Metabolic reprogramming of cancer by chemicals that target glutaminase isoenzymes

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Abbreviations: AKG, alpha-ketoglutarate; AKT, protein kinase B; DON, 6-diazo-5-oxo-L-norleucine; EATC, Ehrlich ascites tumour cells; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinases; GA, glutaminase; GAB, long glutaminase 2 isoform; GAC, short glutaminase isoform; GLUD, glutamate dehydrogenase; Glc, glucose; Gln, glutamine; GLS, glutaminase isoenzyme; GLS2, glutaminase 2 isoenzyme; Glu, glutamate; GS, glutamine synthetase; GSH, glutathione; HCC, hepatocellular carcinoma; 2HG, 2-hydroxyglutarate; IDH1/2, isocitrate dehydrogenase isoenzymes; KGA, long glutaminase isoform; KRAS, V-Ki-ras2 Kirsten rat sarcoma; LGA, short glutaminase 2 isoform; ME1/2, malic isoenzymes; MEK, mitogen-activated protein kinase kinase; mTORC1, mammalian target of rapamycin complex 1; NF-κB, nuclear factor-kappa B; OXPHOS, oxidative phosphorylation; PC, pyruvate carboxylase; PDA, pancreatic ductal adenocarcinoma; PI3K, phosphatidylinositide 3-kinase; PKM2, pyruvate kinase M2 isoform; PPP, pentose phosphate pathway; ROS, reactive oxygen species; RT-qPCR, real-time quantitative polymerase chain reaction; STAT1, signal transducer and activator of transcription 1

ABSTRACT

Background: Metabolic reprogramming of tumours is a hallmark of cancer. Among the changes in the metabolic network of cancer cells, glutaminolysis is a key reaction altered in neoplasms. Glutaminase proteins control the first step in glutamine metabolism and their expression correlates with malignancy and growth rate of a great variety of cancers. The two types of glutaminase isoenzymes, GLS and GLS2, differ in their expression patterns and functional roles: GLS has oncogenic properties and GLS2 has been described as a tumour suppressor factor.

Results: We have focused on glutaminase connections with key oncogenes and tumour suppressor genes. Targeting glutaminase isoenzymes are included into the different strategies aimed at deactivate the rewiring of cancer metabolism. In addition, we found a long list of metabolic enzymes, transcription factors and signalling pathways dealing with glutaminase. On the other hand, an important number of chemicals have been described as isoenzyme-specific inhibitors of GLS and/or GLS2 isoforms. These molecules are being characterized as synergic and therapeutic agents in many types of tumours.

Conclusion: This review states the metabolic pathways that are rewired in cancer, the roles of glutaminase isoforms in cancer, as well as the metabolic circuits regulated by glutaminases. We also show the plethora of anticancer drugs that specifically inhibit glutaminase isoenzymes for treating several sets of cancer.

Keywords: Cancer metabolism, Combinatory therapy, Glutaminase isoenzymes, Glutamine, Glutaminase inhibitors, Metabolic reprogramming

1. Background

Established metabolic changes occur in glycolysis and glutaminolysis of tumour cells [1,2]. Consequently, both processes are top targets for cancer therapy [1]. Metabolic reshuffle mainly consists in the capacity of tumour cells to perform aerobic glycolysis (Warburg effect) [3], and to use glutamine (Gln) as an efficient fuel to produce energy, biosynthetic precursors and antioxidant capacity [4]. In fact, Gln participates in redox homeostasis, macromolecular synthesis, and cancer signalling [4]. Cancer cells carry out aerobic glycolysis because most tumour cells grow under hypoxic conditions and Warburg effect supports rapid proliferation, even at low oxygen concentration, by diverting glycolytic carbons to biosynthetic processes such as the pentose phosphate pathway (PPP) [3]. The use of Gln for energetic, biosynthetic and regulatory purposes is needed in fast growing and proliferating cancer cells [4]. Therefore, targeting glucose (Glc) and Gln cancer metabolism has shown great promise in recent years [3,4,5]. Therapeutic approaches also include pharmacological blockade of Glc [3] Gln [4] transport in preclinical models [2]. Early clinical-trial results show promise for targeting cancer-related biochemical pathways because tumours display a reprogramming of metabolism (Table 1) that facilitates growth but addicts them to key enzyme activities for glycolysis and glutaminolysis [6,7].

Table **1**. Metabolic reprogramming in cancer.

Key altered metabolic pathways	**Critical enzymes**	**References**
Glycolysis	PKM2, LDH, PC	1,3,4-7
TCA cycle	CS, ACO, IDH1/2, SDH, FH	1,4,7
Glutaminolysis	GLS, GLS2, GLUD, AST	1,4-7
Lipids biosynthesis	IDH1/2, ME	1,4,5
Nucleotides biosynthesis	GLS, GLS2, AST	4,7
Pentose phosphate pathway	G6PDH	1,3,4,7
Hexoxamines circuit	GS	4
Amino acids biosynthesis	GLS, GLS2, GLUD, AST, ALT	4,7
GSH biosynthesis	GLS, GLS2, GCS	4,6

ACO, aconitase; ALT, alanine transaminase; AST, aspartate transaminase; CS, citrate synthase; FH, fumarate hydratase; G6PDH, glucose-6-phosphate dehydrogenase; GCS, gamma-glutamylcysteine synthetase; GLS, glutaminase isoenzyme; GLS2, glutaminase 2 isoenzyme; GLUD, glutamate dehydrogenase; GS, glutamine synthetase; GSH, glutathione; IDH, isocitrate dehydrogenase; LDH, lactate dehydrogenase; ME, malic enzyme; PC, pyruvate carboxylase; PKM2, pyruvate kinase M2 isoform; SDH, succinate dehydrogenase; TCA, tricarboxylic acid

Because Gln metabolism is altered during malignant transformation, imaging strategies targeting Gln are providing a useful tool into tumour biology [8]. Advancement of Gln-based imaging into clinical practice should soon make it possible to differentiate tumours that take up Gln from those that do not [4]. Tumour cells have metabolic requirements that differentiate them from normal cells and render them vulnerable to drugs targeting these processes [9-13]. Hence, deregulated energetics/reprogramming energy metabolism has been designated as a hallmark of cancer and a relevant characteristic acquired during multistep development of human tumours [4-7,14]. The vast majority of cancer cells use simultaneously glycolysis and oxidative phosphorylation (OXPHOS) for bioenergetics requirements [5], although ratio differs among cancer types [15], patients [16], and heterogeneous tumours which display distinct metabolic environments [17]. In fact, the ability of Gln to support ATP production is strongly reduced in some types of cancers having lowered OXPHOS activity [18]. However, increased demand for nitrogen and exploitation of glycolysis or tricarboxylic acid (TCA) cycle intermediates

for biosynthesis and NADPH production, are among emerging hallmarks of cancer metabolism [19]. Sustained growth and survival of most cancer cells rely upon a metabolic rewiring (Fig. 1), characterized by an enhanced Glc intake and a stimulated utilization of Gln by reductive carboxylation [6]. The terms "Glc-addicted" and "Gln-addicted" cancer cells have been coined to stress the importance of these two metabolites in tumour biology [20]. It is well known that many cancer cells become "highly addicted" to Gln for supporting their accelerated growth and proliferation [21].

