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Alone and together: current approaches to targeting glutaminase enzymes as part of anti-cancer therapies



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Metabolic reprogramming is a major hallmark of malignant transformation in cancer, and part of the so-called Warburg effect, in which the upregulation of glutamine catabolism plays a major role. The glutaminase enzymes convert glutamine to glutamate, which initiates this pathway. Inhibition of different forms of glutaminase (KGA, GAC, or LGA) demonstrated potential as an emerging anti-cancer therapeutic strategy. The regulation of these enzymes, and the molecular basis for their inhibition, have been the focus of much recent research. This review will explore the recent progress in understanding the molecular basis for activation and inhibition of different forms of glutaminase, as well as the recent focus on combination therapies of glutaminase inhibitors with other anti-cancer drugs.

Plain language summary: Many strategies exist to inhibit cancer progression, from chemotherapy to more targeted therapies that exploit differences between tumors and healthy tissue. One such targeted strategy involves inhibition of the enzyme glutaminase, which converts glutamine obtained from the bloodstream into nutrients that fuel tumor growth. Research into glutaminase is ongoing, with regulation of the enzyme, and novel molecular approaches to inhibit its activity, being key focus areas. Here, we review recent progress on targeting glutaminase enzymes for anti-cancer therapy, including several approaches in which glutaminase inhibitors are combined with inhibitors of other cancer-relevant targets, to increase the overall effectiveness of the treatment.

Tweetable abstract: Inhibition of the enzyme glutaminase is a promising approach to cancer therapy, which draws on unique metabolic differences between tumors and healthy tissues. This review examines recent progress developing such inhibitors.

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Cancer cells frequently undergo metabolic reprogramming to satisfy the increased demand for ATP and biosynthetic precursors characteristic of malignant growth. This phenomenon, termed the 'Warburg effect', results in enhanced glucose consumption, with the sugar being predominantly converted to lactate regardless of the availability of oxygen, i.e. 'aerobic glycolysis'. This effectively uncouples glycolysis from the tricarboxylic acid (TCA) cycle. Despite this uncoupling, cancer cells require the TCA cycle to turn, both to produce synthetic intermediates and to generate 'high-energy' molecules, and thus require an alternative source of synthetic material as input. Isotope tracing [1–5] showed that in many aggressive cancers including glioblastoma, breast cancer, pancreatic cancer, and liver cancer, glutamine catabolism is the major contributor to the TCA cycle instead of glucose. These cancer cells in effect become addicted to glutamine consumption to replenish metabolic intermediates for energy production and biogenesis. One of the simplest pathways by which glutamine is introduced to the TCA cycle runs through glutaminase. Glutaminase (EC 3.5.1.2), or L-glutamine amidohydrolase, is a mitochondrial enzyme that initiates glutamine catabolism by catalyzing the conversion of glutamine into glutamate and ammonia (Figure 1A), with

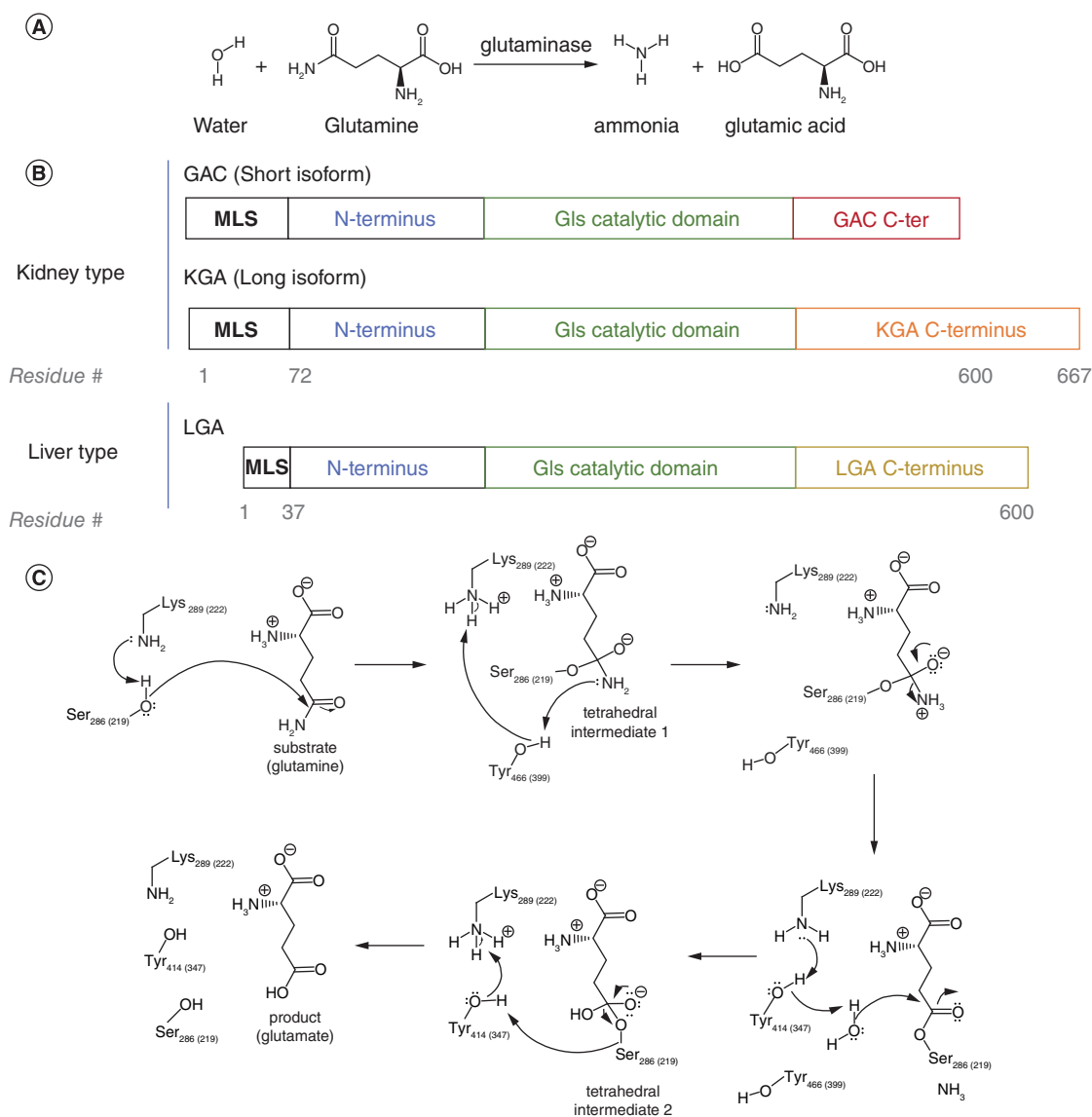


Figure 1. Glutaminase family of enzymes share high degree of homology in sequence and catalytic mechanism. (A) Glutamine hydrolysis as catalyzed by glutaminase enzymes. **(B)** Glutaminase enzymes are encoded by two independent genes, *GLS* and *GLS2*. **(C)** Mechanism of the hydrolytic reaction of the substrate, glutamine, as catalyzed by the active site residues in glutaminase enzymes. All three active forms of glutaminase share the same active site residues, which are numbered according to human GAC (and human LGA). C-ter: C-terminus; MLS: Mitochondrial localization sequence.

glutamate then being converted to the TCA cycle intermediate α -ketoglutarate by glutamate dehydrogenase, or by a variety of reversible transaminases [6]. Glutaminase can thus serve as an essential gateway enzyme to support glutamine addiction. The roles of glutaminase in various cancers have been extensively reviewed in the last few years [7–9]. In this review, we will instead focus on a how this class of enzymes is regulated, along with recent strategies being deployed to inhibit this key onco-supportive enzyme.

Two different genes, encoding for different isozymes of glutaminase, exist in mammals (Figure 1B). The two genes, *GLS* and *GLS2*, were initially identified in different tissues based on their varying responses to changes in pH [10]. Both *GLS* and *GLS2* play critical roles in the altered metabolism observed in cancer cells, and their differential upregulation in various physiological contexts has been a topic of interest for many researchers. The *GLS* gene, located on chromosome 2, is predominantly expressed in kidney, brain, small intestine, and is expressed at lower levels in at least 20 other tissues [11]. Through alternate splicing, *GLS* gives rise to the isoform KGA and

its truncated C-terminal variant GAC. The difference in the C termini results in a higher specific activity for GAC, which might explain why it is more frequently overexpressed in aggressive cancers [12,13]. The *GLS2* gene, from which the isozyme LGA is derived, is located on chromosome 12 and is expressed mainly in the liver [11]. The glutaminase enzymes all have significant structural homology and have an identical mechanism of enzymatic hydrolysis (Figure 1C). However, the isozymes are not identical in their biological activities, and research is ongoing to examine the differences between *GLS* and *GLS2*, which we will outline below.

Metabolic reprogramming has been well established as a major hallmark of cancer cells, and efforts to inhibit glutaminase activity in the past decade have proven to be potentially effective strategies in halting malignant growth [14,15]. Several classes of small molecules that target glutaminase enzymes, ranging from substrate mimetics to allosteric inhibitors, have been identified and optimized. Extensive structure-function characterization efforts, mainly using x-ray crystallography, have revealed several important structural elements of glutaminase that regulate the activity of the enzyme, providing insights into the mechanism of inhibition. In this review, we will discuss the recent progress in understanding the molecular basis for activation and inhibition of different forms of glutaminase. In addition, we will address new research efforts in using glutaminase inhibitors, especially the recent trend toward combination therapies with other anti-cancer drugs.

Controlling the differential expression of glutaminases in cancer cells

Oncogenes regulating glutaminase expression

The basis for the tissue distribution of the different forms of glutaminase is a topic of continuing study. Glutaminase expression can be radically different from one tumor to another (Figure 2 [16,17]A). The choice between whether a cell expresses *GLS* and *GLS2* can have important consequences for cancer progression [18], and so a great deal of effort has been put into understanding the different regulation pathways for either isozyme. The preferential expression of specific forms of glutaminase has been found to be significantly influenced by a number of different factors (Figure 2B). In the hormone-sensitive prostate cancer cell lines LNCaP and LAPC4, KGA expression is promoted by the androgen receptor, and the expression of KGA is reduced when androgen receptors are inhibited during hormone therapy, reducing the cells' ability to satisfy their glutamine addiction. However, glutamine metabolism is ultimately restored through the upregulation of GAC expression [13]. The switch to the GAC isoform, which has higher catalytic activity, results in increased glutaminolysis and hyperproliferation that are independent of the androgen receptors, thus allowing the development of therapeutic resistance for androgen deprivation therapy. This expression of GAC was in fact driven by the oncogenic transcription factor c-Myc, suggesting that c-myc-dependent cancers might also preferentially promote the expression of GAC and become sensitive to glutamine inhibitors.

