Glutaminase isoenzymes in the metabolic therapy of cancer

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Abbreviations: DON, 6-diazo-5-oxy-L-norleucine; EATC, Ehrlich ascites tumour cells; ERK, extracellular signal-regulated kinases; GA, glutaminase; GAB, long glutaminase 2 isoform; GAC, short glutaminase isoform; GDH, glutamate dehydrogenase; Glc, glucose; Gln, glutamine; GLS, glutaminase isoenzyme; GLS2, glutaminase 2 isoenzyme; Glu, glutamate; GS, glutamine synthetase; GSH, glutathione; 2HG, 2-hydroxyglutarate; IDH1, isocitrate dehydrogenase-1; KGA, long glutaminase isoform; LGA, short glutaminase 2 isoform; ME1/2, malic enzyme; NF-κB, nuclear factor-kappa B; OXPHOS, oxidative phosphorylation; PC, pyruvate carboxylase; PDA, pancreatic ductal adenocarcinoma; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase isoform 3; PI3K, phosphatidylinositide 3-kinase; PKB, protein kinase B; PKM2, pyruvate kinase M2 isoform; ROS, reactive oxygen species; RT-qPCR, real-time quantitative polymerase chain reaction; STAT1, signal transducer and activator of transcription 1

ABSTRACT (word count: 100)

Altered cellular metabolism is a hallmark of cancer. Cancer cells express isoforms of metabolic enzymes that may constitute therapeutic targets. Glutaminase controls glutamine metabolism and their expression correlate with malignancy of tumours. 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. Selective genomic and epigenomic intervention over glutaminase affects the metabolic reprogramming of cancer. This review highlights the molecular metabolic vulnerabilities in various types of cancer, to be used for biomarker development, drug design, and in personalized oncology.

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

1. Introduction

Just enough taking a look at recent publications in highest impact factor journals [1] to figure out that glutamine (Gln) tumour metabolism is a very hot topic in oncology and an intense area of research where scientists are trying to identify novel therapeutic targets [2] as well as biomarkers for next-generation imaging [3]. Cancer cells have peculiar metabolic characteristics. Because of this, tumours are vulnerable to drugs that target cancer metabolism [4]. In fact, reprogramming energy metabolism has been designed as a hallmark of cancer and a key feature of human tumours [5]. Most cancer cells use both glycolysis and oxidative phosphorylation (OXPHOS) together to produce ATP, but ratio differs among cancer types [6], patients [7], and even heterogeneous tumours [8]. Hence, the ability of Gln to generate ATP is deeply reduced in many types of cancers having diminished OXPHOS activity [5]. In addition, use of TCA cycle intermediates for biosynthesis and NADPH production, as well as increased requirement for nitrogen are emerging hallmarks of cancer metabolism [4]. On the other hand, glucose (Glc) and Gln are the two main fuels used for cancer to support their strongly increased rate of proliferation [9]. Accordingly, many metabolic pathways favour the elevated glycolysis, and also the enhanced glutaminolysis in cancer [10]. Gln is a signalling molecule that regulates essential metabolic routes indispensable for growth and antioxidant defence against oxidative stress [11]. Additionally, Gln metabolism maintains fatty acid synthesis using Glc-derived carbon and TCA cycle intermediates as biosynthetic precursors [5]. Both Gln uptake and glutaminase (GA) activity proportion glutamate (Glu) for transamination reactions and/or supporting of TCA cycle, which also promotes to amino acid synthesis [12]. Thus, Gln is hydrolysed by GA to generate glutamate which is consecutively transformed to α-ketoglutarate, and then converted to 2-hydroxyglutarate (2HG) by isocitrate dehydrogenase-1 (IDH1) [13]. A metabolic hallmark of many brain tumours is *de novo* production of elevated levels of the oncometabolite 2HG [7].

Therapeutic strategies targeting Glc and Gln are promising approaches to treat Glc/Gln-addicted tumours with good specificity [10]. However, this therapy will habitually elicit metabolic stress, and resistance to therapy, regular characteristics of aggressive tumours [9]. Of note, impairment of mitochondrial function and induction of glycolysis does not decrease the magnitude of Gln metabolism for tumour cell proliferation [11]. Warburg effect (high rates of Glc intake and lactate discharge in the presence of oxygen) is a common consequence/cause responsible for carcinogenesis, and therefore a target key to be utilized as a cancer biomarker, as well as in cancer treatment [5].

