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Is *MGMT* Gene Silencing an Opportunity for Enhanced Metformin Action in Glioblastoma Cells?

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Abstract

Isocitrate dehydrogenase 1 (*IDH1*)-wild type glioblastoma (GBM) constitutes about 12-15% of primary central nervous system tumors. The 5-year survival remains dismal at less than 5% due to the limited options available in the management of GBM patients. Metformin (*N, N*-dimethyl biguanide), a medication used primarily in the management of patients with type 2 diabetes mellitus, showed anti-proliferative actions in the management of various tumor cells, including GBM. The temozolomide (TMZ)-based genotoxic management of GBM patients elicits DNA damage response (DDR) pathways that limit the efficacy of TMZ and induce resistance. Tumors with a *methylated O⁶-methylguanine-DNA-methyltransferase* (*MGMT)* promoter status may be more dependent on DDR pathways due to the reduced DNA repair capability. Metformin can reinforce an integrative approach in GBM management in conjunction with TMZ by inhibiting several key DDR pathways of metabolism and growth. In addition to inhibition of the mitochondrial complex I, uncoupling glycolysis from oxidative phosphorylation, metformin AMP-activated Protein Kinase (AMPK)-mediated inhibition of Akt signaling can manipulate multiple key metabolic cellular processes. Our commentary proposes that metformin may exert an asymmetrical effect, preferentially inhibiting GBM cells with *MGMT* promoter methylation, by exploiting differences in metabolism that extend to glycolysis, oxidative phosphorylation, lipolysis, sphingolipid metabolism, and amino acid transport and sensing. We also highlight an emerging role for metformin in epigenetic modification through regulation of homocysteine metabolism and 2-Hydroxyglutarate bioavailability. In this commentary, expanding on our recent study showing that metformin treatment is associated with improved survival in GBM patients with a methylated *MGMT* promoter, we explore how the targets of metformin in GBM may differ depending on the *MGMT* methylation status. Our commentary invites a better examination of the action of metformin in GBM and highlights its therapeutic potential in conjunction with the molecular signature of GBM.

Keywords: Glioblastoma, Metformin, MGMT, AMPK, Molecular determinants

Abbreviations:

GBM: Glioblastoma Multiforme; *MGMT: O⁶-methylguanine-DNA-methyltransferase*; *m-MGMT:* methylated-*O6-methylguanine-DNA-methyltransferase* promoter status; *u-MGMT*: unmethylated-*O⁶-methylguanine-DNA-methyltransferase* promoter status; TMZ: Temozolomide; MTF: Metformin; DDR: DNA Damage Response; IRS-1: Insulin Receptor Substrate 1; PI3K: Phosphatidylinositol 3-Kinase; Akt: Protein Kinase B; PDK1: 3-Phosphoinositol-Dependent Protein Kinase 1; mTORC1: Mechanistic Target of Rapamycin Complex 1; 4EBP1: Eukaryotic Translation Initiation Factor 4E-Binding Protein 1; p70S6K: p70 Ribosomal S6 Kinase; AMPK : AMP-Activated Protein Kinase; RAS: Rat Sarcoma; RAF: Rapidly Accelerated Fibrosarcoma; MAPK: Mitogen-Activated Protein Kinase; EGFR: Epidermal Growth Factor Receptor; *IDH1*: *Isocitrate Dehydrogenase 1*; HMG-CoA: 3-Hydroxy-3-Methylglutaryl-Coenzyme; S1P: Sphingosine-1-Phosphate; PKM2: Pyruvate Kinase M2; HIF-1α: Hypoxia-Inducible Factor 1 α; Bcl-2: B-cell lymphoma 2 (a family of proteins regulating cell death); Bax: Bcl-2-associated X protein; PTEN: Phosphatase and Tensin Homolog; PIP2: Phosphatidylinositol (4,5)-Biphosphate; PIP3: Phosphatidylinositol (3,4,5)-Triphosphate; PDGFRα: Platelet-Derived Growth Factor Receptor Alpha; HK2: Hexokinase 2; ATP: Adenosine Triphosphate; SAH: S-adenosylhomocysteine; SAM: S-adenosylmethionine; SNAT2: System A Amino Acid Transporter 2; LAT1: Leucine-Amino Acid Transporter 1; CAT1: Cationic Amino Acid Transporter 1; V-ATPase: Vacuolar-type H+-ATPase; RAG GTPases: Ras-Related GTPases; Rheb: Ras Homolog Enriched in Brain; TSC: Tuberous Sclerosis Complex; NF-E2: Nuclear

Factor Erythroid 2 (related to cyto-protective genes); KEAP1: Kelch-Like ECH-Associated Protein 1; Nrf2: Nuclear Factor Erythroid 2-Related Factor 2; HMG-CoA: 3-Hydroxy-3-Methylglutaryl-Coenzyme A; NCT: National Clinical Trial; HART: Hypofractionated Accelerated Radiotherapy; CLIC1: Chloride Intracellular Channel-1; ATGL: Adipose Triglyceride Lipase; HSL: Hormone-sensitive Lipase; LEGO: Laboratory Engineered Glioblastoma-like Organoid

Commentary

The use of metformin (*N*, *N*-dimethyl biguanide) in cancer therapy stemmed from epidemiological studies that demonstrated a reduced cancer risk in diabetic patients treated with metformin [1]. Later studies on breast cancer demonstrated that the survival benefit attributed to metformin is not mediated through improved glycemic control, but rather by direct anti-proliferative effects on tumor cells [2]. Increased interest in the anti-oncogenic properties of metformin prompted preclinical studies which suggested that metformin may exert its anti-tumor effects in various cancers [3], including glioblastoma (GBM) [4].

