

Glutamine addiction: a new therapeutic target in cancer

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Most cancers depend on a high rate of aerobic glycolysis for their continued growth and survival. Paradoxically, some cancer cell lines also display addiction to glutamine despite the fact that glutamine is a nonessential amino acid that can be synthesized from glucose. The high rate of glutamine uptake exhibited by glutamine-dependent cells does not appear to result solely from its role as a nitrogen donor in nucleotide and amino acid biosynthesis. Instead, glutamine plays a required role in the uptake of essential amino acids and in maintaining activation of TOR (target of rapamycin) kinase. Moreover, in many cancer cells, glutamine is the primary mitochondrial substrate and is required for maintenance of mitochondrial membrane potential and integrity and for support of the NADPH production needed for redox control and macromolecular synthesis.

Cancer cells can be addicted to glutamine

The advent of molecular cancer genetics diverted biologists away from studies of cancer cell metabolism. However, two recent sets of observations have begun to stimulate interest into how signal transduction is integrated with metabolism. First, several oncogenes and tumor suppressors have been linked to the regulation of metabolic processes [1–3]. Second, the efficacy of several cancer therapies has been associated with their effects on metabolism [4–7]. One of the implications of these studies is the synergistic potential of combining pharmacologies that target signal transduction components with those that target metabolic pathways.

One of the oldest conundrums in cancer biology is Warburg's observation that cancer cells tended to take up more glucose and produce more lactic acid than normal tissue [8]. These observations led Warburg to hypothesize that cancer resulted from the regression of cells to the more primitive metabolism exhibited by proliferating single cell eukaryotes [9]. Recent studies have implicated oncogenic activation of glucose uptake as the cause of the 'Warburg effect' [2]. Constitutively activated components of phosphoinositide 3-kinase (PI3K) signaling can directly stimulate levels of glucose uptake and metabolism that exceed the capacity of the cell to use glucose in support of bioenergetic and macromolecular synthesis. When this occurs, the cancer cell secretes excess glycolytic metabolites in the form of lactic acid. In some tumors, this seemingly wasteful metabolism of glucose is mirrored by a similarly inefficient metabolism of glutamine [10,11]. Such cancer cells, in fact,

cannot survive in the absence of exogenous glutamine and exhibit 'glutamine addiction' [12]. Most cancer researchers have viewed the switch of glutamine from being a nonessential amino acid (NEAA) to an essential amino acid (EAA) as an artifact of *in vitro* culture. However, recent studies have suggested that glutamine is a key substrate required for anabolic growth of mammalian cells. This review addresses the role of glutamine in cell growth, the signaling pathways that regulate the cellular utilization of glutamine and potential therapeutic strategies that might exploit the dependence of certain cancer cells on glutamine.

Glutamine provides nitrogen for protein and nucleotide synthesis

The growing cancer must synthesize nitrogenous compounds in the form of nucleotides and NEAAs. Glutamine is the obligate nitrogen donor in as many as three independent enzymatic steps for purine synthesis [phosphoribosylpyrophosphate (PRPP) amidotransferase, phosphoribosylformylglycinamide (FGAM) synthetase, GMP synthetase] and in two independent enzymatic steps for pyrimidine synthesis (carbamoyl phosphate synthetase II, CTP synthetase) [13–15]. In these reactions, glutamine donates its amide (γ nitrogen) group and is converted to glutamic acid (GA) in the process.

GA is the primary nitrogen donor for the synthesis of the NEAAs [13,15] (Figure 1). The transaminases transfer the amine group from GA (originally the α -nitrogen of glutamine) to α -ketoacids. The α -ketoacids used to generate NEAAs are the carbon catabolites of glucose or glutamine: pyruvate, 3-phosphoglycerate, oxaloacetate, and glutamic acid gamma-semialdehyde, which are used to synthesize alanine, serine, aspartate, and ornithine, respectively. Serine is a precursor for glycine and cysteine biosynthesis, ornithine is a precursor for arginine biosynthesis, and aspartate is a precursor for asparagine biosynthesis (glutamine obligately donates its amide group to this reaction). GA contributes its carbon skeleton and nitrogen to the synthesis of proline. Tyrosine is the only NEAA that is not derived from glucose or glutamine; it is directly produced from the EAA phenylalanine.