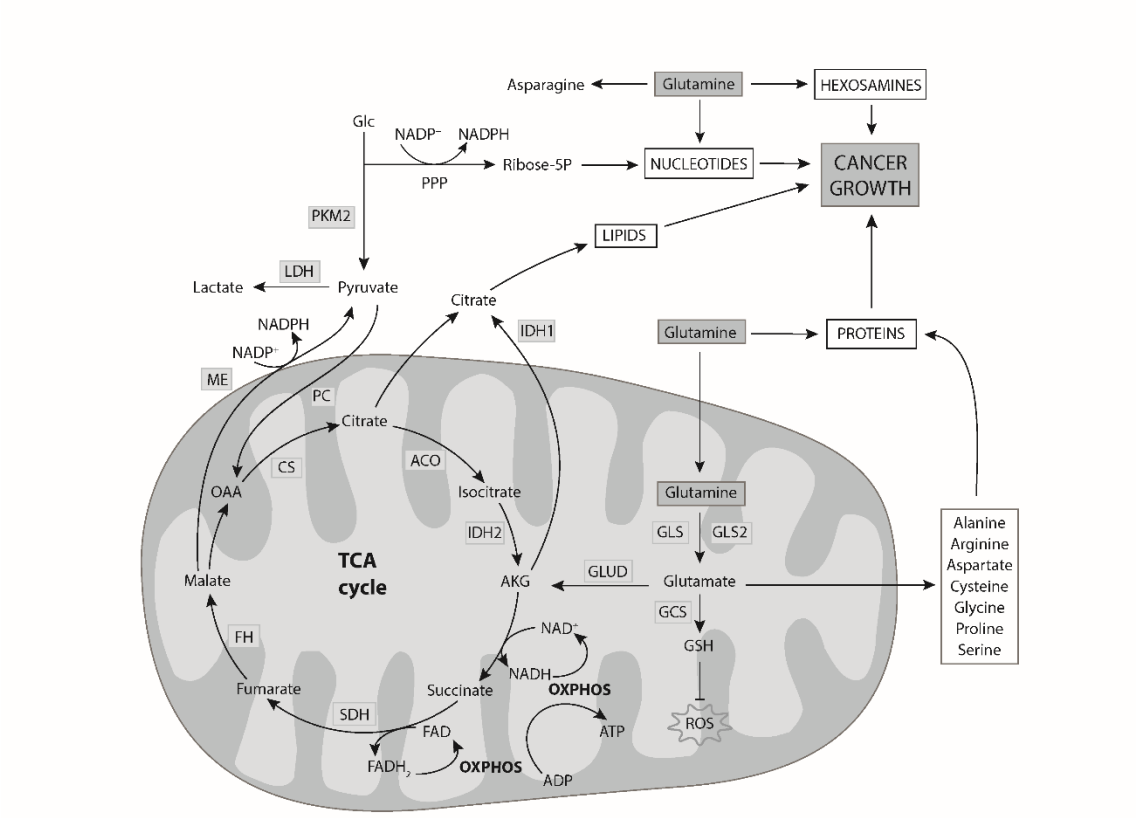

Figure 1. Main metabolic pathways that are rewired in cancer cells. Gln affords its α-nitrogen, γ-nitrogen and carbon skeleton to provide synthetic and energetic objectives. The γ-nitrogen from glutamine's amide group is used for synthesis of asparagine, hexosamines and nucleotides. Glu carries most of glutamine's α-nitrogen to produce NEAAs. Alanine and aspartate are formed by transamination reactions between Glu and OAA and pyruvate, respectively. Biosynthesis of other NEAAs (serine, glycine, cysteine, arginine, proline) also need the amino group from Glu. This amino acid is one of the three amino acids that form GSH through GCS, together with cysteine and glycine (also Glu derivatives). GSH is the main intracellular antioxidant and fights against ROS from high metabolic regime of tumour cells. One of the malignant characteristic of cancer metabolism is the high speed for the conversion of Glu in AKG, via GLUD, and the oxidation of its carbon backbone in the mitochondria, leading to ATP production by OXPHOS. Mutations in genes encoding ACO, IDH, SDH, SDH, FH and CS are noted in many cancers, generating oncometabolites which increase glycolysis, glutaminolysis (through GLS and GLS2), and ROS production. IDH1 activity can participate in the synthesis of citrate (from Gln, Glu, and then AKG) and acetyl-CoA for lipid biosynthesis. Other rewired metabolic enzymes are represented in this figure (LDH, PKM2, PC, and ME). Higher LDH and PKM2 activities are a hallmark of proliferative cancers. PC allows metabolic plasticity using Glc-derived pyruvate instead of Gln for anaplerosis. Malate can be converted in pyruvate by ME to generate extra

NADPH. A significant amount of glycolytic intermediates is diverted to PPP for production of NADPH and nucleotides. AKG, alpha-ketoglutarate; ACO, aconitase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CS, citrate synthase; FH, fumarate hydratase; GA, glutaminase; GCS, gamma-glutamylcysteine synthetase; Glc, glucose; Gln, glutamine; GLS, glutaminase isoenzyme; GLS2, glutaminase 2 isoenzyme; Glu, glutamate; GLUD, glutamate dehydrogenase; GSH, glutathione; IDH1/2, isocitrate dehydrogenase isoenzymes; LDH, lactate dehydrogenase; ME, malic enzyme; NEAAs, non-essential amino acids; OAA, oxaloacetate; OXPHOS, oxidative phosphorylation; PC, pyruvate carboxylase; PKM2, pyruvate kinase M2; PPP, pentose phosphate pathway; ROS, reactive oxygen species; SDH, succinate dehydrogenase; TCA, tricarboxylic acid.

Gln supports cell defences against oxidative stress by generating high amounts of the antioxidant glutathione (GSH) [22]. Additionally, Gln metabolism provides the cells with a source of NADPH production to support fatty acid synthesis making Glc-derived carbon and TCA cycle intermediates to act as biosynthetic precursors [23]. Proliferating cancer cells take up Gln and convert it to glutamate through a variety of de-amidation and transamidation reactions, most notably glutaminase (GA), that both support TCA cycle and contributes to amino acid synthesis [24]. Thus, Gln is processed by GA to generate glutamate which is later turned to alpha-ketoglutarate (AKG), and then converted to 2-hydroxyglutarate (2HG) by mutant isocitrate dehydrogenase-1 (IDH1) [25]. A metabolic hallmark of numerous brain tumours is *de novo* synthesis of high levels of the oncometabolite 2HG, exclusively in the form of the D-enantiomer (D-2HG), by the mutant IDH1 enzyme through the NADPH-dependent reduction of AKG [16].

In theory, therapies aimed at curtailing Glc and Gln utilization could be efficacious in treating "Glc/Gln-addicted" tumours with relative specificity [26]. However, this approach will invariably trigger metabolic stress, which is often observed in highly aggressive tumours, and correlates with poor patient survival and resistance to therapy [27,28]. Cancer cells with impaired mitochondria use Gln-dependent reductive carboxylation rather than oxidative metabolism as the major pathway of citrate formation and fatty acid synthesis, being a common cellular response to defective mitochondrial metabolism [29]. Interestingly, Warburg effect has been designated like an effect/reason for malignancy [30], and consequently a key target to be exploited as a cancer biomarker [31], as well as in tumour therapy [32]. Nevertheless, there is a remarkable number of other metabolic circuits in cancer that complement the elevated glycolysis, and also the enhanced glutaminolysis [33]. Different strategies for changing the peculiar metabolism of the cancer have been used with therapeutic intention, mainly (i) targeting the synthesis of macromolecules (lipids [4], proteins [5], nucleic acids [23]), (ii) blocking mitochondrial metabolism (OXPHOS [5], TCA cycle [19]), and (iii) targeting glycolysis (Glc transport [2], lactate [26], PKM2 [28]). Stage of development and key preclinical findings of the above and other procedures have been reviewed in detail by Vander Heiden [6]. Among targets, Gln [2,20,23] and GA [21,22,24] have gained successful attention in last years.

2. Glutaminases

The GA protein family members are encoded by two paralogous genes, *Gls* and *Gls2* [11]. As described in Table 2, proteins GLS and GLS2 have different characteristics, including kinetic and immunologic properties [34]. Exon 18, which codes for the C-terminal region of both proteins, shows the lowest sequence similarity (29.4%). This region of human GLS2 proteins has been demonstrated to be involved in the recognition of PDZ (PSD95\Dlg\ZO1) domains-interaction modules [35]. Therefore, the most significant differences between human GLS and GLS2 proteins are located in regions involved with protein–protein interplay and organelle

targeting, which may help to account for their distinct role and regulation [34]. The abundance of a particular GA mRNA specie may significantly switch in accordance with metabolic interactions with the microenvironment, the tissue or the developmental state of the tissue [36]. The physiological meaning of the existence of different species of mRNA coding for GA is not fully understood. However, it is tempting to speculate that each transcript may represent a specific target for different stimuli. GA is the enzyme responsible for catalyzing the conversion of Gln to Glu, representing the first step in Gln metabolism (glutaminolysis) [34]. Under physiological conditions GA is a glutamine amidohydrolase, a genuine hydrolytic enzyme releasing the γ-glutamyl group and ammonium into the media, having an essential role in the glutamatergic synaptic function in brain [37]. Ammonium can induce autophagy [34]. In malignancies, GA provides cancer cells with elevated levels of energy to meet their accelerated growth and proliferation, and also with molecules for synthesis of proteins, nucleic acids and lipids [35].