2. Glutaminase isoenzymes

Glutaminase (GA, EC 3.5.1.2) catalyse the transformation of Gln to Glu, being a key step for glutaminolysis [11]. This reaction endues tumour cells with high amounts of energy to support their accelerated metabolism, and also with biosynthetic precursors for generation of biomolecules, i.e.: protein, nucleic acids and lipids [12]. The GA isoenzymes are encoded by *Gls* and *Gls2* genes [2]. Proteins GLS and GLS2 have different kinetic, immunologic, and molecular characteristics. Relevant differences between human GLS and GLS2 involve organelle targeting and protein-protein interactions. This fact explains their different function and regulation [11]. The abundance of each isoform may change depending upon the tissue type, environment, or the developmental or metabolic state of the cells [12].

The human *GLS* gene has 82 kb and 19 exons, and is located in chromosome 2 [14]. Two transcripts arise by alternative splicing of this gene: KGA, firstly found in kidney, composed by 18 exons, and GAC, which uses only the first 15 exons [12]. The mammalian transcripts KGA and GAC are mitochondrial [11]. In their inactive states, KGA and GAC are dimeric species [14]. *In vitro*, KGA or GAC can be activated utilizing inorganic phosphate, which stimulates the formation of an active tetramer [2]. KGA is strongly inhibited by the end-

product Glu. GAC is mainly expressed in some types of tumour with preference to KGA [14]. Additionally, the ratio GAC/KGA augmented in lung tumours compared with healthy lung tissue [12]. GAC upregulation through miRNAs appears to be a mechanism by which cancer cells acquire specific benefices for using alternative sources of carbon favouring their adaptation to changing metabolic environments [14]. The Rho GTPase mediated activation of GAC is dependent on nuclear factor-kappa B (NF-κB), which disrupted mitochondrial GA activity inducing posttranslational modifications [15].

The human *GLS2* gene has 18 kb and 18 exons, is located in chromosome 12, and is a target of p53 [11]. Two transcripts have been characterized from the mammalian *Gls2* gene: the long transcript called GAB, and the short transcript LGA that lacks exon 1 and was discovered in rat liver [16]. GLS2 is activated by low phosphate levels and marginally inhibited by Glu [12]. Although GAs are traditionally mitochondrial enzymes, GLS2 have been localized in nuclei [14], suggesting that GLS2 participate directly or as a co-regulator in the modulation of gene expression [11]. Therefore, GLS2 isoforms might have essential impact upon the fine regulation of Gln metabolism [14]. GLS2 have been found in human cancers: colorectal, hepatoma cells, lymphocytic cells, myeloid cells, and breast cancer cells [12]. Interestingly, GLS2 was higher expressed in less malignant cancers [11]. Conversely, GLS2 expression was scarce in hepatocellular carcinomas and glioblastomas, the most aggressive brain tumours, showing high levels of GLS [14]. When GLS2 is increased major amounts of Gln, OXPHOS activity and glutathione (GSH) levels were found [12]. Moreover, those cells were more efficiently protected against reactive oxygen species (ROS) and their deleterious effect on DNA, lipids and proteins [11].

3. Glutamine metabolic reprogramming in cancer

Reprogramming of Gln metabolism is a hallmark of cancer [10]. Rewiring Gln metabolic routes affords an important advantage during initiation and progression of tumours [11]. This process is mediated through the transcriptional regulation of multiple key metabolic enzymes and signalling pathways linked to GA, including IDH1/2, glutamate dehydrogenase (GDH), malic enzyme (ME1/2), pyruvate carboxylase (PC), protein kinase B (PKB), phosphatidylinositide 3-kinase (PI3K), signal transducer and activator of transcription 1 (STAT1), and extracellular signal-regulated kinases (ERKs) [17]. To date, intense focus is placed on inhibiting metabolic enzymes as alternative or parallel targets for probable cancer treatments [4]. This strategy might help accost the heterogeneity among cancer cells and their different regulation by diverse GA-related enzymes or protein complexes, i.e.: IDH1 [7,18], Rho GTPases [15], KEAP1 [19], MYC [6,16,20], KRAS [21,22], mTORC1 [23], HIF-1 [9,24], NOTCH1 [25], and pyruvate kinase M2 isoform (PKM2) [4].

