (1:1000, ProteinTech cat#10547-1-AP) and B-actin (1:1000, Cell Signaling cat#4970) were performed by western blot.

Statistical Analysis

Statistical analyses were conducted using GraphPad PRISM 10. One-way ANOVA was employed to compare the means of two or more independent groups, assessing whether the population means were significantly different. Two-way ANOVA was used for multiple comparisons. T-tests were applied for dependent analyses. In all analyses, there was considered a significance of 5%.

Results

Loss of MTAP affects the response of immune cells to the tumor

A macrophage polarization assay was performed on macrophages treated with MTA, as well as on T98G MTAP $^{+/+}$ and $^{-/-}$ tumor cells. TPH-1 cells were induced to transform into macrophages (**Figure 1A-B**). Monocytes are cells that grow in suspension (**Figure 1A**), but when they transform into macrophages they adhere to the plaques (**Figure 1B**). MTA treatment induced the polarization of M2 macrophages (CD38-/EGR2+/Arg1+), which are related to tumor progression (**Figure 1C**). In MTAP$^{-/-}$ tumors, MTA treatment increased the expression of genes related to M2 polarization (EGR2 and ARG1) and decreased the expression of genes related to M1 (CD38 in > MTA concentrations and IRF5) (**Figure 1D**).

In the ADCC assay, NK cells are incubated with drugs and antibodies to analyze whether they will affect the cytotoxicity of NK cells on tumor cells T98G MTAP $^{+/+}$ and $^{-/-}$. For this assay, in addition to DMSO, the IgG antibody, which does not have a specific target, and the anti-GD2, highly expressed in T98G cells, antibody Dinutuximab were used as controls. Treatment with the drugs MTA and 2-FA was also performed. In MTAP $^{+/+}$ cells, the addition of NK cells alone is sufficient to cause cytotoxicity, and the addition of MTA and 2-FA potentiated this effect (**Figure 1G**). In MTAP -/- cells, NK cells had a cytotoxic effect only when combined with Dinutuximab (**Figure 1G**). This demonstrates that the presence of MTA in the microenvironment and in the treatment are, in some way, inhibiting the activation

of NK cells.