One mechanism by which c-Myc drives glutaminase expression works through microRNAs (miRNAs), a class of short, non-coding RNAs. For example, c-Myc has been shown to select for the expression of *GLS* over *GLS2* in P493-6 lymphoma cancer cells and PC3 prostate cancer cells by repressing the microRNAs miR-23a and miR-23b [19]. These microRNAs bind to the *GLS* gene, but not the *GLS2* gene, and thus decrease *GLS* transcription. Expression of another transcription factor in the same family, N-Myc, correlates with *GLS2* mRNA expression in neuroblastoma cells. Mirroring this finding, glutamine deprivation was more damaging to *MYCN* amplified Kelly cells than to *MYCN* nonamplified SHEP cells, and siRNA silencing of n-Myc in the Kelly cells reduced the sensitivity to glutamine withdrawal [20].

In some cases, radically different genes drive isozyme preference in different categories of cancer effecting the same tissue. For example, glutaminase isozyme expression is distinct for luminal versus basal breast cancer [4]. *GLS2* is expressed in luminal-subtype breast cancer cell lines (MDA-MB-453, T-47D, BT-474, MCF7 cells), and its expression is driven by the luminal-transcription factor GATA3. However, in basal-subtype breast cancers, such as the MDA-MD-231, TSE, Hs 578T, and HCC38 cell lines, the *GLS2* promoter is methylated and therefore protein expression is suppressed. These cancer cells instead upregulate *GLS* expression by utilizing the c-Jun oncogenic transcription factor. The reverse was observed in liver tumors where *GLS2* expression was increased upon knockout of *GLS* [2].

One interesting pathway by which some oncogenes regulate glutaminase expression is by preventing its degradation. For example, *GLS2* transcript levels can be protected from degradation, resulting in higher *GLS2* expression compared with *GLS*. Investigation of TE13 and TE1 esophageal squamous cell carcinoma cells revealed that the RNA-methylating enzyme METTL3 enhances the stability of the *GLS2* transcript in a YTHDF1/IGF2BP2-mediated m6A-dependent manner [21]. *GLS* is regulated in a similar matter by Sirtuin 5 (Sirt5). In MDA-MB-231 or MDA-MB-468 breast cancer cells, and in A549 lung adenocarcinoma cells, silencing of SIRT5 resulted in a

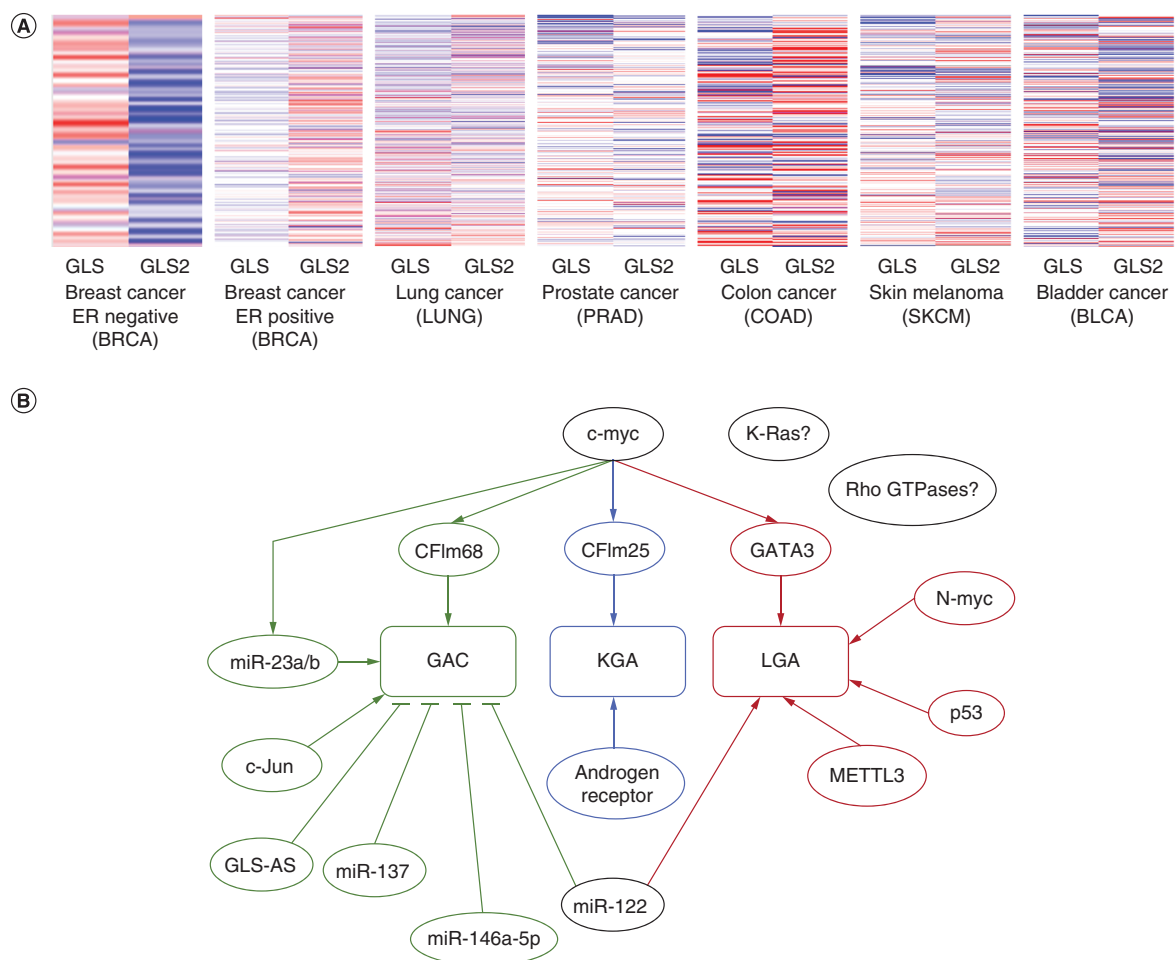


Figure 2. Regulated expression of different glutaminase isozymes. (A) Heat map for differential expression levels of GLS and GLS2 in the most prevalent cancers in the USA [16]. Expression levels are normalized to the respective cancer type study. Relative increase in gene expression is indicated by the red color and relative decreases are in blue. The results shown are based wholly or in part upon data generated by the TCGA Research Network: www.cancer.gov/tcga, and the heat maps were prepared using Xena Browser, analyzed online on 17 September 2022, <https://xenabrowser.net/>. **(B)** Many oncogenic factors and transcriptional factors are involved in controlling the differential expression of specific forms of glutaminase in different types of cancer. Whether the regulation is activating, or inhibiting, is indicated by different arrowheads.

significant decrease in *GLS* expression [22]. It was determined that this occurs because SIRT5 desuccinylates *GLS* at residue Lys164. This, in turn, prevents ubiquitination of *GLS* at Lys158, resulting in reduced ubiquitination-driven protein degradation.

There are many other cases where oncogenic signals triggered by Rho-GTPases [14], K-Ras [5,23], and c-Myc [24,25] upregulate glutamine metabolism, but little is known about the specific glutaminase isoforms or isozymes involved in these signaling pathways. Such studies tend to rely upon examining the effects of glutamine deprivation in cell culture models, tracking the fate of isotope-labeled glutamine, or measuring ammonia production (a by-product of glutamine hydrolysis by glutaminase). Clearly, more work is required to both fully understand the signaling pathways which lead to glutaminase expression and degradation, and to characterize which form of glutaminase is most involved in any given instance of glutamine dependence.

Role of non-coding RNAs

As mentioned above, miRNA could help oncogenes such as c-myc control the expression of specific isozyme of glutaminase at the transcript level. Besides miR-23a/b, another direct binder to the 3'-UTR of glutaminase mRNAs is miR-122, the most abundant miRNA in the liver. This miRNA suppresses *GLS* level while increasing *GLS2*

level [26], as shown through western blot analysis of *GLS* and *GLS2* expression. A clear reduction in *GLS* expression and an increase in *GLS2* level was observed in the liver of liver-tumor-bearing mice, as well as in human primary hepatocellular carcinoma cell line EC4 when miR-122 is introduced. miR-137 plays a similar role in melanoma by targeting *GLS* and thus acts as a tumor suppressor, as shown in A375 and SK-MEL-1 cells and accompanying mouse xenograft model [27]. Other miRNAs have been found to regulate glutaminase expression through different modes. For example, in chronic myeloid leukemia, miR-146a-5p can target ubiquitin-specific protease 6 (USP6), which in turn affects the level of ubiquitination and hence degradation of *GLS* using K562-R cells [28]. A more comprehensive screen using glioblastoma cell lines (LN229 and T98G) also highlights other miRNAs and their specific association with either *GLS* or *GLS2* in order to select for specific expression of each isozyme [29].

In addition, long non-coding RNAs (lncRNAs) could also influence the expression of the *GLS* gene. For example, the alternative splicing of *GLS* or the GAC:KGA ratio can be controlled at the pre-transcriptional level by long non-coding RNA (CCAT2) and polyadenylation factors (CFIm25 and CFIm68) [12]. The long non-coding RNA CCAT2 has two alleles G and T. The G allele preferentially binds to CFIm25, and the complex can then associate with the poly(A) site within intron 14 of the *GLS* pre-mRNA to selectively direct splicing toward generating the GAC transcript. The T allele of CCAT2 on the other hand binds with higher affinity to CFIm68 and selects for the production of the KGA transcript. In colorectal cancer, the G allele of CCAT2 is overexpressed [12], and thus upregulates GAC expression to promote metastases and cell proliferation. However, GAC is not always the ultimate choice to support malignant growth in aggressive cancers. Triple-negative breast cancer cells (MDA-MB-231) utilize both GAC and KGA [14,30] and removal of either isoform is sufficient to reduce glutamine catabolism [30]. In pancreatic cancer, it has been found that *GLS* expression is regulated by a nuclear-enriched antisense lncRNA (GLS-AS) at the post-transcriptional level [31]. Specifically, GLS-AS can inhibit *GLS* transcription by ADAR1/Dicer-dependent RNA interference, and hence pancreatic cancer cells such as BxPC-3 or PANC-1 cells show low expression of GLS-AS as these cells are dependent on glutaminase activity and glutamine catabolism.