2.1. GLS

The human *GLS* gene (cited in some publications with the incorrect term *GLS1*: see HUGO Gene nomenclature, http://www.genenames.org/), spans 82 kb and splits into 19 exons [35]. Two different transcripts arise by alternative splicing of this gene: the KGA transcript, originally found in kidney, integrated by 18 exons and formed by joining exons 1 to 14 and 16 to 19, and the GAC transcript which appears by alternative splicing and comprises only the first 15 exons [36]. In humans, *GLS* is located in chromosome 2 [35]. The mammalian proteins encoded by the KGA and GAC transcripts are distinctly mitochondrial [36]. In their inactive states, KGA and GAC exist primarily as dimeric species [37]. KGA and GAC can be activated via the addition of inorganic phosphate (Pi), which is thought to stimulate the formation of an active tetramer [11]. Thus, GLS is activated by high phosphate levels, but strongly inhibited by its end-product Glu (Table 2). The tetramer-dimer equilibrium is concentration-dependent; concretely, for the GAC isoform, it has been shown to occur also in the absence of Pi just by raising the enzyme concentration, which argues in favour of GAC having a greater tendency to oligomerize when compared to KGA: the unique C-terminal sequence of GAC seems to be determinant for oligomerization [36]. The GLS isoform GAC, which differs from KGA only in their C-terminal region, seems to be predominantly expressed in certain types of tumour with preference to KGA [36]. In fact, GAC was first cloned and characterized from human colon cancer cells, while normal kidney and brain tissues were the sources of most mammalian KGA [35]. Additionally, the GAC/KGA ratio increased in lung tumours compared with matched normal lung tissue, mostly due to a significant downregulation of KGA because GAC expression levels were similar in tumour and normal tissue [34]. Furthermore, transient knockdown of GAC had a greater effect on tumour cell growth than silencing of KGA, reinforcing the view of GAC playing a more relevant role than KGA in cancer [36]. MiRNAs-mediated GAC regulation could be a strategy used by tumours to gain selective advantages to utilize distinctive sources of carbon stimulating their adjustment for altering metabolic environments [35]. In relation with signalling pathways controlling GLS isoforms, a synergistic cross-talk between KGA-mediated glutaminolysis and epidermal growth factor (EGF)-activated Raf-MEK-ERK kinases module was reported in human 293 T cells [38]. The increase in GLS activity mediated by the Raf-MEK-ERK module was found to be phosphorylation dependent, because specific inhibition of kinases or co-expression of protein phosphatase PP2A completely abrogates the enhanced KGA activity [34]. Whether this regulation of *GLS*-encoded KGA isoform by Raf-MEK-ERK signalling could be targeted in cancer cells awaits further elucidation [38]. Knocking down both GLS isoforms, KGA and GAC, in Rho-transformed cells disrupted mitochondrial GA activity. Activation of GAC through Rho GTPase was shown to be dependent on nuclear factor-kappa B (NF-κB), which probably induces posttranslational modifications in the GAC protein, maximizing its enzyme activity in cancer cells

[39]. GLS can be regulated by over a number of other signalling pathways, transcription factors and microRNAs as detailed in Fig. 2.

Table **2**. Main differential characteristics among human glutaminase isoenzymes.

Gene	*GLS*	*GLS*	*GLS2*	*GLS2*
Isoform name	KGA	GAC	GAB	LGA
Transcript length (nts)	4348	3183	2408	2026
No. Exons	18	15	18	17
Protein name	GLS	GLS	GLS2	GLS2
Protein length (aas)	669	598	602	565
Approximate subunit molecular mass (kDa)	68 and 66	58	63	56
Pi dependence	High	High	Low	Low
Ammonia activation	No	No	Very low	Strong
Glutamate inhibition	Strong	Strong	Moderate	No

2.1. GLS2

The human GLS2 gene is located in chromosome 12 [34], and it has a length of approximately 18 kb and splits into 18 exons [40]. Two transcripts have been identified from the mammalian *Gls2* gene: the canonical long transcript termed GAB, formed by joining all 18 exons of the gene, and the short transcript LGA that lacks exon 1 and was originally identified in rat liver [41]. Human GAB transcript was isolated as a cDNA clone from ZR-75 breast cancer cells encoding a protein of 602 amino acids, which is 67 amino acids longer than rat liver LGA protein [35]. The recombinant human GAB protein was expressed as a precursor protein of 66 kDa and a mature, processed form, of about 63 kDa, arises after cleavage of the first 38-39 amino acids at the N-terminal sequence [37]. In humans, the *GLS2* gene is located in chromosome 12 [34]. GLS2 is activated by low phosphate levels and slightly inhibited by Glu [35]. Cells with heightened GLS2 levels showed enhanced Glu concentrations, increased OXPHOS activity, and high GSH level [34]. Furthermore, overexpression of GLS2 afforded protection from reactive oxygen species (ROS)-induced apoptosis or from DNA damage [35].

Although GAs have been traditionally considered as mitochondrial enzymes, GLS2 has been localized in nuclei [37]. This location and its interactions with PDZ proteins suggest that the role of this protein may go beyond GA activity, for example regulation of gene expression patterns regarding cancer metabolism [34,37]. Therefore, GLS2 family isoforms might have essential impact upon the fine regulation of Gln metabolism in cancer [37]. GLS2 have been found in human colorectal tumour cells, human hepatoma HepG2 cells, medullar blood mononuclear cells from patients suffering from leukaemia, KU812F human myeloid cells, and MCF7 and ZR-75 human breast cancer cells [35]. Interestingly, GLS2 mRNA was consistently higher in slow-growing adenomas than in rapidly proliferating carcinomas [34]. Furthermore, GLS2 expression is downregulated in hepatocellular carcinomas (HCC) and highly malignant glioblastoma, i.e.: in glioblastomas (WHO grade IV), the most malignant brain tumours, high levels of GLS, and only traces or lack of GLS2 transcripts were found [35]. GLS2 has been identified as a target for *p53* tumour suppressor gene [34]. Although traditionally considered as a gene which triggers apoptosis or senescence to prevent tumour progression, other described roles for p53 protein also include the maintenance of normal metabolism and survival pathways allowing cells to adapt to various types of metabolic stress [37]. *P53* is the first identified tumour

suppressor gene involved in the regulation of Gln metabolism by activation of GLS2 expression [34]. Although increase in GLS2 abundance was triggered by specific stresses that activate p53, basal GLS2 levels were also regulated by p53 under non-stressed conditions [35]. In fact, *GLS2* was confirmed to be a p53 target gene in both non-tumour and tumour cells [37]. Additional transcription factors, signalling proteins and microRNAs dealing with GLS2 are depicted in Fig. 2.