Glutamine activates TORC1 signaling

The contribution of glutamine to amino acid biosynthesis establishes it as a key ingredient for the protein translation needs of cancer cells. A further role for glutamine in cancer cell protein translation stems from observations

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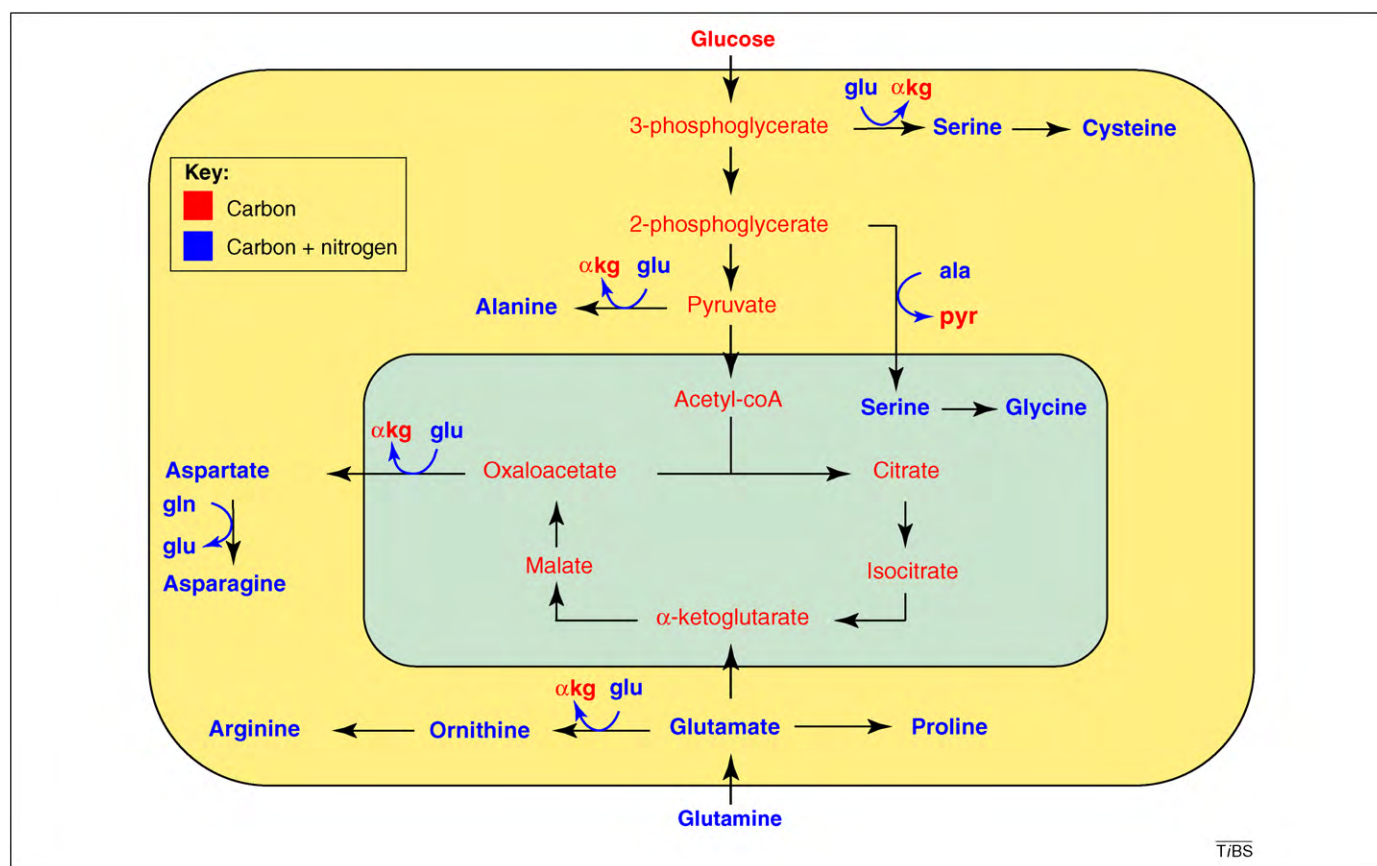


