Kidney Cancer 3 (2019) 15–29
DOI 10.3233/KCA-180043
IOS Press

Review

The 'Achilles Heel' of Metabolism in Renal Cell Carcinoma: Glutaminase Inhibition as a Rational Treatment Strategy

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Received 4 September 2018
Accepted 10 December 2018

Abstract. An important hallmark of cancer is 'metabolic reprogramming' or the rewiring of cellular metabolism to support rapid cell proliferation [1–5]. Metabolic reprogramming through oncometabolite-mediated transformation or activation of oncogenes in renal cell carcinoma (RCC) globally impacts energy production as well as glucose and glutamine utilization in RCC cells, which can promote dependence on glutamine supply to support cell growth and proliferation [6, 7]. Novel inhibitors of glutaminase, a key enzyme in glutamine metabolism, target glutamine addiction as a viable treatment strategy in metastatic RCC (mRCC). Here, we review glutamine metabolic pathways and how changes in cellular glutamine utilization enable the progression of RCC. This overview provides scientific rationale for targeting this pathway in patients with mRCC. We will summarize the current understanding of cellular and molecular mechanisms underlying anti-tumor efficacy of glutaminase inhibitors in RCC, provide an overview of clinical efforts targeting glutaminase in mRCC, and review approaches for identifying biomarkers for patient stratification and detecting therapeutic response early on in patients treated with this novel class of anti-cancer drug. Ultimately, results of ongoing clinical trials will demonstrate whether glutaminase inhibition can be a worthy addition to the current armamentarium of drugs used for patients with mRCC.

Keywords: Kidney cancer, renal cell carcinoma, treatment, glutamine, metabolism, metabolic reprogramming, glutaminase, glutaminase inhibitor, biomarkers

INTRODUCTION

Renal cell carcinoma (RCC) most commonly occurs due to loss of the von-Hippel Lindau (VHL) tumor suppressor, which leads to stabilization of the transcription factor hypoxia-inducible factor (HIF). Constitutive HIF signaling results in extensive metabolic reprogramming, including a shift towards increased glutamine utilization, which suggests that RCC may be particularly susceptible to interfering with glutamine metabolism. Therefore, a review of glutamine metabolic pathways in RCC was undertaken. Glutaminase is a mitochondrial enzyme that converts glutamine to glutamate. Here we review the role of glutamine in cellular metabolism, preclinical evidence of addiction of RCC cells to glutamine and glutaminase activity, and inhibitors of glutaminase as a novel strategy for the treatment of RCC.

GLUTAMINE - A CELL'S 'MULTI-PURPOSE FUEL'

Glutamine is a non-essential amino acid that is synthesized and metabolized by all cells in the body. It participates in a wide array of physiological functions

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ISSN 2468-4562/19/$35.00

throughout the human body, not simply in cancer. This fact will be especially important for later discussions of early failed attempts at targeting the glutamine pathway for anti-cancer treatment. Hence, we will first review the role of glutamine in organismal and cellular physiology.

Glutamine is the most abundant amino acid in the plasma, where it serves as a universal carrier for the inter-organ transport of carbon and nitrogen - in essence, functioning as a 'wildcard' amino acid for the human body. The majority of glutamine is generated *de novo* by skeletal muscle, adipocytes, and the lungs, which maintain organism-wide glutamine homeostasis [8, 9]. When glutamine demand exceeds the biosynthetic capacity of the body such as during wound repair or sepsis, glutamine becomes an essential amino acid [10, 11].

In cells, it is used as 'fuel' for the biosynthesis of other amino acids, metabolites, nucleotides, lipids, proteins, and for generating energy in the form of adenosine triphosphate (ATP) [12–17]. Hence, rapidly dividing cells typically use the largest quantities of glutamine due to the high demand for the building blocks of macromolecules and for energy, including epithelial cells of the small intestine (enterocytes), immune cells (e.g. activated lymphocytes), and ultimately, cancer cells [18, 19]. If intracellular *de novo* synthesis is inadequate to meet the cellu-