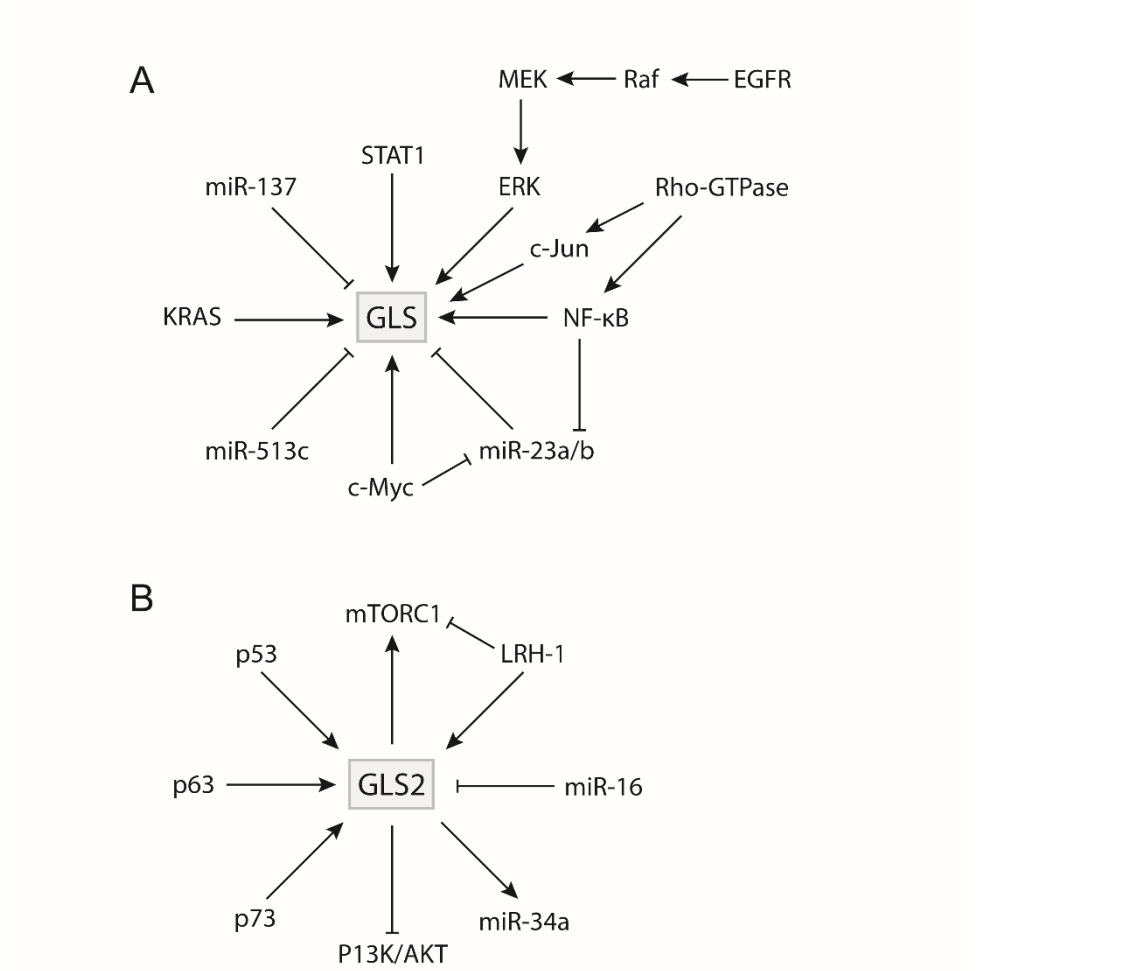

Figure 2. Regulatory network modulating glutaminase isoenzymes. **A.** GLS expression is activated by transcription factor such as c-Myc and STAT1. Conversely, it is inhibited by microRNAs (miR-23a/b, miR-137 and miR-513c). Rho-GTPase activates both NF-κB and c-Jun to stimulate GLS overexpression. Loss of KEAP1 induces GLS via KRAS. Finally, Raf-MEK-ERK signalling node regulates GLS through EGF activation. **B.** GLS2 expression can be activated by tumour suppressor factors p53, p63 and p73, as well as by the nuclear receptor LRH-1. GLS2 is negatively regulated by miR-16, whereas GLS2 upregulates miR34a maturation. On the other hand, GLS2 blocks the PI3K/AKT signalling pathway, but stimulates mTORC1. AKT, protein kinase B; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinases; GLS, glutaminase isoenzyme; GLS2, glutaminase 2 isoenzyme; KRAS, V-Ki-ras2 Kirsten rat sarcoma; LRH-1, nuclear receptor liver receptor homolog 1; MEK, mitogen-activated protein kinase kinase; miR, microRNA; mTORC1, mammalian target of rapamycin complex 1; NF-κB, nuclear factor-kappa B; PI3K, phosphatidylinositide 3-kinase; STAT1, signal transducer and activator of transcription 1.

3. A plethora of essential functions for glutamine and glutaminases

Glutamine is not only a key amino acid for biosynthesis and bioenergy purposes but also essential as a signalling molecule [34]. Metabolic reprogramming of Gln provides a selective advantage during tumourigenesis [42]. Many metabolic enzymes, transcription factors, and signalling pathways are involved in the rewiring of Gln metabolism [21-24]. There is a long list of proteins dealing with GA which have been intensively tackled in recent years (Table 3).

Table **3**. Relationship of metabolic enzymes, signalling proteins and transcription factors with glutaminase or with glutamine metabolism.

Protein	Working mechanism	References
IDH1	Evoking reductive carboxylation of glutamine	43-46
Rho-GTPase	Enhancing GLS activity via NF-κB	39
Rho GTPase	Promoting p53 via GLS2	43,47
KEAP1	Loss of KEAP1 enhances KRAS-dependent glutaminolysis	48
N-MYC	Activating GLS2, but not GLS, to increase glutamate levels	49
c-MYC	Activating aminotransferases via ROS	50
c-MYC	Suppressing miR-23a/b and inducing GLS	51
c-MYC	Inducing Gln addiction	52
AST, ALT	Supplying a source of anaplerotic carbon for the TCA cycle when GLS/GLS2 are inhibited	53
GLUD	Supplying a source of anaplerotic carbon for the TCA cycle when GLS/GLS2 are inhibited	6,44
PC	Providing an alternative route to replenish the TCA cycle when GLS/GLS2 are inhibited	6,45
KRAS	Gln-derived aspartate is converted into OAA by GOT1	53,54
mTORC1	Activating autophagy via GLS2	55
mTORC1	Gln reactivates mTORC1 during amino acid starvation	56
HIF-1	Modulating Gln metabolism via PI3K/AKT	57
HIF-1	Connecting glycolysis and glutaminolysis	58
NOTCH1	Inducing glutaminolysis via GLS overexpression	59
p53	Inducing GLS2 to enhance mitochondrial respiration	43,60
STAT1	Activating GLS transcription through interaction with p300	61
PTEN	Degradation of GLS via ubiquitination	62
ERK	Regulating GLS by phosphorylation	38

ALT, alanine transaminase; AKT, protein kinase B; AST, aspartate transaminase; ERK, extracellular signal-regulated kinases; GLS, glutaminase isoenzyme; GLS2, glutaminase 2 isoenzyme; GLUD, glutamate dehydrogenase; GOT1, aspartate transaminase; HIF-1, hypoxia-inducible factor 1; IDH1, cytosolic isocitrate dehydrogenase; KEAP1, Kelch-like ECH-associated protein 1; KRAS, V-Ki-ras2 Kirsten rat sarcoma; mTORC1, mammalian target of rapamycin complex 1; NF-κB, nuclear factor-kappa B; OAA, oxalacetate; PC, pyruvate carboxylase; PI3K, phosphatidylinositide 3-kinase; PTEN, phosphatase and tensin homolog; ROS, reactive oxygen species; STAT1, signal transducer and activator of transcription 1.

Although the importance of ROS in cell transformation and tumour maintenance has been already established, the relative contribution of different Gln-related metabolic circuits to redox regulation in cancer is not fully understood [27]. GA catabolizes Gln mainly for ATP and GSH synthesis and its reduction affects growth and apoptosis presumably through depletion of

ATP and augmentation of ROS, respectively [18,63]. Noteworthy, GLS2 expression affects certain p53 target gene(s) or some specific function of p53, which leads to the modulation of ROS-associated apoptosis [64]. Overexpressing miR-23a in leukemic cells impaires Gln use and induces mitochondrial dysfunction leading to cell death. In fact, miR-23a targets GLS mRNA and inhibits expression of GLS protein [65], showing new insights into potentially druggable objectives against cancer. Many studies suggest that GA can regulate cancer development in leukaemia [59], glioma [66], neuroblastoma [67], melanoma [68], as well as in liver [15], colorectal [69], colon [70], pancreas [58], ovarian [71], kidney [34], bladder [72], lung [73], and breast [74] cancers.

Enhanced c-Myc activity was sufficient to drive Gln metabolism and to impair cell survival in low Gln conditions [52]. Oncogene c-Myc also dictates the expression of the GLS gene through effects on the miRNAs miR23a and miR23b, de-repressing GLS translation [51]. Surprisingly, N-Myc, an essential MYC family member, promotes conversion of Gln to Glu in MYCN-amplified neuroblastoma cells by directly activating GLS2, but not GLS, transcription [49].

Simultaneous expression of GA transcripts was always found in leukaemia cells isolated from medullar blood of patients and in breast cancer cell lines [75]. Nonetheless, fully developed lymphocytes from the medullar blood of a patient suffering aplasia did not express GLS transcripts and showed a 15-fold multiplication of GLS2 transcripts. Above results state that GLS correlates with increased rates of proliferation, whereas prevalence of GLS2 appears to be related with quiescent or differentiated cell states in some models [75]. Although few structural information has been reported so far for GLS2 [76], probably because GLS2 isoforms (GAB and LGA) are considerably more unstable, labile and refractory to purification than GLS isoenzymes [77], it has been shown that *Gls2* expression can be regulated by the p53 family member p73 in cancer cells [78], driving GSH synthesis [79], and having a key regulatory function on the homeostatic control of physiological oxidative balance during cancer transformation [80].

