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Dysregulation of glutaminase and glutamine synthetase in cancer

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ABSTRACT

Besides fast glucose catabolism, many types of cancers are characterized by elevated glutamine consumption. Medical oncology pursuits to block specific pathways, mainly glycolysis and glutaminolysis, in tumor cells to arrest cancer development. This strategy frequently induces adaptive metabolic resistance that must be countered. Combination therapy is an anticancer synergistic tool to overcome both cancer growth and resistance mechanisms. Dysregulation of glutaminase and glutamine synthetase are key events that allow anabolic adaptation of tumors. Several specific drugs that inhibit metabolic enzymes dealing with glutamine metabolism have been able to eliminate some neoplasms. Targeting the tumor microenvironment can be also another essential factor to be taken into account when single or combined cancer metabolic therapy fails.

1. Introduction

Glutamine (Gln) is a key molecule in cancer beyond its role as a critical amino acid for biosynthetic purposes [1]. Gln has essential functions to provide amino acids, lipids, nucleotides, hexosamines, and polyamines, but also to render metabolic energy (ATP), to be used as a pleiotropic cell signaling molecule, or to highlight several cancer tracking pathways [2] (Fig. 1). Besides, Gln is indispensable to generate glutathione (GSH), the most important intracellular antioxidant molecule [3]. Cancer cells frequently increase oxidative damage in response to changes of the metabolic circuits [2]. Oxidative damage and induction of the apoptotic pathway in cancer cells are activated by reactive oxygen species (ROS), that trigger changes in mitochondrial membrane function, and act as mediators between mitochondria and apoptosis [1]. In fact, the inner mitochondrial membrane potential is severely altered by both Gln and GSH levels [4]. While glutamate (Glu) provides precursors for GSH production (Fig. 1), which contribute to modulate the adequate oxidative status of cells [5], partial oxidation of Gln through glutaminolysis evades oxidative phosphorylation (OXPHOS) and attenuates excess of ROS that otherwise are toxic to cells [6]. Simultaneously, Gln can be essential for the protection of metabolic enzyme α-ketoglutarate dehydrogenase (KGDH) from inactivation by ROS [1].

Mitochondria is the physical network where Gln carries out most of its substantive and regulatory capacities (Fig. 1). Gln/Glu ratios are controlled by glutaminase (GA) and glutamine synthetase (GS), also called γ-glutamyl:ammonia ligase (GLUL) [7]. Gln can fuel tricarboxylic acid (TCA) cycle through its conversion to Glu by GA, and subsequently to α-ketoglutarate (AKG) by aspartate transaminase (AST) and alanine transaminase (ALT), as well as by glutamate dehydrogenase (GLUD) [2,7]. AKG enters the TCA cycle, generating reduced nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide ($FADH_2$), which are used to produce an important source of ATP via electron transport and OXPHOS [8]. OXPHOS refers to the formation of ATP from ADP and phosphate by complex V (ATP synthase) using the proton gradient generated across the inner mitochondrial membrane [9]. Thus, mitochondrial OXPHOS is a key pathway for ATP generation in many cancer sets [10].

Gln has a key role as precursor of other anabolic processes such as pentose phosphate pathway (PPP), generating reduced nicotinamide adenine dinucleotide phosphate (NADPH) [9]. Besides, Gln is involved in facilitating cell building biomolecules (Fig. 1). In addition, several cancer types, like pancreatic cancer and non-small-cell lung cancer (NSCLC), can utilize proteins from outside or inside the cell (i.e.: autophagy) to generate Gln and other amino acids to feed the TCA cycle for cell progression [11]. Usually, cancer cells appear to enhance both glycolysis and OXPHOS simultaneously, relative to surrounding normal cells [3]. Besides, TCA cycle can also be fueled by pyruvate carboxylation, which generates oxaloacetate (OAA) from glucose (Glc) [11]. The discovery of mutations in genes that encode succinate dehydrogenase (SDH), fumarate hydratase (FH) or isocitrate dehydrogenase (IDH1 and IDH2) in some types of cancer points out the key role of these genes as tumor suppressors (SDH, FH) or oncogenic factors