Since GA catabolizes Gln generating ATP and GSH synthesis (Fig.1), GA reduction influences proliferation and cell death conceivably through ATP decline and ROS production [11]. These findings support that Gln catabolism through GA is critical for cell proliferation, and provides protection against oxidative stress [12]. ROS are indispensable in cell transformation and tumour maintenance but they must be finely regulated. Gln metabolic reprogramming are essential in cancer redox control, although mechanisms are not still fully understood. ROS biology can affect gene transcription (hydroxymethylation is associated to transcriptomic changes), targeting cell growth and apoptosis signalling pathways. Additionally, miR-23a expression, which blocks GLS translation, sensitizes leukaemia cells to ROS, providing new clues into new undiscovered drug targets against cancer [11]. Accordingly, GA overexpression seems to be a hallmark exhibited by many tumours [12].

Figure 1. Glutamine (Gln) is a key piece in metabolic reprogramming of cancer. Gln is not only a biosynthetic precursor of non-essential amino acids, nucleotides and lipids but also a fuel to produce ATP and a signalling molecule. Gln is a fundamental brick in metabolism, TCA cycle and the substrate for glutaminase isoenzymes (GLS and GLS2). A plethora of main metabolic enzymes (IDH1), oncogenes (c-Myc, KRAS), tumour suppressor factors (p53), transcription factors (HIF-1), signalling pathways (mTORC-1, Rho-GTPases), processes (OXPHOS, glycolysis, apoptosis), essential antioxidants (GSH) and bioenergetic sources (ATP) are tightly related with this multi-faceted amino acid. ATP: adenosine triphosphate; GSH: glutathione; HIF-1: hypoxia-inducible factor-1; IDH1: isocitrate dehydrogenase 1; mTORC-1: mammalian target of rapamycin complex 1; OXPHOS: oxidative phosphorylation; Rho-GTPase: Ras homologous protein family guanosine triphosphate dependent.

Very recent findings state that the expression of *Gls* is upregulated and correlates with pathological factors in leukaemia, glioma, neuroblastoma, melanoma, as well as in liver, colon, pancreas, ovarian, kidney, bladder, lung, and breast cancers [4,6,9,17,24,25]. Studies on experimental and human tumours pointing at both glutamine synthetase (GS) and GA disclosed a common pattern: a knockdown of GS expression along with an overexpression of GA. Differential expression of GA isoenzymes in cancer has been investigated to settle its role in the tumour transformation and the possibility of its clinical use as an outstanding factor [11]. Co-expression of GA transcripts was found in leukaemia cells and in several breast cancer cell lines [26]. However, lymphocytes from the medullar blood of a patient suffering aplasia did not express GLS transcripts and showed a 15-fold increase of GLS2 transcripts. Taken together, the data suggest that GLS up-regulation links with augmented ratios of tumourigenesis, while predominance of the GLS2 isoforms seems to be related with resting, quiescent or differentiated cell states [14]. Differently, it has been reported that oncogenes influence Gln metabolism and that tumour genetics can determine cellular dependence on Gln for survival. Oncogene c-Myc indirectly regulates the protein expression of the *GLS* gene through effects on miR23a/b and, thus, derepressed *GLS* translation, supplying Gln oxidation in the mitochondria [20]. Conversely, it has been shown that *Gls2* expression can be regulated by the p53 family member p73 in cancer cells, driving GSH synthesis, and having a key regulatory function on the homeostatic control of oxidative equilibrium during cancer transformation [11]. These observations stimulate renewed efforts to understand metabolic reprogramming in tumours and to develop anticancer strategies targeting GA isoenzymes.

4. Glutaminase as an oncogenic or a tumour suppressor factor

Glutaminase is associated with both oncogenes and tumour suppressor factors (Fig. 2). It has been found an increased Gln catabolism in Myc-induced liver tumours allied with

suppression of GS and switch from *Gls2* to *Gls* expression [6]. In contrast to liver tumours, Myc-induced lung tumours display increased expression of both *GS* and *Gls*, and accumulate Gln. These results suggest that the metabolic profiles of tumours depend on both the genotype and tissue of origin and have implications regarding the designing of medication targeting tumour metabolism [6]. Pertinent to this goal, it has been found that inhibition by antisense technology of GLS, enables reversion of the transformed phenotype of Ehrlich ascites tumour cells (EATC) *in vitro* [12] and makes tumour cells incapable to grow *in vivo* due to their inefficacy to avoid the host immune response [11]. Of note, it has been demonstrated a similar tumour regression by overexpressing the Gls2-encoded GAB isoenzyme in human glioblastoma [14]. Stably transfected T98G cells with the full GAB cDNA coding sequence were evaluated for the effects of transfection on proliferation, migration and survival [11]. The transfected cells (T98-GAB cells) showed almost 50% reduction in these main physiological parameters [12]. Microarray analysis revealed a significantly changed expression of 85 genes in T98-GAB. Microarray data were confirmed by real-time quantitative polymerase chain reaction (RT-qPCR) analysis for several genes coding proteins related to cell growth, cellular proliferation, cell migration, or oncogenesis: FNDC3B was upregulated, and S100A16, CAPN2, TIMP4, and MGMT were downregulated [11]. Furthermore, in view of the potential function of GLS2 in the adjustment of gene transcription, it has been hypothesized that its shortage has implications for the physiology of glia-derived tumours, triggering them toward more aggressive conditions [14].