4. Conflicting roles of glutaminase isoenzymes

Glutaminase isoforms can be ascribed to oncogenic or antitumour roles (Fig. 3). Although their function depends on tissue and cell type, enhanced Gln catabolism in Myc-induced liver tumours have been associated with curtailment of Gln synthetase (GS) and switch from *Gls2* to *Gls* expression [15]. These findings are being used to design of therapies targeting Gln metabolism [15]. Antisense experiments on GLS (KGA isoform) allows reversion of the cancer phenotype of Ehrlich ascites tumour cells (EATC) *in vitro* [81], showing marked changes in their morphology while losing their tumourigenic capacity because of the development of an effective anti-tumour immune response [82]. Analogous effect was achieved by overexpressing the GLS2-encoded GAB isoenzyme in human glioblastoma cells, supporting a role for this isoform in transcriptional regulation indeed [83]. Furthermore, in view of the presumed role of GLS2 in modulation of gene transcription, it has been hypothesized that its deficit has implications for the physiology of glia-derived tumours, perhaps driving them toward malignant phenotype. To address this question, stably transfected T98G cells with the full GAB cDNA coding sequence were assessed for the effects of transfection on basic physiological parameters: proliferation, migration and survival [84]. The transfected cells (T98-GAB cells) showed a 40% decrease of cell survival, a 45% reduction of cell migration and a 47% decrease in the proliferation index [35]. Microarray inspection showed a significantly altered expression of 85 genes in T98-GAB, but not in sham-transfected or control cells. Microarray findings were confirmed by real-time quantitative polymerase chain reaction (RT-qPCR) analysis for various genes coding oncoproteins: FNDC3B was overexpressed, and S100A16, CAPN2, TIMP4, and MGMT were inhibited [83].

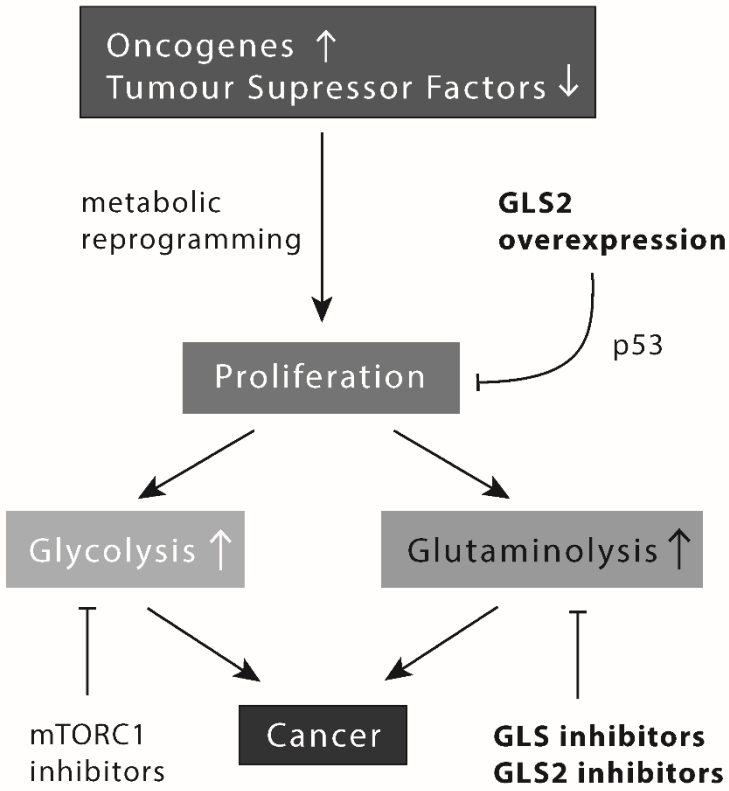

Figure 3. Metabolic reprogramming of cancer by isoform-specific glutaminase-inhibitors. As mTORC1-inhibitors are the best chemical tool for repressing glycolysis, glutaminase inhibitors are the most important key to block glutaminolysis. Glutaminase isoforms KGA and GAC (GLS isoenzymes) are oncogenic factors. Glutaminase isoforms LGA and GAB (GLS2 isoenzymes) are context-dependent tumour suppressor factors. GLS isoforms overexpression has been described in many types of cancers. Conversely, GLS2 isoforms are downregulated in several types of malignancies. In these cancers, overexpression of GLS2 can help to hamper cancer, inducing cells to differentiate instead of to proliferate. GLS; glutaminase isoenzyme; GLS2, glutaminase 2 isoenzyme; mTORC1, mammalian target of rapamycin complex 1.

Silencing of KGA isoform in SFxL and LN229 human glioma cell lines activates the pyruvate carboxylase (PC) pathway to allow cells to use Glc-derived pyruvate preferably than Gln for anaplerosis [9]. Interestingly, induction of PC during chronic suppression of Gln metabolism may prove to be a mechanism of resistance to therapies targeting glutaminolysis [85]. In those models, down-regulating *GLS* or overexpressing *GLS2* produces similar settings and proteins involved showed equivalent activity: c-Myc and Bcl-2 were diminished and Bid was augmented [84]. However, in other tissues or cell types GLS2 was over-regulated showing oncogenic properties [86]. Combination of GA modulated expression with arsenic trioxide induce a synergistic effect suppressing malignant properties of cancer cells [84]. In addition, GLS2 is a key player in the cell differentiation, particularly neuronal differentiation [78]. GLS2 increases cellular levels of GSH and NADH and decreases ROS levels in cells, acting as an essential component in mediating these two unique functions of p53 in the regulation of energy metabolism and antioxidant defence [87]. Furthermore, the p63/GLS2 axis might be an

important pathway regulating ROS-dependent apoptosis in the absence of functional p53 [88]. Above results agree with our own findings relating *GLS2* overexpression and cancer regression [89]. Overall, emerging evidence states that cancer cells have much more complex metabolic requirements than previously appreciated, and that numerous pathways complement Glc- and Gln-dependent metabolism [90]. It is presently unknown how GA isoenzymes may undergo such different roles in tumour biology. Understanding of genetic pathways involved in the development and progression of every type of cancer may help define novel therapeutic targets and identify treatment regimens that are very likely to provide clinical benefit to patients [59, 91]. Although there are multiple compensatory metabolic networks in cancer cells upon perturbation of Gln metabolism, targeting these compensatory pathways may have therapeutic utility [92]. For example, cytosolic reductive carboxylation operates as substitute for malate-aspartate shuttle, tightly coupling the oxidation of Gln with glycolysis and likely enabling glycolytic flux for ATP generation and biomass synthesis [93]. Although it is accepted that GA is a mitochondrial enzyme, using newly generated isoform-specific antibodies, we have found a differential intracellular immunolocalization of GLS and GLS2 isoforms [94]. In fact, there was a different subcellular distribution: GLS isoform was always present in mitochondria while GLS2 appeared in two different locations, mitochondria and nucleus [95]. Moreover, the ability of the C-terminus of GLS2 to interact with PDZ domain-containing proteins was also demonstrated [96], pointing out to the regulation of gene expression as one of its potential nuclear roles [97].

5. Anti-cancer combinatory therapy might include switching glutaminase expression

Glutaminase is a very hot spot in metabolic reprogramming in cancer to be exploited for cancer therapy. First drug used to target GLS was the irreversible Gln-competitive inhibitor 6-diazo-5-oxy-L-norleucine (DON) [98]. However, DON lacks selectivity and has various characterized targets [99]. In last years, numerous new small molecules have been found to inhibit GA isoenzymes (Fig. 4).

Dibenzophenanthridine-968, 5-[3-bromo-4-(dimethylamino)phenyl]-2,3,5,6-tetrahydro-2,2-dimethyl-benzo[a]phenanthridin-4(1H)-one, is an allosteric regulator of GLS isoform that induces apoptosis [100,101]. Molecule 968 blocks oncogenic transformation induced by various Rho GTPases in fibroblasts, and the growth of human breast cancer and B lymphoma cells, without affecting normal cells [102]. Other investigators further showed that oncoproteins such as AKT and ErbB2 were substantially downregulated, thus suggesting that GA inhibition might be a more effective epigenetic therapy than the use of histone deacetylase inhibitors, which tend to have a broader impact on cells [103]. Huang et al. utilized dibenzophenanthridine-968 while testing the hypothesis that Gln metabolism via GA in cancer cells is more responsible for the control of intracellular pH (via ammonia release) than for providing inputs to the citric acid cycle [104]. Probably, the most important GA inhibitor was discovered in Curthoys' lab in 2007 [105]. It is bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl (BPTES) [25], a specific molecule that targets GLS isoforms over the GLS2 isoenzymes [100]. The derived molecule 2-(pyridin-2-yl)-N-(5-(4-(6-(2-(3-(trifluoromethoxy)phenyl)acetamido)pyridazin-3-yl)butyl)-1,3,4-thiadiazol-2-yl)acetamide (CB-839) was orally administered to mice and it did not modify blood cell count parameters in a xenotransplantation model of breast cancer [106]. In cell lines and patient samples harbouring IDH1/IDH2 mutations, CB-839 reduced production of oncometabolite 2HG, inducing differentiation [107]. CB-839 is getting excellent results in clinical trials against several types of malignancies [106]. Compound 2-phenyl-N-(5-(4-((5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)amino)piperidin-1-yl)-1,3,4-thiadiazol-2-yl)acetamide (named as UPGL00004) that strongly inhibits the growth of triple-negative breast tumour cells [108], occupies the same binding site

as CB-839 or BPTES. All three inhibitors regulate the enzymatic activity of GAC via a similar allosteric mechanism, working as promising anticancer molecules [109].