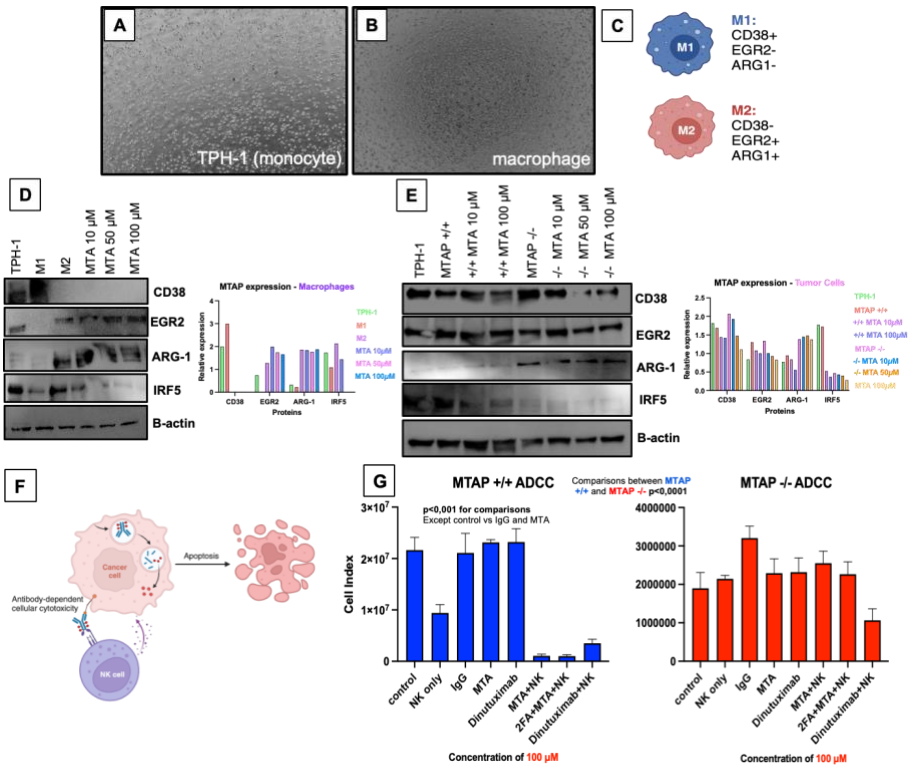


Figure 11 – Representation of the transformation of monocytes (A) into macrophages (B) and analysis of proteins related to the polarization of M1 and M2 macrophages into macrophages (C) and tumor cells (D) treated with MTA. NK cell-mediated cytotoxicity schematic (E) in MTAP $^{+/+}$ and MTAP $^{-/-}$ tumor cells (F). *MTA induces macrophage polarization to M2, inducing a pro-tumorigenic state, and reduces NK cells cytotoxicity.*

The expression of cytokines in the supernatant of HCMEC cells treated with 100µM of MTA and adenosine and T98G MTAP $^{+/+}$ and $^{-/-}$ were measured by ELISA. CCL5 expression was significantly reduced in HCMEC treated with MTA ($p<0.01$) (**Figure 2A**), while in the tumor cells there were no differences in expression of CCL5 between cells MTAP $^{+/+}$ and $^{-/-}$ (**Figure 2B**). Regarding CCL23, HCMEC treated with MTA also had a decreasing in expression ($p<0.01$) (**Figure 2C**), while MTAP $^{-/-}$ tumor cells had an increasing ($p<0.01$) (**Figure 2D**). To investigate if MTA was affecting cancer cell signaling and immune pathways, western-blot was performed to check the expression of phospho-TBK1, AKT and ERK, related to the activation of cancer signaling pathways and STING, a signaling responsible for the activation of interferons in HCMEC cells. HCMEC cells were treated with MTA and ADU, a STING agonist, for 24 hours, where MTA seems to increase the expression of phosphorylated TBK1 and ERK, while inhibiting STING pathway.

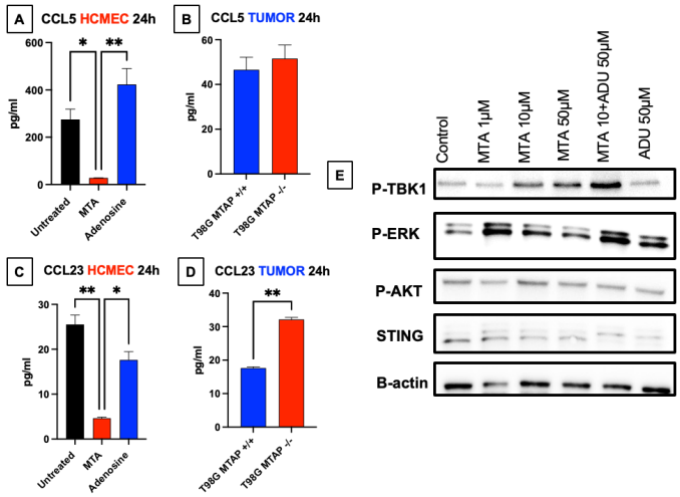
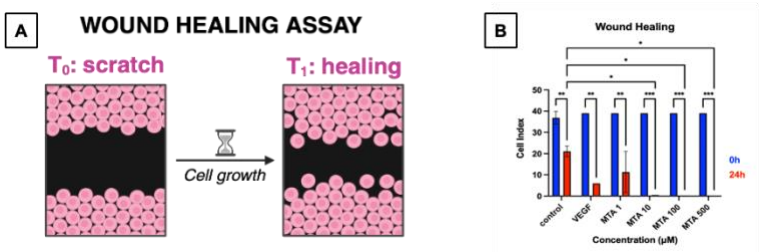


Figure 12 – Expression of the cytokines CCL5 and CCL23 in the supernatant of cells HCMEC (A-C) and T98G MTAP $^{+/+}$ and $^{-/-}$ (B-D), and the expression of proteins related to signaling pathways in HCMEC cells treated with MTA and ADU (E). *CCL5 expression was significantly reduced in HCMEC cells treated with MTA ($p < 0.01$), while no differences in CCL5 expression were observed between MTAP $^{+/+}$ and $^{-/-}$ tumor cells. For CCL23, MTA treatment resulted in a significant decrease in expression in HCMEC cells ($p < 0.01$), while MTAP $^{-/-}$ tumor cells showed a significant increase in expression ($p<0.01$). Additionally, MTA appeared to enhance the expression of phosphorylated TBK1 and ERK while inhibiting the STING pathway in HCMEC cells.*

MTA accumulation may modulate angiogenesis in MTAP$^{-/-}$ tumors

The wound healing migration analysis showed that the addition of MTA to HCMEC cells increased the migration and proliferation of cells, which closed the fissure after 24h ($p<0.05$-0.001) (**Figure 2B-C**). The same occurred in the transwell migration analysis, where the addition of MTA increased the invasion of HCMEC cells ($p<0.001$) (**Figure 2E-F**).