Localization regulation

The localization of each form of glutaminase might also provide insights into their preferential expression. Prediction algorithms, such as TPPred3 [32] and TargetP 2.0 [33], when performed on KGA, GAC, and LGA have consistently predicted that each localizes to the mitochondria. Specifically, when analyzing the full-length cytosolic precursors of the glutaminase isoforms, these servers predict that GAC and KGA share a cleavable mitochondrial localization signal peptide at the start of the N-terminus based on matching cleavage site motifs (i.e. cleavage after the initial 45 amino acids – TPPred3, and 72 amino acids – TargetP 2.0). LGA is also predicted to have an N-terminal signal peptide (cleavage after the initial 21 amino acids – TPPred3, and 40 amino acids – TargetP 2.0). Predictions using the entire sequence (BAR 3.0 [34] and ESLPred [35]) also show similar results. Interestingly, one experimental study provided evidence showing that GAC localizes to the mitochondria while KGA remains in the cytosol in MDA-MB-231 and SKBR3 breast cancer cells, PC3 and DU145 prostate tumor cells and A549 lung cancer tumor cells, using cell fractionation approach, western blot analysis, and immunofluorescence [36]. Similarly, another study that examined LGA expression in human hepatocellular carcinoma, glioblastoma, neuroblastoma cells, and monkey COS-7 cells using immunocytochemistry and subcellular fractionation also suggested that LGA can also localize to the nucleus besides the mitochondria [37]. Therefore, experimental approaches beyond sequence analyses of the localization of the various glutaminases for different contexts of cancer progression might be important in offering an alternative explanation for their differential regulation in various types of cancer cells.

Molecular basis for activation of glutaminase

The glutaminase enzymes have a number of key features that relate to their activity. The N- and C-termini are poorly resolved in crystal structures to date, but are believed to contribute to the regulation of these enzymes, possibly by interacting with binding partners, or by facilitating the formation of large multimeric structures [38]. By comparison, in-depth structural data is available for the bifurcated catalytic domain, many features of which are well conserved among the glutaminase isozymes. Considering *GLS* and *GLS2*, sequence alignment with BLAST shows 88% similarity for the catalytic domains from Ile221 to Arg544 in GAC/KGA and Ile154 to Arg477 in LGA. Structure alignment of the catalytic domains of KGA (PDB: 3czd) and LGA (PDB: 4bqm) is shown in Figure 3A. Specifically, all three glutaminases are highly similar in their catalytic sites, with each containing a Ser-Lys-Tyr catalytic triad (Figures 1C & 3B). GAC, KGA, and LGA have all been found to self-associate to form

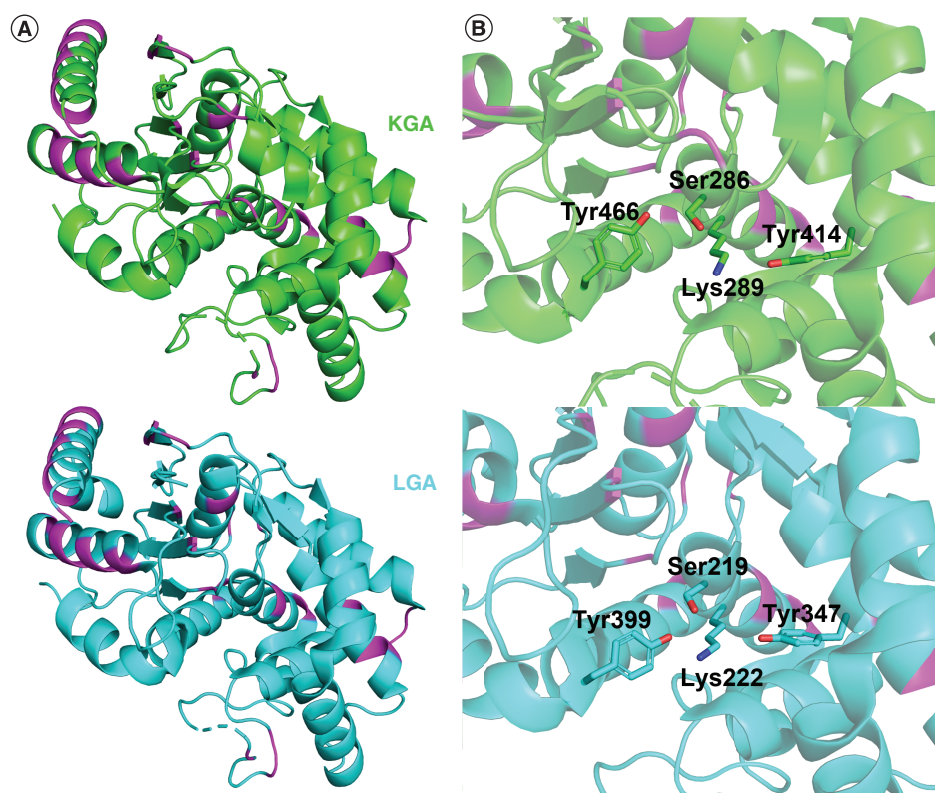


Figure 3. GLS and GLS2 share highly conserved active sites. **(A)** Alignment of the catalytic domains of KGA (green, PDB: 3czd) and LGA (cyan, PDB: 4bqm). The few differences are highlighted in magenta. **(B)** A closer look at the substrate-binding pocket, with the catalytically active residues shown as sticks.

Isoform	K_s for P_i (mM)	k_{cat} (s^{-1})	K_M for glutamine (mM)	Cooperativity
GAC	80 ± 8	22 ± 2	1.4 ± 0.4	Michaelis-Menten kinetics
KGA	76 ± 7	10 ± 1	1.9 ± 0.4	Michaelis-Menten kinetics
LGA	8 ± 1	2.5 ± 0.3	4.0 ± 0.2	Positive cooperativity

Table adapted from DeLaBarre *et al.* [39].

dimers and tetramers in solution (Figure 4A). The dimers are held together mainly by the salt bridges between Asp386 and Lys396. Important structural elements such as the ‘activation loop’ (Gly315 to Glu325 in GAC/KGA and Gly248 to Glu258 in LGA) [39] and the ‘lid region’ (Tyr249 to Lys255 in GAC/KGA and Tyr182 to Lys188 in LGA) [40,41] have been implicated in regulating the activation of GAC and LGA, and as discussed below are also implicated in small-molecule inhibitory mechanisms (Figure 4B). Despite the close similarity between the various isozymes, several small molecules have been found which can discriminate between *GLS* and *GLS2*, as discussed further below.

The transition from dimers to tetramers increases the affinity of glutaminase enzymes for glutamine [36,40,41], which correlates with increased catalytic activity. All three forms of glutaminase exhibit maximal catalytic activity in the presence of the allosteric activator inorganic phosphate (P_i). The activation by phosphate [39] differs markedly between *GLS* and *GLS2*, as shown in Table 1. The efficient catalytic turnover of GAC [13] is thought to be a driving force for the preferred expression of this form by many cancer cells, and thus its mechanism of activation has been extensively studied. Phosphate drives the formation of GAC tetramers *in vitro* [36], and the formation of an active tetramer is essential for maximal catalytic activity [42,43]. It has recently been suggested that the presence of phosphate activates GAC through a conformational shift in the ‘lid region’ of the enzyme. Specifically, residue Tyr249 forms a ‘lid’ which is able to cover the glutamine binding site of the enzyme, and thus converts from a ‘closed’

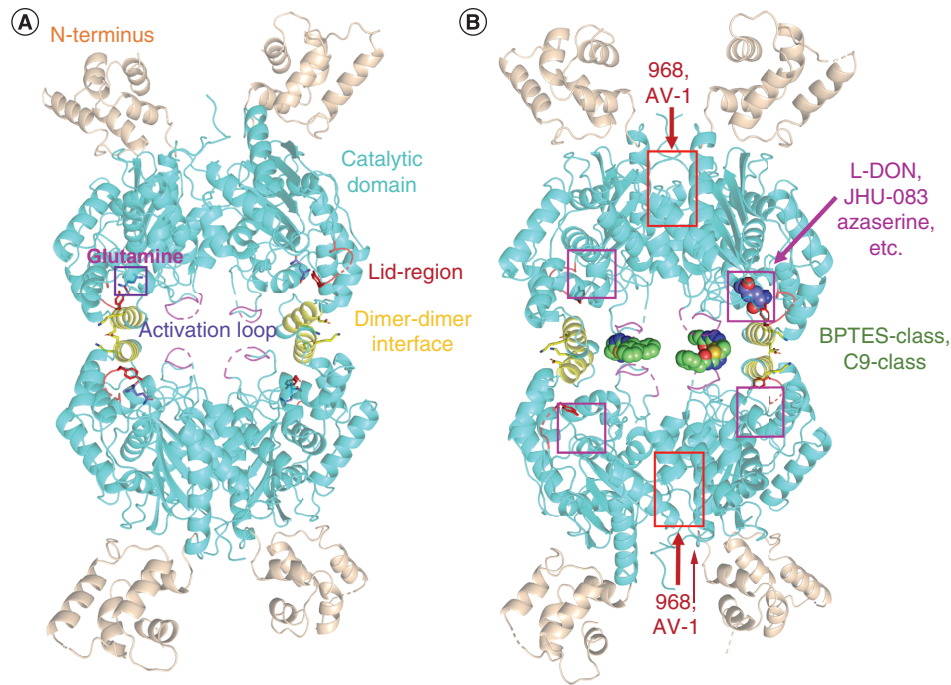


Figure 4. Important structural elements of glutaminase. (A) Glutaminase monomers assemble into dimers and tetramers, with the crystallographic assembly shown. Different important structural units are highlighted in different colors (PDB: 7sbm). **(B)** Binding sites for glutaminase inhibitors are shown, all determined by crystallography except for 968 and AV-1 (which were determined by molecular modeling).

conformation which covers the active site and blocks substrate entry or product exit, to an open conformation where the residue projects away from the site. The conversion between the two positions of Tyr249 is essential for high affinity glutamine binding and maximal catalytic turnover in the presence of phosphate [41]. The tetramer is generally thought to be the biologically meaningful glutaminase multimer, but addition of large amounts of phosphate can lead to even higher-order structures [36,38]. It remains unclear however why phosphate induces the formation of these higher-order oligomers of GAC in solution, and how this impacts enzyme activation [38,44]. Enhanced catalytic activity can also be achieved by mutating Lys320 in the activation loop of GAC to Ala, which yields a constitutively active enzyme with activity matching that of GAC (wt) in the presence of phosphate, and also results in oligomer formation [43]. In contrast, KGA and LGA do not show the same capability for forming higher order oligomers [38]. In fact, LGA only forms tetramers in the presence of phosphate [40].