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Abbreviations

AKG	α-ketoglutarate
ALDH1L2	aldehyde dehydrogenase 1 family member L2
ALT	alanine transaminase
AMPK	adenosine monophosphate-activated protein kinase
AST	aspartate transaminase
ASCT2	alanine/serine/cysteine transporter 2
BSO	L-buthionine-(S,R)-sulfoximine
CDK	cyclin-dependent kinase
CQ	chloroquine
CRC	colorectal cancer
CTH	cystathionine gamma-lyase
DBZ	dibenzazepine
DHA	dihydroartemisinin
DON	6-diazo-5-oxo-L-norleucine
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinase
ESCC	esophageal squamous cell carcinoma
5-FU	5-fluorouracil
FH	fumarate hydratase
GA	glutaminase
GAB	long glutaminase 2 isoform
GAC	short glutaminase isoform
GBM	glioblastoma
GCLC	γ-glutamate-cysteine ligase catalytic subunit
GCLM	γ-glutamate-cysteine ligase regulatory subunit
GCS	γ-glutamylcysteine synthetase
GLUD	glutamate dehydrogenase
Glc	glucose
Gln	glutamine
GLS	glutaminase isoenzyme
GLS2	glutaminase 2 isoenzyme
Glu	glutamate
GLUL	γ-glutamyl:ammonia ligase
GOT	glutamate-oxaloacetate transaminase
GS	glutamine synthetase
GSH	glutathione
GSI	γ-secretase inhibitor
GST	glutathione S-transferase
G-TPP	gamitrinib triphenylphosphonium
HCC	hepatocellular carcinoma
2HG	2-hydroxyglutarate
HIF-1α	hypoxia-inducible factor-1α
IDH1/2	isocitrate dehydrogenase isoenzymes
KGA	long glutaminase isoform
KGDH	α-ketoglutarate dehydrogenase
KRAS	V-Ki-ras2 Kirsten rat sarcoma
LAT2	L-type amino acid transporter 2
LGA	short glutaminase 2 isoform
MAPK	mitogen-activated protein kinase
ME1/2	malic isoenzymes
mTORC1	mammalian target of rapamycin complex 1
MTX	methotrexate
MSO	L-methionine sulfoximine
NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NSCLC	non-small-cell lung cancer
OAA	oxaloacetate
OXPHOS	oxidative phosphorylation
PC	pyruvate carboxylase
PDA	pancreatic ductal adenocarcinoma
PET	positron emission tomography
PI3K	phosphatidylinositide 3-kinase
PKM2	pyruvate kinase M2 isoform
PPP	pentose phosphate pathway
PTEN	phosphatase and tensin homolog
ROS	reactive oxygen species
SLC	solute carrier
SCC	squamous cell carcinoma
SDH	succinate dehydrogenase
SNAT2	sodium-coupled neutral amino acid transporter 2
T-ALL	T-cell acute lymphoblastic leukemia
TCA	tricarboxylic acid
TAMs	tumor-associated macrophages
TNBC	triple negative breast cancer
TXNIP	thioredoxin-interacting protein
YAP	Yes-associated protein

(IDH1/2) [12]. Of note, such mutations in IDH1/2 produce the oncometabolite 2-hydroxyglutarate (2HG), which impairs histone demethylation and blocks the cell differentiation [13]. However, GAs y GS, which are not mutant oncoproteins, are the main enzymes that regulate glutaminolysis and mitochondrial function in cancer [1,7,14]. In this mini-review, we will discuss both the dysregulation of GAs and GS in cancer, as well as the targeting of these metabolic enzymes as a valuable tool to fight against cancer.

2. Glutaminases and cancer

2.1. Glutamine metabolism

Increased metabolism of Gln is a hallmark of cancer [15]. Fifty years ago, the Gln analog 6-diazo-5-oxo-L-norleucine (DON) was tested as an antineoplastic agent [16]. Unfortunately, promising results became useless because of its high toxicity. However, later experiments proved the effects of DON (and another Gln analog, acivicin, Fig. 2) as synergistic molecules inhibiting GA and cancer growth [17]. Nowadays, many and less toxic agents have been described as valuable drugs to inhibit GA [18]. Cancer metabolism might be described as a large city underground map, and when a pathway is blocked another can be activated to reach the energetic and biosynthetic requirements of tumor cells. The only hope is to block several related pathways to synergistically arrest cancer development. Among synergistic tools against cancer, GA has become a key target in the metabolic therapy of cancer.

