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Aerobic Glycolysis: A Novel Target in Kidney Cancer

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Introduction

Renal cell carcinoma (RCC) accounts for approximately 95% of neoplasms arising from the kidney. Despite the availability of several new agents for the treatment of advanced kidney cancer since late 2005, mortality from kidney cancer remains fairly stable.[1] With the exception of high dose interleukin-2, agents used to treat advanced kidney cancer are not curative and seldom lead to long-term durable responses. This underscores the need to continue the search for new and effective RCC therapies.

While once considered a single disease, several histologic subtypes of kidney cancer characterized by classic morphologic features have been established. Individual subtypes of kidney cancer are characterized by distinct genetic alterations and metabolic properties, and therefore one therapy is not likely to be effective for all tumor types. With improved understanding of the unique biology of clear cell RCC (*VHL* inactivation), agents targeting key components of the VHL-Hypoxia inducible Factor (HIF)- vascular endothelial growth factor (VEGF) axis have been developed and form the mainstay of therapy against this subtype of kidney cancer.[2] Applying this strategy in patients with non-clear cell RCC is not supported by similar biologic rationale and at this time the clinical benefit of VEGF-targeted therapy in these cancers appears to be modest.[3] Recent insights into the biology of kidney cancer suggest that dysregulated metabolic pathways play an important role in many subtypes of this disease.[4] An improved understanding of the metabolic changes underlying different forms of kidney cancer is likely to lead to innovative therapeutic strategies for the management of this disease.

1) Aerobic Glycolysis in Cancer

Warburg Effect—Glycolysis is a multistep process where glucose is converted to pyruvate. Most differentiated human tissues oxidize pyruvate to acetyl-coA by pyruvate dehydrogenase for entry into the mitochondrial Krebs cycle, the primary means of energy (ATP) production via oxidative phosphorylation. In the absence of oxygen, cells are able to generate ATP, albeit less efficiently, by converting pyruvate into lactate by the enzyme lactate dehydrogenase (LDH). In the early half of the 20th century, Dr. Otto Warburg first noted that compared to normal cells, cancer cells consumed large amounts of glucose and produced lactate even in the presence of oxygen (aerobic glycolysis). This phenomenon was

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named the 'Warburg effect" and was hypothesized to be caused by mitochondrial dysfunction, thereby rendering the cells deficient in oxidative phosphorylation and dependent on aerobic glycolysis for generation of ATP.[5] During his Nobel Laureate Meeting Lecture in 1966, Warburg concluded that "the prime cause of cancer is the replacement of the respiration of oxygen in normal body cells by a fermentation of sugar."[6] The validity of this theory has been much debated over the last several decades, and while several lines of evidence suggest that tumors do indeed preferentially use aerobic glycolysis, support for a concomitant or causative defect in mitochondrial function is lacking in all but a few models.[7]

Critics of the Warburg effect initially argued that glycolytic shift in cancer resulted from tumor hypoxia, with a consequent switch to *anaerobic* glycolysis and enhanced lactate production. As solid tumors frequently outgrow their oxygen supply, anaerobic glycolysis is a common occurrence. Induction of hypoxia inducible factor (HIF) acts to restore oxygen balance by induction of glycolysis, erythropoiesis, and stimulation of angiogenesis.[8, 9] Whether tumor glycolysis happened independently of hypoxia (the Warburg effect) was a big question. Over the last few years, interest in this theory has been rekindled by evidence suggesting that oncogene activation or loss of tumor suppressor function could lead to a metabolic shift towards aerobic glycolysis.[10-12]

Role of HIF in glycolysis—Several cancers are associated with HIF overexpression despite normoxia. HIF1α is believed to play a key role in the induction of glycolysis, and stabilization of this protein either due to hypoxia or oncogenic alterations can promote glycolysis.[13] In clear cell kidney cancer, *VHL* alteration by mutation or hypermethylation inhibits ubiquitination and subsequent proteosomal degradation of HIF alpha subunits.[9, 14-17] Other mechanisms of normoxic HIF stabilization include *ras* activation, accumulation of Krebs cycles substrates such as fumarate, as well as activation of the PI3K/AKT/mTOR pathway.[18] While HIF expression plays an important role in the regulation of glycolysis, there are multiple mediators of this process. Mechanisms of HIF-independent aerobic glycolysis have been demonstrated in cancer and were first linked to activation of *c-Myc*.[12] Overexpression of *c-Myc* results in increased expression of several genes that promote glycolysis such as *LDH*-A, PKM2, GLUT-1 and hexokinase-2.[12, 19, 20] Other alterations in oncogenenic pathways such as PI3K/AKT/mTOR also appear to be key mediators of a glycolytic switch in cancer.[10]