In the tube formation analysis, it was found that MTA induces tube formation in HCMEC cells even at low concentrations such as 10 nM, and that increasing the MTA concentration also increases tube formation ($p<0.0001$) (**Figure 3B-C**). The same was true in the transwell migration analysis, where the addition of MTA increased the migration of HCMEC cells (**Figure 2E-F**). Cells HCMEC under hypoxia and treated with MTA have higher VEGF levels compared to the control and the cells treated with MTA under hypoxia (**Figure 2G**).



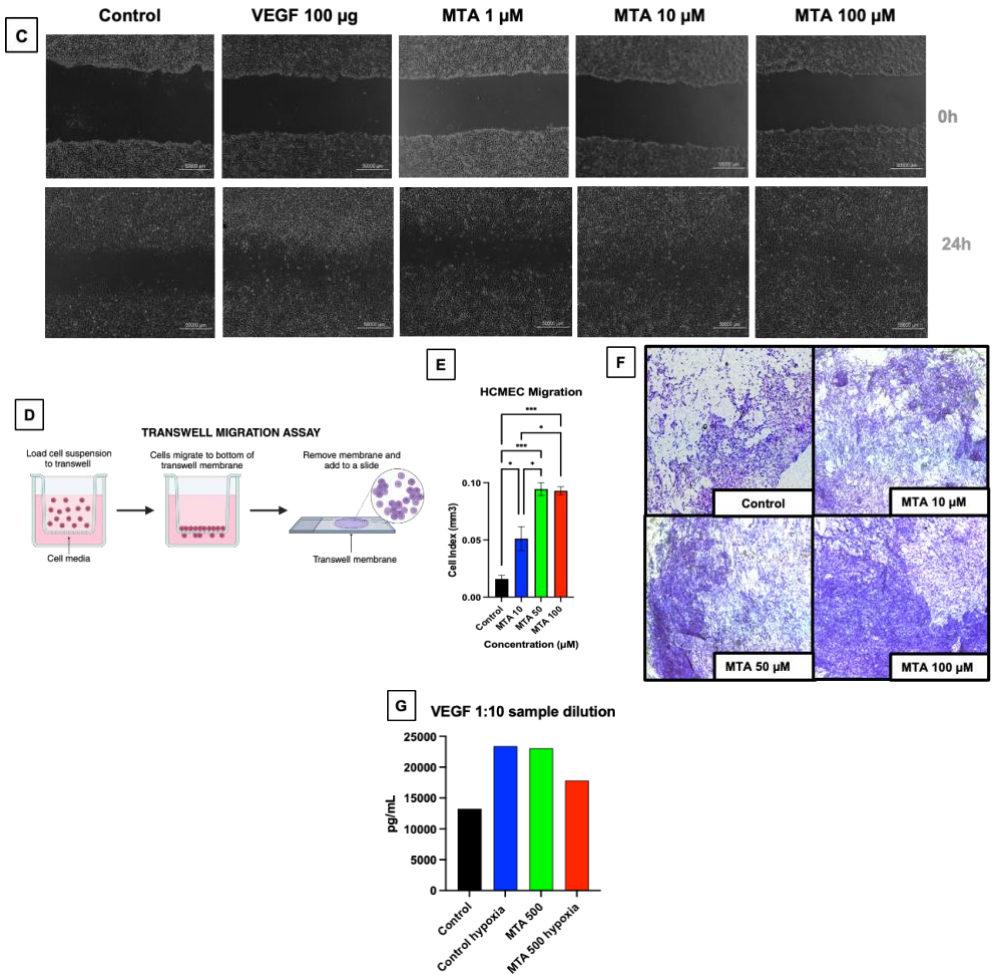


Figure 13 – Wound healing assay schematic (A) showing the migration (B-C) and transwell assay schematic (D) showing the migration (E-F) of treated HCMEC cells after 24h. VEGF concentration in HCMEC untreated and treated with MTA in hypoxia or not (G). *The migration of HCMEC cells increases in the presence of the metabolite MTA (p<0.001), and cells HCMEC under hypoxia and treated with MTA have more VEGF.*