2.2. Glutaminase isoenzymes

GA (EC 3.5.1.2) is the enzyme responsible for catalyzing the conversion of Gln to Glu, and represents the first step in Gln metabolism (glutaminolysis). This process endows cancer cells with high levels of energy to cover their accelerated growth and proliferation, and also with biosynthetic precursors for synthesis of protein and nucleic acids [19]. To date, intense focus is placed on inhibiting GA as an alternative or parallel target for potential cancer treatment regimens, as this strategy might help to tackle the heterogeneity among cancer cells [18]. Human GA proteins are encoded by two paralogous genes named *GLS* and *GLS2*. Two isoforms derived from each GA gene have been so far identified in humans. The transcripts known as KGA and GAC arise by alternative splicing of the *GLS* gene, whereas two GLS2 transcripts were identified from the *GLS2* gene: the canonical long transcript termed GAB isolated from human breast cancer cells, and the short transcript LGA, which was originally identified in rat liver [19].

GLS has been shown to be associated with Gln addiction in tumors and has oncogenic properties whereas GLS2 has been described as a

context-dependent tumor suppressor factor [18]. The heterogeneity of tumor-induced changes in the expression of key metabolic genes—like *GLS* and *GLS2*—suggests that different tumors might have differential requirements of glutaminolysis. These results suggest that the metabolic profiles of tumors are likely to depend on both the genotype and tissue of origin and have implications regarding the design of therapies targeting tumor metabolism [14]. Among chemicals targeting GLS, BPTES (Fig. 2) has been extensively used against several types of tumor cells [2]. Some promising results have been obtained using both chemical (BPTES) and genetic (*Gls* gene silencing) inhibition of GLS in HCC and xenografts [20]. Further, specific GLS inhibition has triggered synergistic effects in the therapy of different cancers, as described below.

2.3. Rewiring glutaminolysis by combination therapy

Metabolic reprogramming of cancer cells rests on overexpression of key metabolic enzymes in order to upregulate determinant pathways such as glycolysis, glutaminolysis and fatty acid synthesis. Combined inhibitory treatment with lodinamine, DON and orlistat targeted hexokinase-2, GLS and fatty acid synthase, respectively. This synergistic therapy exhibits a significant degree of cell viability inhibition in the human colon cancer SW480 cell line, and in mice *in vivo*, showing good tolerance and overcoming the mechanisms of resistance to standard treatment [21].