As an interesting contrast, at higher enzyme concentrations, LGA shows positive cooperativity for glutamine catalysis while both GAC and KGA show classical Michaelis-Menten kinetics [40]. The positive cooperativity indicates that LGA binds glutamine at reduced affinity at low concentrations of substrate, only to undergo a switch to a higher affinity state that is driven by an increase in substrate concentration, resulting in a lower overall K_M (Table 1). The different responses to substrate binding and catalysis might account for the differences in the mechanism of activation for these enzymes, offering a basis for the preferential expression of each glutaminase in varying contexts. As a result, it may be possible that new competitive inhibitors can be developed to better compete with glutamine in targeting LGA, given the isozyme's reduced affinity for the substrate at low concentration. Further mechanistic investigation of substrate binding in LGA can provide a potential avenue for the development of effective competitive inhibitors in LGA.

Glutaminase can also be activated by several other bivalent anions such as sulfate [42], though to a lesser extent. Isolation of intact mitochondria also shows an enhanced rate of glutamine hydrolysis in the presence of ADP, ATP, citrate, and tricarboxylic acid [45,46]; however, these effects have not been investigated with recombinant enzymes. Determination of the local concentration of inorganic phosphate at the sites where the glutaminase enzymes localize, along with further studies investigating the metabolic profiles in cancer cells with enhanced glutaminase activities, will help to shed light on the physiological mechanisms of glutaminase activation.

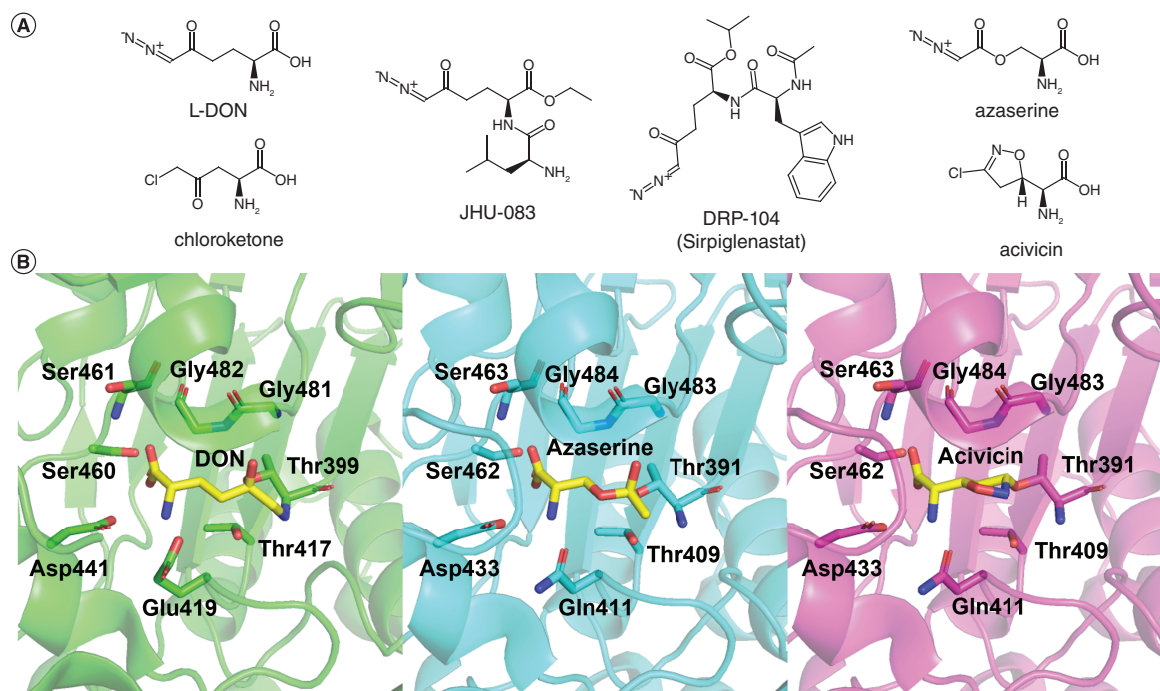


Figure 5. Glutamine analogs and their conserved mechanism of action at the glutamine-binding site. (A) Structures of various glutamine analogs have shown inhibition of glutaminase activity **(B)** Alignment of the active sites of γ -glutamyltranspeptidase when bound to different glutamine mimetics showed highly similar interactions between the analogs and the catalytically active residues. From left to right is the structure of *Bacillus licheniformis* γ -glutamyltranspeptidase with DON (green, PDB: 5Y9B), *Escherichia coli* γ -glutamyltranspeptidase with azaserine (cyan, PDB: 5Z8I), *Escherichia coli* γ -glutamyltranspeptidase with acivicin (magenta, PDB: 2Z8K).

Molecular basis for glutaminase inhibition

Competitive inhibition by glutamine analogs

Efforts targeting glutaminase enzymes, mainly GAC and KGA, have found success using both substrate mimetics and small-molecule allosteric inhibitors [47,48]. Glutamine analogs share structural similarities with the substrate but typically contain other functional groups in place of the amide moiety, and they bind directly to the catalytic active site (Figure 4B). 6-diazo-5-oxo-L-norleucine (DON) and its prodrugs JHU-083 and DRP-104, azaserine, chloroketone, acivicin, and their derivatives function as competitive inhibitors versus the substrate, either through an irreversible or reversible binding mechanism (Figure 5 [48]A). Among these compounds, DON exhibits the lowest IC_{50} value with the recombinant glutaminase enzymes compared with the other analogs [49], and thus has been the focal point for optimization. The hydrolysis of glutamine catalyzed by GAC/KGA involves a nucleophilic attack on the acyl carbon by Ser286 in the enzyme active site, and subsequent proton transfer from Tyr466 facilitates the deamidation/hydrolysis reaction. The same nucleophilic attack occurs at the acyl carbon in DON, but without subsequent hydrolysis. Instead, the diazo group is released as Ser286 forms a covalent bond with the remainder of the small molecule, locking in the suicide inhibition. Co-crystallization with the enzyme (PDB: 4O7D) shows that this glutamine mimetic binds in a highly specific manner to the active site, making hydrogen bonds with Tyr249, Gln285, Ser286, Asn335, Glu381, Asn388, Tyr414, Tyr466 and Val484 [49]. Aside from DON, there have not been any direct structural characterizations for glutaminase and other glutamine mimetics. However, a closely related enzyme, γ -glutamyltransferase, which also catalyzes glutamine hydrolysis, has been crystallized with DON [50], azaserine [51], and acivicin [51] in three independent structures (PDB: 5Y9B, 2Z8I, and 2Z8K respectively). The similarities in these structures suggest that the glutamine mimetics are all binding to γ -glutamyltransferase in a similar manner (Figure 5B), and presumably the same would be true for *GLS*. Unfortunately, these structures also demonstrate how glutamine analogs have significant off-target effects. Beyond *GLS* and γ -glutamyltransferase, glutamine mimetics inhibit many other enzymes that hydrolyze glutamine [52], including but not limited to asparagine synthetase, CTP synthetase, NAD^+ synthetase, and carbamyl phosphate synthetase.

Table 2. Current clinical trials with CB-839.

Indication	Trial No. (NCT....)	Phase	Status (Number of patients)	Ref.
NF1 positive tumors & nerve sheath tumors	03872427	II	Active – Recruiting (108)	
Hematological tumors	02071888	I and II	Completed (25)	
CB-839 was well tolerated at and above effective doses Dosing reduction from TID to BID with food improved the PK profile and resolved LFT elevations				[61]
Healthy patients (PK/formulation study)	02944435	I	Completed (14)	
Leukemia	02071927	I	Completed (43)	
Observations included CB-839 being generally well tolerated for prolonged periods of BID dosing with food, and reductions in marrow and peripheral blast counts in acute leukemia populations				[62]
Solid tumors, multiple indications	02071862	I	Completed (210)	
Rapid absorption and clearance with a half-life of about 4 hours, inhibition was targeted in tumors.				[63]
Additional details on the trials are provided when available (trials marked with *). BID: Twice a day; LFT: Liver function test; PK: Pharmacokinetics; TID: Three-times a day.				

Despite its anti-tumor effects [15,52], DON showed high levels of gastrointestinal toxicity and was removed from clinical trials after phase II as patients experienced severe nausea and vomiting [52]. For this reason, efforts have been made to create prodrugs based on DON that might bypass the gastrointestinal tract by adding protecting groups on both the amine and carboxylate functional groups [53]. With this strategy, the prodrugs showed increased stability in human plasma, swine liver and intestinal homogenates. Further, one of the better studied such prodrugs, JHU-083, has been shown to be effective in models of brain cancer [54], with 10 μ M JHU-083 significantly slowing the growth of several different MYC-driven medulloblastoma cell lines, including one derived from a PDX tumor. Similar results were obtained with another pro-drug, JHU-395 [55]. These compounds also showed improved human tumor cell-to-plasma partitioning and are better directed to the central nervous system. The compound DRP-104, currently in a clinical trial, has shown promising results with lower toxicity compared with DON, and sustained glutaminase inhibition in CT26 murine colon carcinoma models [56].