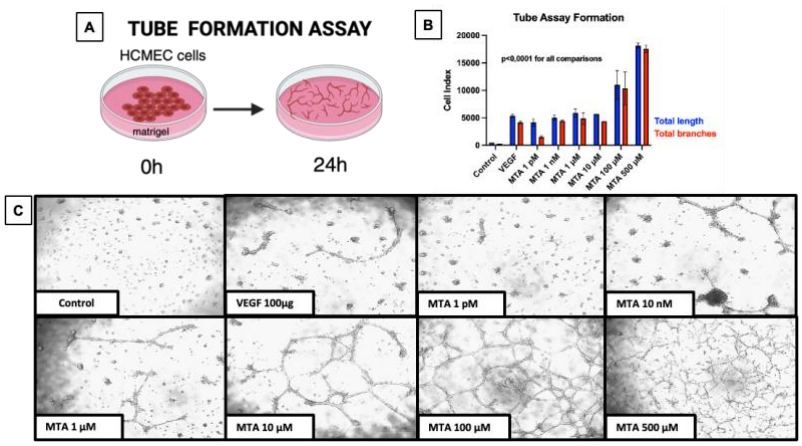


Figure 14 – Tube formation assay schematization (A) showing the tubes formed by HCMEC

cells after 24h of treatment (B-C). *MTA increases the formation of tubes in HCMEC, and this increasing is proportional to the amount of MTA added (p<0.0001).*

Is MTA modulating angiogenesis through ADORA receptors?

We hypothesized that MTA may be stimulating ADORA receptors and inducing angiogenesis in MTAP$^{-/-}$ tumors. Thus, we analyzed the expression of the ADORA2A and ADORA2B in HCMEC and tumor cells. There were no significant differences between expression of the genes in HCMEC (**Figure 5A**), although the expression of ADORA2A decreased in tumor cells under hypoxia (**Figure 5B**). In the tube formation assay, HCMEC were cultured with conditionate media previously treated with Adenosine and MTA. MTA conditionate media increased the formation of tubes (p<0.0001), and when ADORA receptors inhibitors were add, the tube formation decreased in both treatments, MTA and Adenosine, when compared with the cells with treatment only (p<0.0001) (**Figure 2C**). In addition, conditioned media from tumor cells MTAP$^{-/-}$ increased the tube formation (p<0.0001) (**Figure 2D**).