Noteworthy, the pleiotropic effect of c-Myc in cancer growth and

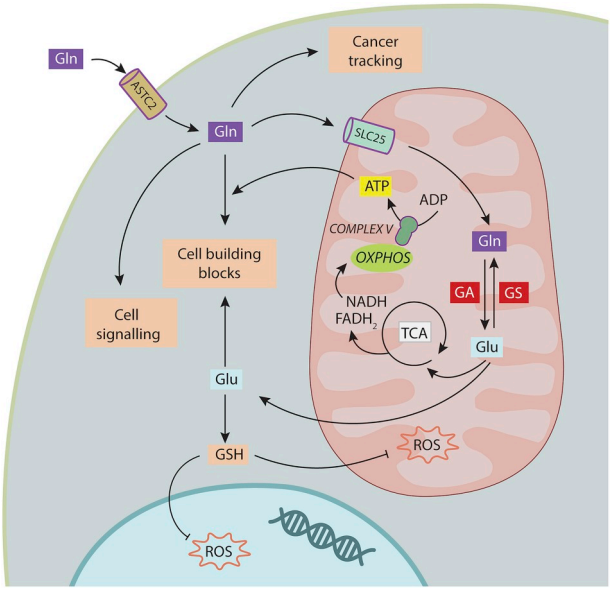

Fig. 1. Glutamine (Gln) as a pleiotropic molecule in cancer. Gln has key roles generating cell building blocks (i.e.: amino acids, proteins, fats, carbohydrates, and nucleotides), energy (ATP), buy also can work as a cell signaling molecule, and like a cancer tracking agent. Gln is equally essential because it is used for the synthesis of GSH, the most important non enzymatic antioxidant in cells, that fights against oxidative damage countering ROS in mitochondria and nucleus. GA (GLS and GLS2) converts Gln into Glu, and GS catalyzes the opposite process. Both metabolic enzymes regulate Gln/Glu ratio. Glu is transformed into AKG, that enters TCA cycle to produce NADH, $FADH_2$ and ATP. The human complex V, or mitochondrial ATP synthase, is the 5th multi subunit of OXPHOS complex used to generate ATP in the inner mitochondrial membrane. AKG, α-ketoglutarate; ASCT2, alanine/serine/cysteine transporter 2, human Gln/neutral amino acid transporter (also called SLC1A5); FADH2, reduced flavin adenine dinucleotide; GA, glutaminase; Gln, glutamine; GLS, glutaminase isoenzyme; GLS2, glutaminase isoenzyme 2; Glu, glutamate; GS, glutamine synthetase; GSH, glutathione; NADH, reduced nicotinamide adenine dinucleotide; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species, SLC25, solute carrier 25, a mitocondrial transporter for Gln; TCA, tricarboxylic acid cycle.