Aerobic Glycolysis and Macromolecule Generation—Cells require a constant pool of macromolecules such as proteins, lipids, and nucleotides to support growth and proliferation. Cells with a high proliferative rate, such as tumor cells, acquire many alterations that help satisfy their exaggerated metabolic needs. Glucose and glutamine are the major source of carbon and nitrogen molecules necessary for macromolecule synthesis. Aerobic glycolysis, by engendering a high glucose flux into cells, may help provide an abundant supply of the necessary substrates for macromolecule synthesis. Krebs cycle components such as citrate are important intermediaries in the synthesis of macromolecules from glucose and glutamine. In cells where entry of glucose into the Krebs cycle is limited (as with inactivation of key Krebs cycle enzymes such as fumarate hydratase), glutamine, by

a process known as glutaminolysis, serves as the major source of biosynthetic intermediaries (Figure 2). Approaches aimed at targeting tumor glutaminolysis are being actively explored and may complement strategies designed to interfere with glycolysis.

2) What Drives Aerobic Glycolysis in Kidney Cancer

Interruption of the Krebs Cycle—At least two forms of hereditary kidney cancer appear to arise as a result of germline inactivating mutations in genes encoding Krebs cycle enzymes. The resulting incapacitation of the Krebs cycle forces the cells to use glycolysis as the major source of energy production. While these provide perhaps the best examples of the Warburg effect in human cancer, other tumors including sporadically occurring renal tumors may have similar metabolic dysfunction (such as mutations in isocitrate dehydrogenase in CNS tumors and leukemias). Mitochondrial mutations in complex I have also been described in oncocytoma, the most common benign renal tumor.[21] Additionally, somatic alterations in mitochondrial genes have also been reported in RCC.[22, 23]

a) Hereditary Leiomyomatosis Renal Cell Carcinoma (HLRCC): Hereditary Leiomyomatosis and Renal Cell Cancer (HLRCC) is an autosomal dominant condition characterized by the development of cutaneous and uterine leiomyomas and kidney cancer.[24-28] Germline inactivating mutations in *Fumarate Hydratase (FH)* are responsible for HLRCC.[29] This key enzyme is responsible for the conversion of fumarate to malate in the Krebs cycle. Loss of FH activity leads to impairment of oxidative phosphorylation and reliance on aerobic glycolysis to meet the bioenergetic needs of the cell.[30] Approximately 15-30% of individuals affected with HLRCC develop kidney cancer that is a clinically aggressive papillary variant with large orangiophilic nuclei and a characteristic perinucelar halo.[26, 28, 31]

b) Succinate dehydrogenase Deficiency: Hereditary Paraganglioma (PGL) is a syndrome manifested by head and neck PGL and pheochromocytoma (PCC). Germline alterations in genes that encode the subunits of succinate dehydrogenase (SDH), are associated with this entity.[32-34] SDH, an enzyme located on the inner mitochondrial membrane, consists of SDHA, SDHB, SDHC, and SDHD subunits. SDH catalyzes the oxidation of succinate to fumarate in the Krebs cycle and serves as complex II in the electron transport chain. The identification of germline mutations in *SDHB* in familial kidney cancer patients led to the realization that RCC is another disease manifestation.[35, 36] Renal tumors associated with this syndrome demonstrate impaired oxidative phosphorylation with reliance on aerobic glycolysis, and are clinically aggressive.[37, 38] Tumors associated with SDHB appear to have a distinct morphology with cuboidal cells having “bubbly, eosinophilic cytoplasm” with indistinct cell borders.[39]

Upregulation of HIF—Inactivating mutations in *VHL* and upregulation of HIF are the hallmark of both sporadic and hereditary clear cell renal cell carcinoma (ccRCC).[14, 16, 17] Increased intracellular HIF, particularly HIF-1α, leads to transcriptional upregulation of a variety of genes including several-GLUT1, PFK2, PDH, LDH-A- that promote aerobic glycolysis.HIF-1α can also be upregulated by VHL-independent mechanisms. Tumors associated with HLRCC and those resulting from SDH mutations express high levels of HIF

1α.[40, 41] In patients with HLRCC, biallelic inactivation of FH leads to accumulation of fumarate. Similarly, in cancers associated with SDH mutations, intracellular levels of succinate accumulate. Both fumarate and succinate appear to interfere with the hydroxylation of HIF by HIF prolyl hydroxylases (HPHs), leading to intracellular HIF accumulation (Figure 1).