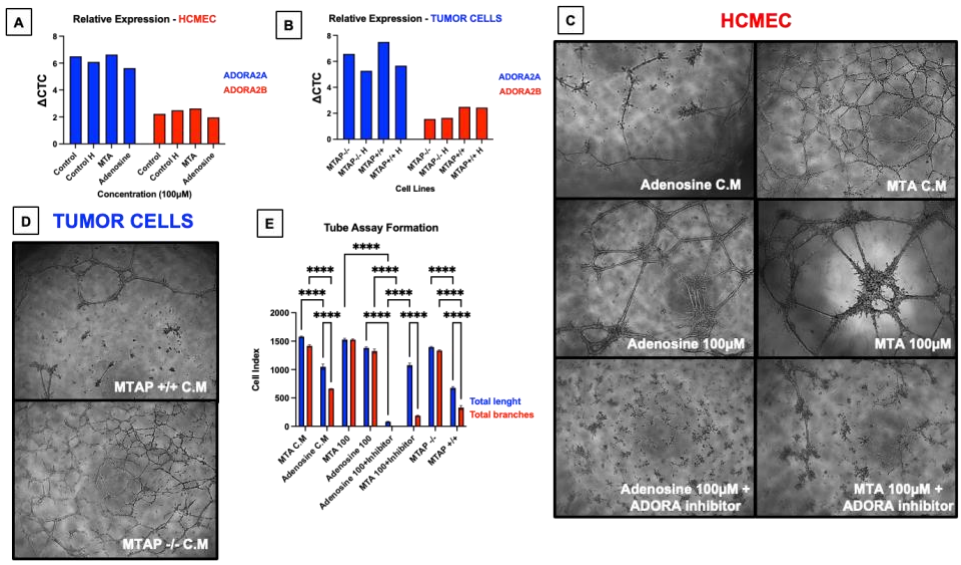


Figure 15 – Expression of ADORA2A and ADORA2B in HCMEC (A) and tumor cells MTAP $^{+/+}$ and $^{-/-}$ (B). Tube formation assay in HCMEC cells treated with Adenosine and MTA conditionate media, Adenosine and MTA at 100μM with and without ADORA inhibitor (C), and tumor cell MTAP +/+ and -/- conditionate media. *Although there were no significant differences in the expression of ADORA2A/B, the use of ADORA inhibitor decreased the formation of tubes in HCMEC cells treated with MTA or Adenosine (p<0.0001).*

Discussion

It is well known that MTA accumulation in MTAP$^{-/-}$ tumors is a potent inhibitor of methylosome formation by arginine N-methyltransferase-5 (PRMT5) and leads to increased sensitivity in these cells to PRMT5 depletion, which is essential for key processes related to carcinogenesis, such as cell proliferation, metastasis, and resistance to apoptosis[2]. However, little has been studied about the

effects of MTA accumulation on cancer development and progression in addition to its role in modulating immune cells. In several neoplasms, $MTAP^{-/-}$ tumors are more metastatic and have a worse prognosis when compared to $MTAP^{+/+}$ tumors[4,13,14], therefore, we speculate that MTA accumulation could also be modulating angiogenesis. For this purpose, we performed functional analyses using brain endothelial cells treated with MTA at different concentrations, as well as a tube formation assay. Our study found that MTA accumulation can modulate the TME through inactivation of immune cells and promotion of angiogenesis.

In this study, we found that MTA suppresses the activation of NK cells, and this is in line with a study that found that MTA blocks cytotoxicity and cytokine production in NK cells by interfering with the NK cell receptor activation signaling cascade[15]. In addition, we confirmed that MTA accumulation induces the polarization of macrophages to M2. M1 macrophages (CD38+/EGR2-/Arg1-) are usually activated by interferon-gamma (IFN-γ) and lipopolysaccharide (LPS), which are typical signs of bacterial infection or invading pathogens. They are considered pro-inflammatory macrophages, involved in the initial immune response against infections[12]. They play an important role in eliminating pathogens, such as bacteria and viruses, and in inducing inflammation[12]. M1 macrophages may play a protective role against cancer, through the production of inflammatory cytokines, inhibiting tumor growth and inducing cell death by apoptosis. M2 macrophages (CD38-/EGR2+/Arg1+) are activated by anti-inflammatory cytokines, such as IL-4, IL-13, and IL-10[12]. They are anti-inflammatory macrophages and promote tissue repair and have also been implicated in fibrous diseases and cancer, where their function of promoting repair and angiogenesis can facilitate tumor progression[12].

CCL5 (C-C motif ligand 5), also known as RANTES (Regulated upon Activation, Normal T Expressed and Secreted), is a chemokine involved in immune cell recruitment and inflammation[16]. It plays a pivotal role in both the immune response and the tumor microenvironment (TME). CCL5 is known to attract various immune cells, including T cells, monocytes, eosinophils, and dendritic cells to the site of inflammation or tumor growth[16]. Therefore, the significant decreased in CCL5 expression in cells treated with MTA shows how MTA might inhibit CCL5 to avoid the immune response. CCL23 (C-C motif ligand 23), also known as MIP-3, is a chemokine that plays a role in the recruitment and activation of immune cells, particularly T cells and monocytes, to sites of inflammation or infection[17]. CCL23 may also attract monocytes and macrophages, which can differentiate into tumor-associated macrophages (TAMs). Thus, the increase of CCL23 in $MTAP^{-/-}$ tumor cells can be due to the recruitment of macrophages and polarization to M2 caused by MTA accumulation.