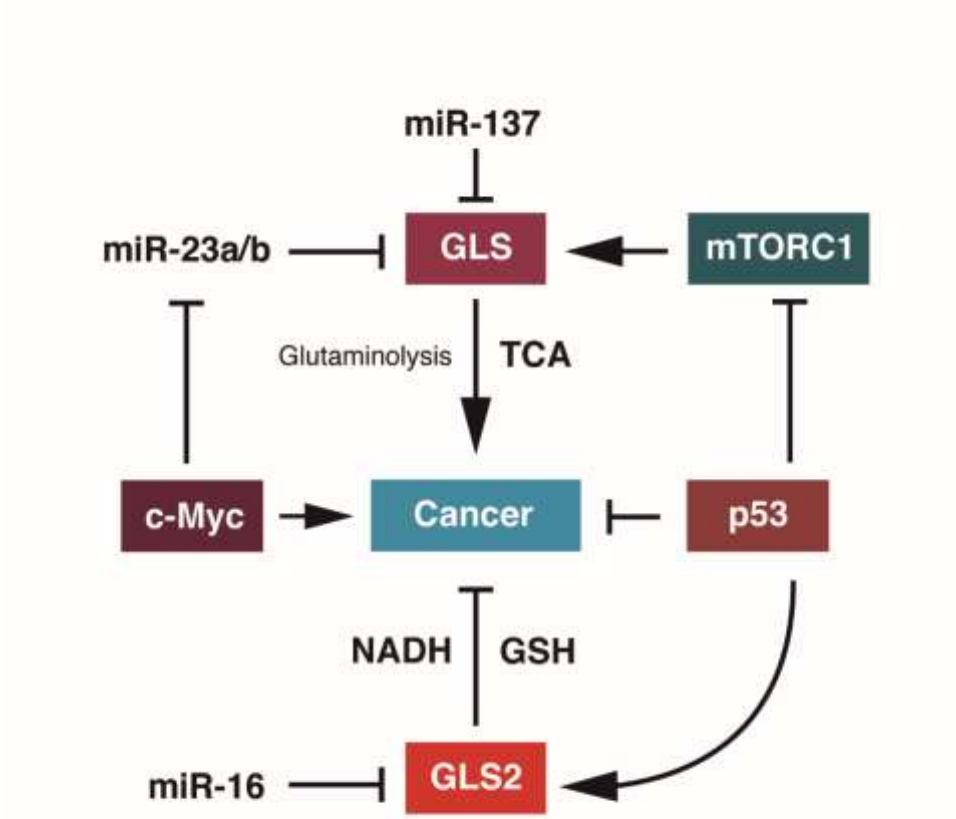

Figure 2. Glutaminase family isoenzymes GLS and GLS2 can display opposites roles in cancer. GLS isoforms are overexpressed in many types of cancers, whereas prevalence of the GLS2 isoforms seems to be related with more differentiated cell states. In fact, GLS is positively activated by mTORC-1 facilitating both the synthesis of macromolecules and the supply of energy through glutaminolysis and TCA cycle. On the other hand, GLS2 is a mediator of p53's role in energy metabolism and antioxidant defence through GSH and NADH levels. Hence, while oncogene c-Myc regulates GLS through miR23a and miR23b, GLS2 is regulated by several p53-family tumour suppressor factors. In addition, GLS and GLS2 are downregulated by miR-137 and miR-16, respectively. GSH: glutathione; miR: microRNA; mTORC-1: mammalian target of rapamycin complex 1; NADH: reduced nicotinamide adenine dinucleotide; TCA: tricarboxylic acid.