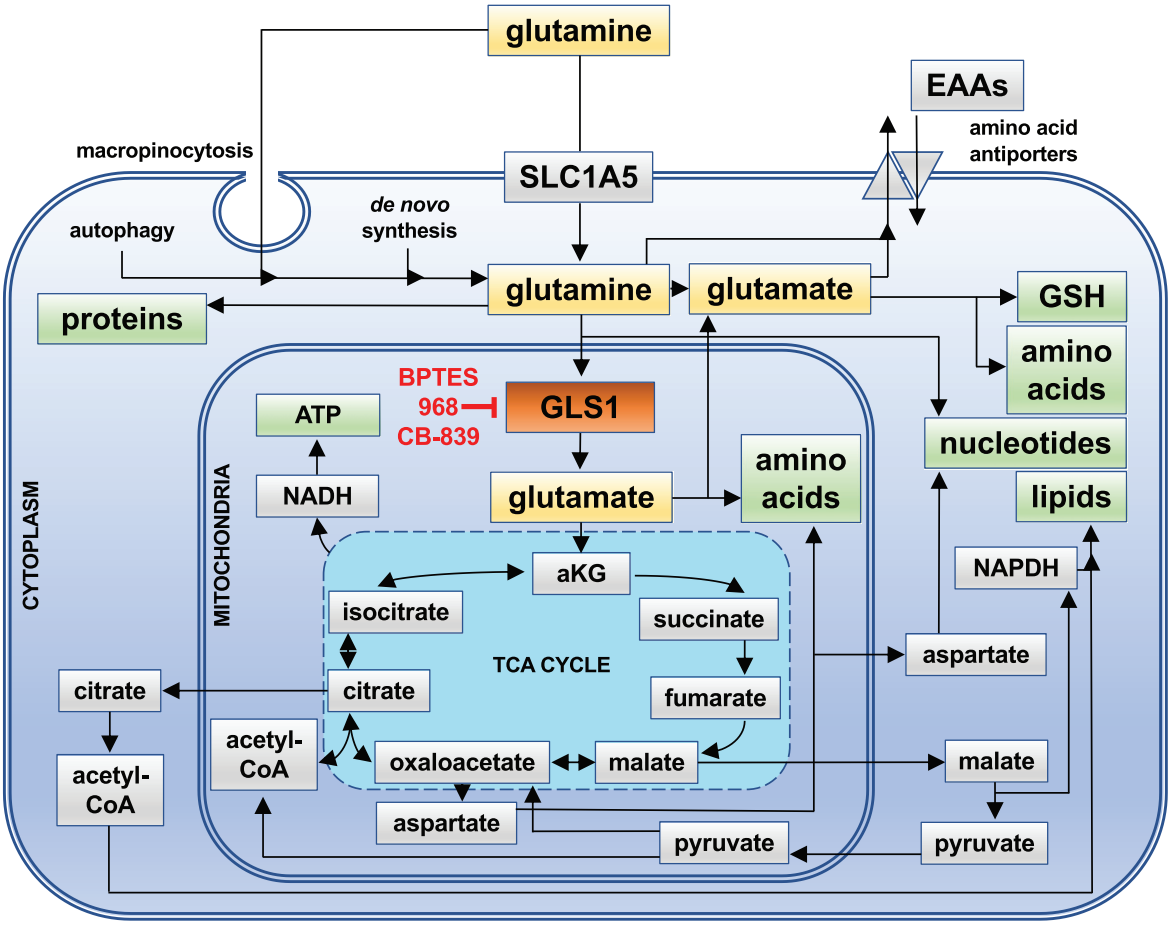

Fig. 1. Cellular Uptake Routes and Intracellular Utilization of Glutamine. Glutamine (yellow) is either synthesized by cells *de novo*, taken up through the solute carrier 1A5 (SLC1A5), or derived from the intracellular breakdown of macromolecules (autophagy). Glutamine and its metabolic product, glutamate (orange), can be exported from cells in exchange for other essential amino acids (EAAs). Intracellularly, they are involved in a wide range of metabolic pathways that serve to generate other amino acids and glutathione (GSH) as well as precursors for the biosynthesis of nucleotides and reducing equivalents in the form of nicotinamide adenine dinucleotide (NADH) and NADH phosphate (NADPH) for generating energy and lipids. Most of glutamine is utilized by mitochondria, where the enzyme glutaminase 1 (GLS1, red) or glutaminase 2 (GLS2, not shown) convert glutamine into glutamate, which in the form of alpha-ketoglutarate (aKG) can enter the tricarboxylic acid cycle (TCA cycle). GLS1 is the target of glutaminase inhibitors such as BPTES, 968, and CB-839.

lar demand for glutamine, then it can be imported into the cytoplasm via glutamine transporters of the solute carrier (SLC) family (in particular SLC1A5, see Fig. 1), macropinocytosis (the uptake of large vacuoles of extracellular fluid by endocytosis), or even released through the intracellular breakdown of macromolecules (autophagy) [20–22].