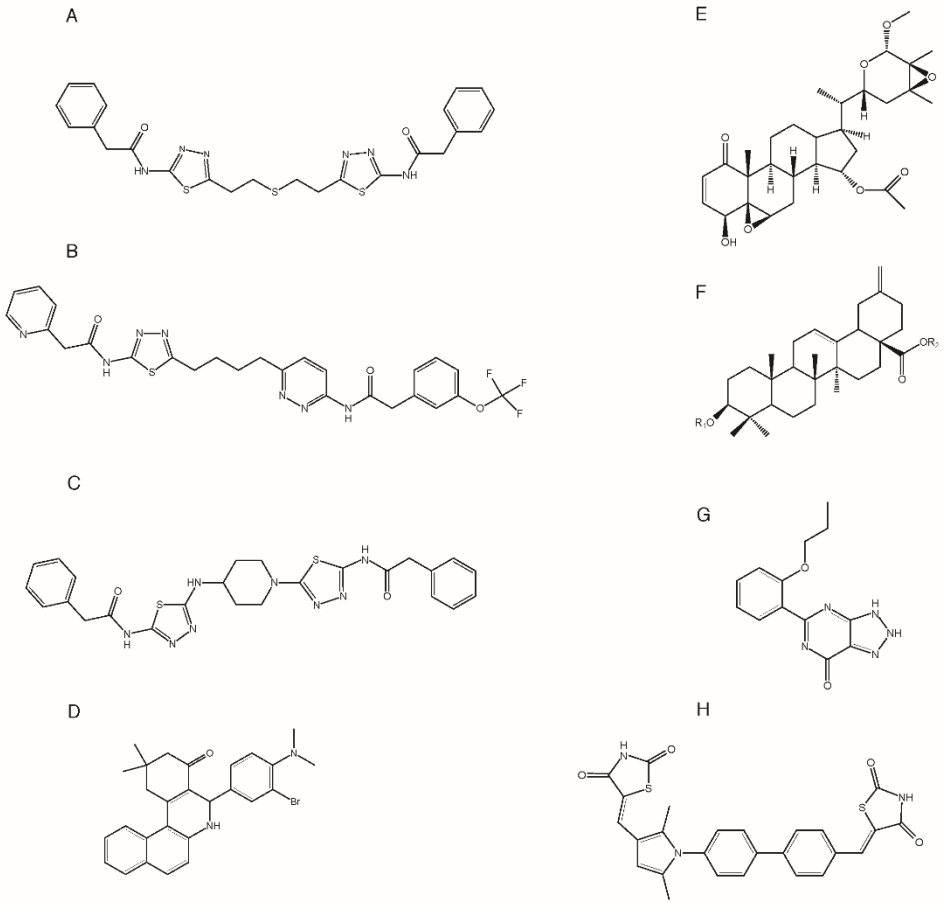

Figure 4. GLS isoenzyme-specific inhibitors. A: BPTES, B: CB-839; C: UPGL00004; D: dibenzophenanthridine-968; E: physapubescin K; F: brachyantheraoside A8; G: zaprinast; H: thiazolidine-2,4-dione compound#5.

(*E*)-5-((4'-(3-((Z)-(2,4-Dioxothiazolidin-5-ylidene)methyl)-2,5-dimethyl-1H-pyrrol-1-yl)-[1,1'-biphenyl]-4-yl)methylene)thiazolidine-2,4-dione is depicted in Table 4 as compound 5. (*E*)-5-((4'-(3-((Z)-(2,4-Dioxothiazolidin-5-ylidene)methyl)-2,5-di(thiophen-2-yl)-1H-pyrrol-1-yl)-[1,1'-biphenyl]4-yl)methylene)thiazolidine-2,4-dione is compound 6 in Table **4**. Both molecules block GLS and GLS2, but with different selectivity and sensitivity [110]. Inhibition of GLS by these molecules severely impacts the proliferation and survival of many cancer cell lines, but without having negative consequences on healthy cells [111]. They represent a significant advance in the development of anticancer therapeutics targeting Gln metabolism.

While BPTES is reasonably potent *in vitro* (IC_{50} ~60 nM), higher concentrations are needed to produce an effect on GLS in cells (IC_{50} ~20 μM)[54]. Moreover, the hydrophobic nature and low solubility of benzophenanthridines poses some challenges to the physiological delivery of the inhibitor molecules [106]. Multiple BPTES-derivatives, with similar IC_{50}, include truncated

compounds, molecules containing a pyridazine replacement, others containing an aminopyridin, aminopyrimidin, or pyrrole[2,3-c]pyridazine ring, or compounds with a cycloalkane, aminopyrrolidine, pyrrolidine, piperidine, oxypiperidine or oxypyrrolidine linker [112].

Table **4**. Low toxicity non-glutamine mimetic molecules that inhibit glutaminase isoenzymes. IC_{50} are given in nM.

Compound	GLS	GLS2
BPTES	(10-80) [106,107]	64 x 10^3 [116]
CB-839	30 [108, 109]	(10-35) x 10^3 [116]
UPGL00004	29 [109]	(10-35) x 10^3 [109]
Dibenzophenanthridine-968	(4-19) x 10^3 [101]	–
Physapubescin K	663-989 [113, 114]	–
Brachyantheraoside A8	610 [114]	-
Zaprinast	150 [115]	–
Thiazolidine-compound#5	102-381 [109]	1.2 x 10^3 [109]
Thiazolidine-compound#6	50 [109]	102 [109]
Ardisianone (Lee-AV1)	2.1 x 10^3 [114,116]	280 [114,116]
Lee-AV2	3.9 x 10^3 [114,116]	290 [114,116]
Lee-AV8	2.9 x 10^3 [114,116]	260 [114,116]

Physapubescin K is other GLS-specific inhibitor. Chemically, 6-hydroxy-15-(1-{2-methoxy-1,6-dimethyl-3,7-dioxabicyclo[4.1.0]heptan-4-yl}ethyl)-2,16-dimethyl-3-oxo-8-oxapentacyclo[9.7.0.0^{2},^{7}.0^{7},^{9}.0^{12},^{16}]octadec-4-en-13-yl acetate, blocked Gln metabolism in SW1990 cells and inhibited the tumour growth in a SW1990 xenograft mouse model [113]. Another natural product, brachyantheraoside A8 inhibited HCC1806 cell migration by inducing cancer cell apoptosis through the modulation of Bax and Bcl-2 [114]. Zaprinast is 5-(2-propoxyphenyl)-1H-[1,2,3] triazolo[4,5-d]pyrimidin-7(4H)-one. Treatment with this molecule reversed histone hypermethylation and soft-agar growth of IDH1-mutant cells, and treatment of Gln-addicted pancreatic cancer cells reduced growth and sensitized cells to oxidative damage [115].

The search for specific compounds that block GLS2 activity has been hampered [37], and little drug-discovery effort has been directed toward GLS2 (Fig. **5**). Some benzoquinones isolated from *Ardisia virens* or *Ardisia kusukuensis* showed potency against recombinantly expressed GLS2, while exhibiting up to tenfold higher affinity for GLS2 than for GLS isoenzymes [55]. Further, they provided evidence that these molecules bind to a novel site on GLS2, distinct from the equivalent binding sites for BPTES, dibenzophenanthridine-968 or DON on GLS. Notably, 1-(5-methoxy-3,6-dioxocyclohexa-1,4-dien-1-yl)pentadecan-2-yl acetate (compound Lee-AV), also named ardisianone, induces apoptosis in human prostate cancers through mitochondrial damage stress, leading to the inhibition of mTOR/p70S6K pathway, downregulation of Bcl-2 family members, degradation of survivin, and activation of caspase cascades [116]. Alkyl benzoquinones ardisianone-derivatives (Lee-AV2 and Lee-AV8; Fig. **5**) have shown similar potential [113].