Experiments using SFxL and LN229 human glioma cell lines with silenced GLS-encoded KGA isoform shows how tumour cells activate PC circuit to permit cells to utilize Glc-derived pyruvate rather than glutamine for anaplerosis [3]. Tumour cells having silenced GLS

expression showed lower survival ratios and a reduced GSH-dependent antioxidant capacity. Similar observations were made in T98-GAB cells [27]. Hence, control of GA isoenzyme expression may demonstrate to be a vital tool to modify both metabolic and oxidative stress in cancer therapy [11]. Accordingly, *in vivo* experiments showed human glioblastomas displaying unexpected metabolic complexity, oxidizing Glc via pyruvate dehydrogenase and the TCA cycle [28]. It has been shown that cancer cells may stop proliferation either by silencing *GLS* or by up-regulating *GLS2* genes, in agreement with their apparently contrary roles in cancer [12]. Thus, blocking *GLS* or overexpressing *GLS2* induced less c-Myc and Bcl-2 expression, as well as major proapoptotic Bid expression [27]. In conclusion, the control of GA isoenzymes expression may become a therapeutic strategy for gliomas and other cancers [11]. Interestingly, ROS generation by treatment with arsenic trioxide synergizes with either GLS knocking-down or GLS2 overexpression to cut down aggressive features of tumour cells [27]. Nevertheless, this behaviour is not universal and there are some types of cancers where *GLS2* is upregulated (cervical, colon and some lung cancers) [11,29]. Besides, this overexpression was related with therapeutic resistance [24]. On the other hand, *GLS2* can be transcriptionally regulated by several tumour suppressor and stress-related proteins, including p53, p63, and p73 [11], which agree with our hypothesis linking *GLS2* overexpression and cancer recession in some tumours [12]. However, GLS2 has been also described with proliferative properties in other cancers, pointing at its context-dependent role in malignancies [29]. The heterogeneity of the expression of key metabolic genes –like *Gls* and *Gls2*– suggests that different tumours might have differential requirements of glutaminolysis, reflecting the tumour specific relationship between oxidative stress, Gln metabolism and ROS-dependent apoptosis [10]. It has been found that inhibition of GLS selectively diminished cell proliferation and survival of osteogenic sarcoma MYC-expressing cells. This inhibition was enhanced by using the mitochondrial-targeted antioxidant Mito-Vitamin E. These results highlight that GLS and mitochondrial ROS might be attractive targets for cancer therapy in MYC-driven tumours [17].

Thus, synergistic metabolic treatments are vital strategies against many types of cancer [24]. In an illuminating study of mice harbouring T-cell acute lymphoblastic leukaemia, inhibition of GLS and reduction of autophagy markedly and synergistically enhance the antileukaemic effects of anti-NOTCH1 therapy [25].

Glutamine metabolic reprogramming evokes multiple compensatory metabolic networks [5]. In pancreatic cancers, these countervailing pathways include autophagy, micropinocytosis and uptake of free amino acids released by stromal cell. Targeting these compensatory circuits is having a rising value in modern treatments against cancer [30]. Accordingly, gemcitabine-resistant pancreatic ductal adenocarcinoma (PDA) cells increase Gln import becoming more Gln-dependent to growth. Hence, Gln can be used by CTP synthetase to proliferate [24]. However, Gln analogous acivicin diminish CTP availability and Glc import, showing that cancer metabolic reprogramming connects fuelling by Glc and Gln [31]. On the other hand, several lines of evidence implicate GLS2 isoforms in the regulation of transcription: GLS2 was found in the neuronal and astrocytic nuclei [32]. Furthermore, overexpression of *Gls2* in tumour cells leads a revolution in their transcriptional programme [11]. The interaction of *Gls2* with PDZ domain-containing proteins might explain its nuclear translocation [14]. The presence of GLS2 isoforms in certain cell nuclei has not been explained, although transcriptional regulation has been proposed as a revolutionary road toward a new nuclear function [33].

5. Targeting glutaminase isoenzymes as a valuable tool against cancer

Among metabolic targets that could be utilized for cancer therapy GA isoenzymes are increasing value because their association to the metabolic reprogramming of cancer [11]. In fact, it has been suggested that glutaminase inhibition might be a more effective epigenetic therapy than the use of other drugs, which have larger consequences on cells and organisms