The intracellular fate of glutamine can be classified into two major categories: **cytoplasmic** and **mitochondrial** (see Fig. 1). In the cytoplasm, glutamine can be used for either protein and nucleotide biosynthesis, or for export by antiporters where it serves as a 'currency' in exchange for other amino acids. Moreover, cytoplasmic glutamine-derived glutamate is an important precursor for many amino acids, hexosamines (precursors for the glycosylation of signaling proteins), and glutathione - a major reducing agent for radical oxygen species (ROS) [12]. In the mitochondria, **glutaminase 1 and 2 (GLS1** and **GLS2)** convert glutamine to glutamate and ammonia. Glutamate then becomes a primary commodity for amino acid biosynthesis or is converted into alpha-ketoglutarate (aKG), which enters the tricarboxylic acid (TCA) cycle - a central hub in cellular metabolism that can interconvert many precursors of biosynthetic reactions (see Fig. 1). Through the TCA cycle, glutamate provides precursors for the biosynthesis of amino acids, nucleotides, lipids, and reducing equivalents in the form of NADH (required for the synthesis of ATP by oxidative phosphorylation), and NADPH (needed for lipid and nucleotide biosynthesis, and the regeneration of cytoplasmic glutathione). Lastly, glutamine-derived glutamate can 'fuel' the TCA cycle by generating acetyl-CoA via malate and pyruvate.

Hence, glutamine is a major source of carbon, nitrogen, and electrons for virtually all cellular metabolites, macromolecules, and energy.

GLUTAMINE- AND GLUTAMINASE-ADDICTION IN RCC - WHEN GLUCOSE CAN'T DO THE JOB

In this section, we will review the molecular mechanisms proposed for the dependence on extracellular glutamine and glutaminase activity in RCC. This overview ultimately provides therapeutic rationale for targeting this specific metabolic pathway.

Increased glutamine uptake and elevated expression of glutaminase have been recognized as hallmarks of proliferating tumor cells *in vitro* and *in vivo* since the 1950s [23–30]. Subsequent studies in RCC cells confirmed that glutamine is consumed at high rates *in vitro* [31–33].

For the most common subtype of kidney cancer, clear cell RCC (ccRCC), tumors are consistently reported to have higher levels of glutamine and glutamate compared to normal kidney tissue in addition to increased expression of glutamine importers such as SLC1A5 [34–43]. Early glutamine deprivation studies demonstrated that some cancer cell lines are dependent on glutamine even under glucose-replete conditions [44]. Additional studies involving genetic perturbations further demonstrated that many tumors, including RCC, are dependent on glutaminase activity, thus implying that addiction to glutamine is a consequence of the increased need for glutamate [32, 45–56].

Glutaminase, the mitochondrial enzyme that converts glutamine to glutamate, exists as two isoenzymes, GLS1 and GLS2, encoded by the genes, *GLS1* and *GLS2* [57]. GLS1 has two splice variants, kidney-type glutaminase (KGA) and a shorter, more active variant, glutaminase C (GAC) [58, 59]. Both splice variants are widely expressed across tissues with especially the GAC variant frequently expressed at higher levels in tumor cells compared to corresponding normal cells [41, 48, 49, 51, 52, 59–68]. Interestingly, in most ccRCC tumors, expression levels of GLS1 seem not to be significantly changed, though expression of the more active GAC variant of GLS1 is slightly increased relative to the KGA variant in ccRCC cell lines [12, 31–33, 69]. GLS2 is predominantly found in the liver, brain, and pancreas and, like GLS1, has not been reported to be elevated in RCC [61].

The fact that glucose and glutamine are both abundant resources for cellular metabolism under normal *in vitro* culture conditions, and that both 'fuel' the same metabolic pathways through the TCA cycle, raises the question: why do RCC and other cancer cells become dependent on glutamine in the presence of glucose? The oncogenic transcription factor HIF, which is often activated in cancer cells by hypoxia in poorly perfused regions of solid tumors or by the activity of other oncogenes, plays a central role in this phenomenon.