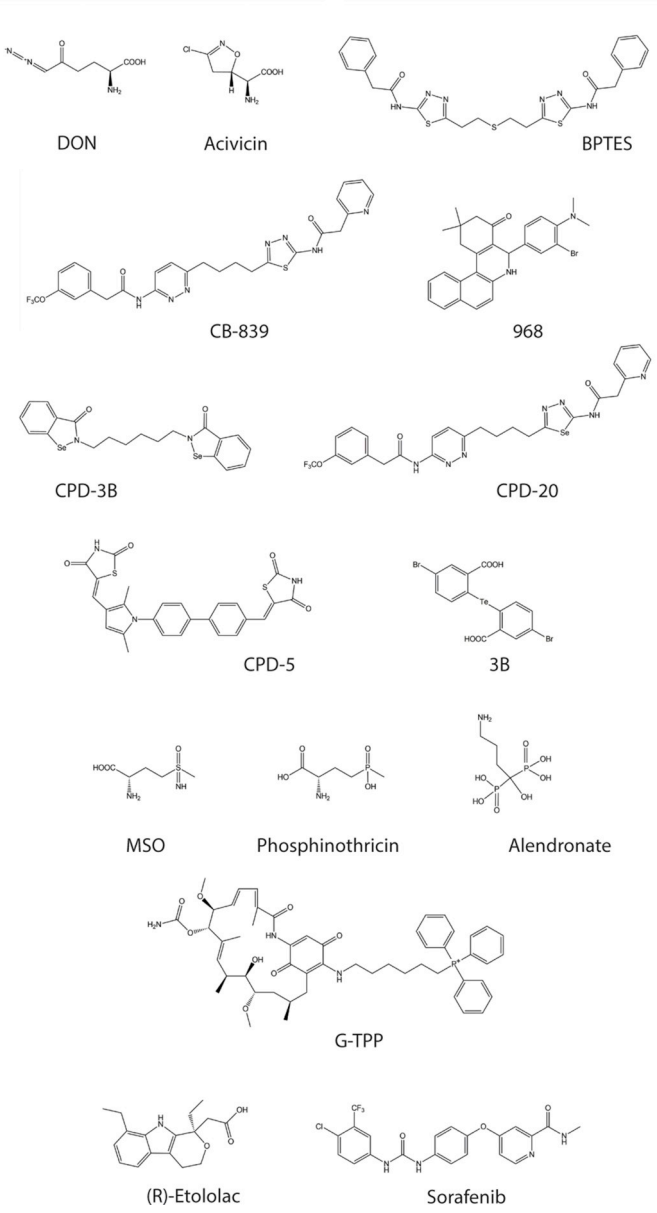

Fig. 2. GLS and GS inhibitors, with anticancer properties, described in literature. DON: (5S)-5-amino-1-diazonio-6-hydroxy-6-oxohex-1-en-2-olate. Acivicin: (2S)-amino[(5S)-3-chloro-4,5-dihydro-1,2-oxazol-5-yl]ethanoic acid. BPTES: bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide. CB-839: 2-(pyridin-2-yl)-N-(5-(4-(6-(2-(3-(trifluoromethoxy)phenyl)acetamido)pyridazin-3-yl)butyl)-1,3,4-thiadiazol-2-yl)acetamide. 968: 5-(3-bromo-4-(dimethylamino)phenyl)-2,2-dimethyl-2,3,5,6-tetrahydrobenzo[a]phenanthridin-4(1 H)-one. CPD-3B: 2,2′(hexyl)bis(benzo[d] [1,2]selenazol 3(2H)-one. CPD-20: bis-2-(5-phenylacetamido-1,2,4-selenadiazol-2-yl)ethyl sulfide. CPD-5: 1-(4-bromophenyl)-2,5-diphenyl-1H-pyrrole-3-carbaldehyde. 3B: 4,4′-dibromo-2,2′-tellurodibenzoic acid. MSO: (2S)-2-amino-4-(S-methylsulfonimidoyl)butanoic acid. Phosphinothricin: 2-amino-4-methylphosphinobutyric acid. Alendronate: (4-amino-1-hydroxybutylidene)bisphosphonic acid. G-TPP: 6-[[(4E,6E,8S,9S,10E,12S,13R,14S,16R)-9-carbamoyloxy-13-hydroxy-8,14-dimethoxy-4,10,12,16-tetramethyl-3,20,22-trioxo-2-azabicyclo[16.3.1]docosa-1(21),4,6,10,18-pentaen-19-yl]amino]hexyl-triphenylphosphanium. (R)-Etololac: 2-[(1R)-1,8-diethyl-4,9-dihydro-3H-pyrano[3,4-b]indol-1-yl]acetic acid. Sorafenib: 4-[4-[[4-chloro-3-(trifluoromethyl)phenyl]carbamoylamino]phenoxy]-N-methylpyridine-2-carboxamide.

Glc uptake is dependent on the increased rate of glutaminolysis. Thus, GLS inhibition by GLS inhibitor CB-839 (Fig. 2) led to a reduction of Glc uptake in PC3 prostate cancer cells. Consequently, synergistic effect of

repression of thioredoxin-interacting protein (TXNIP) to block Glc uptake and the inhibition of GLS through CB-839 can be used as therapeutic tools against prostate cancer [22]. In highly Gln-dependent triple negative breast cancer (TNBC) basal subtype cell lines, treatment with compound CB-839 leads to an activation of the integrated stress response pathway, given that GLS inhibition mimics a nutrient-starvation situation. Furthermore, CB-839 also leads to a decreased mammalian target of rapamycin complex 1 (mTORC1) activity, since it depends on nutrient availability. Combined treatment of CB-839 and mTORC1 inhibitor AZD8055 aims to exploit the connection between Gln metabolism and mTORC1 activity pathways, being especially promising in highly glutaminolysis-dependent tumor cells and resulting in a synergistic inhibition of *in vitro* cell growth [23]. In fact, in lung squamous cell carcinomas (SCCs) a similar pattern has been recently found. The mTORC1 inhibitor MLN128 suppresses glycolysis by targeting mTORC1, but cancer maintains its high rate of anabolic growth by adaptive Gln metabolism through GSK3α/β pathway upregulating c-Myc and c-Jun. GLS inhibition by compound CB-839 allowed to overcome that acquired metabolic resistance to MLN128. Interestingly, this combined and synergistic treatment promises not only to reduce growth of human lung SCC, but also in other tumor types that share a similar metabolic signature as head and neck squamous cell carcinoma and osteosarcoma [24]. Very recently, THZ1, a covalent inhibitor of cyclin-dependent kinase 7 (CDK7), suppressed the proliferation and inhibited the migration of several human NSCLC cell lines, blocking the glycolysis pathway. Noteworthy, combined treatment of THZ1 with compound CB-839 had a great synergistic effect [25].