[9]. For many years, the predominant drug used to target GLS directly was 6-diazo-5-oxy-L-norleucine (DON). DON acts as an irreversible glutamine competitive inhibitor. Although effective against glutaminase, DON is not selective and has several verified targets and many undesirable effects [34]. More recently, several new small molecules have been discovered that inhibit GLS and GLS2 isoenzymes (Fig. 3). One of these molecules is dibenzophenanthridine-968, formal name 5-[3-bromo-4-(dimethylamino)phenyl]-2,3,5,6-tetrahydro-2,2-dimethyl-benzo[a]phenanthridin-4(1H)-one, which was discovered by Cerione´s laboratory and was shown to be an allosteric regulator of both GLS and GLS2 isoforms [34,35]. The inhibitory potential of dibenzophenanthridine-968 has been described in a number of cancer cell lines in culture, as well as in a mouse xenograft model [36]. The second important glutaminase inhibitor to be described during the past few years is bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl (BPTES) [13], a symmetrical molecule specific for GLS over the GLS2 isoforms [34]. The BPTES molecule inhibitor selectively inhibit GLS, prolonging survival of a genetically engineered mouse model of liver cancer [37]. The related 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, better known as CB-839 is a more potent inhibitor of GLS than BPTES and it is currently being administered to humans in phase 1 clinical trials for solid tumour, lymphoid, and myeloid cancers [34]. The newest molecule 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 (UPGL00004) which inhibits the growth of triple-negative breast cancer cells, and like CB-839, more potently blocks the enzymatic activity of GLS (compared with BPTES), is an outstanding promising therapeutic compound to be used against many types of cancer [38].

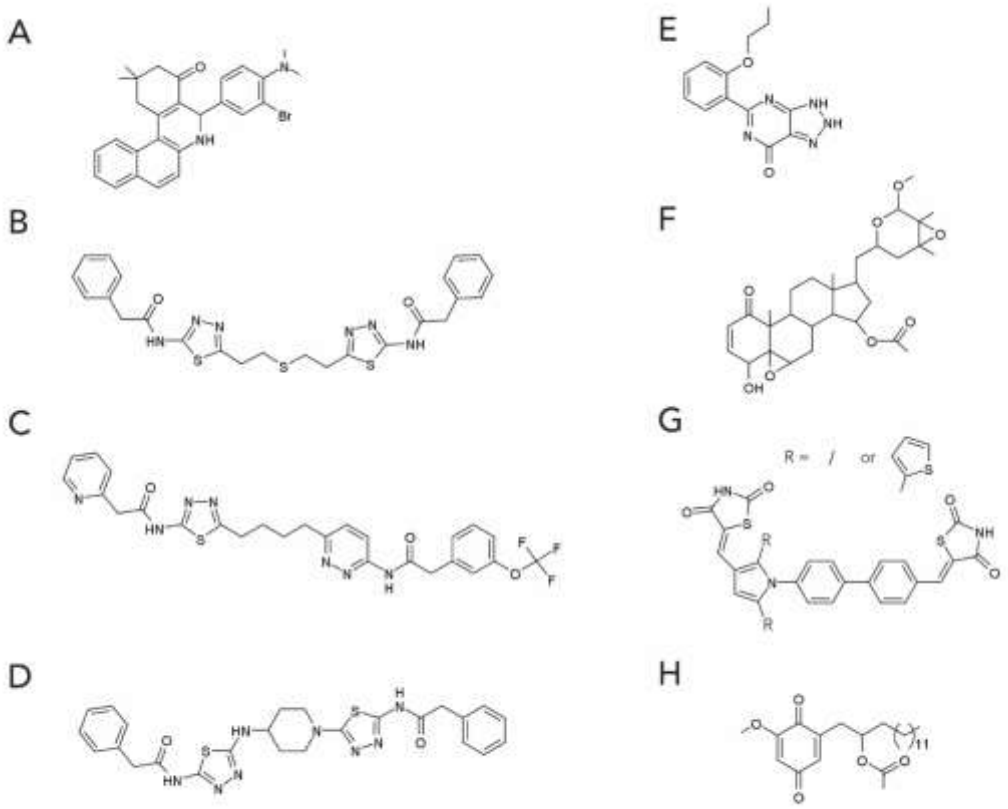

Figure 3. Specific, non-glutamine mimetics, GLS and GLS2 inhibitors. A: Dibenzophenanthridine-968; B: BPTES, C: CB-839; D: UPGL00004; E: zaprinast; F: physapubescin K; G: thiazolidine-2,4-dione compounds, compound#5 (R= methyl), or compound #6 (R= 2-methylthiophene); H: AV-1, also known as ardisianone. Compounds A-F target GLS over GLS2 with a selectivity index > 10^3. G compounds target both GLS and GLS2 with a low selectivity index. H targets GLS2 more selectively.