In ccRCC, loss of VHL and fructose-1,6-bisphosphatase 1 (FBP1) tumor suppressor functions - fundamental features of this subtype of RCC - results in increased HIF activity in virtually all cancer cells, independently of hypoxia [70–72]. HIF reprograms cellular metabolism and increases glucose

uptake and glycolysis via increased expression of glucose transporters and glycolytic enzymes. Moreover, HIF also shifts the TCA cycle from predominant glucose utilization to a predominantly glutamine-fuelled system *in vitro* and *in vivo* [31–33, 52, 73–77]. In normal cells, glucose-derived carbons enter the mitochondria via pyruvate, which drives the TCA cycle in a 'clockwise' fashion (see Fig. 1). However, even though ccRCC cells show increased glycolysis, increased HIF activity leads to inhibition of pyruvate dehydrogenase (PDH) and pyruvate carboxylase (PC) activity, thus resulting in drastically decreased entry of glucose-derived carbon into the TCA cycle [37, 39, 78, 79]. In consequence, these cells 'fuel' the TCA cycle primarily in a 'counterclockwise' direction by glutamine-derived glutamate via reductive carboxylation of aKG to isocitrate (see Fig. 1). Glutamine-addicted cancer cells operate the TCA cycle in this 'reverse' direction to generate citrate and malate for lipid biosynthesis, and oxaloacetate for nucleotide biosynthesis, while deriving most of their energy from glycolysis [31, 33, 74–77]. HIF activation in ccRCC is both necessary and sufficient for increased glutamine utilization and dependence, both by limiting the ability of glucose to 'fuel' the TCA cycle and by reprogramming glutamine metabolism to provide the requisite macromolecules needed for sustaining the increased needs of rapidly dividing cells [31, 32, 77].

The degree of metabolic reprogramming and glutamine dependence in ccRCC increases as tumors become more advanced and aggressive and accumulate genetic alterations in other oncogenes and tumor suppressor genes, for example in phosphoinositide 3-kinase (PI3K), c-MYC, or p53 [35, 39–41, 47, 48, 52, 80–89].

Interestingly, HIF activation does not seem to influence the expression or protein levels of glutamine transporters or GLS1 [32, 90, 91]. This suggests that even normal cells already have the capacity to use glutamine as the major 'fuel' for the TCA cycle, yet prefer to use glucose. Cancer cells in which HIF is activated, by contrast, lose the ability to use glucose as major TCA cycle 'fuel', thus developing a particular dependence on glutamine. This may explain why interfering with glutamine utilization via glutaminase inhibition negatively impacts cancer cells preferentially compared to normal cells.

Metabolic programming and glutamine dependence in non-clear cell RCC are less well studied. While genetic alterations have been clearly described for papillary type 1 and chromophobe RCC, the metabolic landscapes have not yet been characterized [92–97].

In hereditary leiomyomatosis and renal cell cancer (HLRCC) syndrome, where patients develop kidney tumors with papillary type 2 histology, RCC cells lack a functional enzyme in the TCA cycle, fumarate hydratase (FH), which converts fumarate to malate (see Fig. 1) [98]. FH deficiency causes an accumulation of fumarate, which stabilizes HIF protein [99, 100]. *In vitro*, these cells demonstrate increased glycolytic activity and reliance on both glucose and glutamine for cell proliferation and survival [74, 101–104].

Similarly, in familial and sporadic forms of succinate dehydrogenase (SDH) deficient RCC, there is a lack of the functional enzyme, SDH, which converts succinate to fumarate in the TCA cycle. Similar to FH-deficient cells, SDH-deficient cells also demonstrate increased HIF activity as well as increased glycolysis and glutamine utilization [105–112].

Finally, a c-MYC driven mouse model of collecting duct RCC (cdRCC), a particularly aggressive form of RCC arising from the epithelial cells of the renal collecting duct system, demonstrated upregulated glutamine utilization [41]. The c-MYC pathway is also activated in a subset of ccRCC tumors and in some rare forms of RCC caused by chromosomal translocations [35, 113]. Thus, as in ccRCC, glutaminase inhibition may be a viable drug target even for some non-clear cell RCC.

In summary, extensive metabolic reprogramming is a common theme in all forms of RCC. Glutamine-derived glutamate plays a central role in the TCA cycle to generate sufficient amounts of intermediates required for the biosynthesis of lipids, nucleotides, and other amino acids, as well as NADPH for maintaining redox homeostasis. The limited ability of ccRCC cells - and any cancer cells with activated HIF - to use glucose to drive the TCA cycle provides a plausible explanation for increased glutamine dependence in ccRCC and other cancers, as this metabolic strategy provides alternative carbon and nitrogen sources for generating macromolecules [114–116].