Figure 1. Glucose and glutamine can provide the carbon and nitrogen for the synthesis of the nonessential amino acids. High throughput glucose and glutamine (gln) uptake provide the growing cell with a large pool of carbon and nitrogen for the biosynthesis of the nonessential amino acids. Compounds containing carbon, but not nitrogen, are shown in red, whereas those containing carbon and nitrogen are shown in blue. Carbon precursors derived from glycolysis (3-phosphoglycerate, 2-phosphoglycerate, pyruvate) and glutaminolysis (OAA, GA γ -semialdehyde) serve as the carbon substrate for amino acid biosynthesis. Glutamine-derived GA (glu) donates its amine group to these carbon substrates to produce nonessential amino acids (serine, alanine, aspartate, ornithine) and α -ketoglutarate (α kg). Alanine (ala) serves as the amine donor to produce serine and pyruvate (pyr) in the mitochondrion for the synthesis of glycine. Glutamine provides the carbon and nitrogen for the synthesis of proline, ornithine and arginine. Glutamine can also serve as a direct nitrogen donor in the synthesis of asparagine from aspartic acid.

that a master regulator of protein translation, the mammalian target of rapamycin complex (mTORC)1, is responsive to glutamine levels.

The evolutionarily conserved and rapamycin-sensitive mTORC1, a master regulator of cell growth, activates protein translation and inhibits macroautophagy in response to amino acid abundance and growth factor signaling, among other stimuli [16]. In the presence of sufficient amino acids, growth factor signaling through the insulin-like growth factor (IGF)–PI3K–Akt or the extracellular signal-regulated kinase (ERK)–ribosomal protein S6 kinase (RSK) pathways activates mTORC1 by releasing the RHEB (Ras homolog enriched in brain) GTPase from repression by the tumor suppressors tuberous sclerosis (TSC)1 and TSC2. The importance of sufficient amino acid levels for TORC1 activation was initially recognized in yeast, in which amino acid starvation recapitulated the effects of treatment with the TORC1 inhibitor, rapamycin, in suppressing TORC1-induced protein translation and inducing autophagy. In yeast, TORC1 is responsive to both glutamine and EAA levels [17].

Substantial evidence supports the role of amino acids in the activation of mTORC1-dependent signaling [18–22]. Of the EAAs, leucine seems to produce the most acute response from mTORC1; however glutamine is also necessary for maximum mTOR activation, for reasons

that remained unknown until recently [18,23]. To better understand the mechanism through which glutamine synergizes with EAAs to regulate mTORC1 activation, Nicklin *et al.* [24] studied a cell line in which mTORC1 activation was dependent on the presence of both glutamine and EAAs. Those authors found that a portion of the glutamine taken up through solute carrier family (SLC)1A5 (also called ASCT2) glutamine importer was rapidly exported through the bidirectional amino acid transporter SLC7A5 (also called LAT1) in exchange for the uptake of extracellular EAAs. Knockdown of SLC1A5 in these cells impaired glutamine uptake and export, EAA uptake and mTORC1 activation, suggesting that glutamine uptake and export is required for EAA activation of mTORC1.

Although glutamine is a crucial contributor to the NEAA pool incorporated into newly translated proteins, that study demonstrated that a portion of the glutamine imported into cells is not used for anabolic metabolism. Rather, glutamine is shuttled out of the cell in exchange for EAAs that directly activate TORC1 for the initiation of protein translation and cell growth. Of course, these EAAs can also be directly incorporated into newly synthesized proteins. As such, the uptake and subsequent export of glutamine serves both as a signal to mTORC1 and as a source of EAAs to promote protein translation.