HIF can also be upregulated by activation of the mTOR pathway. mTOR is a key regulator of cell growth and proliferation and is commonly upregulated in many cancers. In clear cell RCC, mutations in an upstream regulator of mTOR function (*PTEN*) are infrequently observed.[42, 43] Decreased expression of PTEN in the absence of a discernible mutation is common and is believed to contribute to an activated downstream AKT/mTOR pathway.[42, 44] Mutations in TSC1 and TSC2 can also upregulate mTOR and have been associated with renal angiomyolipomas and kidney cancer. The mTOR pathway is also activated in tumors associated with BHD, a hereditary kidney cancer syndrome resulting from germline inactivating mutations in *folliculin (FLCN)* and characterized by bilateral multifocal renal tumors of varied histology.[45, 46]

Reactive Oxygen Species—Reactive oxygen species (ROS) are oxygen free radicals characterized by unpaired electrons. These highly reactive molecules are byproducts of normal oxidative metabolism and function in normal homeostasis and signaling. However with increased levels of ROS, cellular damage and dysfunctional signaling can ensue. In HLRCC, increased cellular glucose levels stimulate NADPH-mediated ROS production.[47] The increased levels of ROS can directly stabilize HIF1, contributing to a metabolic shift towards aerobic glycolysis.[48, 49] In HLRCC, ROS are believed to stabilize HIF1 by direct inhibition of HPHs.[47]

In SDH, increased levels of ROS may contribute to HIF1 stabilization and tumorigenesis.[50] In yeast models, loss of SDH function generates increased levels ROS and the accumulation of oxidized proteins.[51] However data from SDH knockdown models in mammalian cells demonstrates that increased levels of ROS do not occur.[41]

NRF2/KEAP1—Although HIF is upregulated in both VHL null and FH deficient kidney cancer, these two entities demonstrate significant histological and clinical differences. In a bid to account for these differences, several groups have investigated the role of HIF-independent pathways in FH associated renal tumors. Animal models attempting to recapitulate HLRCC have been developed using conditional FH (−/−) knockout mice, which develop large renal cysts and renal failure.[52] Crossing these mice with HIF1 or HIF2 knockout mice fails to ameliorate this renal phenotype, suggesting that in this model, the renal abnormalities associated with FH loss are independent of HIF 1 and HIF 2.[53] Analyses of renal cysts from these FH-/mice demonstrate an aberrant anti-oxidant response pathway that may contribute to HLRCC associated renal tumorigenesis. At least two groups investigating the mechanism by which FH loss leads to upregulation of anti-oxidant genes have demonstrated that elevated intracellular fumarate leads to post-translational modification of Kelch-like ECH-associated protein 1(KEAP1). KEAP1 is a regulator of Nuclear factor (erythroid-derived 2)-like 2 (NRF2), a master transcriptional regulator of the response pathway to oxidative stress. Under normal conditions, KEAP1 is believed to bind

to and ubiquinate NRF2, resulting in its degradation. The interaction between KEAP 1 and NRF2 is disrupted following succination of the former in the presence of elevated levels of fumarate, leading to intracellular accumulation of NRF2 and activation of antioxidant signaling pathways.[53, 54] Recent evidence suggests that NRF2 is an important regulator in cancer cell metabolism. Mitsuishi and colleagues recently demonstrated that activation of this pathway results in the redirection of glucose and glutamine into anabolic pathways such as glycolysis and the pentose phosphate pathway.[55] In addition to HLRCC tumors, dysregulation of the KEAP1/NRF pathway appears has also been reported in the morphologically similar sporadic papillary type II RCC.[54]

Other Oncogenic Pathways—The PI3K/AKT/MTOR and myc proto-oncogene pathways are activated in different subtypes of kidney cancer. Both pathways play critical roles in the altered cellular metabolism seen in kidney cancer. Myc has been shown to upregulate the expression of various pro-glycolytic enzymes such as PKM2, hexokinase 2, and LDH-A. In addition, both the glucose transporter GLUT-1 and the glutamine transporter ASCT2 are regulated by Myc. The AKT/mTOR pathway stimulates glycolysis directly by upregulating several key glycolytic enzymes and indirectly by translational upregulation of HIF.