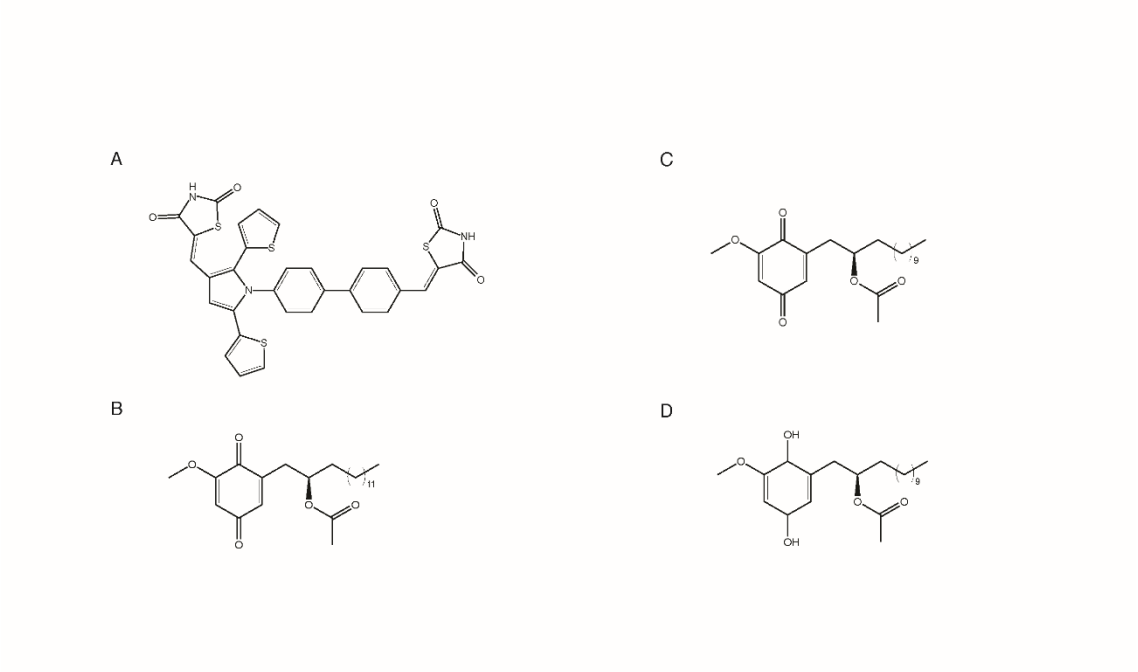

Figure 5. GLS2 isoenzyme-specific inhibitors. A: thiazolidine-2,4-dione compound #6; B: alkyl benzoquinone Lee-AV, also known as ardisianone; C: alkyl benzoquinone Lee-AV2; D: alkyl benzoquinone Lee-AV8.

On the other hand, GLS2 negatively regulates the phosphatidylinositide 3-kinase/protein kinase B (PI3K/AKT) signalling and shows tumour suppression activity in human HCC [117] and glioma cells [118]. Epigenomic downregulation of GLS2 in these liver cancer cells is caused by promoter hypermethylation, consistent with observations in human glioblastoma [119] and colon cancer, inhibiting cancer cell growth and colony formation ability through induction of cell cycle arrest [120]. Importantly, a recent study implicated GLS2 in miRNA regulation through Dicer stabilization, upregulation of miR-34a and repression of Snail and metastasis in HCC cells [121]. Notably, while individual knockdown of GLS2 or p53 dramatically promoted the migration and invasion of Huh-1 and HepG2 cells, simultaneous knockdown of GLS2 and p53 did not display a clear effect on the migration and invasion of these cells [47]. These results demonstrate that GLS2, which binds to Rac1, is an important mediator of p53 in suppressing cancer metastasis. Moreover, GLS2 expression is significantly elevated in MYCN-amplified neuroblastomas in comparison with non-amplified ones, correlating with unfavourable patient survival. In aggregate, these results reveal a novel mechanism deciphering context-dependent regulation of metabolic heterogeneities, uncovering a previously unsuspected link between MYC, GLS2 and tumour metabolism [49]. Consequently, the use of a combination of inhibitors of GLS and GLS2 may be a new action plan for treatment of some types of cancer. Deeper research is necessary to polish this combination therapy, supplying a novel anti-cancer strategy.

6. Conclusions and perspectives

Functions of Gln in cancer metabolism include the biosynthesis of essential lipids, citrate, and GSH, the latter of which is the key anti-oxidant molecule to combat oxidative stress [122]. Cancer-associated isoenzymes as well as their signalling pathways are new targets in anti-cancer therapeutics [7,8,12,18,34,92]. Moreover, dual targeting using siRNA of both GLS

isoforms was more effective at sensitizing cancer cells to cisplatin than targeting either GAC or KGA alone [123]. Positive regulators of GA such as STAT1, c-Myc, NF-κB and ERK allow opportunities to inhibit well-studied signalling proteins while simultaneously negatively impacting Gln metabolism. Likewise, negative regulators of GA represent potential pathways that would allow similar indirect metabolic modulation [124]. In-depth knowledge of the signalling processes leading to cell proliferation, differentiation or death, is crucial to understanding the mechanisms of cancer development [125]. Given the complex interplay between oncogenic signalling and metabolic rewiring, new metabolic and non-metabolic functions of Gln in cancer cells will be uncovered [126]. Future analysis of inherited defects of glutaminolytic enzymes, molecular characterization of mitochondrial Gln carriers and GA, as well as generation of transgenic animal models will provide further insights into the specific actions of Gln [127]. In this regard, posttranscriptional modification of GAC (phosphorylation) correlates with poor survival rate of lung cancer patients [128]. Approaches targeting connected nodes exemplify future precision medicine strategy to beat cancer [129]. The identification of metabolic weaknesses of cancer has been used to create efficient methods to fight against tumour cells, but there are still challenges to overcome in optimizing molecules that target cancer metabolism in real patients [130]. However, approaches focusing on a handful of metabolites have already been used successfully in humans to measure gluconeogenesis and the urea cycle *in vivo*, and to compare metabolism between tumours and surrounding tissue, leading to a deeper understanding of metabolic individuality, and for finding new targets [131]. Even though it is not feasible to model all the extrinsic factors that operate in the body, it has been described that a more heterogeneous resistance can be shaped [132].

Tumour cells metabolize Glc, Gln, lactate, pyruvate, hydroxybutyrate, acetate, and fatty acids at much higher rates than normal cells. Their intercompartmental transfer is used in clinical trials to study the efficacy of drugs targeting cancer metabolism [133]. Metabolic vulnerabilities might be targeted therapeutically [134]. Over the last years, considerable efforts by many groups have been invested to identify the genetic signature of diverse mechanisms of chemoresistance accounting for the so-called “resistome” present at each moment in tumour life for every patient [135]. In personalized oncology, synergistic treatment have included GLS inhibitor CB-839 together with NADPH:quinone oxidoreductase-bioactivatable drugs (β-lap, ARQ761) to induce cell death at well-tolerated doses [136]. Other synergistic strategies include GLS inhibitors and the stabilizer of the topoisomerase-DNA complex doxorubicin. This combination suppressed breast and pancreas cancer [110]. GLS-dependent anticancer chemical compounds are not limited to specific GA inhibitors (Fig. **6**). Very recently, the natural sesquiterpene lactone costunolide (3a*S*,6*E*,10*E*,11a*R*)-6,10-dimethyl-3-methylene-3,3a,4,5,8,9-hexahydrocyclodeca[*b*]furan-2(11aH)-one) was characterized as a potent agent decreasing viability and proliferation of HCT116 cells [137]. The inhibitory mechanism is p53-dependent and blocks promoter activity of GLS. Importantly, costunolide did not affect human intestinal epithelial cells. Of note, multiple treatment inhibiting critical enzymes regulating glycolysis (hexokinase), glutaminolysis (GLS), and synthesis of lipids (fatty acid synthase), with lonidamine, DON and orlistat, respectively, showed excellent results in many types of tumour cells [138]. Synergistic medication against cancer has very recently used the natural compound pancratistatin (or its analogous SVTH-6), mitocans that target cancer cell mitochondria to selectively induce apoptosis, with piperlongumine, that targets the stress response to ROS [139]. When apoptosis resistance is presented, excellent results have been achieved using gossypol, epigallocatechin-3-gallate, UMI-77, triptolide and selinexor [140]. Recent advances in cancer therapy (inhibitors being in clinical trials) include the targeting of Glc transporters, hexokinase, pyruvate kinase M2 isoform (PKM2), GLS, and IDH [141]. Targeting Gln transport *via* ASCT2 inhibition is another promising therapeutic approach for treatment of some types of cancer [142]. Other studies have identified a new link between tumour cell metabolism and the invasive behaviour of tumour cells, and indicate that drugs capable of interfering with glutamate release

may be useful in combatting tumour progression [143]. Interestingly, miR-137 acts as a tumour suppressor in melanoma by targeting GLS, thus regulating tumour cell proliferation and Gln catabolism (Fig. **2**). Low levels of miR-137 associate with poor survival of melanoma patients [144]. Future research must show whether miR-137 is a therapeutic target for other solid tumours. Of interest, suppression of nuclear receptor liver receptor homolog 1 (LRH-1) target both glucolysis (via mTORC1) and glutaminolysis (via GLS2) to inhibit tumourigenesis of HCC [145].