Phosphorylated TBK1 (TANK-binding kinase 1) plays an important role in the regulation of immune response, inflammation, and cancer progression. TBK1 is primarily involved in the activation of various signaling pathways, particularly the

NF-κB and IRF3 (Interferon Regulatory Factor 3) pathways, which are crucial for the immune response to infections and the regulation of inflammation[18]. TBK1 signaling also plays a role in shaping the tumor microenvironment (TME) by modulating immune cells in the TME, including macrophage. Phosphorylated ERK (Extracellular signal-Regulated Kinase) plays a pivotal role in the regulation of several key cellular processes, including cell proliferation, survival, differentiation, and migration[19]. ERK is a part of the MAPK (Mitogen-Activated Protein Kinase) signaling pathway. In cancer, the activation of ERK through phosphorylation is particularly significant, as it can influence tumor progression, metastasis, and response to therapies[19]. The STING (Stimulator of Interferon Genes) pathway is a critical part of the innate immune system, playing a significant role in detecting DNA from pathogens or damaged cells, and initiating immune responses[20]. Many tumors acquire mechanisms to evade STING activation, thereby escaping immune surveillance, allowing them to escape immune destruction[20]. There is an increasing in the expression of phosphorylated TBK1, ERK and STING in HCMEC treated with MTA. Altogether, the results show how MTA promotes invasion of the immune system and a colder tumor environment, showing that $MTAP^{-/-}$ are not good candidates to respond to immunotherapies. A study demonstrated that the loss of MTAP is associated with an immunosuppressive profile in GBM[5].

Angiogenesis is a biological process that stimulates the formation of new capillaries from existing blood vessels. It occurs both physiologically, such as during embryogenesis, wound healing, and immune responses[8], and pathogenically, as seen in conditions like neoplasias[6]. The *in vitro* analysis showed that the addition of MTA to HCMEC cells increased the migration and proliferation of cells and induces tube formation. MTA appears to affect the activity of cells in the TME, potentially triggering the release of pro-angiogenic factors that promote angiogenesis. Fibroblasts, which are crucial for tissue repair and remodeling, also play a role in angiogenesis by secreting various cytokines and growth factors[7]. Fibroblasts exposed to MTA undergo molecular reprogramming associated with cancer aggressiveness and gain the ability to secrete purine derivatives that promote macrophage polarization[21]. MTA can affect the modulation of macrophage function[5], impacting their inflammatory properties and the secretion of factors that regulate angiogenesis[12]. MTA appears to act as a signaling molecule that can influence endothelial cells, fibroblasts, and macrophages to promote or inhibit angiogenesis. Here, we found that MTA treatment increases VEGF in HCMEC cells. Under hypoxia, cells typically activate the hypoxia-inducible factor 1 (HIF-1) pathway[22]. HIF-1 stimulates the transcription of various genes, including VEGF, which plays a critical role in promoting angiogenesis[22]. MTA treatment can further potentiate this response, leading to higher VEGF levels in HCMEC cells by influencing adenosine signaling, which may play a role in vascular endothelial growth. However, cells in hypoxia treated with MTA might have lower VEGF levels

because MTA could interfere with the HIF-1 pathway , alter adenosine signaling , or induce cellular responses that reduce VEGF expression. MTA may shift the cell's focus from angiogenesis to other survival or stress-related responses under hypoxia. Further research is needed to fully understand the complex mechanisms by which MTA influences angiogenesis and how these effects might be harnessed or mitigated in various clinical scenarios.

Adenosine receptors (ADORA - A1, A2A, A2B, A3) are key regulators of many physiological functions. They mediate the effects of adenosine, influencing processes such as vasodilation, angiogenesis and inflammation. A study found ADORA2B as the specific receptor and signaling pathway for the MTA metabolite in melanomas[23]. The ADORA2B receptor stimulates angiogenesis by inducing VEGF and eNOS in human microvascular endothelial cells[24]. We hypothesized that MTA could be activating ADORA receptors, thereby promoting angiogenesis in $MTAP^{-/-}$ tumors. While no significant differences were observed in the expression of ADORA2A/B, treatment with an ADORA inhibitor reduced tube formation in HCMEC cells treated with either MTA or adenosine. Therefore, more analyses need to be performed to confirm ADORA receptor as a target receptor of MTA that is stimulating angiogenesis in HCMEC cells. To the best of our knowledge, there are no studies relating MTA accumulation with increasing in angiogenesis in tumors to date.