3) Targeting aerobic glycolysis

A variety of rational ‘metabolic’ targets have been proposed and are being evaluated in preclinical/clinical studies. These targets include critical components of aerobic glycolysis as well pathways involved in biosynthesis of macromolecules (Figure 3). The following discussion is restricted to those targets considered relevant to kidney cancer.

Glucose Uptake—Cellular glucose uptake is regulated by trans-membrane glucose transporters (GLUT receptors). Different genes encode for GLUT isoforms, which have variable distribution based on tissue type. Cancer cells frequently upregulate GLUT expression in an attempt to increase glucose uptake, especially important for cells that rely on aerobic glycolysis. Inhibiting this process could hinder cell proliferation by “glucose starvation” and make them more susceptible to cell death. This approach may be a viable option in clear cell RCC as a recent study demonstrated that *VHL* deficient cell lines demonstrate synthetic lethality with GLUT1 inhibition as did cancers with obligate aerobic glycolysis such as those associated with FH and SDH mutations.[56] Inhibitors of glucose uptake are currently in development.

PKM2—Pyruvate kinase (PK) is the last enzyme involved in glycolysis, catalyzing the dephosphorylation of phosphoenolpyruvate (PEP) to pyruvate with the generation of a molecule of ATP. Several isoforms of PK have been identified and the expression of these isoforms is tissue-dependent. The PKM gene has two splice variants (M1 and M2) varying by the presence of one exon.[57] The M2 isoform is present in embryonic tissues and in cells requiring rapid glucose turnover including muscle and brain cells. This enzyme is unique in that it can exist in both dimeric and tetrameric forms.[58] In cancer tissue, PKM2 is preferentially expressed and is important to tumor metabolism and proliferation.[59-61] The ratio of the dimeric to tetrameric forms determines enzyme activity and whether PEP is

converted to pyruvate or is shuttled into biosynthesis of other molecules such as amino acids and nucleic acids.[62]

PKM2 is being explored as a therapeutic target in cancer cells dependent on aerobic glycolysis. Knockdown of PKM2 decreases *in vitro* cell proliferation and glucose metabolism and also inhibits growth of xenografts.[63] Small molecule inhibitors targeting PKM2 are currently in development.[64]

Hexokinase—Hexokinase is the first enzyme involved in the glycolytic pathway and catalyzes the phosphorylation of glucose into glucose-6-phosphate. Once phosphorylated, glucose-6-phosphate cannot exit the cell. 2-deoxy-D-glucose (2DG) is a glucose analog that is taken up by cells via the same mechanisms mediating glucose uptake. Once in the cell, 2DG is phosphorylated by hexokinase. However, after this step, the resultant metabolite cannot be processed by glucose-6-phosphate isomerase, the second enzyme in glycolysis. Accumulation of phosphorylated-2DG leads to inhibition of hexokinase. Treatment with 2DG has long been known to inhibit *in vitro* tumor growth.[65] 2DG inhibits *in vitro* growth of UOK262, an *FH* deficient kidney cancer cell line derived from a patient with HLRCC. The agent has been evaluated in phase I and II studies. A case report of a patient with metastatic HLRCC treated with 2DG was recently reported; unfortunately, the patient did not appear to derive any clinical benefit.[66] Treatment with 2DG may not be effective as monotherapy, as escape mechanisms such as activation of the PI3K/AKT pathway may overcome inhibition of hexokinase.[67] Other pharmacologic mechanisms aimed at inhibiting hexokinase have been explored. Inhibitors such as 3-Bromopyruvate and Lonidamine can inhibit hexokinase function and may be a useful strategy for tumors relying on aerobic glycolysis.[68] Lonidamine was in clinical development in the United States but these trials were terminated due to modest clinical activity. However, this drug was approved in Italy for cancer therapy over 20 years ago.[68]