In the setting of GBM, the tumor O^6-methylguanine-DNA-methyltransferase (*MGMT*) methylation status and the use of temozolomide (TMZ)-based chemotherapy may reshape our understanding of the action of metformin in GBM cells. Metformin may serve to enhance the cytotoxic response to radiation and TMZ treatment [5,6]. DNA damage response (DDR) pathways induced after DNA damage from radio-chemotherapy facilitate GBM cell therapy resistance. By limiting the survival benefit from these pathways, metformin may augment the immediate effect of adjuvant therapy and delay GBM recurrence.

The action of metformin in GBM cells is mediated through multiple pathways, including the phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B)/ mechanistic target of rapamycin complex 1 (mTORC1), AMP-activated protein kinase (AMPK), RAS/RAF/mitogen activated protein kinase (MAPK), and mitochondrial signaling pathways [7]. However, the primary mechanism of action involves activation of AMPK, a central regulator of cellular energy homeostasis [8]. By inhibiting mitochondrial respiratory chain complex I, metformin increases the AMP/ATP ratio, leading to AMPK activation [8]. AMPK activation inhibits mTORC1 signaling, which plays a crucial role in regulating cell growth, proliferation, and survival in cancer cells [9], including GBM [10]. Among other GBM DDR pathways [5], alteration of the epidermal growth factor receptor (EGFR)/PI3K pathway is considered a key feature of TMZ resistance in GBM [11]. Ideally, knowledge of interactions between molecular signatures and specific DDR pathway dependence emerges from comprehensive bottom-up approaches that delineate pathways related to specific GBM molecular clusters under treatment conditions [12,13]. Nonetheless, knowledge of this connection may be incidental by identifying off-label anti-proliferative effects of medications with well-studied mechanisms of action, such as metformin.

We recently demonstrated in a retrospective study on *Isocitrate dehydrogenase 1* (*IDH1*)-wild type GBM patients that metformin use is associated with survival in patients with tumors with *MGMT* promoter methylation (m-*MGMT*) when compared to patients with tumors that showed absence of *MGMT* promoter methylation (u-*MGMT*) [14]. In this study, we excluded patients with low-grade gliomas or patients diagnosed with a pre-existing malignancy. Due to inherent biochemical differences (some listed here), we also excluded patients with *IDH-1* or *-2* mutation. In our patient cohort [14], metformin was mostly administered to nondiabetic patients to control steroid-induced hyperglycemia [14]. Further, in many patients with m-*MGMT* tumors who received metformin, glycosylated hemoglobin levels rarely exceeded 7%. Therefore, our findings indicate that metformin in GBM may have anti-proliferative effects that are independent of the role that metformin plays towards establishing a normoglycemic state.

Our findings renew interest in DDR dependence exploration in GBM and highlight the knowledge gap between known molecular signatures, such as the *MGMT* promoter methylation and related DDR pathway dependence. In this commentary, we aim to further our discussion on the possible mechanism of metformin in GBM cells and how this mechanism may be more effective in m-*MGMT* tumors.

Dependence on DDR pathways is proportional to the level of DNA damage [15]. Therefore, in m-*MGMT* tumor cells, DDR dependence may be increased **(Figure 1A)**. However, without exploiting this increased dependence on DDR pathways, our TMZ-based integrative approaches in the management of GBM may not be optimal. Enrichment annotation of differential protein-coding genes in m-*MGMT* and u-*MGMT* GBM cells showed a distinctive increase in expression of genes in the PI3K/Akt pathway of m-*MGMT* tumor cells [16]. Therefore, in the context of DDR pathway inhibition, the effect of metformin is likely asymmetrical, favoring the m-*MGMT* status. Through its AMPK-dependent and independent actions, metformin can provide a synergistic and/or an additive effect that helps limit the survival benefit by acting as an inhibitor of both cell cycle check points and DDR pathways [17].