ANTI-TUMOR EFFICACY OF GLUTAMINASE INHIBITION IN RCC: PRECLINICAL DATA AND MOLECULAR MECHANISMS

Early studies demonstrated that RCC cells are dependent on glutaminase activity, suggesting that glutamine is required for maintaining intracellular

glutamate supply [32, 45–56]. While it was believed for a long time that glutamine-addicted cancer cells require this glutamate for generating enough energy for proliferation and survival, more recent work suggests that ATP production does not seem to be the major limiting factor for cancer cell proliferation [5]. Rather, glutamine-derived glutamate plays a central role in the TCA cycle to generate enough intermediates required for the biosynthesis of lipids, nucleotides, other amino acids, and NADPH. Which of these intermediates become limiting, leading to impaired cellular functions, in glutamine-addicted cancer cells upon glutaminase inhibition? The development of selective glutaminase inhibitors over the last decade not only revived the interest in glutamine metabolism research in cancer, but also helped develop a better understanding of this central question of glutamine dependence in RCC and other cancers and have been used to study the role of glutaminase as a therapeutic target:

1) Bis-2-(5-phenylacetimido-1,3,4,thiadiazol-2-yl) ethyl sulfide (BPTES) inhibits both the KGA and GAC isoforms of GLS1 by stabilizing the tetramer form of the enzyme in an inactive conformation, but does not inhibit GLS2 [117–120].
2) The bromo-benzophenanthridinone compound 968, a GLS1 inhibitor thought to preferentially target the GAC isoform, binds to the monomeric form of the enzyme, thereby locking GLS1 in its inactive state [121]. Inhibition of GLS2 by 968 has not been investigated [122].
3) CB-839, a newer compound with structural similarity to BPTES, but higher potency and improved bioavailability, inhibits both isoforms of GLS1 (KGA and GAC), but not GLS2 [123].

Glutaminase inhibitors have anti-proliferative activity *in vitro* and *in vivo* in mice in a wide range of cancers including RCC [32, 33, 41, 49, 52, 53, 55, 64, 67, 68, 123–139]. *In vitro*, the effects of glutaminase inhibition generally mimic those of glutamine deprivation, suggesting that the mitochondrial fates of glutamine are most critical for cell proliferation and survival. However, these effects vary with cancer type and cell line being studied: in some cases cells become quiescent, whereas in others cells undergo apoptosis [32, 33]. Okazaki et al. investigated the molecular mechanism how GLS1 inhibition impairs proliferation of VHL-deficient RCC cells and found nucleotide biosynthesis and redox homeostasis to be the limiting functions that are mediated by GLS1 in RCC cells [33]. Inhibition of GLS1 by BPTES or CB-839 impaired the ability of VHL-deficient RCC cell to generate aspartate, required for the biosynthesis of pyrimidine nucleotides. GLS1 inhibition in these cells leads to DNA replication stress, more frequent DNA double-strand breaks (DSBs), increased ROS levels and decreased glutathione levels. Furthermore, Sun et al. showed that the growth defect of cancer cells upon glutamine depletion could be rescued by the addition of lipids or citrate [77]. Hence, *in vitro*, anti-proliferative effects of GLS1 inhibition in VHL-deficient RCC cells do not result from impaired energy production. Rather, the limited supply of TCA cycle intermediates as building blocks for the biosynthesis of nucleotides, lipids, and glutathione appear to be the limiting factors.

In vivo, tumor xenograft studies showed that treatment with glutaminase inhibitors results in reduced tumor growth in a number of cancer types, including RCC [32, 33, 41, 49, 52, 67, 68, 123, 136, 138]. Interestingly, glutaminase inhibitors can have additive or even synergistic effects with other anticancer drugs. For example, in an RCC xenograft model, CB-839 showed increased efficacy when combined with the poly(ADP-ribose) polymerase (PARP) inhibitor olaparib [33]. In mouse xenografts of the RCC cell line Caki-1, tumor growth was inhibited by treatment with CB-839 alone, but the combination with either cabozantinib or everolimus inhibited tumor growth more effectively than either agent alone (Calithera Biosciences, Inc., unpublished data, see ref. [140, 141]). In the syngeneic CT26 colon carcinoma mouse model, the addition of CB-839 to PD-L1 inhibition increased the number of complete tumor regressions compared to either single agent (Calithera Biosciences, Inc., unpublished data, see ref. [142]). Similarly, in a syngeneic B16 melanoma model, the combination of CB-839 with PD-L1 inhibition more effectively inhibits tumor growth than PD-L1 inhibition alone (Calithera Biosciences, Inc., unpublished data, see ref. [143]). Taken together, preclinical data suggest that CB-839 can have anti-tumor activity in RCC and other cancer types as a single agent and in combination with other agents.