LDH-A—Lactate dehydrogenase (LDH) catalyzes the reversible conversion of lactate to pyruvate. LDH exists in two major isoforms; LDH-A is the isoform that promotes conversion of pyruvate into lactate. As LDH-A is upregulated in solid tumors reliant on glycolysis, inhibition of this enzyme may be a valid therapeutic approach.[69] Knockdown experiments in several models demonstrate decreased *in vitro* cell proliferation and *in vivo* tumorigenicity.[69] HLRCC associated tumors appear to overexpress LDH-A and knockdown of this enzyme in UOK262 limits *in vitro* cell proliferation and *in vivo* growth inhibition.[70]

Haem Oxygenase—Several Krebs cycle intermediates are needed for macromolecule biosynthesis and integrity of this cycle is essential for sufficient NADH production. In cells with Krebs cycle dysfunction, the mechanisms used to generate macromolecules and NADH is not well understood. Cells dependent on aerobic glycolysis are thought to rely on high levels of glutamine metabolism for these biochemical precursors.[71] Experimental modeling and ^{13}C labeling in mouse *FH*-deficient kidney cells determined that these cells utilize an alterative metabolic pathway involving heme biosynthesis and degradation for NADH production. Knockout of heme oxygenase 1, required for heme degradation, disrupts this pathway and is associated with synthetic lethality in FH negative cells and is being explored as a therapeutic target..[72]

Glutaminase—In addition to their reliance on aerobic glycolysis, cancer cells also demonstrate increased glutamine uptake, which serves as the major source of nitrogen for protein and lipid biosynthesis.[71, 73] Interfering with glutamine metabolism may therefore starve the cancer cell of the necessary biosynthetic molecules required for continued growth and proliferation. Cells relying on a high rate of aerobic glycolysis are profoundly sensitive to glutamine withdrawal.[74] Therapeutic strategies aimed at blocking glutamine metabolism by glutaminase inhibition are being explored. Small molecule inhibitors of this enzyme have been shown to block oncogenic transformation in several cell lines without affecting normal cells.[75] In glioma cell lines with a mutated Krebs cycle enzyme (isocitrate dehydrogenase), genetic and pharmacologic inhibition of glutaminase can decrease cell proliferation.[76] While this may be a promising strategy for targeting cancer metabolism, emerging data suggests that cells adapt to overcome glutaminase inhibition by activating alternative pathways for generating substrates for macromolecule synthesis. Further studies are required to determine the role of glutaminase inhibition in the treatment of cancer.[77]

Pyruvate Dehydrogenase Kinase—Pyruvate dehydrogenase (PDH) is a mitochondrial enzyme complex responsible for the decarboxylation and acetylation of pyruvate into acetyl-CoA, the starting point of the Krebs cycle. This complex is tightly regulated by two distinct enzymes, pyruvate dehydrogenase phosphatase and pyruvate dehydrogenase kinase (PDK). Phosphorylation of PDH inactivates the complex and shunts pyruvate towards lactate production in the cytoplasm. Methods aimed at inhibiting PDK are thought to shift metabolism towards the Krebs cycle. Sodium Dichloroacetate (DCA) is a pyruvate analogue that promotes entry of pyruvate into the Krebs cycle and has been extensively studied for cancer treatment. Cells with defects in the electron transport chain appear to be more sensitive than cells less reliant on glycolysis.[68] The agent is in early clinical trials and appears to be well tolerated.

Angiogenesis Inhibitors—Prior to 2005 immunotherapy was the sole systemic modality available to patients with advanced kidney cancer. Unfortunately, only a small proportion of patients with clear cell RCC appear to benefit from immunotherapy while non-clear cell RCC are generally not responsive.[78, 79] Newer agents targeting the VEGF or mTOR pathways show only modest activity in papillary RCC.[80] Thus, no effective strategy exists for papillary variants of RCC. In a phase II study of erlotinib in papillary RCC, a low overall response rate was observed, although the median survival of 27 months was encouraging.[81]

We hypothesized that combining a VEGF pathway antagonist and an EGFR inhibitor would constrain glucose delivery to tumor cells by targeting its vasculature; targeting a growth factor pathway downstream from HIF would theoretically act in concert with the antiangiogenic approach. This approach was first evaluated in clear cell RCC using using bevacizumab and erlotinib therapy, a combination that was well tolerated.[82] This approach is being currently evaluated in patients with papillary RCC in a phase II trial at the NCI (Trial ID, NCI01130519).

