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Cancer stem cell molecular reprogramming of the Warburg effect in glioblastomas: a new target gleaned from an old concept

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Practice points

- Identification of potential enzyme targets driven by hypoxia in the setting of glycolytic phenotype will delineate the mechanisms that reverse the metabolic shift induced by Warburg effect.
- Inhibition of glucose metabolism may be redirected to oxidative phosphorylation by targeting GLUT1, HK2, PK and PDK.
- GLUT1 represents a viable target for future study in GBM treatment.
- Higher expression of PDK1 in glioblastoma renders PDK1 inhibition as a plausible target for therapy.

Prior targeted treatment for glioblastoma multiforme (GBM) with anti-angiogenic agents, such as bevacizumab, has been met with limited success potentially owing to GBM tumor's ability to develop a hypoxia-induced escape mechanism – a glycolytic switch from oxidative phosphorylation to glycolysis, an old concept known as the Warburg effect. New studies points to a subpopulation of cells as a source for treatment-resistance, cancer stem cells (CSCs). Taken together, the induction of the Warburg effect leads to the promotion of CSC self-renewal and undifferentiation. In response to hypoxia, hypoxia-inducible transcription factor is upregulated and is the central driver in setting off the cascade of events in CSC metabolic reprogramming. Hypoxia-inducible transcription factor upregulates GLUT1 to increase glucose uptake into the cell, upregulates HK2 and PK during glycolysis, upregulates LDHA in the termination of glycolysis, and downregulates PDH to redirect energy production toward glycolysis. This review aims to unite these old and new concepts simultaneously and examine potential enzyme targets driven by hypoxia in the glycolytic phenotype of CSCs to reverse the metabolic shift induced by the Warburg effect.

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Background

Despite multimodal therapeutics, including surgery, chemotherapy and radiotherapy, prognosis for glioblastoma multiforme (GBM) remains poor with a median 15-month survival time [1]. Targeted treatment is hindered by the heterogeneity of GBMs, resulting in cells within the same tumor exhibiting variability in downstream molecular aberrations [2]. Further adding to the complexity of treatment is recent evidence pointing to a newly identified cancer stem cell (CSC) phenotype consisting of a subpopulation of treatment-resistant cells equipped with the capability to

KEYWORDS

- GLUT1 • HK2 • hypoxia
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- Warburg effect

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self-renew and recapitulate the initial tumor [3]. To overcome these challenges, recent studies are directing investigation at upstream molecular mechanisms that are common among all GBM cells for molecular modulation [2].

GBMs are aggressive tumors characterized by uncontrolled vascular and cell growth, hypoxia and large numbers of aberrant gene mutations [3–5]. Targeting angiogenesis with VEGF antibodies, such as bevacizumab, has been met with limited success, suggesting that GBMs develop a means to evade treatment and survive under the hypoxic conditions generated by anti-angiogenesis [3,6]. Furthermore, as GBMs proliferate, they expand beyond their existing blood supply, intensifying the hypoxic environment [7]. Recent studies point to a molecular escape mechanism that exchanges energy production from aerobic mitochondrial oxidative phosphorylation (OXPHOS) to anaerobic cytoplasmic glycolysis [3,7–8]. Interestingly, despite the upregulation of VEGF, complete oxygen restoration does not occur and glycolysis persists in GBMs – paradoxical aerobic glycolysis and the glycolytic phenotype – the 1920s concept of the Warburg effect [7,9]. Examination into the modulation of this molecular reprogramming provides promising new direction in GBM targeted treatment gleaned from an old concept. Here, we discuss potential enzyme targets to reverse the Warburg effect and restore OXPHOS metabolism to eliminate the survival advantage afforded to GBM cells with the glycolytic phenotype.

Reversing the Warburg effect & the glycolytic phenotype

Though OXPHOS metabolism provides more energy with 36 ATPs versus glycolytic metabolism with two ATPs, the glycolytic phenotype provides survival and proliferative advantage to GBMs by decreasing their reliance on aerobic respiration. Energy production terminating in glycolysis augments the levels of lactic acid to induce the breakdown of extracellular interstitial matrix, enabling tumor expansion and metastasis [2,8,10–11]. Furthermore, the glycolytic phenotype confers anti-apoptotic resistance through the direct anti-apoptotic effects of glycolytic enzymes, inhibition of mitochondrial-dependent apoptosis through hyperpolarization, reduction in reactive oxygen species (ROS) and decreased mitochondrial function [2,8,10–11].