Multiple reports demonstrated that metformin may act in 'synergism' with TMZ [18,19]. The synergistic effect of metformin and TMZ may contribute to the improved survival we observed with metformin treatment in m-*MGMT* tumor patients [14], as the TMZ effect is optimal in patients with m-*MGMT* tumors [20,21]. High-grade glioma tumor cells treated with TMZ, and metformin exhibited significantly greater suppression in proliferation than cells treated with TMZ alone [22]. Studies also showed that metformin enhances TMZ effect on TMZ-sensitive GBM cells and overcomes resistance in TMZ-resistant cell lines [19]. In these cells, combination treatment of metformin and TMZ promoted

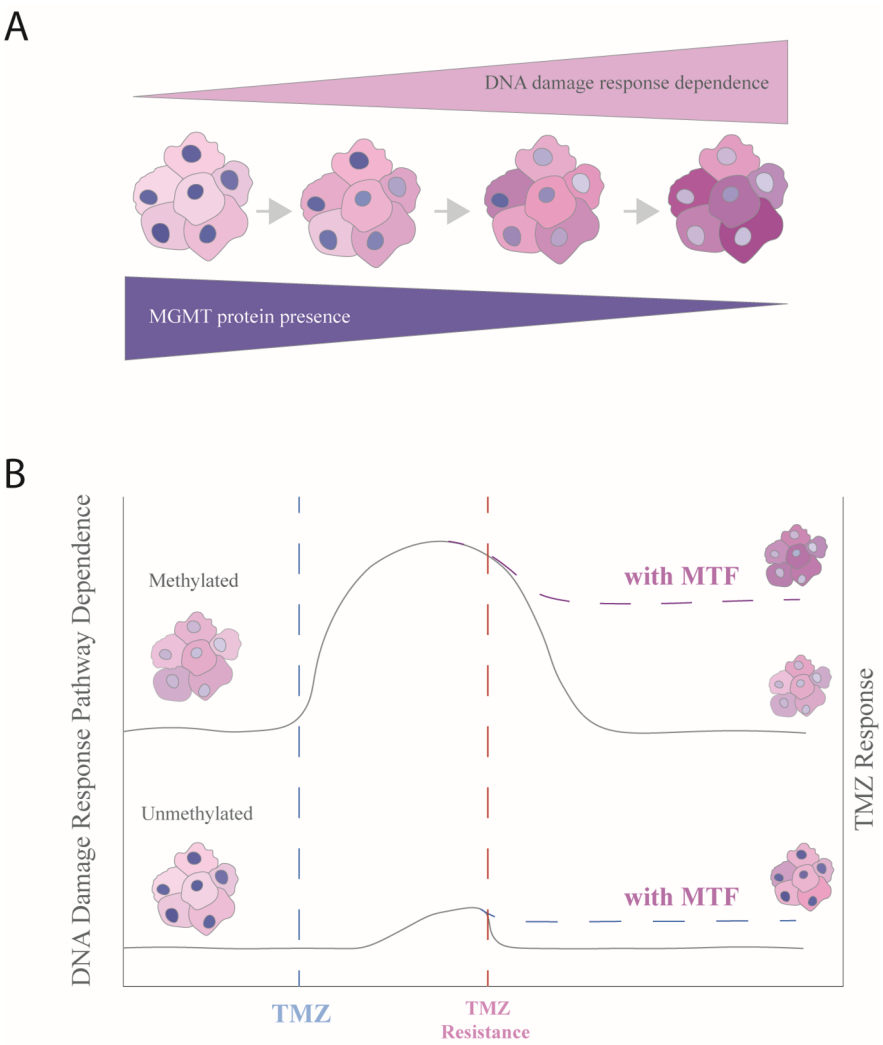

Figure 1: DNA methylation of the *MGMT* promoter and DNA damage response dependence. A) Dynamic and nonuniform changes in DNA methylation of the O^6-methylguanine-DNA-methyltransferase (*MGMT*) promoter over time may not only signify changes to tumor progression but also inform about tumor compensatory pathway dependence under treatment conditions. **B)** Proposed difference between *MGMT* promoter methylated and unmethylated tumors in compensatory pathway dependence and temozolomide (TMZ) response after metformin treatment (MTF) in glioblastoma (GBM). The reduced ability of methylated *MGMT* tumor cells to repair DNA in the face of TMZ alkylating damage increases the dependence of GBM *MGMT* methylated cells on compensatory DNA damage repair pathways. MTF's action in suppression of many compensatory growth pathways through AMP-activated protein kinase (AMPK) can delay the resistance to adjuvant therapy and prolong patient survival. Decreased dependence on compensatory DNA damage response pathways in *MGMT* promoter unmethylated tumor cells may preclude a similar efficacy for metformin in limiting resistance to TMZ.

apoptosis through increased Bax/Bcl-2 ratio [19]. *In vivo* work on tumor-bearing mice demonstrated that combining TMZ and metformin significantly slowed down the growth of TMZ-resistant tumors [19]. Another study indicated that TMZ induces AMPK activation and subsequent p53 activation by phosphorylation at Serine-15 [23]. In turn, metformin can induce AMPK activation and arrest in p53-null cells [24]. These data show that the effect of metformin in GBM cells may not only be synergistic but also additive to the effect of TMZ, irrespective of the actions downstream of AMPK activation.