4) Conclusion

A variety of kidney cancer subtypes are characterized by dysregulated metabolic pathways. The dependence of some tumors on aerobic glycolysis is an Achilles heel that can be exploited in targeted therapeutic strategies. Multiple steps in the glycolytic pathway have been evaluated as possible anti-cancer targets in preclinical studies and some agents have entered early clinical trials for solid tumors. It is hoped that these approaches will spawn novel therapeutic options in the fight against kidney cancer.

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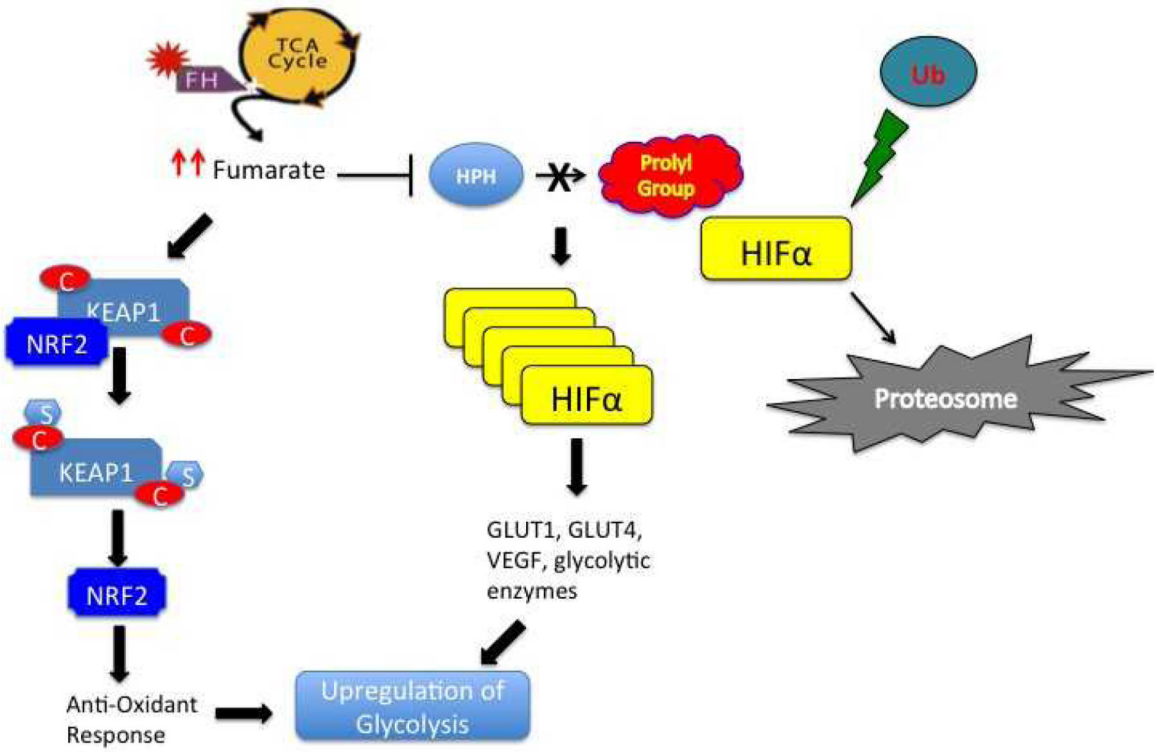

Figure 1.

Proposed mechanisms of tumorigenesis in HLRCC. Due to loss of function of fumarate hydratase, fumarate accumulates, inhibiting HIF prolyl hydroxylases (HPHs). Inactivation of HPH leads to intracellular HIF accumulation and transcription of important mediators of angiogenesis, growth and proliferation. In addition, upregulation of fumarate can lead to post-translational modification (succination of cysteine residues) and inactivation of KEAP1. This results in NRF2 dysregulation and transcriptional activation of several NRF2-dependant genes that mediate the cellular anti-oxidant response, Both HIF and NRF2 contribute to the aberrant metabolic signature associated with FH inactivation.