TARGETING GLUTAMINE METABOLISM IN PATIENTS

Targeting glutamine metabolism can be exploited clinically. Notably, some FDA-approved drugs show off-target effects on glutamine utilization [89]. For example, bacterial L-asparaginases are used to treat acute lymphoblastic leukemia (ALL) and can metab-

olize both asparagine and glutamine and thereby deplete plasma glutamine levels [144]. Sorafenib, an FDA-approved multi-tyrosine kinase inhibitor (TKI) for advanced RCC, has recently been reported to also inhibit the cystine/glutamate antiporter SLC7A11/xCT [145]. Nevertheless, the main mechanism of efficacy of sorafenib and other TKIs is mostly attributed to inhibition of receptor tyrosine kinases.

Rather than relying on off-target effects, attempts to target the glutamine/glutaminase pathway more specifically began as early as the 1980s. Glutamine mimetics such as 6-diazo-5-oxo-L-norleucine (DON), azaserine, and acivicin were developed to competitively bind to glutamine transporters, thereby inhibiting cellular glutamine uptake [146]. Acivicin was tested in a clinical trial for metastatic RCC [147]. These drugs showed high efficacy in preclinical experiments, but turned out to be less potent in patients - acivicin had an objective response rate of 4% in RCC - and were highly toxic to the brain, bone marrow, and gastrointestinal tract [148]. These wide-ranging adverse effects can be understood in the context of glutamine's crucial role in neurotransmitter biogenesis and in the metabolism of rapidly dividing normal cells [17]. The limited efficacy and severe toxicities of these drugs rendered glutamine metabolism a sub-optimal target in cancer therapy for a long time.

The refined knowledge about the importance of glutaminase in cellular glutamine utilization and about the increased dependence of cancer cells on GLS1 revived the interest in targeting glutamine metabolism and led to the development of selective glutaminase inhibitors for cancer therapy. However, the poor solubility of the GLS1 inhibitors BPTES and 968 in aqueous solution and their limited potency have hampered their translation into clinical applications. The availability of structural information about these drugs in complex with GLS1 has led to efforts to rationally evolve BPTES into more potent and stable analogues with better solubility. For example, the BPTES analog UPGL00004 is a more potent inhibitor of the the GAC variant of GLS1 than BPTES and inhibits breast cancer growth in mice when combined with the anti-vascular endothelial growth factor (VEGF) antibody bevacizumab [149, 150]. Other approaches explored delivering the drug by nanoparticles, which improved pharmacokinetics and efficacy of BPTES [151]. In parallel to GLS inhibitors, glutamate dehydrogenase (GLUD) inhibitors have been developed. GLUD catalyzes the reaction that converts glutamate to aKG and ammonia, downstream of GLS. The GLUD inhibitor epigallocatechin gallate (EGCG) is currently being evaluated as adjuvant therapy for colorectal cancer in a phase I clinical trial [152].

Of the next generation GLS inhibitors, CB-839, a newer compound with improved bioavailability, is the only agent that has reached clinical trials so far. It is being tested as single agent or in combination with established therapies in hematologic tumors and in a wide range of solid tumors including RCC. Early data from the phase I trials assessing the safety, pharmacokinetics, and pharmacodynamics indicate that CB-839 is tolerable as a single agent in patients with mRCC and other cancers [153]. Monotherapy with CB-839 in 21 patients with RCC resulted in one partial response (with duration of 356 days) and stable disease in 52% of patients. Moreover, CB-839 also appears to be well tolerated in combination with other drugs, with some patients experiencing tumor stabilization and clinical efficacy [153]. The combination of the mTOR inhibitor everolimus and CB-839 showed a disease control rate of 92% in 24 patients with RCC (with a median of two prior lines of therapy). In 12 patients with advanced RCC treated with the TKI cabozantinib and CB-839 (with a median of 3 prior lines of therapy), overall response rate was 40%, with 100% disease control rate. Whether CB-839 might have clinical efficacy for RCC in randomized trials is currently being tested (see Table 1). Lastly, novel GLS1 inhibitors are in development and also selective GLS2 inhibitors have been reported [154–156]. Glutaminase inhibitors thus represent a new class of drugs to clinically target cancer cell glutamine metabolism with promising early results in the management of mRCC.