However, it is worth noting that the cell models used to study the coadministration of TMZ and metformin are very different on the molecular level (including the *MGMT* status). As we show in this commentary, action of metformin through any one of its vast number of targets may be misinterpreted as 'additive' or 'synergistic' with TMZ without pharmacodynamic evidence. Therefore, until the relationship of metformin and TMZ can be studied in more relevant clinical systems, our current understanding of this relationship may be dependent on a very narrow focus. Prior to the emergence of results

from clinical prospective studies on the relationship between metformin and TMZ, it may be more convincing from an *in vitro* perspective, to show that metformin increases the sensitivity of GBM cells already resistant to TMZ. This notion is less speculative as it does not suggest a dual metformin/TMZ effect. Instead, by increasing the sensitivity of TMZ-resistant GBM cells, metformin may simply be acting through known mechanism to inhibit DDR pathways.

Akt overactivation is considered one of the main forms of GBM TMZ-resistance [25,26]. The increased activation of AMPK and suppression of Akt activation resulting from metformin treatment [27,28] may in part explain the ability of metformin to decrease the resistance to TMZ [29]. However, one report suggested that AMPK activation may be a stress response to anticancer chemotherapy rather than a direct effect of metformin in cancer cells [30]. But with the mounting evidence in favor of a metformin-mediated AMPK activation in tumor cells, it would be difficult to exclude a role for metformin in metformin with TMZ in suppressing the PI3K/Akt pathway. The complimentary role that metformin plays in decreasing resistance would then allow the primary cytotoxic effect of TMZ to be more effective, especially in patients with m-*MGMT* tumors [20] **(Figure 1B)**.

Given the inter-tumor heterogeneity in GBM, metformin may exhibit more or less effect based on the most prominent metabolic pathway driving tumor growth. Classifying metformin as an anti-Warburg therapeutic may be challenging based on its multiple targets **(Figure 2)** and its function in uncoupling glycolysis and oxidative phosphorylation. Metformin directly acts on mitochondria and shifts the balance between coupling and uncoupling reactions in favor of uncoupled reactions by inhibiting complex I-dependent respiration [31]. The inhibition of oxidative phosphorylation by metformin is accompanied by a compensatory increase in glycolysis [32]. In addition, *MGMT* methylation status-based potential differences in metabolism **(Figure 2)** further complicate the role of metformin in relation to GBM.