Glutamine as a mitochondrial substrate

Although the unusually high glucose requirement of cancer cells was described in the 1920s, the essential glutamine requirement of proliferating cells was first highlighted by Eagle in 1955. In studies of the nutritional requirements of cell lines growing *in vitro*, Eagle observed that the glutamine consumption rate of many of the cell lines exceeded the consumption of any other amino acid by 10-fold [12]. The cell lines studied could not proliferate in the absence of exogenous glutamine, and many could not maintain their viability in the absence of glutamine. In 1971, Kovacevic and colleagues observed that the glutamine carbons could be accounted for in the carbon dioxide released by cells, providing evidence that glutamine could serve as a combustible fuel [10]. Subsequent experiments showed that certain cancer cell lines consume glutamine at a rate substantially higher than that of any other amino acid. In order to be oxidized, glutamine must first lose its amide group to form GA, which then loses its amine group to form the tricarboxylic acid (TCA) cycle metabolite, α -ketoglutarate. As mentioned above, the enzymes involved in nucleotide biosynthesis contribute to the conversion of glutamine to GA. The enzyme glutaminase (GLS) also contributes to this activity through the release of the amide group of glutamine as free ammonia. The generated GA can be converted into α -ketoglutarate through the cellular transaminases or through GA dehydrogenase, which catalyzes the NAD(P)⁺-dependent liberation of the amine as ammonia.

Recent studies have extended our understanding of the contribution of glutamine to the intermediary carbon metabolism of cancer cells. Real-time ¹³C NMR studies have shown that a significant fraction of glutamine carbon is converted into lactic acid and secreted from the cell [25]. This seemingly wasteful metabolism of glutamine is analogous to the cancer cell metabolism of glucose observed by Warburg, and is consistent with the observation that the glutamine consumption rate exceeds its rate of incorporation into newly synthesized proteins by 10-fold [25,26]. Conversion of glutamine into lactic acid requires the activity of malic enzyme (ME), which oxidatively decarboxylates malic acid, producing carbon dioxide, NADPH and pyruvate. Nucleotide and lipid synthesis both depend on NADPH for a source of reducing equivalents [1]. High throughput glutaminolysis through the provision of substrate for ME can provide a proliferating cell with a significant fraction of its NADPH needs [25].

Glutamine contributes to macromolecular synthesis by means other than the production of NADPH. Real-time ¹³C NMR studies in a glioblastoma cell line have shown that glutamine contributes the majority of the cellular OAA pool [25]. OAA is the obligate substrate that condenses with acetyl-coA to form citrate, which can donate acetyl-coA groups for the synthesis of cholesterol and fatty acids [27] and for the modification of chromatin structure [28]. In providing the cancer cell with a source of OAA, glutamine provides anaplerosis, the refilling of the mitochondrial carbon pool. Replenishment of the mitochondrial carbon pool by glutamine provides the mitochondria with precursors for the maintenance of mitochondrial membrane potential, and for the synthesis of nucleotides, proteins

and lipids. When first discovered, the high rate of aerobic glycolysis in cancer was felt to reflect mitochondrial dysfunction; however, a significant body of evidence suggests that mitochondrial respiratory capacity is maintained in cancer [29]. The available data on mitochondrial glutamine metabolism in cancer cells supports the indispensable nature of mitochondrial metabolism to the physiology of many cancer cell types. In nontransformed, nonproliferative tissues, such as pancreas, liver, kidney, muscle and brain, cells are reported to rely upon the activity of pyruvate carboxylase [30–33], for production of OAA through pyruvate carboxylation. This activity enables these cell types to use glucose for their anaplerotic needs. By contrast, ¹³C NMR studies do not support the presence of pyruvate carboxylase activity in several cancer cell types [25,34]. The mechanism through which pyruvate carboxylase activity is suppressed in these cell types is an area of active investigation, as is the connection between pyruvate carboxylase activity and glutamine dependence.