Inhibition by allosteric inhibitors

The ability to specifically inhibit the glutaminase enzymes greatly advanced upon the development of small-molecule allosteric inhibitors for GAC/KGA. These compounds are also more potent than the glutamine analogs, and mainly target two distinct allosteric sites on the enzyme: the dimer-dimer interface and the monomer-monomer interface. Kinetics studies thus far suggest that the allosteric inhibitors of glutaminase are either uncompetitive or noncompetitive [57,58].

BPTES class

The first class of inhibitors are all derived from the initial hit compound bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl) ethyl sulfide (BPTES) (Figure 6A). Among these is CB-839, which was optimized for better potency from BPTES [59], and is currently the lead compound for this class, being in phase II clinical trials (Table 2). A second BPTES derivative, IPN60090 [60], has also entered clinical trials (Supplementary Table 1, see Glutaminase inhibitors and conventional therapies), but is considerably less well studied.

Despite being the lead compound in clinical trials, CB-839 shows higher calculated logP (a measure of lipophilicity) and lower lipophilic efficiency (LipE, a measure of inhibitory potency relative to lipophilicity, and often used as a predictor of long-term clinical success) than BPTES [64], and also requires an as-yet unexplained long pre-incubation times to achieve maximum potency [57]. Thus, other derivatives are being developed to improve the pharmacokinetics of the compounds in this class, including for example the UPGL series of allosteric inhibitors or IPN60090 [60], which substitute a heterocyclic ring in place of the flexible core of BPTES or CB-839 in order to constrain the conformational space of the molecules and reduce the entropic penalty for binding, while simultaneously improving metabolic stability and increasing LipE [65]. Moreover, improvement in the delivery strategy for BPTES, such as encapsulation of the compound in dense poly(ethylene glycol) nanoparticles, also showed enhanced drug exposure and lower liver toxicity in mice bearing orthotopic pancreatic tumors [66,67].

The BPTES class of compounds are highly selective for *GLS* [39]. The difference in potency seems to be dictated by the activation loops of GAC/KGA and LGA. Extensive structural characterizations using x-ray crystallography have been carried out for compounds in this class. It has been found that these compounds occupy the space

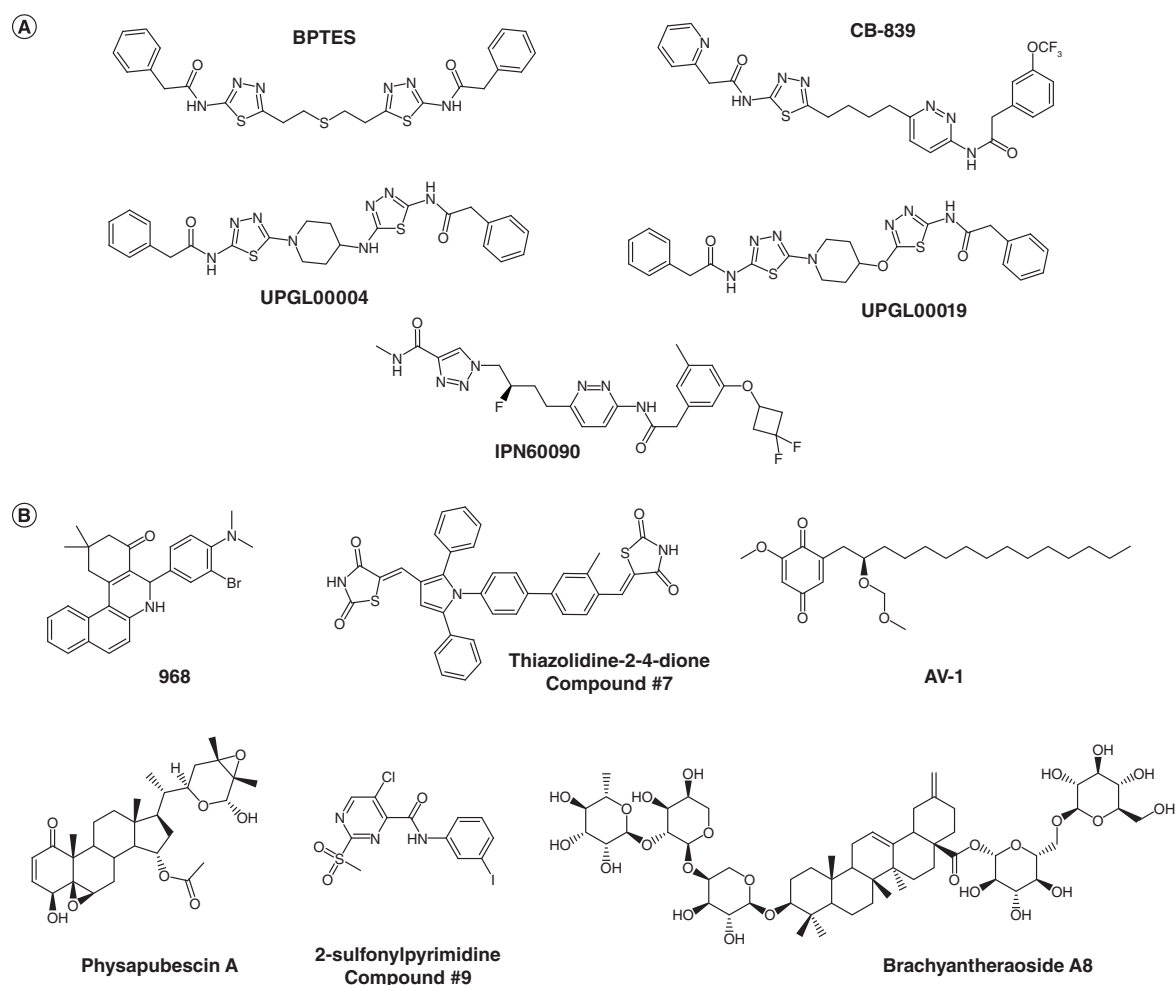


Figure 6. Structures of the small-molecule allosteric inhibitors that target glutaminase enzymes. **(A)** Structures of representative compounds from the BPTES class that specifically target GLS. **(B)** Structures of other allosteric inhibitors that can target GLS and GLS2.

between two adjacent activation loops of GAC and KGA, which span from residues Gly315 to Glu325. Important hydrogen bonds that stabilize the enzyme-drug interactions involve the backbones of Leu323, Phe322, and Tyr394. By binding near the dimer-dimer interface of the glutaminase tetramers, these compounds can stabilize an inactive tetrameric conformation of the enzyme [42]. However, despite the knowledge of this general mechanism, traditional cryo-cooled x-ray crystallography has not been able to explain the differences in potency among the compounds in the BPTES class.

Serial room-temperature x-ray crystallography, which circumvents the cryo-cooling step traditionally used in x-ray crystallography in order to examine molecules in a more natural conformation, has helped to provide important insights into the chemical determinants underlying the differences in potency between BPTES-type molecules by capturing distinct conformations of the inhibitors [64]. When comparing BPTES ($IC_{50} = 371$ nM) with the more potent UPGL00004 compound ($IC_{50} = 29$ nM), it was observed that BPTES forms a more extended, linear conformation compared with the cup-shaped conformation of UPGL00004. This linear conformation demonstrates that even when bound to the enzyme, the less potent inhibitor molecule is more flexible and undergoes additional conformational orientations which do not make optimal contacts with the protein binding site, before eventually settling into an energetically favored conformation that resembles that of the more potent inhibitors. Moreover, these two compounds share a number of hydrogen bonds with the activation loop, but the average B-factor for BPTES is much higher than that of UPGL00004, further indicating that the interactions between BPTES and glutaminase are less stable. Indeed, an effort in which macrocyclic molecules were prepared that maintained the

cup-like orientation even with a flexible molecular core did show improvements in the IC₅₀ compared with parent compound BPTES, though the addition of the various alkyl linkers also worsens the pharmacological properties [68].

The development of fluorescence spectroscopic read-outs for the binding of inhibitor molecules to the glutaminase enzymes have also contributed greatly to our understanding of their mechanisms of action. For example, the substitution of a tryptophan residue for Phe327 at the activation loop showed that the BPTES-class of compounds compete with inorganic phosphate in modulating the activation loop conformation [42]. This tryptophan mutant, together with another tryptophan substitution for Tyr466 in the active site of GAC, has provided valuable tools for assaying the binding affinities of different compounds in this class of inhibitors [42,64]. Additional mechanistic insights into how different amino acids in the activation loop contribute to the binding of the BPTES-class of inhibitors have also been obtained using these fluorescence readouts. For example, they have shown that mutating Lys320 to an alanine abolished the binding of the UPGL00019 to GAC, indicating that the lysine side chain interacts with the terminal phenyl rings of the inhibitor. This is further support for the previously identified high B-factors for Lys320 and these terminal rings in the co-crystal structure of GAC and e.g. UPGL00019 or CB-839, and strongly suggests that one important role for the terminal aromatic rings in these molecules is to flexibly maintain a binding contact with a mobile Lys320.

This class of allosteric compound has been shown to be effective in a number of different cancers. The compounds have been most heavily studied in the context of triple-negative breast cancer, where potent compounds such as CB-839 [57] or UPGL00004 [69] can inhibit the growth of models such as MDA-MB-231 or Hs578T with nanomolar potency. However, CB-839 especially has been widely studied in other contexts, and has been demonstrated to be effective against e.g. A549 and H460 lung tumor cells [70], Cal-27 tongue cancer cells [71], and U226 Bzr multiple myeloma cells [72]. These are only some of the tumor classes that have been successfully inhibited *in vitro* by BPTES-class allosteric regulators of *GLS*, and it is likely that more will be discovered in the coming years.

Benzophenanthridinone class

Another group of allosteric inhibitors is comprised of molecules derived from benzophenanthridinone, with the lead compound being 968 (Figure 6B). This compound was first used to show the importance of GAC activity for oncogenic transformation in cell culture, as well as for the growth and survival of cancer cell lines and tumor growth in mice [14], and it has since been shown to exhibit a slight selectivity (<= twofold) for inhibiting LGA, compared with GAC [4]. 968 and its optimized analogs all contain a 'hotspot ring' (generally an ortho-bromo, N-methyl-amino-phenyl moiety or a structurally similar group). This so-called hotspot ring was used to precipitate its target, GAC, from oncogenic transformed NIH 3T3 cell lysates [14]. Using GAC molecules labeled with fluorescent reporter groups, it was shown by FRET that 968 uses an inhibition mechanism distinct from that of BPTES. Specifically, 968 does not interfere with the dimer-dimer interaction within the glutaminase tetramers. Instead, it was reported to preferentially bind to the monomeric form of GAC, with the requirement that it associates with the enzyme prior to the binding of activators, as it engages and stabilizes an inactive state. 968 is also able to inhibit the catalytic activity of the LGA isozyme though the mechanism of inhibition has not been characterized in detail as with GAC [4].