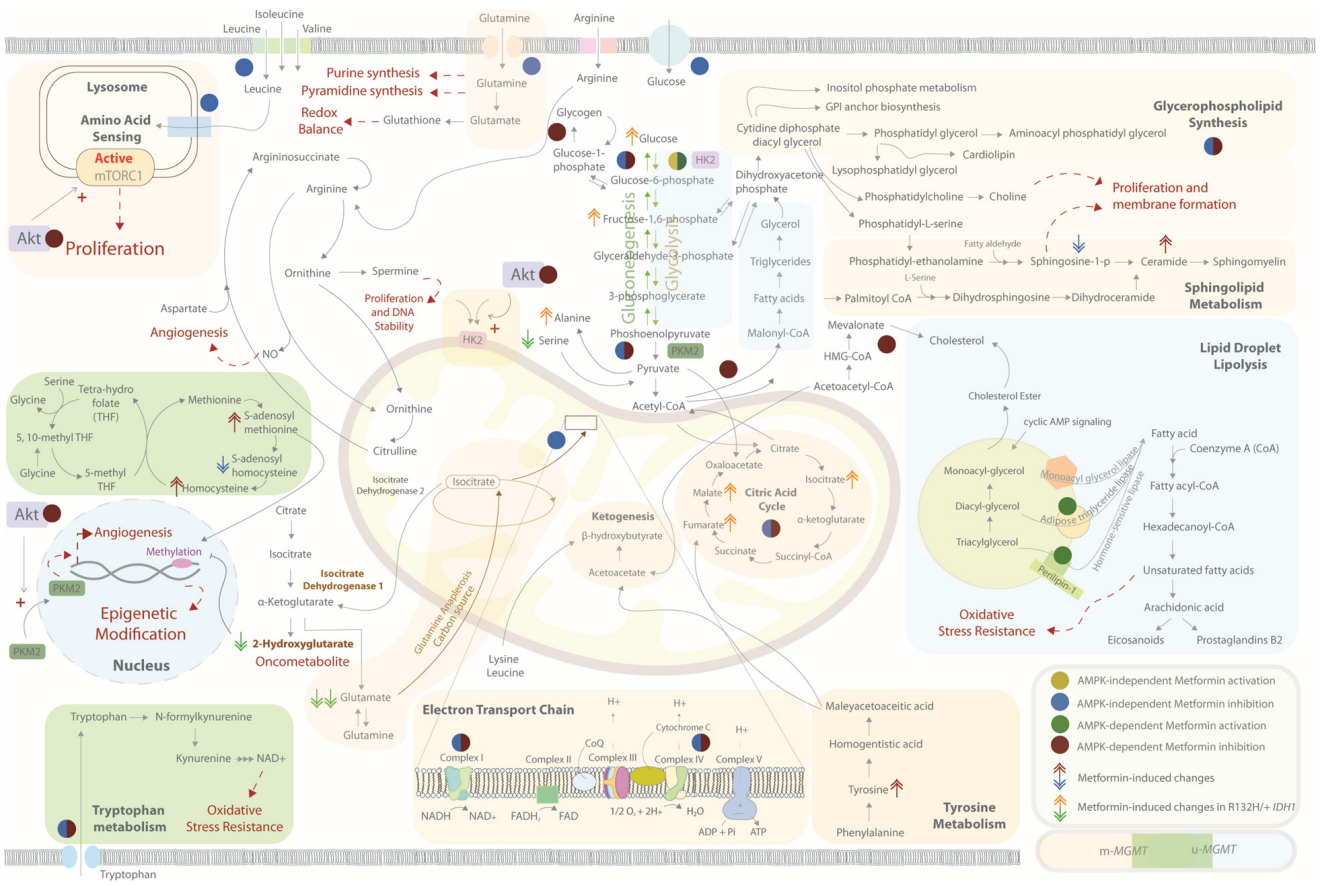

Figure 2: **Potential *MGMT* methylation status-based differences in metabolism and metformin targets**. Schematic detailing reported methylated (faded orange) and unmethylated (faded blue) O^6-methylguanine-DNA-methyltransferase (*MGMT*) promoter differences in metabolism and how metformin can regulate these respective targets. Faded green represents cellular processes that are reported to be important in glioblastoma cells without specific indication of *MGMT* status. HK2: Hexokinase 2; PKM2: Pyruvate Kinase M2; u-*MGMT*: *MGMT* promoter unmethylated; m-*MGMT: MGMT* promoter methylation.

The increased PI3K/Akt signature in m-*MGMT* tumor cells [16] suggests that the Akt-mediated effects on glucose metabolism may be more pronounced in m-*MGMT* tumor cells. Akt induces glucose dependence and promotes aerobic glycolysis in GBM cells [33]. Loss of PTEN in GBM cells and/or DDR dependence can ensure heightened Akt activation through constitutive activation of receptor tyrosine kinases, EGFR signaling, or platelet derived growth factor receptor α (PDGFRα) [34]. Interestingly, *PDGFRA* gene amplification or gain of function was found in 22% of progressive m-*MGMT* tumor compared to only 7.5% of u-*MGMT* tumors [35]. Further, Akt activation recruits hexokinase 2 (HK2) to the mitochondrial surface **(Figure 2)**, which helps in coupling glucose metabolism to oxidative phosphorylation. This is accomplished by phosphorylating glucose molecules using intramitochondrial ATP and preventing apoptosis by stabilizing cytochrome c [36]. In DDR-dependent m-*MGMT* tumor cells, metformin inhibition of Akt may prevent HK2 action and impact to a greater extent the proliferation of m-*MGMT* tumor cells.

Furthermore, metformin can inhibit pyruvate kinase M2 (PKM2) [37], the enzyme responsible for the conversion of phosphoenolpyruvate (PEP) to pyruvate. This potential function for metformin in GBM cells may be counterintuitive as PKM2 deletion was reported to accelerate mammary tumor growth [38], possibly by slowing down glycolysis and rerouting metabolites towards macromolecule formation necessary for tumor growth [39]. However, EGFR overactivation, a predominant event in GBM, and phosphorylation of PKM2 can induce translocation of PKM2 to the nucleus to act as a coactivator of hypoxia-inducible factor 1α (HIF-1α) [40] **(Figure 2)**. Although PKM2 activation and its role in metabolism are considered desirable for favorable clinical outcomes, the anti-proliferative role of metformin may impact the oncogenic epigenetic functions of PKM2 in the context of EGFR amplification **(Figure 2)**. From this angle, metformin use may be most beneficial with EGFR amplification and Akt activation, which are DDR events that may be targeted in TMZ-resistant m-*MGMT* tumor cells.

One study demonstrated more dysregulated metabolites of glycerophospholipid, sphingolipid, and tyrosine metabolism in the m-*MGMT* tumors compared to the u-*MGMT* status [16]. According to analysis of *KEGG* pathways these changes are related to changes in oxidative phosphorylation and biosynthesis of amino acids in the m-*MGMT* tumors compared to the u-*MGMT* tumors [16]. Metformin significantly decreased the synthesis of several glycerophospholipids [41] essential for membrane production of rapidly growing tumor cells [42]. Further, sphingosine-1-phosphate (S1P), which is produced during complex sphingolipid catabolism, stimulates proliferation and survival in GBM [43]. Metformin upregulates the genes coding enzymes that mediate conversion of S1P to sphingosine and downregulates ceramide degradation [43] **(Figure 2)**. This shift away from S1P promotes apoptosis and antiproliferative effects [43]. This further highlights how metformin may have a greater effect in GBM cells with a m-*MGMT* status.