BIOMARKERS FOR SENSITIVITY AND RESPONSE TO GLUTAMINASE INHIBITORS IN RCC

The early clinical results with CB-839 and the increasing number of novel glutaminase inhibitors and drug combinations on the horizon pose an opportunity and need for establishing biomarkers for patient stratification, therapeutic response, and resistance in parallel with developing novel glutaminase inhibitors. Developing assays for the activity of GLS1 protein as the actual drug target of GLS1 inhibitors will be vital to understand GLS1 regulation

Table 1
Ongoing Clinical Trials of Glutaminase Inhibitor CB-839 in Solid Tumors

clinicaltrials.gov Identifier	Title	Phase
NCT02071862	Study of the Glutaminase Inhibitor CB-839 in Solid Tumors	Phase I (ref. [153])
NCT02771626	Study of CB-839 in Combination With Nivolumab in Patients With Melanoma, ccRCC and NSCLC	Phase I/II
NCT03163667	Study of CB-839 With Everolimus vs. Placebo With Everolimus in Patients With RCC (ENTRATA Trial)	Randomized Phase II
NCT03428217	Study of CB-839 With Cabozantinib vs. Placebo With Cabozantinib in Patients With Metastatic RCC (CANTATA Trial)	Randomized Phase II

Table 2
Modes of Regulation of GLS1

Mode of GLS1 regulation	Effect	Reference
Transcription	STAT1 induces expression of GLS1	[157]
Pre-mRNA splicing	RNA-binding proteins regulate GLS1 alternative splicing	[56, 69, 158, 159]
mRNA stability	GLS1 mRNA contains a pH-responsive stability element	[160]
Protein translation	c-MYC and NF-κB activity induce translation of the KGA isoform of GLS1 by inhibiting expression of the translational inhibitory microRNAs 23a and 23b	[161]
Post-translational modification	the GAC isoform of GLS1 is activated in cells transformed by diffuse B-cell lymphoma protein (Dbl; a GEF for Rho GTPases), likely by phosphorylation;	[49]
	the KGA isoform of GLS1 is activated, likely through phosphorylation, in response to EGF stimulation through the RAF-MEK-ERK signaling pathway;	[162]
	GLS1 is inactivated through desuccinylation by Sirtuin 5, which is overexpressed in some lung cancers	[163,164]
Protein localization	in neurons, the KGA isoform of GLS1 localizes to neurite terminals or mitochondria depending on the activity of BCL2/adenovirus E1B 19 kd-interacting protein (BNIP) family members	[165,166]
Enzymatic activity	GLS1 is activated by inorganic phosphate *in vitro*, with the GAC isoform of GLS1 showing the strongest increase in activity; glutamate, but not ammonia, inhibits GLS1	[8, 51, 167]
Protein degradation	the KGA isoform of GLS1 is ubiquitinated by the anaphase-promoting complex (APC)-CDH1 E3 ubiquitin ligase complex and subsequently degraded;	[168, 169]
	the GAC isoform of GLS1 is degraded by the LON protease upon diphenylarsinic acid (DPAA) treatment	[170]

in RCC and to develop biomarkers for stratifying patients. Interestingly, beyond transcriptional regulation, GLS1 is regulated at virtually every post-transcriptional level (see Table 2). Gene expression analysis alone may therefore provide only limited information on the activation status of GLS1 and hence sensitivity to GLS1 inhibitors. The findings that GLS1 inhibition in RCC cells leads to depletion of the intracellular glutamate pool, impaired synthesis of TCA cycle intermediates and thereby to increased DNA replication stress, more frequent DNA double-strand breaks, impaired glutathione synthesis, and increased ROS levels, suggests that markers in these cellular pathways may be explored as biomarkers for response to glutaminase inhibitors in RCC patients [171].