GLS inhibition using BPTES potentiated anti-cancer effect when combined with DNA damage by 5-fluorouracil (5-FU), that blocked pyrimidine synthesis in NSCLC. Dual BPTES and 5-FU treatment demonstrated a great synergistic effect in cell death induction [26]. These authors propose that the synergistic mechanism of the dual therapy against GLS and thymidylate synthetase (EC 2.1.1.45) is connected with the inactivation of carbamoyl phosphate synthetase II (EC 6.3.5.5). Moreover, BPTES pretreatment has been shown to have similar effects to Gln deprivation in basal-type TNBC cell line HCC1937. Inhibition of Gln metabolism via BPTES pretreatment also synergized with DNA targeting therapy by cisplatin, another DNA-damaging drug. Combined treatment sensitized HCC1937 cells to subtoxic dose of cisplatin and resulted in reduced proliferation and increased apoptosis. In contrast, in a claudin-low TNBC cell line, BT-549, combination of BPTES plus cisplatin showed almost no improvement compared to DNA-targeting therapy alone, which could be attributable to higher GLS expression levels in BT-549 cells, and therefore a higher dose of BPTES would be required. These results suggest that combination of BPTES plus DNA-damaging drugs could be a potential therapeutic strategy in TNBC, but efficacy and drug concentration needed will be dependent on cell intrinsic characteristics [27]. Mutated NOTCH1 is a common alteration in T-cell acute lymphoblastic leukemia (T-ALL), driving cell growth through regulation of multiple pathways, one of which is glutaminolysis. NOTCH1 signaling directs Gln metabolism towards TCA cycle, promoting Gln utilization as a source of carbon in NOTCH1-induced T-ALL. Dibenzazepine (DBZ) is a γ-secretase inhibitor (GSI) which effectively inhibits NOTCH1. However, DBZ treated cells overexpressing GLS increased their Gln utilization, which suggests GLS overexpression could be a potential mechanism of resistance upon NOTCH1 inhibition. Combined treatment of T-ALL cell lines with DBZ and BPTES showed strong synergistic effects impairing cell growth, thus sensitizing cells to NOTCH1 inhibitory treatment, with a dominant role for glutaminolysis over glycolysis. *In vivo* models of T-ALL xenografted mice administered with combined treatment of BPTES plus DBZ showed a deep tumor growth inhibition [28]. On the other hand, the tumor suppressor phosphatase and tensin homolog (PTEN, EC 3.1.3.67) phenotype also appeared as a determinant factor for combined DBZ plus BPTES treatment efficacy, since *Pten*-deleted T-ALL cells showed no response to GSI alone nor combined with GLS inhibition therapy. Hence, both GLS and PTEN phenotype could act as predictive factors of the efficacy of the combined inhibitory treatment in T-ALL [28].