Other GBM molecular determinants may be related to metformin function. Gain-of-function mutations in *IDH* gene result in the transformation of α-ketoglutarate to (D)-2-hyroxyglutarate (D-2-HG) leading to hypermethylation of DNA and histone, affecting the coding of tumor suppressor genes and oncogenes in lower-grade gliomas and *IDH* mutant grade IV astrocytoma tumors [44]. In *IDH1* mutant tumor cells, metformin blocks carbon entry into the citric acid cycle by blocking complex I of the ETC downstream of glutamine anaplerosis to α-ketoglutarate [45] **(Figure 2)**. Cancer cells grown in the presence of glutamine but absence of glucose are more sensitive to the effect of metformin [46], indicating that cells reliant on oxidative phosphorylation may be more prone to the effects of metformin. We believe that the link between DDR pathways and oxidative phosphorylation may suggest that the negative metformin effect on glutamine anaplerosis is likely more effective in m-*MGMT* GBM cells than on their u-*MGMT* counterparts.

p53 is also related to the biological effects of metformin. Metformin was found to decrease the levels of p53 through proteosome-mediated degradation to induce apoptosis in HeLa cells [47]. However, other studies demonstrated that the metformin effect is mediated through AMPK-linked phosphorylation and activation of p53 [48]. Although the latter scenario is likely more relevant for the effect of metformin in GBM, the relationship with *MGMT* promoter methylation is not clear. However, two studies on GBM found a higher frequency of *TP53* mutations with *MGMT* promoter methylation [49,50]. Furthermore, using laboratory engineered glioblastoma-like organoid (LEGO), one study reported that metformin has no significant effect on the growth of *PTEN and TP53*-null when compared to wild-type LEGOs [13]. Together these observations indicate that p53 may in part mediate the actions of metformin in m-*MGMT* GBM tumor cells.

Metformin is also known to suppress expression of amino acid transporters in liver cells limiting branched amino acid (leucine, isoleucine, and valine) transport [51] **(Figure 2)**. Metformin suppresses uptake of branched amino acids, arginine, and glutamine by reducing availability of system A transporter (SNAT2) with the leucine antiporter (LAT1) and Na^+-independent cationic amino acid transporter 1 (CAT1) [51]. In contrast, there is evidence to suggest that metformin causes a mild increase in cellular tyrosine levels [51]. m-*MGMT* cells rely heavily on amino acids like glutamine and branched chain amino acids to sustain their growth and survival under stress. Branched amino acids are necessary in mTORC1 signaling and growth [52]. By limiting the availability of branched amino acids, such as leucine [51], metformin may disrupt an intricate process of lysosomal amino acid sensing that is largely AMPK-independent [53]. Lysosomal amino acid sensing plays a crucial role in mTORC1 activation through ensuring the docking of mTORC1 to the lysosomal surface, through regulation of vacuolar ATPase complex (V-ATPase) assembly and activity [54]. The recruitment of

mTORC1 from the cytoplasm to the lysosomal surface enables PI3K/Akt/Rheb pathway-mediated activation of mTORC1. Therefore, metformin may act in a dual fashion to suppress both mTORC1-activating arms: the amino acid-dependent recruitment of mTORC1 to the lysosomal surface and the AMPK-regulated activation of mTORC1 after lysosomal surface docking **(Figure 3)**. This ensures optimal suppression of mTORC1 and decreases the chances of resistance in tumor cells. Remarkably, the inhibition of mTORC1 in u-*MGMT* GBM cells did not result in a reduction in MGMT protein availability, despite significant reduction in global protein synthesis rates [55]. This demonstrates that MGMT synthesis and function in tumor cells may not be dependent on mTORC1 signaling and that *MGMT* gene methylation is the primary method for *MGMT* expression reduction. Furthermore, these findings indicate that after TMZ and radiation treatment, u-*MGMT* tumors may be less reliant on DDR pathways.

A general view of **Figure 2** shows the dependence of m-*MGMT* tumor cells on the mitochondria, the main target of metformin. However, metformin may also target cellular processes that are evidenced to be more related to u-*MGMT* tumorigenicity. For example, metformin was shown to reduce GBM cell viability through inhibition of the chloride intracellular channel-1 (CLIC1) current, inducing G1 arrest in GBM stem cells [56]. Remarkably, CLIC1 bioavailability was elevated in *IDH*-wild type and in u-*MGMT* tumors [57], indicating that the metformin effect may be more pronounced with *MGMT* promoter unmethylation. Other actions of metformin may be more controversial in u-*MGMT* cells. Metformin activates,