High expression of GS is found in the majority of human glioblastomas, that withdraw carbons from the TCA cycle to synthesize Gln *via* GS, being other metabolic target in cancer [146]. Even better, a benzo-selenazol that acts as a dual inhibitor of GLS and glutamate dehydrogenase (GLUD) causing cell death is an early promise as an effective anti-tumour agent with minimal toxicity [147]. Simultaneous inhibition of glycolysis and glutaminolysis has also produced excellent results against gastric [148], colon [149], breast [150], lung cancer [151], and glioblastoma [152]. First and second approaches target PKM2 and GLS, either by using specific inhibitors (shikonin and BPTES, respectively) [148], either by knocking-down PKM2 and GLS expression [149]. Third, the allosteric mTORC1 inhibitor everolimus and the catalytic mTORC1 inhibitor AZD8055 synergistically with CB-839 inhibit growth in triple-negative breast cancer cell lines [150]. Next, combinatorial strategy targets glycolysis using mTORC1 inhibitor sapanisertib (also called compound MLN128), and glutaminolysis using GLS inhibitor CB-839, in lung squamous cell carcinomas, head and neck squamous cell carcinoma and osteosarcoma [151]. Last, competitive mTORC1 inhibitor PP242 (also named torkinib) showed a specific and synergistic effect combined with CB-839 against glioblastoma, without side effects in healthy brain (cortex and hippocampus), liver, and kidney [152]. Differently, chloroquine, an autophagy inhibitor, inhibits compound 968-induced autophagy and sensitizes cells to this GLS inhibitor [153]. Similarly, dihydroartemisinin achieves a great synergistic effect with compound CB-839, inducing high ROS, to evoke apoptosis in HCC, sparing normal cells [154].

Activation of glutaminolysis for Gln-dependent anaplerosis has been found to be a key resistance mechanism, in certain types of malignancies including leukaemia [59], pancreas [136], and lung [151] cancer. Therefore, a combined therapy targeting GA enzymes has shown to be very effective in these cases, where the effects of known drugs are countered by the adaptive Gln catabolism [155]. In this sense, it is urgently needed to identify the biological basis of both addiction to glutaminolysis (usually involving GLS upregulation) and the Gln-independent status shown by some cancer cell types.

In spite of combined metabolic inhibition getting excellent results, further characterization of pharmacokinetic properties of the mix, as well as designing optimal vehicles to deliver the drugs towards target cells, need to be attained [156]. Besides, to identify whether the same synergies exist in individualized patients will be critical to ensure a therapeutic index. Cancer biomarker discovery is now a very active area of research, which benefits from technology developments in epigenomic, metabolomic and proteomic instrumentation [125]. Importantly, proteomics and metabolomics analysis after GLS specific inhibition can predict responsiveness to combinatorial treatment [92], and GLS itself can be a key biomarker for some types of cancers [157]. Additionally, compound CB-839 has entered Phase II clinical trials in combination with other medical drugs like paclitaxel, capecitabine and nivolumab [158]. Results from this studies are expected for the development of new patents and treatments [159]. Striking metabolic plasticity makes necessary a carefully executed *in vitro* and *in vivo* analyses to unravel the metabolic adaptations in response to metabolic perturbations [160]. By studying the modalities of adaptation, scientific community will be able to develop novel and more effective therapeutic targets and novel diagnostic biomarkers [161].

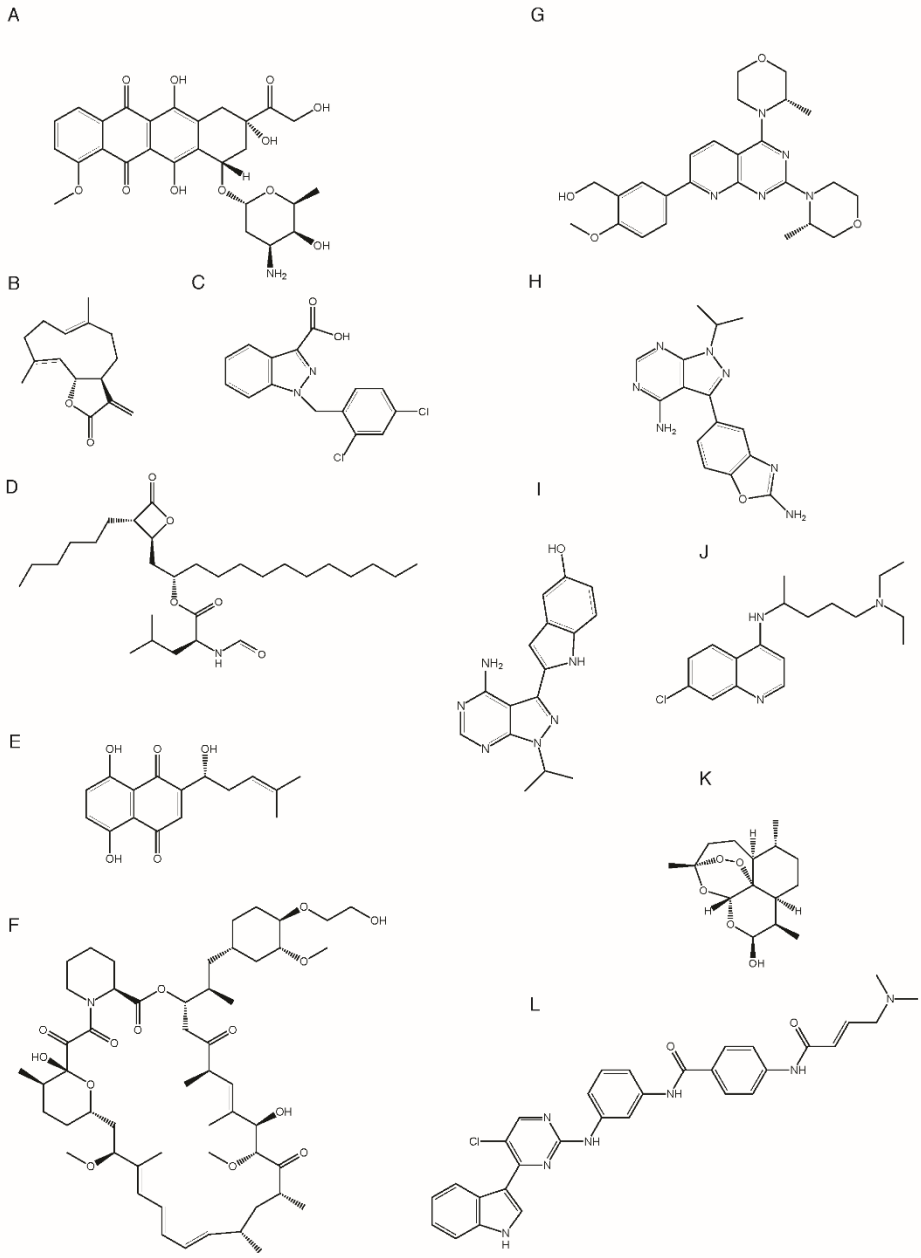

Figure 6. Synergic drugs with glutaminase inhibitors. A: doxorubicin; B: costunolide; C: lonidamine; D: orlistat; E: shikonin; F: everolimus; G: AZD8055; H: sapanisertib; I: torkinib; J: chloroquine; K: dihydroartemisinin.

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Conflict of interest

The authors declare no conflict of interest, financial or otherwise.

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