A novel class of thiazolidine-2,4-dione compounds capable of selectively inhibiting GLS and/or GLS2 activity, *in vitro* and *in vivo*, have been discovered and developed: (*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 (compound#5, more selective for GLS isoforms), and (*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 (compound#6 having similar selectivity index for GLS and GLS2, but more sensitivity). Both molecules have demonstrated to be effective in reducing tumour size *in vitro* and *in vivo* (using a human pancreatic xenograft tumour model) [39]. Moreover, the hydrophobic nature and low solubility of these drugs presents important disadvantages to the physiological distribution of the inhibitor compounds [11]. Neither dibenzophenanthridine-968 nor BPTES, CB-839, UPGL00004 or thiazolidine-2,4-dione compounds are glutamine mimetics, suggesting that it may be possible to inhibit GLS specifically without disrupting other aspects of glutamine metabolism [40]. Given the high degree of toxicity observed with glutamine mimetics, the discovery of allosteric inhibitors of glutaminase is a huge progress in the developing of antitumour molecules targeting glutamine metabolism [40].

Of interest, other new GLS-specific inhibitor, 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 (physapubescin K), exhibited potent proliferation inhibitory effects on several human cancer cell lines, such as SW1990 and HCC827-ER [41]. On the other hand, 5-(2-propoxyphenyl)-1H-[1,2,3]triazolo[4,5-d]pyrimidin-7(4H)-one (zaprinast,) a phosphodiesterase 5 inhibitor, reduced growth and sensitized glutamine-addicted pancreatic cancer cells to oxidative damage by inhibiting GLS [42]. One essential point of glutaminase inhibition is that the small molecules dibenzophenanthridine-968, BPTES, CB-839, UPGL00004, zaprinast, and physapubescin K have been found to be relatively nontoxic. In the case of dibenzophenanthridine-968, for example, a drug dose that significantly inhibits the growth of tumour cells has been shown to have small effect upon non-transformed cells [15]. Nevertheless, while BPTES is reasonably potent *in vitro* (IC_{50} ~60 nM), higher concentrations are needed to produce similar effect *in vivo* (IC_{50} ~20 μM) [17].

The pursuit for specific molecules targeting GLS2 interferes with the fact that relevant structural information on GLS2 isoforms from X-ray diffraction data and/or NMR studies has not yet been published [14]. Consequently, few groups have focused their research toward the targeting of GLS2 isoforms. Several alkyl benzoquinones isolated from *Ardisia virens* or *Ardisia kusukuensis* had submicromolar activity against GLS2 (tenfold higher affinity for GLS2 than for GLS isoforms). Thus, 1-(5-methoxy-3,6-dioxocyclohexa-1,4-dien-1-yl)pentadecan-2-yl acetate (compound AV-1), also known as ardisianone, induces lead mitochondrial collapse, apoptosis and downregulation of survivin, a relevant prosurvival protein in numerous tumour types [34]. Further work is needed to investigate the metabolic consequences of GLS2 inhibition/overexpression. An understanding of genetic and epigenetic circuits dealing with this isoenzyme will be useful in developing combination therapies to augment the effects of glutamine reprogramming, providing new class of anticancer medication [9].

6. Conclusions

Glutamine is required for the synthesis of nucleotides, hexosamines, lipids, citrate, non-essential amino acids, and GSH, the most important intracellular antioxidant molecule generated by cells to fight against oxidative stress [10]. Therefore, inhibition of glutamine pathway decreases pyrimidine synthesis (indispensable for DNA formation), and an extensive failure to combat oxidative stress [11]. The key role of glutaminolysis in cancer development was known from long time ago; however, in last ten years a strong revival has arisen in scientific community and a clear relationship between GA isoform expression and oncogenes and tumour suppressor genes has been demonstrated [12]. Hence, GA-associated isoenzymes and their interacting partners and signalling pathways are now potential new targets for cancer therapy [30]. Multiple targeting has reached better results in both inhibiting cancer growth and proliferation, and effectively sensitizing tumour cells to other drugs [24,25]. GA

isoenzymes are druggable and the recent years have seen an intensive interest from research groups and the pharmaceutical industry in designing and using new molecules to target GLS and GLS2 isoenzymes [38,40,43]. Nevertheless, only a few inhibitors have been described, and most of them lack solubility and/or specificity to be adequate for clinical trials [34,38]. Therefore, it is urgently needed to find out novel strategies to target GA isoenzymes. Besides, technology developments in epigenomics, metabolomics and proteomics instrumentation are fundamental tools for the discovery of novel cancer biomarker associated to the metabolic reprogramming of Gln [8].