Dihydroartemisinin (DHA), a semisynthetic derivative of artemisinin which has been shown to have potent anticancer activity by augmenting intracellular ROS has been assayed together with the GLS inhibitor compound 968 (Fig. 2) yielding a decreased antioxidant capacity via disrupting redox homeostasis. Compound 968 and DHA cooperatively induced excessive intracellular ROS resulting in severe synergistic apoptosis in hepatocellular carcinoma (HCC) cells, thus increasing antitumor efficacy while sparing normal cells [29]. Metformin, a biguanidine that interferes with Glc metabolism by activating adenosine monophosphate-activated protein kinase (AMPK) pathway, causes an inhibitory effect on mTOR pathway. Ribavirin (1-β-D-ribofuranosyl-1, 2, 4, -triazole-3-carboxamide) is a well-known agent against several RNA and DNA virus. One commonly used drug against colorectal cancer (CRC) is oxaliplatin. Of note, treatments using metformin and ribavirin enhanced the synergistic effect of oxaliplatin when combined with the anti-GLS specific compound 968 for CRC therapy [30]. CB-839 plus phenformin/metformin combined treatment synergistically reduced tumor burden *in vitro* and *in vivo*, also overcoming CDK4/6 inhibitor resistance in esophageal squamous cell carcinoma (ESCC) cells, which overexpress cyclin D1, supporting that cyclin D1-CDK4/6 drives Gln-addiction through a mechanism including Retinoblastoma protein (Rb) and mTORC1 [31]. Additionally, treatment with 968 greatly reduced tumor growth and migration of NSCLC cells, inhibiting epidermal growth factor receptor (EGFR) and phospho-extracellular signal-regulated kinases 1 and 2 (ERK1/2) expression levels. However, treatment with compound 968 also induced autophagy as a mechanism of resistance for NSCLC cells. Although 968 could inhibit cell growth alone, combined treatment with chloroquine (CQ), a classical antimalarial drug which is also an autophagy inhibitor, resulted in synergistic effects. Since CQ inhibited 968-induced autophagy and sensitized cells to 968, a much lower dose of 968 was necessary to significantly reduce GLS expression and activity levels and effectively inhibit cell growth [32]. Allosteric mTORC1 inhibitor rapamycin, and with higher effect the ATP competitive mTORC1 inhibitor PP242, evoked compensatory increase of Gln metabolism. Combination of PP242 and GLS inhibition by 968 administered to glioblastoma (GBM) patient–derived tumor cells, assayed in xenograft models, blocked tumor growth. Of note, there was no significant induction of cell death in brain, liver, and kidney among mice treated with PP242, compound 968 alone, or in combination. These results demonstrated that GLS inhibition can reverse mTORC1-targeted therapy resistance *in vivo* and that it acted synergistically with PP242 by regulating tumor bioenergetics. Importantly, the cytotoxic effect of the drug combination was enhanced in tumor tissue, rather than in normal tissue [33]. These findings may have important implications for combining mTORC1 kinase inhibitors with GLS inhibition for patients with GBM and possibly other mTORC1-activated cancers. Intriguingly, a dual GLS/GLUD inhibitor completely disrupted mitochondrial function, showing potent anticancer activity in several glioma cell lines with a minimum level of toxicity [34].

2.4. State of the art

New potent GLS inhibitors have been characterized very recently (Fig. 2): (i) the hexylselenium compound described above [34] that also inhibited GLUD, named CPD-3B, and significantly slowed the growth of HCT116 (IC_{50} = 1.20 μM) and H22 (IC_{50} = 0.92 μM) cell lines, as well as liver cancer xenograft models [35], (ii) a selenium derivative of CB-839 (called CPD-20) that inhibited the growth of HCT116 (IC_{50} = 0.009 μM) and H22 (IC_{50} = 6.78 μM) cell lines, as well as liver cancer xenograft models [36], (iii) thiazolidin-2-4-dione derivatives, like CPD-5, that inhibited GLS and GLS2 and slowed down the growth of AsPC-1 (IC_{50} = 34.5 μM) and MDA-MB-231 (IC_{50} = 42 μM) cell lines, as well as inhibited tumor growth in a preclinical mouse model [37], (iv) a tellurodibenzoic acid (named 3B) that strongly inhibited HCT116 cells with (IC_{50} = 2.4 μM) [38]. Nevertheless, targeted therapy induced mechanisms of resistance that make necessary the

development of new combined treatments aiming to achieve synergistic effects, reduce dose and beat resistance [18].

2.5. Perspectives

Recent strategies have been proposed to integrate Gln metabolism into the diagnosis, classification, treatment, and monitoring of some types of cancers [39]. In animal models, a subset of many tumors requires Gln metabolism [40]. This finding implies that approaches to image, quantify, or block Gln metabolism in human cancers could be incorporated into the diagnosis and management of the disease [39], including the use of Positron Emission Tomography (PET) technology [41]. In particular, it is essential to use *in vivo* perioperative administration of isotope-labeled biomarkers (Glc and Gln) to cancer patients to differentiate metabolic pathways between tumors and benign/healthy tissue [42]. Additionally, Gln metabolic studies may help predict which tumors would respond to therapies targeting its metabolism [39].