Structure-activity relationship studies were carried out for the benzophenanthridinone class of inhibitors. A docking study suggested that 968 binds in a hydrophobic pocket between the monomer-monomer interface of GAC, at the junction where the N- and C-termini meet the catalytic domain [58]. This binding position was supported by site-directed mutagenesis that caused a corresponding reduction in 968 potency, but has not been unambiguously verified e.g. by x-ray crystallographic structural determinations, as the low solubility of this compound and its preference for binding to the enzyme monomer (which exists only at relatively low protein concentrations) has hindered crystallization efforts. Other structural approaches have been complicated by the intrinsic absorbance of 968, which shares the same wavelength as tryptophan, rendering biophysical studies similar to those conducted on the BPTES-class compounds challenging. In addition, as the binding affinities of these compounds are reduced with increasing enzyme concentration, characterization methods which require high protein concentrations, such as isothermal titration calorimetry, or surface plasmon resonance, are not ideal for studying this class of glutaminase inhibitors. A newer technique, microscale thermophoresis, might offer an alternative as it requires less protein for measurement [73], but to the best of our knowledge no such study has yet been attempted.

Like in the case of the BPTES-class of inhibitors, 968 has been used to inhibit the growth of a number of different cancers. When originally discovered, 968 was shown to have potency against MDA-MB-231 and SKBR3 breast cancer cells [14]. However, in more recent years, 968 has shown efficacy against HEY, SKOV3, and IGROV-1 ovarian

cancer cell lines [74], A549, Spc-A1, H23, H292, and H1299 non-small cell lung cancer cells [75], and HepG2, 7402, and LM3 hepatocellular carcinoma cells [76]. Crucially, in these studies, 968 was shown to be relatively ineffective at blocking the growth of e.g. human mammary epithelial cells [14], bronchial epithelial cells [75], and EVC304 non-malignant endothelial cells [76], helping to demonstrate the potential therapeutic window for clinical glutaminase inhibitor use.

Other classes of allosteric inhibitors

There are several additional lead compounds from other classes of inhibitors that target glutaminase enzymes, many of which were identified through high-throughput screens (Figure 6B). Thiazolidine-2,4-dione derivatives [77] possess unique structures from the previous classes of molecules, and can inhibit both *GLS* and *GLS2*. Molecular modeling and site-directed mutagenesis studies with KGA suggest that these compounds occupy the active sites of the glutaminase enzymes, likely engaging in a hydrophobic interaction with Arg387, and thus might explain the low selectivity toward the different enzyme forms. A recent effort to create high throughput assays for glutaminase inhibitors using a fluorescence enzyme-coupled read-out identified novel scaffolds with 2-sulfonylpyrimidine/2-thiopyrimidine derivatives for inhibitor design [78]. These scaffolds were found to engage in noncompetitive inhibition versus substrate and to bind and stabilize GAC in a tetrameric state. Structural modeling suggested that the lead compound C9 might bind to the activation loops of GAC similarly to BPTES. However, while BPTES decreases polydispersity of the enzyme upon binding (i.e. caused the enzyme to reduce aggregation), C9 did not exhibit such an effect. Natural-product inhibitors have also been identified, including physapubescin [73] and brachyantheraoside A8 [79]. Interestingly, physapubescin shares structural similarity with the benzophenathridinone class of inhibitors, displaying even lower IC_{50} . However, the interactions of these compounds with the glutaminase enzymes have not been well characterized. Further structural characterizations and structure-activity relationship studies across different classes of compounds might provide new insights into their mechanism of inhibition.

In addition, since the role of LGA in cancer progression has not gained significant attention until recently, inhibitor development specific for this isozyme also warrants additional efforts. Currently, no other molecule besides AV-1 [80], a natural alkyl benzoquinone, can selectively inhibit LGA over GAC/KGA (almost tenfold selectivity against LGA, IC_{50} being 0.28 μM vs IC_{50} being 2.1 μM for KGA). Molecular docking also suggested that this compound binds at a distinct site from DON, BPTES, or 968, but as is the case for most of these allosteric compounds, a definitive binding site has yet to be identified by structural approaches.

Combinational therapies with glutaminase inhibitors & other anti-cancer treatments

Combining the effects of small molecules that target glutaminase

The expression of the different glutaminase isozymes is highly dependent on the type of cancer cells, and upstream regulations could influence the switch from one isozyme to another, as discussed above. Moreover, *GLS2* has been found to compensate for loss of *GLS* in certain cases [2], and in such cases, it may be beneficial to simultaneously inhibit all major glutaminase isozymes at once. The effect of simultaneously inhibiting *GLS* and *GLS2* is most clearly demonstrated with the use of compound 968, an inhibitor that has been shown to inhibit all forms glutaminase (KGA, GAC, and LGA). However, it is important to also note the alternate role of *GLS2* as a tumor suppressor in some contexts, and that we still do not have a clear understanding on the role of glutamine catabolism in these cases. Interestingly, it has also been shown that combining 968 and BPTES can have an additive or synergistic effect in inhibiting the growth of triple-negative breast cancer cells which predominantly express GAC, suggesting that the binding of one class of inhibitors to GAC may enhance the binding of the other class of compounds [58]. Future studies examining the combination of substrate mimics like DON and the BPTES-family of inhibitors might also result in synergistic effects. Such combination approaches could eventually contribute to lowering the treatment dose of each drug and limit off-target effects.

Combining inhibitors of glutaminase & other metabolic enzymes

In cancer cells that are addicted to glutamine, glucose uptake is also frequently highly upregulated to support subsequent aerobic glycolysis, producing high levels of lactate. Inhibition of glycolysis has the potential to synergize with the inhibition of glutamine metabolism. Piperazine-2-one (Glutor), an inhibitor of glucose transporters GLUT-1/-3, has been found to sensitize colon cancer cells (HCT 116) to treatment with CB-839 [81]. The presence of 1 μM Glutor resulted in a GI_{50} value of less than 50 nM for CB839, compared with a GI_{50} value of 12 μM in the absence of Glutor. Conversely, the presence of 5 μM CB-839 reduced the GI_{50} for Glutor by

40-fold to a value of 10 nM compared with a GI_{50} of 428 nM in the absence of CB-839. In addition to blocking glucose uptake, the inhibition of other glycolytic enzymes represents a potential strategy worth exploring. For example, it has been found that under conditions of glutamine deprivation, or upon silencing of *GLS*, glioblastoma becomes dependent on pyruvate carboxylase, which allows cells to utilize glucose-derived pyruvate as an energy source instead of glutamine [82]. Thus, dual targeting of both pyruvate carboxylase and glutaminase could be a viable therapeutic strategy when a tumor acquires resistance to therapies targeting glutamine metabolism.

mTOR is generally considered to be a master regulator of cell function, and hyper-activation of mTOR has been linked to a number of cancers. Inhibition of the mTOR complexes mTORC1 and mTORC2 has proven to be an important synergistic strategy together with glutaminase inhibition, as it was found that inhibition of mTOR resulted in a dependence on glutamine metabolism in lung squamous cell carcinoma [83], renal cell carcinoma [84], and ovarian cancer [85]. In a lung squamous cell carcinoma model that became resistant to the mTOR inhibitor MLN128, the GSK3a/b pathway was activated to upregulate c-MYC and c-JUN expression, which in turn increased the expression of glutaminase enzymes. In ovarian cancer cell lines, *GLS* promoted resistance through STAT3 signaling [85]. The combined use of CB-839 together with an mTOR inhibitor (i.e. MLN128, PP242, or rapamycin) overcame this resistance. Similarly, inhibition of glutaminase sensitized glioblastoma multiforme cells derived from patients and ovarian cancer cell lines to treatments that target mTOR, and the combination of both treatments blocked tumor growth in xenograft models [86]. The combinational therapy might even offer synergistic effects, as shown in renal cell carcinoma cell lines, with the combination index being less than 1, i.e. indicative of synergy [84]. Furthermore, treatment with an Hsp90 inhibitor, in addition to inhibiting mTOR and *GLS*, was shown to induce apoptosis in cell xenograft tumors with tuberous sclerosis complex deficiency [86].

Blocking amino acid uptake has also been found to be an effective strategy to overcome resistance and insufficiency when inhibiting glutamine catabolism alone. A recent study showed that compound V-9302, which inhibits the amino acid transporter ASCT2, sensitizes glutamine-addicted liver cancer cells to CB-839 treatment [87]. Specifically, using a hepatocellular carcinoma xenograft mouse model, the combination treatment resulted in a reduction of glutathione levels, inducing cytotoxic effects and apoptosis in the liver cancer cells, and hindered xenograft growth *in vivo*. Blocking amino acid synthase can also provide an additional level of inhibition when glutamine catabolism is reduced. Dual siRNA-knockdowns of *GLS* in cancer-associated fibroblasts and glutamine synthetase in neighboring stromal cells resulted in a reduction in tumor weight, as well as in the number of tumor nodules, and metastases in a tumor-bearing orthotopic mouse model for ovarian carcinoma [88]. In addition, blocking asparagine catabolism has also been shown to synergize with glutaminase inhibition. This is because under conditions of glutamine deprivation, asparagine can restore glutamine biosynthesis by upregulating glutamine synthetase as well as activate mTORC1 to support glutamine-independent cell growth [89]. Inhibition of asparagine usage can block these processes. For example, treatment with recombinant L-asparaginase, together with the glutaminase inhibitor DON, showed a synergistic reduction of cell proliferation, induced apoptosis, and autophagy in several glioblastoma cell lines (U251, U87, and SF767) [90].

Inhibiting lipid metabolism together with glutaminase activity is another potential combination strategy. Triple-negative breast cancer cells, when treated with 968, underwent further metabolic reprogramming to increase lipid catabolism over time [91]. These cells, upon becoming resistant to glutaminase inhibition, showed increased CPT1 activity levels, an enzyme involved in lipid processing [92]. Therefore, targeting enzymes important for lipid metabolism offers promising potential in overcoming resistance in breast cancer cells.