Two complementary approaches will be critical to establish and validate such biomarkers:

First, serial measurements of these candidate biomarkers in preclinical *in vivo* models will be needed. In human RCC tumors, multi-region metabolic profiling has revealed a remarkable level of spatial heterogeneity of metabolic phenotypes, which can not be modeled sufficiently *in vitro* [42]. Moreover, increased glucose uptake by tumor cells limits glucose availability for tumor-infiltrating lymphocytes in mouse models and this metabolic competition causes T cell exhaustion [172, 173]. It will be important to study whether this also applies to glutamine metabolism in RCC to assess how the interactions of tumor cells with their microenvironment influence response and resistance to glutaminase inhibitors. Thus, *in vivo* model systems of RCC such as patient-derived xenografts that better reflect the complex metabolic landscape and microenvironment of solid tumors are needed to understand the mechanisms of

response and resistance to glutaminase inhibitors in patients [174–178].

Second, in order to validate preclinical biomarkers, serial measurements in patients before and during glutaminase inhibitor therapy will be needed to correlate these biomarkers with clinical outcomes. A wide range of technologies is available to facilitate molecular measurements in patients: mass spectrometry may be useful to measure changes in metabolite abundance in biopsies from tumors treated with glutaminase inhibitors, for example, the depletion of glutamate and accumulation of glutamine [34, 39–42, 179]. Complementary nanotechnologies to allow minimally invasive serial sampling of patients during treatment have the potential to facilitate measurements of protein abundance and activity of cellular signaling pathways in scant clinical tissue specimens and in blood (e.g. circulating tumor cells, circulating RNA or proteins; refs. [180–184] and our own unpublished data). Serial measurements may be a crucial approach to find the best strategy to measure response and resistance early on during therapy, so that patients who do not benefit from glutaminase inhibitors can quickly move on to a different, more effective treatment.

In vivo, novel metabolic imaging using positron emission tomography (PET) tracers of glutamine metabolism have been developed: PET probes [18F](2S,4R)4-fluoroglutamine, [11C]glutamine, and (4 S)-4-(3-[18F]Fluoropropyl)-L-Glutamate (18F-FSPG) allow for glutamine and glutamate tracing in patients and have been tested preclinically and clinically to detect sites of increased uptake of these amino acids in multiple cancers including RCC [138, 185–195]. Since glutaminase inhibition depletes the intracellular glutamate pool, it is possible that tumors in which glutaminase activity is effectively inhibited may also show compensatory increased glutamate uptake. Studies to evaluate whether glutamine or glutamate PET tracers may be useful to detect tumors with increased activity of glutamine and glutamate metabolic pathways are underway. Future work will determine the optimal strategy of combining imaging, genetic, gene expression, protein, and/or metabolic biomarkers to identify the patient population with the highest likelihood to benefit from glutaminase inhibitor treatment.

CONCLUSION

Metabolic reprogramming in RCC changes both glucose and glutamine utilization and renders RCC cells dependent on exogenous glutamine supply. Glutamine is converted to glutamate in mitochondria by the enzyme glutaminase and serves to generate TCA cycle intermediates, which are the building blocks for the biosynthesis of amino acids, lipids, nucleotides, and antioxidants. The addiction of RCC cells to glutamine is an ‘Achilles heel’ in RCC metabolism. Glutaminase inhibitors, which lead to reduced cell proliferation *in vitro* and *in vivo,* may be a way to strike at this ‘Achilles heel’. Early data from clinical trials using the glutaminase inhibitor CB-839 alone or in combination with other drugs to treat metastatic RCC suggest that this novel class of drugs is well tolerated and able to control the disease in some cases. Yet, biomarkers are needed to identify patients who derive the most benefit from glutaminase inhibitors and for detecting therapeutic response and resistance early on during treatment. The combination of *in vivo* models for metastatic RCC, serial measurements in these models and in patients, novel imaging probes, and nanotechnologies to interrogate molecular biomarkers in scant clinical tissue specimens and in blood will help identify and validate such biomarkers.

ACKNOWLEDGMENTS

AF is supported by the Conquer Cancer Foundation, the NIH/NCI (grant 5U54CA199075), and Calithera Biosciences, Inc.

VC is supported by and NIH/NCI T32 grant and a Stanford University Translational Research and Applied Medicine (TRAM) award.

CONFLICT OF INTEREST

AF is the site principal investigator of the glutaminase inhibitor CB-839 clinical trials NCT02771626, NCT03163667, and NCT03428217, and receives salary support from Calithera Biosciences, Inc.

AF is founder of Molecular Decisions, Inc.

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