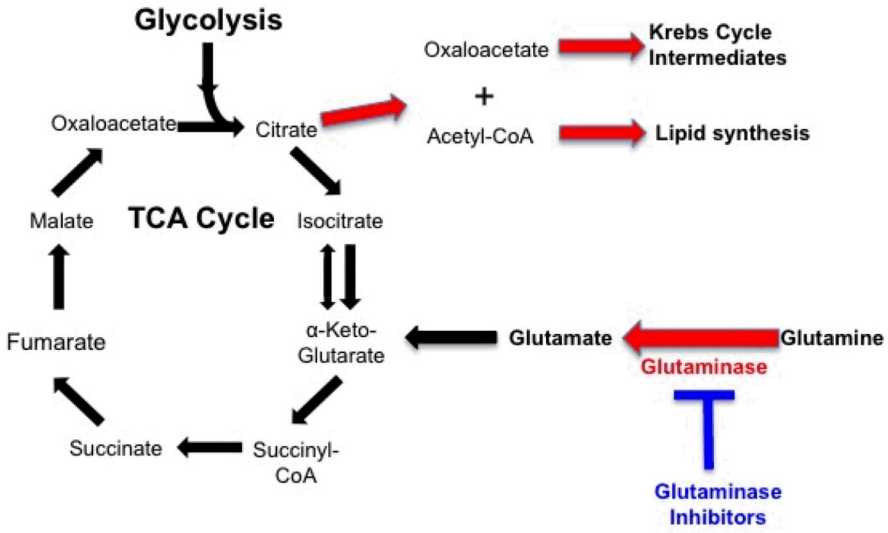

Figure 2.
Macromolecule generation using glutamine as a biologic precursor.

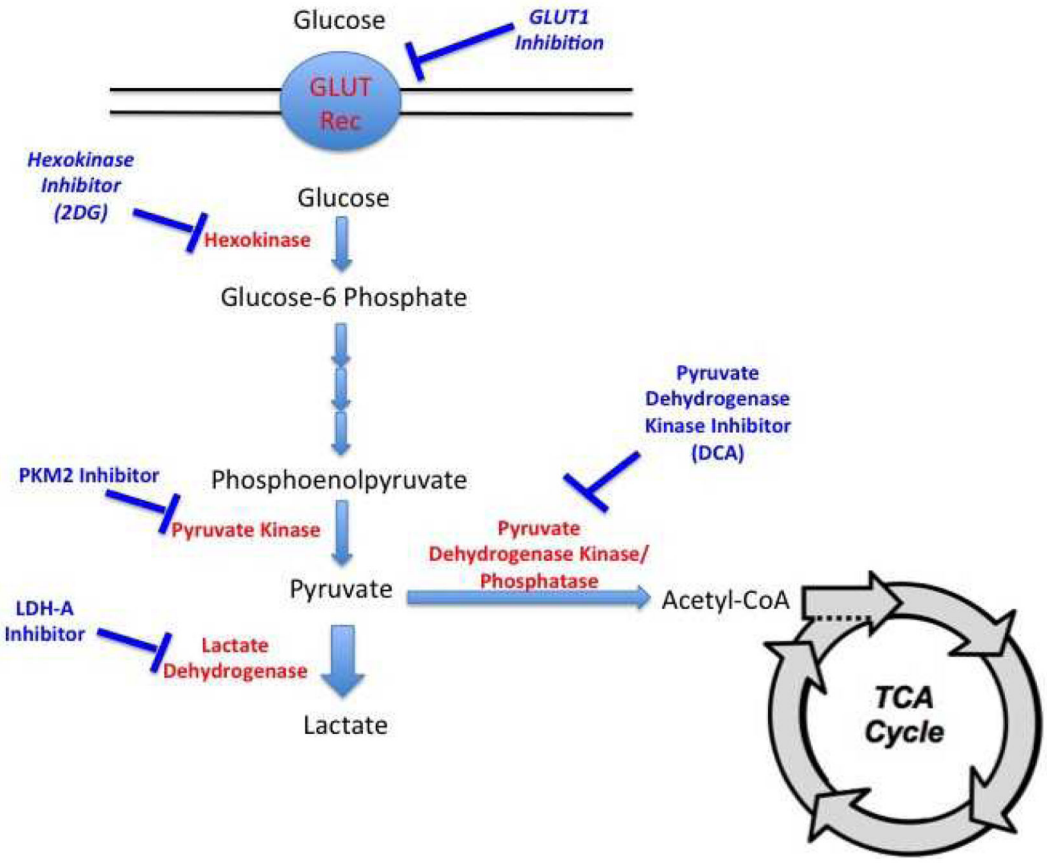

Figure 3.
Metabolic flow of glucose and opportunities for therapeutic intervention in cancer