Combining glutaminase inhibitors with checkpoint inhibitors

One mechanism by which cancer cells survive is by evading the immune system. They frequently do so by targeting receptors on immune cells that send kill-signals to those cells, activating immune checkpoints. Targeting the immune checkpoint via blockade therapy in cancer has gained tremendous interest in the past decade, and clinical trials involving combination therapy between immune checkpoint inhibitors and other cancer treatments have become a preferred strategy due to the enhanced efficacy versus checkpoint inhibitors alone [93]. One such combination approach centers on glutaminase inhibition. Upon activation, T-cells undergo metabolic reprogramming, switching from oxidative phosphorylation to aerobic glycolysis to support the rapid growth and proliferation necessary for an effective immune response [94]. To evade the immune system, cancer cells express PD-L1, which upon interaction with PD-1 on activated T cells impairs glycolysis and glutaminolysis in these cells, and promotes apoptosis [95]. Since immune cells compete with cancer cells in the tumor microenvironment and decrease the tumor-accessible supply of nutrients and oxygen [96], glutamine metabolism becomes an attractive target for combination therapy

with immunotherapy. In fact, inhibition of the PD-1/PD-L1 axis shows synergistic effects when combined with inhibitors of glutamine metabolism in restoring the immune response by T cells on patient-derived melanoma, as well as in a colon carcinoma model, and in clear cell ovarian carcinoma [3,97,98].

In addition to immune checkpoint inhibitors, cell cycle checkpoints have also proven to be promising combinatorial therapeutic targets to use together with glutaminase inhibitors. Upon inhibition or depletion of the key cell cycle regulators CDK4 or CDK6 in colorectal cancer cells, there is an increase in myc expression, leading to an enhancement in glycolysis and mitochondrial glutamine metabolism [99]. Moreover, inhibiting glutaminase activity by CB-839 overcame the acquired resistance of esophageal squamous cell carcinoma to CDK4/6 inhibition [100]. Synergistic reduction of cell growth and tumor burden were observed when using both treatments simultaneously. The dual inhibition of CDK4/6 and glutaminase has now entered clinical trials in combination with CB-839 (Supplementary Table 1, see Glutaminase inhibitors and conventional therapies). Given that cell cycle progression and glutamine metabolism are interconnected, therapeutic strategies that combine cell cycle checkpoint and glutaminase inhibitors offer promising research potential [101].

One particular difficulty in combining glutaminase and immune checkpoint inhibitors, however, is that glutaminase also plays a role in T cell function. For example, in freshly activated T cells [102], *c-Myc* was correlated with the expression of *GLS2*, but not *GLS*, during the cells growth phase prior to initiation of proliferation. Similarly, when CD8⁺ T cells in a lung cancer model were activated by anti-PD1 therapy, *GLS* inhibition with CB-839 was found to inhibit clonal expansion of the activated cells [103]. This mirrors similar findings in which CD4⁺ T cell expansion shows dependence on glutamine and the activity of *GLS* [104]. However, competing research suggests that T cells can adapt to glutamine denial, and that the *GLS* inhibitor JHU-083 in fact leads to metabolic reprogramming resulting in enhanced growth, proliferation and survival [3]. This in turn aligns with research showing that inhibition of CDK4 and/or CDK6 [105] decreased T cell proliferation, but enhanced overall T cell activity and tumor infiltration. Given the many similarities between cancer and immune cell metabolism, these various and sometimes conflicting reports are not entirely surprising. These findings also highlight the importance of selecting the appropriate model systems that encompass glutamine catabolism in both tumor and immune cells. Immune-competent models are particularly important when studying checkpoint inhibitors. But this also limits the studies to highly artificial systems in which human immune systems are implanted into mice, or fully murine systems, which do not necessarily replicate a human cancer. In the end, many complications need to be overcome to allow any combination metabolic & checkpoint therapy to be used in the clinic.

Glutaminase inhibitors & conventional therapies

Many different combinations of traditional therapeutic treatments of cancer have been examined for synergistic effects with glutaminase inhibitors (Supplementary Table 1). Current clinical trials that use a combinatorial approach with glutaminase inhibition include chemotherapies targeting DNA, topoisomerase, proteasome inhibition, PARP inhibition, and growth receptors' inhibition. Enhanced outcomes have also been observed when combining radiotherapy with CB-839 in head and neck squamous cell carcinoma cell lines and xenograft mouse models [71]. For a subset of non-small cell lung cancer having *STK11/LKB1* mutations, *GLS* inhibition was able to overcome resistance to standard radiation [106].

Metformin, a conventional therapy for diabetes, has been found to have synergistic effects with glutaminase inhibitors. Metformin is widely known to primarily limit cellular glucose uptake in diabetic patients [107], but has recently entered phase III clinical cancer trials. A recent study showed that metformin strongly synergized with CB-839 in inhibiting osteosarcoma cell growth and metastasis [108]. Metabolic profiling using [¹³C]glucose and [¹³C¹⁵N]glutamine with orthotopic tumors derived from patients with pancreatic ductal adenocarcinoma showed that a combination of BPTES-nanoparticles and metformin is efficient in reducing tumor cell growth as they complement each other in inhibiting glutamine and glucose metabolism, respectively. Similarly, by promoting aerobic glycolysis and lactate production, metformin also causes prostate cancer cells to be more susceptible to glutaminase inhibition by 968 and BPTES [109]; in a reciprocal fashion, glutamine deprivation sensitizes these cells to metformin treatment.

Complications arising from glutaminase inhibition

Despite the general promise shown to date by glutaminase inhibitors, both when used on cell culture models, animal models, and clinical patients, a number of complications may arise when glutaminase inhibitors are employed. Perhaps preeminent among these is that, in some cases, glutaminase (and particularly *GLS2*) has been suggested

to be a tumor suppressor, rather than a tumor promoter. For example, *GLS2* has been shown to be promoted in some cases by the tumor suppressing transcription factor p53 [110,111]. In hepatocellular carcinomas (HCC), the *GLS2* promoter was found to be hypermethylated in the majority of samples. Reduced expression of *GLS2* in HCC compared with healthy tissue was verified by staining tissue microarrays for protein, and by using real-time PCR to detect *GLS2* mRNA. Overexpression of *GLS2* in Huh1 and Huh7 cells was shown to block PI3K/AKT activation, although the mechanism causing that reduction in activity was not determined [112]. Largely similar effects were demonstrated in T98G glioblastoma cells, which have naturally low levels of *GLS2*, and experienced reduced growth rate upon transient transfection of *GLS2* [113]. *GLS2* has also been shown to promote ferroptosis, an iron-dependent form of programmed cell death in which lipidic peroxides accumulate within cells, in *GLS2*-enriched mouse embryonic stem cells, STAM HCC cells [114], and mouse embryonic fibroblasts [115].

Other challenges include inhibiting compensatory pathways which allow cells in the tumor microenvironment to obtain α -ketoglutarate, or other TCA cycle intermediates, without the involvement of glutaminase [67,116–118]. One such input involves importing pyruvate to form oxaloacetate. This was recently demonstrated in triple-negative/basal-subtype breast cancers, which normally respond well to *GLS* inhibitors [117]. Here, a panel of breast cancer cell lines including MDA-MB-231, MDA-MB 436, and BT549 cells were treated with CB-839 or BPTES in a variety of different culture media. The drug IC₅₀ values were lowest in RPMI-1640 medium, and considerably higher in α -MEM or DMEM media, both of which have a much higher concentration of pyruvic acid. Introduction of sodium pyruvate to RPMI-1640 provided resistance to the drugs. This change in sensitivity was found to depend upon pyruvate carboxylase, which converts pyruvate to oxaloacetate. One could imagine similar effects if cells were provided with other common anaplerotic inputs to enhance formation of e.g. succinyl-CoA or fumarate.

Another compensatory pathway has been shown in a recent study in medulloblastoma where possible input to the TCA cycle from the glutamine transaminase KYAT1 was examined [118]. In this study, KYAT1 was found to transfer a nitrogen group directly from glutamine to α -ketoglutarate, to form α -ketoglutaramate and glutamate. The α -ketoglutaramate was then hydrolyzed to α -ketoglutarate by the omega-amidase, NIT2, for a net conversion of glutamine to glutamate. This set of reaction is sometimes referred to as the ‘glutamine transaminase–amidase’ pathway [67,116,119]. The authors treated D425MED cells with uniformly labeled ($m + 7$) glutamine for two hours, then examined the molecular weight of glutamate in cell lysates. In this case, they found a relatively even amount of $m + 6$ glutamate (presumably from glutaminase activity, which would remove one heavy atom), and of $m + 1$ and $m + 5$ glutamate (the former coming from conversion of KYAT1-formed α -ketoglutarate to glutamate, and the latter from the conversion of α -ketoglutaramate to α -ketoglutarate, then to glutamate). However, when a similar experiment was conducted in mice with flank injections of tumor cells, the $m + 5$ signal almost entirely disappeared, and the $m + 6$ signal decreased. This effect was even more pronounced in orthotopic models, which lost both signals, and had only an $m + 1$ signal, and the authors concluded that this represented a dominance of glutamine consumption by KYAT1. Even though the elimination of the $m + 5$ signal might suggest that the labeled glutamate may have simply arisen from carbon that had gone through the TCA cycle several times, this study does highlight how changing the tumor microenvironment can change its metabolic patterns. Those interested in a more detailed examination of this complicated system, and opinions pertaining to how involved it may be in cancer, are encouraged to examine recent reviews dedicated to the topic [116,119]. Given the multiple ways glutamine is utilized in biological systems, compensatory metabolic pathways such as those described above might interfere with glutaminase inhibition in complex ways, and the potential exists that inhibiting multiple glutaminase-adjacent pathways simultaneously may eventually be shown to have beneficial effects in cancer treatment.