In vitro, hypoxia promotes CSC self-renewal, prevents CSC differentiation [12] and induces the

glycolytic phenotype by upregulating hypoxia-inducible transcription factor (HIF) [7]. HIF is the central driver that sets off the cascade of events in the CSC metabolic reprogramming from OXPHOS to glycolysis. HIF upregulates GLUT1 [13,14] to increase glucose uptake [15]; upregulates HK2 [16] and PK [17] during glycolysis [15]; upregulates LDHA in the termination of glycolysis [15,18] and downregulates PDH to redirect energy production toward glycolysis in the observed glycolytic phenotype (Figure 1) [8]. Preventing the glycolytic phenotype in CSCs can potentially be achieved by reversing the Warburg effect by altering this hypoxia-induced metabolic switch and targeting these specific enzymes to decrease tumor cell viability, while sparing normal cells [11]. Reversing the Warburg effect requires detailed understanding of the underlying molecular mechanisms that occur. Hypoxia induces the upregulation of HIF. In turn, HIF upregulates GLUT1. GLUT1 facilitates the entry of glucose into the cytoplasm of the cell where glycolysis ensues. HK phosphorylates glucose during the first step of glycolysis. PK mediates the production of pyruvate during the last step of glycolysis. At this point, the gatekeeping enzyme, PDH determines whether glucose metabolism will terminate in glycolysis or enter OXPHOS and the tricarboxylic acid (TCA) cycle within the mitochondria [9]. Energy production terminating in glycolysis results in the conversion of pyruvate to lactate by LDHA [18]. Energy production with OXPHOS results in the irreversible conversion of pyruvate to acetyl-CoA through decarboxylation by PDH [19,20]. These pathways introduce multiple mechanisms and enzyme targets: inhibition of hypoxia-induced effects by targeting HIF, inhibition of glucose metabolism by targeting GLUT1, inhibition of glycolysis by targeting HK2 and PK and redirection to OXPHOS by blocking PDH inhibitors.

• Hypoxia-inducible transcription factor

HIF is the principle factor that initiates the cascade of events that leads to the Warburg effect and reprograms OXPHOS to glycolysis in the glycolytic phenotype. Heddleston *et al.* explored the possibility that HIF also reprograms GBM to a secondary phenotype by shifting differentiated nonstem cells to an undifferentiated stem cell phenotype [12]. Both HIF1 α and HIF2 α regulate GBM stem cells VEGF levels. Although glycolytic genes are predominantly,

Figure 1. Cancer stem cell glucose metabolism. Glucose is transported into the cell and undergoes glycolysis. Energy production can terminate in glycolysis or enter oxidative phosphorylation. In cancer stem cells, a metabolic reprogramming occurs to redirect energy production from oxidative phosphorylation to glycolysis – the Warburg effect.

if not exclusively, transactivated by HIF1 α in various cancers [21–23], HIF1 α demonstrates proliferation and survival only in nonstem GBM cells. HIF2 α is found exclusively in the GBM stem cell phenotype [12]. Increased expression of stem cell markers OCT4 (HIF2 α specific target), NANOG (key in maintaining pluripotency in embryonic stem cells) and c-Myc (key in GBM CSC maintenance) is demonstrated in cells containing HIF2 α [12]. Though there are existing inhibitors of HIF2 α , such as small-molecule iron-regulatory protein 1 inhibitors (Table 1) [24] and Mendez *et al.* demonstrated reduced invasive capability of GBM *in vivo* with HIF1 knockdown [25], further studies are needed to elucidate the exact implications of HIF on prohibiting transformation to the CSC and glycolytic phenotypes. Worth mentioning is one clinical trial (Table 2) completed in 2012 that recognized the significance of HIF in GBMs that detected levels of HIF in GBMs with varying degrees of vascularization (Table 3).

• GLUT1

GLUT1 is a member of the GLUT/SLC2A family of transporters and GLUT1 facilitates the

transport of glucose across the cell membrane [31]. HIF1 induces the upregulation of GLUT1, which enables the Warburg effect by elevating the levels of intracellular glucose to facilitate glycolysis and permit tumor progression [9,13,30]. Modulating GLUT1 can lead to reduced levels of glucose and halt the initiation of the Warburg effect [9]. Shibuya *et al.* demonstrated increased sensitivity to GLUT1 in CSCs in comparison to non-CSCs [9]. WZB117 (Table 1), a GLUT1 inhibitor, prevents CSCs from tumor initiation, suggesting that GLUT1 performs a key role in the initiation and self-renewal of GBMs [9]. Though WZB117 was not shown to inhibit tumor progression in existing tumors, some studies demonstrate inhibited tumor progression with silenced GLUT3 in CSCs [9].