In conclusion, the results found in this study corroborate the literature[5,15], which indicate that MTA contributes to a cold immune system, where immune cells, such as NKs are not activated and macrophages polarization is associated with tumor growth. Further studies should be conducted, where MTA inhibition can be used to try to reverse this profile, and tumors respond better to treatments or immunotherapy. $MTAP^{-/-}$ patients, that have a cold tumor response, are not good candidates for immunotherapies, and this may help to find patients who will respond better to treatment and directly impact their survival and prognosis. In addition, MTA accumulation seems to play a significant role in influencing angiogenesis, contributing to a TME that support tumor growth. Understanding the mechanisms behind MTA-induced angiogenesis could provide new therapeutic insights for targeting angiogenic processes in cancer treatment.

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Conclusão

A deleção homozigótica da região chr9p22.1-21.3, frequentemente observada em diversos tipos de câncer, resulta na perda de diversos genes, como *MTAP, CDKN2A e CDKN2B*. *MTAP* é deletado em quase 60% dos casos de GBM do TCGA e associado a um pior prognóstico e características desfavoráveis, como aumento de vias de sinalização celular de cancer e de resposta a inflamação. Em glioblastoma, a perda de MTAP prejudica a proliferação celular e o crescimento tumoral, bem como a invasão e a capacidade de formação de colônias, sugerindo que o acúmulo de MTA que pode levar a um pior prognóstico em tumores $MTAP^{-/-}$. A perda de MTAP também oferece uma janela terapêutica para o desenvolvimento de novos tratamentos direcionados. O antagonista da síntese de purina 2-Fluoroadenina (2-FA) surge como uma estratégia terapêutica promissora para glioblastomas com *MTAP* deletado, podendo ser combinado com o MTA acumulado nos tumores para atenuar a citotoxicidade em células cerebrais normais. 2-FA, assim como MTA, tem capacidade de atravessar a barreira hematoencefálica e ser direcionados para o tumor. Além disso, o acúmulo de MTA influencia a angiogênese e contribui para um microambiente tumoral favorável ao crescimento tumoral. A perda de MTAP pode levar a uma modulação da resposta imune ao tumor, criando uma resposta tumoral "fria", onde células imunes não são ativadas, tornando os pacientes com essa perda menos suscetíveis à imunoterapia. Embora os dados *in vitro* sejam promissores, mais pesquisas são necessárias para avaliar a eficácia e segurança da combinação 2-FA e MTA no tratamento de pacientes com GBM e a modulação de MTA no microambiente tumoral.

Anexos

HOSPITAL de CÂNCER de BARRETOS
INSTITUTO DE ENSINO & PESQUISA

Protocolo: 026/2023

Rua Antenor Duarte Villela, 1331
Bairro Dr. Paulo Prata
Barretos (SP), Brasil
CEP: 14.784-400
Telefone: +55 (17) 3321-6600

CERTIFICADO

Certificamos que o Projeto intitulado ""Avaliação da perda da expressão de MTAP como estratégia de resposta à terapia em tumores cerebrais", sob responsabilidade do Dr. (a) Lucas Tadeu Bidinotto,, na qualidade de orientador e Paola Gyuliane Gonçalves**, na qualidade de aluno de Biologia, Mestre em Ciências – Área Oncologia Molecular vinculado a pós-graduação do Instituto de Ensino e Pesquisa do Hospital do Câncer de Barretos,** foi aprovado pela Comissão de Ética no Uso de Animais do Centro de Pesquisa em Oncologia Molecular do Hospital do Câncer de Barretos, em 18 de Abril de 2023, sob protocolo nº. 026/2023.

O projeto envolve a utilização e manutenção de animais pertencentes ao filo chordata, subfilo vertebrata (exceto o homem), para fins de pesquisa cientifica e encontra-se de acordo com os preceitos da lei n° 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de junho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de experimentação animal (CONCEA).

Espécie/Linhagem: *mus musculus* | Balb/c nude (NU/J)
N° de animais: 48 Macho
Peso/Idade: 8 semanas
Origem: Biotério do Centro de Pesquisa em Oncologia Molecular do Hospital de Câncer de Barretos.

Barretos, 18 de Abril de 2023

Dra. Maria Luiza Nunes Mamede Rosa
Vice Coordenadora – CEUA/CPOM-HCB