Glutaminase inhibitors to date are, largely, less than ideal in terms of aqueous solubility. CB-839, the best-studied clinical candidate, is for instance dosed in mice at an incredible 200 mg/kg BID (oral administration). This particular formulation of the drug relied upon solubilization with 25% w/v hydroxypropyl–cyclodextrin [57]. Another study examined injected UPGL-00004 (1 mg/kg mouse body weight), in a carrier agent consisting of RPMI-1640, 5% DMSO, 5% Cremophor EL, and 5% ethanol [69]. While this was a reduced amount of a comparable drug, it was also in a relatively aggressive injection carrier, depending upon Cremophor EL, a non-ionic surfactant which, despite its long history of pharmaceutical use to solubilize hydrophobic drugs, also has several identified negative health effects associated with it [120–122]. One popular technique to address these problems, briefly mentioned above, is to encapsulate drugs of this class in nanoparticle carriers. Several approaches have been taken here. Elgogary has encapsulated BPTES in biodegradable poly(lactic-co-glycolic acid) (PLGA) nanoparticles coated in a dense polyethylene glycol (PEG) shell, and found that this improved BPTES potency to be nearly identical to CB-839, while preventing the elevation in liver enzymes which accompanies CB-839 treatment, and

can be indicative of toxic side effects [66]. Choi and coworkers took a second approach, creating the core of their nanoparticles from pH-sensing peptides, which precipitate as nanoparticles at pH >7, and solubilize to release their cargo at pH <6.9. When BPTES was enclosed in these pH-peptide nanoparticles, the growth of HCC1806 xenografted tumors was reduced significantly more-so than in the case of treatment with BPTES alone [123]. Despite its comparatively enhanced potency, similar approaches have been taken to CB-839 itself as well. Jian and coworkers used a zeolitic imidazolate framework (ZIF) to form nanoparticles which could deliver both CB-839 and iron, thus inhibiting *GLS* while activating ferroptotic pathways [124]. These ZIF nanoparticles were able to inhibit the growth of MDA-MB-231 breast cancer cells both *in vitro* and *in vivo* in xenograft models. Yet another approach involved encapsulating CB-839 in gold nanoparticles coated with CD133, to direct the nanoparticles to cancer stem cells [125]. Like previous nanoparticles, these were found to be more effective in inhibiting the growth of cancer stem cells compared with the individual components used to synthesize them. These sorts of studies help to show the importance of formulation chemistry, and the improvements to treatment that can be made when solubility problems are overcome.

A final complication is that glutamine hydrolysis by glutaminase also produces ammonia as a byproduct (Figure 1A). Ammonia is both necessary for cell survival and toxic at high concentrations. This is further complicated by metabolic changes cells experience upon hypoxia, which is frequently encountered in tumors, where for example MCF7, HeLa, and 4T1 cells under hypoxic conditions increase storage of nitrogen in orotate and dihydroorotate [126]. Conceivably, any inhibition of glutaminase (a major producer of ammonia) could influence these tightly regulated pathways and result in either cell death or enhanced cell survival, and thus the fate of nitrogen from glutaminase activity is worth monitoring.

Conclusion

The glutaminase family enzymes, which initiate glutamine catabolism in order to satisfy the “glutamine addiction” experienced by many cancer cells as part of the Warburg effect, have shown to be promising targets for cancer therapeutics development. Regulation of these enzymes appears to be complex, and glutamine metabolism feeds into several pathways implicated in various cancer types. As a result, glutaminase inhibition, either alone or as part of combination therapies, can be particularly effective in some cancers. This understanding has driven the development of glutaminase inhibitors in recent years, which has led to several promising clinical trials. Further progress toward the development of potent allosteric inhibitors of glutaminase, with improved efficacy and pharmacokinetics compared to current generation molecules, and ongoing development of potent inhibitors capable of modulating the specific activities of the different glutaminase isozymes, have the potential to greatly improve prognostic outlook for patients with glutamine-addicted cancers.

Future perspective

Targeting glutamine catabolism has provided a promising approach for cancer therapeutics and developing small molecule inhibitors of glutaminase has proven to be an effective strategy. Modulating the expression and activity of the different forms of glutaminase is critical to tuning metabolic pathways in various forms of cancer. Further work optimizing small-molecule inhibitors for glutaminase will be beneficial in being able to selectively inhibit each glutaminase isozyme. A focus on newer structural approaches, such as serial room-temperature crystallography [64] and time-resolved x-ray crystallography [127], may offer new insights into the interactions of various inhibitors with the glutaminase enzymes and further help the development of potent drug candidates. This may be of particular value in the case of allosteric inhibitors, where the inhibitory mechanisms remain less clear, and new opportunities for inhibitor optimization may arise as these mechanisms come into clearer focus.

In addition, exciting possibilities exist regarding the synergistic benefits of combining glutaminase inhibitors with other cancer therapeutics. We suspect that the most important advance here will be due to improvement of model systems, ranging from cell lines, xenografts, to mouse models [128], in which such synergies are studied. Much of the metabolic nature of a tumor model is dependent upon its microenvironment, and changing that microenvironment, from using different cell culture medium to moving from cell culture to whole animal approaches, can greatly affect the potency of metabolic inhibitors such as those discussed here. Similarly, particularly promising approaches, such as combining glutaminase inhibitors with immune checkpoint inhibitors, currently can only be properly investigated in highly complex immune-competent murine model systems. Such models are incredibly powerful, but are also incredibly expensive to deploy, and similarly include ethical complications in their use. As research advances in the generation of *in vitro* models which can more fully reconstitute such systems, studies of glutaminase inhibition will

become both easier, more accurate, and more accessible to scientists globally. In addition, an agreement in the field on standardized methods of pathway analysis to study glutamine catabolism, such as $^{13}\text{C}/^{15}\text{N}$ -glutamine tracing studies and ammonia formation detection, would also unify the various approaches to study the modulatory effects of inhibiting glutaminase in different biological processes.

Given the important roles of glutaminase enzymes in such systems, these enzymes have also been found to have important implications in other diseases related to cancer. For example, as cancer is frequently an age-related impairment, the role of glutamine catabolism is also being studied in senescence. A recent report showed that KGA expression in response to lysosomal membrane damage is essential for maintaining senescence [129]. Glutaminolysis has also been found to modulate cell cycle arrest in human endothelial cells [130]. Future work on controlling cellular fate through metabolic reprogramming with glutaminase inhibition may offer new strategies for regulating senescence [131]. One caveat here, however, is that while glutaminase monotherapy has been generally shown to have a wide therapeutic window between e.g. cancer and non-cancer cells, these studies do show that non-cancer cells may still require intact glutaminolysis in some cases. This becomes doubly problematic when examining synergies between glutaminase inhibitors and other compounds. Indeed, the vast majority of the studies we examined which detail drug synergies did not test these synergies against healthy cell lines, and it is likely that such testing in the future will help to indicate the most promising combination therapies going forward.

Targeting glutamine catabolism might also offer potential therapeutic strategies against viral infection, an interesting possibility in light of the COVID-19 pandemic. Virus infection often results in the metabolic reprogramming of host-infected cells with the upregulation of glutamine catabolism to satisfy the requirements of viral replication. *GLS* inhibition has been found to halt the replication of adenovirus, herpes simplex virus 1 and influenza A in cultured primary cells [132], while α -ketoglutarate was shown to rescue human cytomegalovirus propagation upon glutamine deprivation [133]. Glutamine addiction was also observed in host cells upon infection with hepatitis C, rhinovirus, as well as several other common viruses [134]. Targeting glutamine catabolism in virus-infected host cells might eventually offer an alternative to the current FDA-approved antiviral drugs, which primarily inhibit different components of the virus machinery [135]. The important role of glutamine catabolism is also being established in other therapeutic areas, including allograft acceptance [136], acute lung injury [137], and neural tube defects [138]. And, given the central role of cellular metabolism in biology, we expect that even more disease states will be found to be at least partially driven by glutaminase by the end of the decade. With the importance of glutaminase enzymes, both in cancer and other diseases, and the ever-improving nature of model systems in which to study glutaminase biologically, and technical systems by which to study glutaminase chemically, we anticipate that the development of glutaminase inhibitors, and more generally studies upon the glutaminase enzymes, will be thriving scientific fields for many years to come.

Executive summary

Controlling the differential expression of glutaminases in cancer cells

- GAC, KGA, and LGA are three distinct glutaminase enzymes that are regulated by various oncogenes for differential expression depending on specific types of cancer. Glutaminases share similar catalytic domains but can differ in their mechanisms of allosteric activation and inhibition.
- Major mechanisms that regulate glutaminase expression and activity patterns include transcription factors, long non-coding RNAs, and localization.
- Glutamine catabolism has been established as potentially important therapeutic target. Inhibition of glutaminase is an effective mechanism for inhibiting glutamine catabolism.

Molecular Basis for activation of glutaminase

- Different forms of glutaminase have different binding potency for glutamine and for phosphate, and different turnover rate.
- The detailed basis of glutamine hydrolysis is well understood, but much research remains before there is an understanding of how activating signals are received and turned into enzymatic efficiency.

Molecular basis for glutaminase inhibition

- Glutamine analogs can target glutaminase as competitive inhibitors, and prodrugs are being developed to enhance therapeutic index and reduce systemic exposure.
- Allosteric inhibitors for glutaminase such as the BPTES-class and 968-class of compounds show better specificity and potency than competitive inhibitors, and thus have undergone extensive structure-activity analysis.

Combinational therapies with glutaminase inhibitors & other anti-cancer treatments

- The emergence of combination therapies using glutaminase inhibitors together with other anti-cancer drugs have shown to be an effective strategy in targeting the metabolic programs of different types of cancer. This

includes combining small-molecule inhibitors of glutaminase together, with other metabolic inhibitors, checkpoint inhibitors, and with additional conventional cancer therapies.

Complications arising from glutaminase inhibition

- Several alternate biological pathways exist which either consume glutamine or produce glutamate.
- The role of *GLS2* is not fully understood, and it may act as a tumor suppressor in some situations.
- Current glutaminase inhibitors have some poor pharmacological properties, particularly low aqueous solubility, which are being addressed via formulation chemistry.

Future perspective

- Efforts focusing on optimizing allosteric inhibitors specific for each form of glutaminase will be important in modulating the specific catalytic activities and functions of these enzymes in various types of cancer. These may be advanced via e.g. new approaches to protein structure determination, and more relevant biological model systems.
- Targeting glutamine catabolism might also offer new therapeutic strategies in non-cancer related treatments involving diseases linked to aging, viral infection, and injury recovery.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.4155/fdd-2022-0011

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