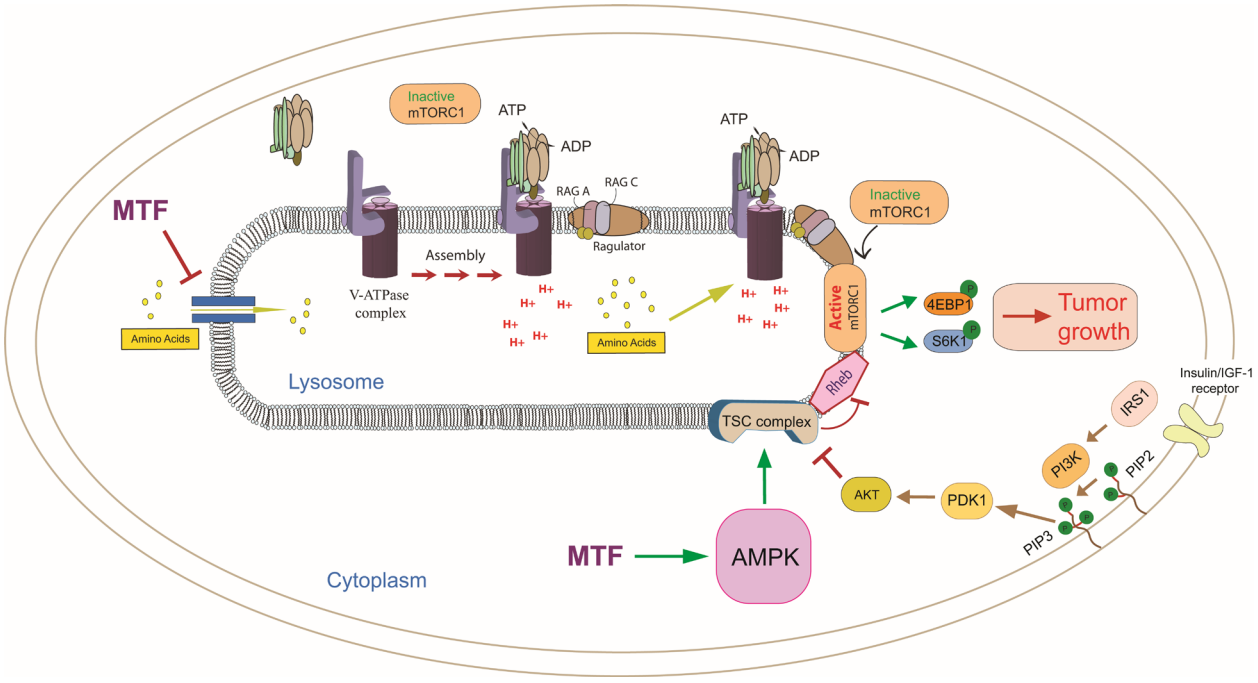

Figure 3: Metformin potential dual efficacy with inhibition of mTORC1. Metformin (MTF) can inhibit the mechanistic target of rapamycin (mTOR) complex 1 through inhibiting amino acid entry into the lysosome and amino acid sensing, a process that utilizes the vacuolar ATPase complex (V-ATPase), the Ragulator, and the RAG GTPases. By preventing amino acid sensing, MTF may disrupt the machinery responsible for the docking of mTORC1 to the lysosomal surface, a step necessary for activation of mTORC1 by the Ras homolog enriched in brain (Rheb). Rheb, a GTP-binding protein, acts as the downstream effector of the phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B) pathway. Growth factor presence activates the insulin receptor substance-1 (IRS-1), which then activates the PI3K and mediates the phosphorylation of phosphatidylinositol (4,5)-biphosphate (PIP2) factor to phosphatidylinositol (3,4,5)-triphosphate (PIP3). This step leads to the activation of protein kinase 3-phosphoinositol dependent protein kinase-1 (PDK1) and to the activation of Akt. Inhibition of the tuberous sclerosis (TSC) complex by Akt disinhibits Rheb and maintains its GTP-bound active form, allowing the activation of mTORC1 on the lysosomal surface. mTORC1 activation leads to the phosphorylation of multiple targets, including the downstream effectors: the eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) and p70S6 kinase to initiate translation and promote tumor growth. This is only possible with the recruitment of mTORC1 to the lysosomal surface after amino acid sensing. Under physiological conditions, this dual activation of mTORC1 ensures mTORC1 activation only with the presence of amino acids necessary for translation. With increased dependence on mTORC1 in glioblastoma cells, metformin may therefore serve as an ideal therapeutic agent to decrease survival and overcome drug resistance.

through AMPK, the adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) activity resulting in fatty acid catabolism [58]. This function may enhance u-*MGMT* cell proliferation, as evidence showed that lipid droplets are likely metabolized more in u-*MGMT* compared to m-*MGMT* tumors [16]. Further, transcription of several critical enzymes important in fatty acid elongation were upregulated in u-*MGMT* tumor cells relative to m-*MGMT* cells, in addition to a surge in unsaturated fatty acids [16]. In particular, unsaturated fatty acids were shown to alleviate oxidative stress [59]. This paradoxical effect by metformin may be offset by the ability of metformin to inhibit cholesterol synthesis [60], an effect tested with the use of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor (Atorvastatin) in u-*MGMT* cells [16]. These data show that the effect of metformin in u-*MGMT* GBM cells requires more scrutiny with some paradoxical roles for metformin in cellular processes critical in u-*MGMT* GBM cell tumorigenesis. This can be contrasted with the role of metformin in m-*MGMT* highlighting how the role of metformin in m-*MGMT* is more straightforward with less projected controversial actions.