Of note, GLUT1 can also perform a role in the treatment of GBM through enhanced drug delivery. One of the challenges of chemotherapy is poor tumor penetration resulting from poor drug delivery due to inability to penetrate the blood–brain barrier (BBB) [32]. The advantage of GLUT1 is its predominant expression on brain capillary luminal surfaces and the choroid plexus and well as its ability to facilitate the transport

Table 1. Glycolytic phenotype inhibitors.

Study (year)	Enzyme	Inhibitor	Ref.
Zimmer <i>et al.</i> (2008)	Hypoxia-inducible transcription factor (HIF)	Iron-regulatory protein 1	[24]
Shibuya <i>et al.</i> (2015)	Glucose transporter 1 (GLUT1)	WZB117	[9]
Zhao <i>et al.</i> (2013)		Phloretin	[26]
Wolf <i>et al.</i> (2011)	Hexokinase 2 (HK2)	3-bromopyruvate	[16]
Pistollato <i>et al.</i> (2010)		2-deoxyglucose	[27]
Neary <i>et al.</i> (2013)		Clotrimazole	[28]
Luo <i>et al.</i> (2012)	Pyruvate kinase M2 (PKM2)	Shikonin Alkannin Compound 3	[15]
Seliger <i>et al.</i> (2013)	Lactate dehydrogenase A (LDHA)	Oxamate	[29]
Michelakis <i>et al.</i> (2010)	Pyruvate dehydrogenase (PDK1)	Dichloroacetate	[8]

of mannose as well as glucose [32]. Exploiting this capability, researchers have utilized mannosylated liposomes to overcome the challenge of penetrating the BBB [32]. Ying *et al.* conjugated a mannose analog, p-aminophenyl- α -D-mannopyranoside (MAN) to the surface of daunorubicin liposomes to improve drug transport across the BBB [32]. Traditionally used in the treatment of colon cancer, phloretin (Table 1), a GLUT1 inhibitor, has shown to produce synergistic effects with daunorubicin [33] and also represents a viable target for future study in GBM treatment.

• HK2

Under hypoxic conditions, HIF and stem cell factor c-Myc activate HK2 [31]. HK2 is a member of HK family of enzymes that mediates the first irreversible, rate-limiting step in glycolysis through the phosphorylation of glucose to glucose-6-phosphate [31]. Moreover, HK2 performs a pivotal role in inhibiting mitochondrial-dependent apoptosis by binding to voltage-dependent anion channels (VDACs) in the mitochondrial membrane [11] and preventing Bax, a pro-apoptotic member of the Bcl-2 family, from binding to voltage-dependent anion channels and heightening cytochrome c release [31]. Recent studies show that reversing the Warburg effect by reducing glycolysis

through silencing of HK2 in GBMs results in decreased expressions of HIF1 α and VEGF in addition to increased sensitivity to radiotherapy and temozolamide [16]. Selected activity of HK2 is supported by one study that demonstrated the addition of the isoform HK1 failed to rescue the glycolytic phenotype despite the restoration of total hexokinase activity [34].

There are several HK2 inhibitors that can be utilized for further study to block the actions of HK2. For example, clotrimazole (Table 1), an antifungal agent used in the study of melanoma [35], detaches HK2 from the mitochondria [28]. 3-Bromopyruvate (Table 1), another inhibitor of HK2, has been shown to augment the efficacy of carmustine on GBM stem cells under hypoxic conditions [36] and inhibit tumor progression *in vivo* [26]. A third agent, 2-deoxyglucose (Table 1), a glucose analog that competitively inhibits glycolytic enzymes, has been shown to influence Warburg effect reversal through HIF1 α degradation in GBM cells and enhanced PDH pyruvate dehydrogenase to direct energy production to OXPHOS and to induce differentiation in CSCs [27]. However, it is important to note that prospective studies face a challenge in selectively targeting HK2 due to lack of specificity for HK2 and systemic toxicity [34] in existing agents, such as 3-bromopyruvate and 2-deoxyglucose [9].

Table 2. Clinical trials.