Oncogenic levels of c-MYC regulate glutamine metabolism

As mentioned, glutamine is the obligate nitrogen donor for nucleotide synthesis. Five enzymatic steps in the synthesis of purines and pyrimidines use glutamine as a source of nitrogen. Recent studies using quantitative reverse transcriptionase (RT)-PCR and chromatin immunoprecipitation (ChIP) in multiple cell systems have suggested that c-MYC (Myc) binds and transactivates 11 genes involved in nucleotide biosynthesis [35]. Myc is a basic helix–loop–helix zipper (bHLHZ) protein that heterodimerizes with the small bHLHZ protein MAX, and exerts both activating and repressing transcriptional effects [36]. Of the five enzymatic steps utilizing glutamine, three (PRPP amidotransferase, carbamoyl phosphate synthetase II, CTP synthetase) are directly regulated by Myc at the transcriptional level [37]. Carbamoyl phosphate synthetase II, a rate-limiting glutamine-dependent enzyme involved in pyrimidine synthesis, is also regulated via epidermal growth factor receptor (EGFR)-dependent mitogen-activated protein kinase (MAPK) phosphorylation [38] and by caspase-dependent degradation [39].

Cancer cells take up and metabolize glucose and glutamine to a degree that far exceeds their needs for these molecules in anabolic macromolecular synthesis. Commonly occurring oncogenic signal transduction pathways initiated by receptor tyrosine kinases or Ras engage PI3K–Akt signaling to directly stimulate glycolytic metabolism [1,2]. Oncogenic levels of Myc have recently been linked to increased glutaminolysis through a coordinated transcriptional program [26,40,41]. Myc activation/amplification is one of the most common oncogenic events observed in cancer, and is known to drive the progression of human lymphomas [42,43], neuroblastoma [44] and small cell lung cancer [45]. Quantitative RT-PCR and ChIP experiments support the binding of Myc and the transcriptional activation of two high affinity glutamine transporters: SLC38A5 (also called SN2) and SLC1A5 (ASCT2), the transporter required for glutamine-dependent mTORC1 activation [24,26]. In addition to facilitating glutamine uptake, Myc promotes the metabolism of imported

glutamine into GA and ultimately into lactic acid [26]. Whether the tendency of Myc to complement Ras and PI3K-Akt [46,47] is related to the interdependence of glutamine and glucose metabolism in support of cell growth remains unclear.

The importance of glutamine metabolism for the MYC-amplified cell has been highlighted by a recent demonstration that Myc also influences post-transcriptional regulation of glutamine catabolism. In a screen for Myc-regulated mitochondrial proteins, Gao *et al.* [41] found that GLS protein levels were significantly upregulated in Myc-overexpressing cells, yet the *GLS* mRNA expression level did not correlate with the increased protein levels, leading the authors to hypothesize that Myc regulates GLS through a post-transcriptional mechanism. Using an algorithm-based approach, the authors found that the microRNA miR-23a/b repressed GLS translation through binding its 3' untranslated region (UTR). Notably, the authors had previously identified miR-23a/b as a strong target of Myc transcriptional repression. This study further links Myc overexpression to the cellular ability to catabolize glutamine to GA, thereby providing cells with a large pool of carbon for anaplerosis and NADPH production. Whether other oncogenic signaling pathways also contribute to the regulation of glutamine metabolism remains to be determined.

Myc-induced metabolic reprogramming triggers cellular dependency on exogenous glutamine as a source of carbon for the maintenance of the mitochondrial membrane potential and macromolecular synthesis. Indeed, glutamine depletion kills transformed cells in a Myc-dependent manner [26,40]. The cell death induced by glutamine starvation can be rescued by the overexpression of BCL-2, BCL-x_L or a dominant negative form of caspase-9, implicating the intrinsic apoptotic pathway as the mechanism of cell death [26,48]. Substitution of glutamine with a membrane-permeable form of α -ketoglutarate, pyruvate or OAA also rescues this death [26,48]. However, consistent with the importance of glutamine as an obligate source of nitrogen, neither overexpression of anti-apoptotic proteins nor addition of TCA cycle intermediates can rescue the proliferation defect observed in glutamine-starved cells [26]. Nevertheless, glutamine depletion of Myc-transformed cells leads to a profound reduction in the levels of TCA cycle metabolites despite abundant extracellular availability of glucose, supporting the importance of glutamine in the maintenance of mitochondrial anaplerosis [48]. These findings suggest that Myc transformation might also suppress the ability of tumor cells to use glucose as an anapleurotic substrate, perhaps through upregulation of lactate dehydrogenase (LDH)-A.