An emerging role for metformin places it as a factor regulating metabolic processes and conversions that can impact nuclear functions, such as epigenetic modification. This potential function for metformin in GBM cells is very interesting (especially from the standpoint of this commentary) as metformin may be able to contribute to DNA methylation (potentially making it both a contributor to and a beneficiary of *MGMT* promoter methylation). For example, metformin increases homocysteine levels and decreases *S*-adenosylhomocysteine (SAH), a strong inhibitor of *S*-adenosylmethionine (SAM), promoting global DNA methylation [61]. Additionally, metformin decreases 2-HG production, an inhibitor of DNA methylation [62]. In turn, DNA methylation regulates multiple factors involved in glycolysis, including glucose transporter 1, lactate dehydrogenase, PKM2, HK, and GAPDH [63], demonstrating an important link between DNA methylation and the Warburg effect. The nuclear factor erythroid 2 (NF-E2)-related factor 2 (Nrf2) is a transcriptional activator of many cyto-protective genes, and its high expression was noted in aggressive tumors [64]. Methylation of the negative regulator of NrF2, *kelch-like ECH-associated protein 1* (*KEAP1*) gene promoter region, decreases Nrf2 degradation and activates glucose 6-phosphate dehydrogenase, transketolase, IDH, and other enzymes critical in glycolysis bypass pathways, such as the pentose phosphate pathway [65]. On the other hand, DNA methylation can disrupt the α-ketoglutarate-dependent prolyl-hydroxylase/von Hippel-Lindau oxygen sensing pathway which promotes aerobic glycolysis through disinhibition of HIF, a factor shown to be downregulated by methylation-sensitive hypoxia response elements [66]. In addition, mitochondrial DNA is also amenable to methylation leading to oxidative phosphorylation dysfunction [67]. Together, these observations demonstrate the complexity of the relationship between DNA methylation and the tumor metabolic profile.

Non-*MGMT* DNA methylation may limit or improve the benefit from bypass glycolytic and angiogenesis pathways in u-*MGMT* GBM cells. Interestingly, a 31-CpG methylation signature involving glycolysis and angiogenesis identified u-*MGMT* GBM cells with increased TMZ sensitivity [68]. However, lack of associations between *MGMT* promoter methylation and methylation of other metabolism-related elements [65], highlights *MGMT* promoter methylation as a pivotal event with relation to GBM metabolism. In this light, *MGMT* promoter methylation may reprogram the metabolic dependence in tumor cells by increasing the dependence on glycolytic coupling and mitochondrial energy production. Consequently, non-*MGMT* DNA methylation regulation of the Warburg effect becomes secondary to effects that target mitochondrial function in m-*MGMT* GBM cells. Mitochondrial function with *MGMT* promoter methylation becomes the new *Achille's heel* that can be effectively targeted with metformin.

In conclusion, metformin represents a promising adjunctive therapy for GBM based on its multifaceted mechanisms of action **(Figure 2)** and synergistic effects with TMZ and conventional therapies. Preclinical studies provided compelling evidence supporting the anti-tumor efficacy of metformin in GBM models, while retrospective clinical data suggest a potential survival benefit in GBM patients [14]. Given the potential of metformin to complement with standard GBM therapies, prospective studies were initiated albeit with contradicting preliminary outcomes. Metformin efficacy as a neo-adjuvant agent in conjunction with TMZ and hypofractionated accelerated radiotherapy (HART) in GBM was assessed in our institution [69]. This phase 2 (NCT02780024) study has thus far confirmed the safety and tolerability of metformin and reported favourable outcomes on the 50 GBM patients recruited [69]. To the contrary, a pooled analysis from 1731 GBM patients concluded that solitary metformin treatment or in combination with TMZ were not associated with improved survival [70]. More studies, such as the phase 2 pilot study investigating the role of ketogenic diets and metformin treatment on GBM patient outcome (NCT04691960) and another trial studying the effect of metformin in addition to TMZ and radiotherapy (NCT04945148), will help resolve the conflicting data.

To our knowledge no study has previously focused on how metformin may exploit biological differences based on *MGMT* promoter methylation status. This knowledge would enhance the translation of preclinical findings into clinical practice, especially with the advent of readily available DNA methylation-based central nervous system tumor classifier tools in diagnostic settings [12]. Ongoing and future clinical trials will be pivotal in determining the clinical efficacy, safety, and optimal use of metformin in the management of GBM, ultimately aiming to prolong survival and enhance quality of life for patients with this devastating disease. Understanding the role of metformin in GBM will harness work on other combinatorial approaches involving EGFR inhibitors, PI3K inhibitors, or immunotherapies.

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