Enzyme	Identifier	Phase	Status
HIF	NCT01200134: HYPONCO – Hypoxia in Brain Tumors	Phase II	Completed July 2012
PDK1	NCT00540176: The Safety and Efficacy of DCA for the Treatment of Brain Cancer	Phase I	Completed March 2014
PDK1	NCT00566410: A Phase I, Open-Labeled, Single-Arm, Dose Escalation, Clinical and Pharmacology Study of Dichloroacetate (DCA) in Patients With Recurrent and/or Metastatic Solid Tumors	Phase I	Active, not recruiting

Table 3. Glycolytic enzymes involved in glycolytic phenotype.

Study (year)	Glycolytic enzyme	Glucose metabolism pathway	Function	GBM progression	Ref.
Shibuya <i>et al.</i> (2015)	Glucose transporter 1 (GLUT1)	Glycolysis	Transports glucose	Upregulation	[9]
Wolf <i>et al.</i> (2011)	Hexokinase 2 (HK2)	Glycolysis – initial step	Phosphorylates glucose	Upregulation	[16]
Yang <i>et al.</i> (2012)	Pyruvate kinase M2 (PKM2)	Glycolysis – final step	Phosphorylates ADP	Upregulation	[30]
Valvona <i>et al.</i> (2015)	Lactate dehydrogenase A (LDHA)	Glycolysis	Converts pyruvate to lactate	Upregulation	[18]
Velpula <i>et al.</i> (2013)	Pyruvate dehydrogenase (PDK1)	TCA entry	Inhibits pyruvate dehydrogenase	Upregulation	[4]

• PKM2

The final rate-limiting step of glycolytic metabolic pathway terminates in production of pyruvate and is mediated by PK. PK converts phosphoenolpyruvate (PEP) into pyruvate by dephosphorylation to generate ATP [31]. The M1 isoform of PK observed in normal adult differentiated tissue (PKM1) [10,37]. The M2 isoform (PKM2) is not expressed in normal adult brain [38] observed during embryonic development and regulates the transition between the G1-S phase through the regulation of cyclin D1 [2,10,37,39]. For the first time Kefas *et al.* demonstrated the expression of PKM2 in GBM stem cells. Further, the targeting of PKM2 in GBM stem cells by miR-326 decreased the metabolism, intolerance for oxidative stress accompanied by decrease in ATP levels. Inhibition of PKM2 was lethal to glioma stem cells and without toxic effects to normal human astrocytes [38]. GBMs express significantly higher levels of PKM2 compared with normal brain [10] as a result of an isoform switch from PKM1 to PKM2 [17]. Distinct from PKM1, HIF directly targets PKM2 and enhances tumor progression [40] by suppressing mitochondrial function [2,10,37] through the reduction of ROS production [41], and inducing downstream processes that upregulate c-Myc transcription [12] in a positive feedback loop that amplifies the expression of GLUT1, LDHA [30] and HIF [40].

Christofk *et al.* demonstrated reversal of the Warburg effect through the reduction of glycolysis by replacing the PKM2 isoform with the PKM1 isoform [10,37]. Other inhibitory mechanisms include PKM2 inhibitors, such as shikonin, alkannin, compound 3 (Table 1) and siRNA [41]. Shikonin and alkannin demonstrate greater efficacy in inhibiting PKM2 in comparison to compound 3 [41] and exhibit >50% selective inhibition of PKM2 in comparison to PKM1 [42]. siRNA has been shown to decrease tumor progression in xenograft mice [41]. Though other studies have shown that PKM2

knockdown inhibits GBM proliferation and survival [38], PKM2 has not yet been showed to be correlated patient outcome and should be further delineated in future studies [17].

• LDHA

Following glycolysis, LDHA, a member of the LDH family of tetrameric enzymes [31], mediates the conversion of pyruvate to lactate and the conversion of nicotinamide adenine dinucleotide (NAD)H to NAD⁺ [18]. While HIF induces the upregulation of LDHA, LDHA establishes a positive feedback loop with HIF1 resulting in heightened concentrations of lactate [18]. Elevated lactate levels induce production of TGF- β and is associated with tumor angiogenesis, migration [29], proliferation and treatment resistance [18]. Furthermore, elevated lactate levels generate an acidic environment which promotes CSCs induction [18] and self-renewal, as suggested by Zhang *et al.* who demonstrated significant correlation between LDHA and Oct-4, an embryonic stem cell gene involved in self-renewal in intestinal-type gastric cancer [18].