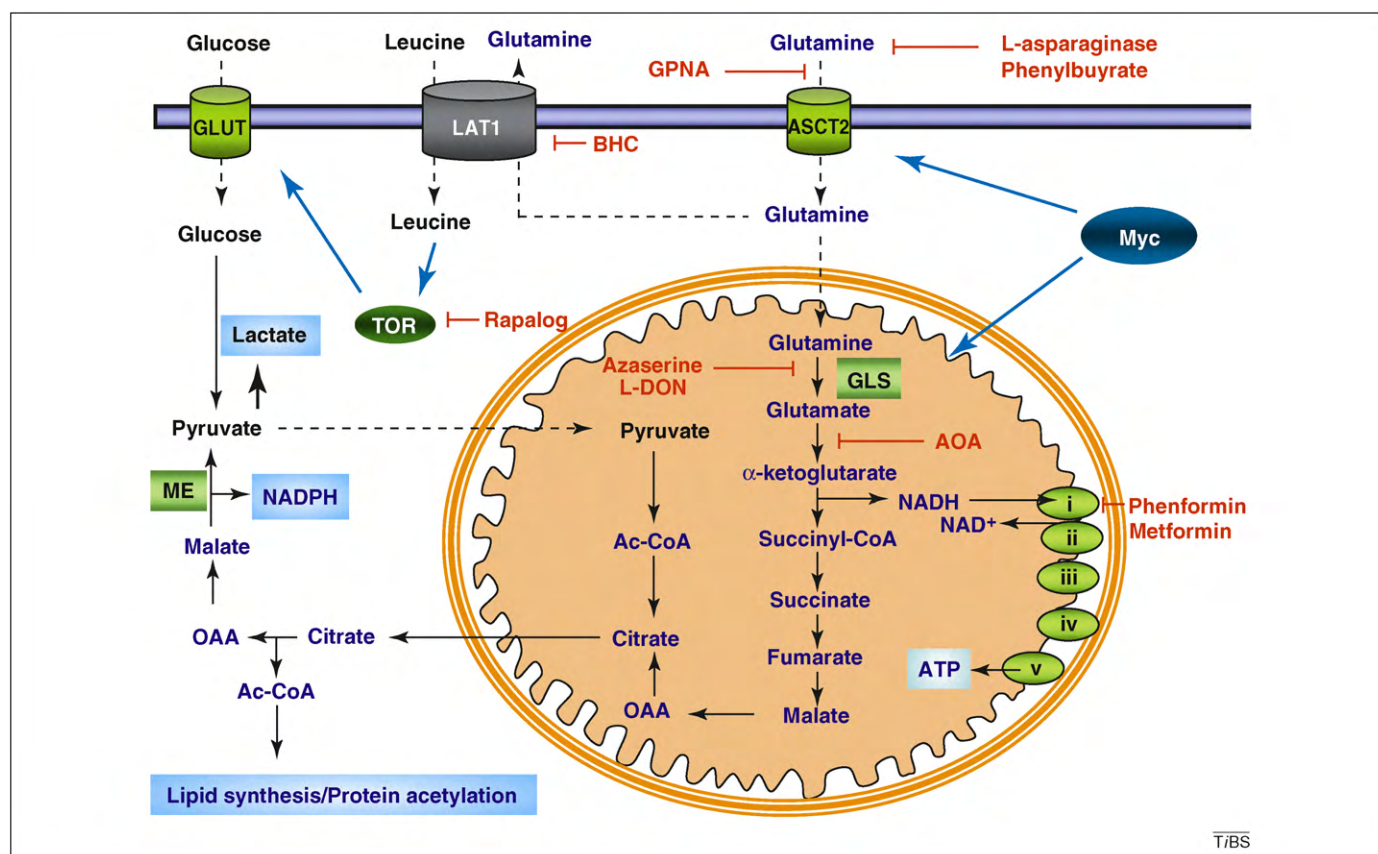


Figure 2. Pharmacologic targets in glutamine metabolism. Myc enables cancer cells to maximize glutamine uptake from the extracellular space through upregulation of the glutamine importer, ASCT2. Uptake of glutamine can be suppressed both by depletion of glutamine from the extracellular space with L-asparaginase and phenylbutyrate or through inhibition of ASCT2-dependent uptake with L- γ -glutamyl-*p*-nitroanilide (GPNA). Once glutamine enters the cell, it can be metabolized through glutaminolysis to provide NADPH or exported to facilitate TOR kinase activation. Myc enables conversion of glutamine into GA via upregulation of GLS, an enzyme whose activity can be inhibited by treatment with L-DON and azaserine. Transamination of GA to α -ketoglutarate can be inhibited with AOA. Mitochondrial metabolism of α -ketoglutarate leads to the production of citrate, its cleavage into OAA, reduction of OAA into malate and oxidation of malate to pyruvate via ME to produce pyruvate and NADPH. The high rate of glutamine metabolism through successive steps into OAA establishes glutamine as the primary anapleurotic substrate. This metabolism requires the regeneration of NAD⁺ through the electron transport chain, a process that can be inhibited with the biguanides, phenformin and metformin. The use of glutamine as a substrate for the amino acid exchanger LAT1 can be suppressed with BCH treatment, and TOR activity can be suppressed by rapamycin treatment.

Targeting glutamine addiction: past, present and future

A wide variety of human cancer cell lines has shown sensitivity to glutamine starvation, including cells derived from pancreatic cancer, glioblastoma multiforme, acute myelogenous leukemia and small cell lung cancer [49]. Experiments in the 1950s showed that the compounds 6-diazo-5-oxo-L-norleucine (L-DON) and azaserine, isolated from a species of *Streptomyces*, have significant activity as glutamine analogues [50]. Later, another glutamine analogue, acivicin, was also isolated. Research into the glutamine dependency of cell lines *in vitro* spurred the testing of these compounds as therapeutics. Preclinical testing of all three agents showed a significant cytotoxic effect against certain tumor types, both in culture and