Gln metabolism is now a very hot topic in tumour biology and strategies leading to block the higher Gln metabolism in cancer are crucial issues to tackle cancer development [7]. Signalling processes to be targeted also include post-translational modification of GLS [44], as phosphorylation, which is responsible for the major glutaminase activity in lung tumour tissues and cancer cells. In fact, phosphorylation levels are inversely correlated to the survival of non-small cell lung cancer patients [45]. Thus, these findings raise exciting expectations regarding the use of glutaminase inhibitors as a powerful anticancer therapeutic strategy. Like c-Myc and p53 [46], *Gls* is an oncogenic factor and *Gls2* may work as a tumour suppressor gene and/or as a scavenger metabolic-stress gene. Dual targeting of both isoenzymes could be a pivotal therapeutic implement in the near future in some types of cancer and/or for some patients in personalized oncology.

7. Future directions

Employment of omics technologies are a very active area which is contributing very much to progress in cancer research. One of the challenges in cancer metabolomics is to analyse the impact of tumours themselves on metabolic flux *in vivo*; that is, to utilize efficient and sensitive methods to evaluate the transfer of carbon and nitrogen along metabolic

pathways in live patients [3]. Combined metabolomics-metabolic flux studies have immense value because they result in a quantitative and comprehensive readout of the variation in metabolism, to gain a deeper understanding of metabolic individuality, to be used in personalized oncology [8]. The search for new and suitable targets is complex by the high plasticity of the metabolic network that can induce compensatory biosynthetic pathways to produce the limiting metabolites, as well as the exchange of metabolites between cancer cells and the surrounding tissues [10].

On the other hand, therapy resistance is a significant problem in cancer, especially in heterogeneous cancers, that are composed of a large percentage of highly proliferative cancer cells and a very small percentage of dormant or sleeping cells that can fuel cancer [30]. Additionally, malignant cells metabolize Glc, Gln, and other substrates with much more avidity than their non-tumour equivalents. However, the metabolic phenotype of cells within tumours is heterogeneous, although distinct from that of their normal counterparts [47]. A fundamental emerging question is whether metabolic vulnerabilities also display such unexpected regional heterogeneity within individual tumours. This would have higher consequences for the prospects of metabolic therapy in cancer [8]. Synergic and personalized treatments are a clear opportunity to selective killing of cancer cells [21], i.e.: combining GLS inhibition (by using CB-839) plus redox-controlling drugs has been used to evoke apoptosis at non-toxic dosage of these drugs in patients suffering from PDA [48]. Another combined treatment of GLS inhibitors (thiazolidine-2,4-dione compounds and BPTES) with doxorubicin (which interferes with the function of DNA) resulted in a synergistic effect for removing breast and pancreatic carcinoma cell proliferation [39]. Hence, synergic treatments should include drug cocktails [22,39], and/or targeting key players like (i) apoptosis [25], (ii) glutamine [1] and glutamate [49] transport, (iii) microRNAs [12], (iv) GDH [50], (v) GS [11], (vi) GLS [24], and (vii) GLS2 [14]. For example, a benzo-selenazol dimer functions as a specific glutaminolysis inhibitor that efficiently disrupted the mitochondrial membrane potential leading to apoptosis. Importantly,

this novel dual GLS/GDH inhibitor did not show growth inhibition of the non-tumour cells, and demonstrated to be an excellent candidate antitumour drug without side effects [50].

Although multiple metabolic inhibition is a promising strategy, identifying whether synergies exist in personalized patients will be necessary to guarantee a clinical use. Importantly, epigenomics, proteomics and metabolomics study after GLS specific inhibition can foretell responsiveness to combinatorial treatment [30]. In conclusion, because cancers have remarkable metabolic plasticity a precise *in vitro* and *in vivo* analyses can help understand the metabolic adjustments to metabolic disturbances. Indeed, recent attempts yielded several successful treatments targeting Gln metabolism [30,48]. Understanding the molecular basis of metabolic reprogramming, and the analytical systems to be used for its characterization (including tracing of labelled metabolites and metabolite imaging) will improve integration of metabolic data with genomics, transcriptomics, and proteomics, through systems biology, to accelerate therapeutic research in cancer [51].

Acknowledgements

J.M.M., J.A.C-S., and J.M. are supported by SAF-2015-6401-R from Ministerio de Economía y Competitividad. Thanks are due to Ralph J DeBerardinis for helpful suggestions.

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