Yang *et al.* demonstrated reversal of the Warburg effect with silenced LDHA, which resulted in decreased tumor progression and the reduction in lactate production, thus compelling the cells to utilize OXPHOS as an energy source [16,18] and enabling mitochondria apoptosis through ROS production [18]. Oxamate (Table 1), a pyruvate analog [18], has been used to competitively inhibit LDHA [29] in treatment-resistant breast cancer and has been shown to increase the sensitivity of breast cancer cells to paclitaxel [18]. In GBMs, Seliger *et al.* demonstrated oxamate decreased tumor migration in a dose-dependent fashion [29]. Worth noting are recent studies that report LDHA is paradoxically inhibited in the glioma subtype, isocitrate dehydrogenase [18,43]. However, the isocitrate dehydrogenase subtype, is characterized by increased survival, improved prognosis and insidious tumor progression [18].

• PDK1

Entry into the TCA cycle is regulated by the gatekeeping enzyme PDH [9] and is downregulated under hypoxic conditions, redirecting energy production away from OXPHOS and toward glycolysis. PDK1, an inhibitor of PDH, phosphorylates PDH to inhibit the entry of pyruvate into the TCA cycle [3]. Inhibition of PDH uncouples OXPHOS and results in reduction of ROS [21]. High expressions of PDK1 observed in GBM in comparison to normal brain [3] rendering PDK1 inhibition a plausible target for therapy.

Dichloroacetate (DCA) (Table 1) is a small-molecule orphan drug that reverses the Warburg effect through the inhibition of PDK1 through dephosphorylation [3–4,7,9–10]. Traditionally used as a metabolic modulator in the treatment of hereditary mitochondrial disease and lactic acidosis, DCA also has the ability to penetrate the BBB [3,8,11,44]. DCA's efficacy is linked to sensitization to mitochondrial-dependent apoptosis by restoration of OXPHOS, mitochondrial hyperpolarization and heightened release of cytochrome c, resulting in enhanced generation of ROS and activation of caspases [34]. Worth mentioning is the regression of the tumor without unwanted toxicity in normal cells [3,8,11,44]. Of note, the glycolytic phenotype can also result postradiotherapy through the uncoupling of OXPHOS driving a metabolic shift to glycolysis, resulting in radioresistance and elevated levels of PDK [45]. By reversing the glycolytic phenotype through the inhibition of PDK, DCA has been shown to provide synergy to the effects of radiotherapy by resensitizing GBM cells to G2–M cell cycle arrest induction and intensifying radiotherapy ROS-induced DNA damage [45]. The translation of DCA utilization into clinical oncology is in progress with clinical trials (Table 2). Interestingly, the isoform PDK2, a ubiquitously expressed isoenzyme, mimics the effects of DCA when silenced [11] and may be a worthy target to investigate in the future along with PDK1. To our knowledge, PDK3 has not been studied in brain tumors, though it has been studied in other cancers, such as melanoma [46]. PDK3 represents a worthwhile enzyme target for investigation because of its unique lack of inhibition by elevated levels of pyruvate, unlike PDK1, PDK2 and PDK4 [47]. As well, existing studies of PDK4 signify need for further exploration as overexpression of PDK4 has demonstrated decreased cell proliferation [48].

Conclusion & future prospects in therapy

Alteration or reprogramming cellular metabolism introduces new opportunities in GBM diagnostics and therapeutics garnered from the long-standing concept of the Warburg effect. Though molecular modulation presents a new avenue to explore, further elucidation is needed to translate these potential benefits into clinical oncology. Future studies must consider inhibition of glycolysis is not selective for GBM cells and may have adverse effects on noncancer cells in tissues that are reliant on glycolysis as their energy source, such as the brain and skeletal muscle [2].

GLUT1 is an appealing target enzyme for further investigation because it is predominantly expressed in CSCs. Likewise, PKM2 is a viable target enzymes because it is not required for the survival and proliferation of transformed astrocytes and provides an advantage in its selectivity for GBM cells without toxicity to normal cells [38]. LDHA inhibition may represent a challenge because existing inhibitors exhibit low potency and the high doses of existing agents are required to achieve desired effects [18]. In addition, studies of LDHA inhibition in brain tumors has been limited and also faces the challenge of drug delivery across the BBB [18].

Future prospects in upcoming treatments can center on combination therapy to link successive inhibitory metabolic mechanisms together. For example, Papandreou *et al.* suggests combination therapy with LDHA inhibition and PDK1 inhibition to obstruct pyruvate conversion to lactate and redirect pyruvate to OXPHOS metabolism, respectively [20]. Though there are challenges to molecular reprogramming, existing studies show promise and warrant further investigation and we also emphasize that further studies are needed to explore the value/importance of inhibiting specific glycolytic enzymes to decrease tumor burden.

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