in mouse xenograft models [14]. Although all three of these agents showed clinical activity, their use was discontinued because of dose-limiting neurotoxicity, gastrointestinal toxicity and myelosuppression [14]. All three compounds show their greatest activity in inhibiting the glutamine-dependent enzymatic steps in nucleotide biosynthesis [51]. In fact, acivicin has only a minor effect on glutaminolysis, while significantly reducing glutamine-dependent nucleotide biosynthesis. These studies demonstrate that glutamine mimetics are unduly toxic. Current investigations seek to preserve the glutamine metabolism required for normal tissue physiology while impairing the glutamine addiction of cancer (Figure 2).

Suppressing cancer cell glutamine uptake

Numerous studies have detected an upregulation of high affinity glutamine transporters in cancer [52]. One primary example, SLC1A5 (ASCT2), is a direct target of the Myc oncoprotein [26] and is upregulated in a host of cancers [52]. L- γ -glutamyl-p-nitroanilide (GPNA), one of a panel of SLC1A5 inhibitors [53], can inhibit glutamine uptake and inhibit glutamine-dependent mTOR activation *in vitro* [24].

Suppressing glutamine-dependent anaplerosis

Studies using the transaminase inhibitor amino-oxyacetic acid (AOA) have suggested that the major route through which glutamine-derived carbon enters the TCA cycle in Myc-transformed cells is through transamination [54]. These studies have suggested that GA dehydrogenase, the principal enzyme converting GA into α -ketoglutarate in the pancreas, is not the rate-determining step in proliferating cells. Recent data suggests that AOA could in fact be a promising cancer therapeutic. AOA treatment produced a cytostatic effect on the growth of a breast cancer cell line in a mouse xenograft model without any obvious dose-limiting toxicities [55]. AOA treatment has also shown a cytotoxic effect on a glutamine-dependent MYC-amplified glioblastoma cell line *in vitro* while having no significant effect on a paired Myc-deficient line [26]. These studies suggest that selective inhibition of one component of glutamine metabolism (glutamate transamination) might reproduce the anti-cancer effects but not the nonspecific toxicity of wholesale inhibition of glutamine metabolism.

Inhibiting complex I

Glutamine-dependent cancer cells undergo dramatic metabolic reprogramming, resulting in reprogramming

of mitochondria to produce anabolic precursors from glutamine. The entry and flux of glutamine through the TCA cycle requires the continual regeneration of mitochondrial NAD⁺ through the activities of the mitochondrial electron transport chain. It has been a longstanding hypothesis that the biguanides activate AMPK through inhibition of the mitochondrial respiratory chain [56–59]. Metformin has shown promise in slowing the growth of cancer cells *in vitro* and xenografted tumors *in vivo* [60–63]. Epidemiological studies also show a reduced incidence of cancer in patients treated with metformin compared with diabetics treated with other drugs [64]. However, therapeutic levels of metformin exhibit the greatest mitochondrial effects in the liver, and further studies are needed to determine the extent to which the *in vivo* efficacy of these agents is due to liver-dependent effects, such as lowering of blood glucose concentration and/or tumor-specific effects on glutamine metabolism.

Targeting glutamine-dependent mTOR activation

As previously noted, a portion of the glutamine imported into cancer cells via SLC1A5 is directly exported through the SLC7A5–SLC3A2 (LAT1–4f2hc) complex. The subsequent import of EAAs, such as leucine, activates the mTORC1 kinase. *In vitro* treatment of cancer cells with L- γ -glutamyl-p-nitroanilide (GPNA; an inhibitor of SLC1A5) or with 2-aminobicyclo-(2,2,1)heptanecarboxylic acid (BCH; an inhibitor of SLC7A5–SLC3A2) blocks the glutamine-dependent activation of mTORC1 and induces autophagy [24]. The *in vivo* utility of this approach for the treatment of glutamine-addicted/mTORC1-dependent cancers is under investigation.

Enzymatic lowering of blood glutamine levels

L-asparaginase (Elspar (Merck & Co. Inc.) or Oncaspar (Enzan Inc.)) hydrolyzes asparagine into aspartic acid and ammonia, and is a cornerstone of treatment for pediatric acute lymphoblastic leukemia (ALL) [65]. This treatment is believed to be successful because ALL cells are incapable of synthesizing asparagine *de novo*. L-asparaginase also possesses significant GLS activity, and is capable of hydrolyzing glutamine to GA and ammonia [66]. L-asparaginase significantly depletes glutamine levels, and studies have confirmed that the success of treatment correlates with glutamine depletion [49,66]. However, L-asparaginase treatment in adults has shown significant toxicity [67]. More work is needed to clarify the potential role for L-asparaginase therapy for the treatment of childhood and adult glutamine-addicted cancers.

An alternative agent that could be used to deplete plasma glutamine is phenylbutyrate [Buphenyl], (Ucyclyd Pharma 8125 N. Hayden Road, Scottsdale, AZ 85258-2463, USA) or Ammonaps (Swedish Orphan), an FDA approved pharmacologic for the treatment of hyperammonemia in patients with congenital urea cycle disorders [68]. Pharmacologic doses of phenylbutyrate lead to a significant depletion of plasma glutamine levels [69]. In humans, phenylbutyrate spontaneously breaks down to form phenylacetate, which is conjugated with glutamine by the hepatic enzyme phenylacetyl coenzyme A:glutamine

acyltransferase to yield phenylacetylglutamine. This latter product is then excreted in the urine [70].

Concluding remarks

There is increasing evidence to suggest that oncoproteins can directly reprogram tumor cell metabolism, rendering the cells addicted to certain nutrients in a way non-transformed cells are not. However, whether alterations in cancer metabolism can be safely targeted therapeutically remains to be determined. In this review, the importance of glutamine metabolism for cancer growth and viability is highlighted and the possibility of developing therapies that can exploit glutamine metabolism for therapeutic gain considered. Undoubtedly, like other targeted therapies being developed, therapies directed against glutamine metabolism will be most effective in tumors that display glutamine dependence. Whether glutamine derivatives can be used to investigate tumor metabolism *in vivo* in a manner analogous to the way the glucose analog, fluorodeoxyglucose, has been developed for positron emission tomography, is currently under investigation. If successful, such studies will help establish whether glutaminolysis is a tissue culture artifact or a potential Achilles' heel of cancer cells.

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