Further work is needed to investigate the metabolic consequences of GLS silencing and GLS2 inhibition/overexpression. Of interest, aldehyde dehydrogenase 1 family member L2 (ALDH1L2), cystathionine gamma-lyase (CTH), and glutathione S-transferase (GST), all proteins dealing with the oxidative stress response protein network, are upregulated in response to GLS inhibition [43]. Hence, although CB-839 treatment had antiproliferative activity in PDA cell lines, it had not antitumor activity in a genetically engineered mouse model of PDA, due to resistance to GLS inhibition, explained by compensatory metabolic pathways. An understanding of genetic and epigenetic circuits dealing with GA isoenzymes will be useful in developing combined and synergistic therapies to augment the effects of Gln metabolism reprogramming, providing new class of anticancer medication [18].

Indeed, recent analyses yielded several interesting combinatorial approaches that showed efficacy. In the above described PDA model an adaptive response to oxidative stress compensated CB-839 effect. Because of the lack of potent inhibitors of CTH, L-buthionine-(S,R)-sulfoximine (BSO) was elected to inhibit γ-glutamate-cysteine ligase catalytic subunit (GCLC), and γ-glutamate-cysteine ligase regulatory subunit (GCLM), which are downstream of CTH and essential for GSH synthesis [43]. Interestingly, combinatorial BSO and CB-839 treatment in CB-839-resistant cells decreased proliferation. Also methotrexate (MTX), albendazole and MG-132 showed synergistic effect against cancer growth with CB-839 in PDA cells. Similarly, GLS inhibition by BPTES sensitizes PDA by lowering anti-oxidant defenses and increasing NQO1-induced ROS damage [44]. In fact, PDAs upregulate GLS, mitochondrial glutamate-oxaloacetate transaminase 2 (GOT2), and cytoplasmic glutamate-oxaloacetate transaminase 1 (GOT1) to support redox balance following rapid proliferation and growth, as a consequence of metabolic rewiring driven by mutant V-Ki-ras2 Kirsten rat sarcoma (KRAS) [44]. On the other hand, combination of a pyruvate kinase M2 (PKM2) inhibitor (shikonin) plus BPTES decreased the proliferation of several hypoxia-resistant gastric cancer cell lines, as well as in xenografted tumor *in vivo* [45]. This fact suggests that PKM2 inhibitor and GLS inhibitor might be useful for several subtypes of aggressive gastric cancers. Inhibiting GLS and PKM2 was also a successful strategy against oxaliplatin-resistant CRC *in vitro* and *in vivo* [46].

3. Glutamine synthetase

3.1. Glutamine synthetase history and tumor microenviroment

GS (EC 6.3.1.2, also known as GLUL) is a ATP-dependent metalloenzyme containing three divalent cations (Mg^{2+} or Mn^{2+}) that combines ammonium and Glu into Gln [47]. In mammals, GSII (from now on GS) is the most common type of GS, forming a homodecamer structure (monomer is around 350 amino acid) organized in two pentameric rings, coordinated through divalent cations [48]. As usually referred, GLUL gene codes for GS protein, that is also critical for endothelial cells motility and migration, contributing to angiogenesis through Rho GTPases, in development and disease [49].

Dysregulation of metabolic enzymes in cancer depends on multiple factors, including some elements modulating tumor microenvironment (TME) [50]. TME consists in different cell types surrounding tumor cells, such as immune cells, fibroblasts, endothelial cells, and adipocytes [51]. In addition, TME is a target for therapeutics to overcome immunotherapy resistance [52]. TME also includes lowered pH, differential oxygenation (hypoxia), higher levels of immune-modulatory metabolites, as well as stress by Glc and Gln [2]. Besides, heterogeneity among tumors is a main characteristic of each type of cancer [18,53]. Accordingly, increased Gln catabolism in mouse liver tumors was associated with decreased levels of GS and the switch from GLS2 to GLS. In sharp contrast, MYC-induced NSCLC tumors display increased expression of both *GLUL* and *GLS* and accumulate Gln [14]. Although immersed in this very complicated scenario, GS is a critical metabolic activity because it can influence cancer circuits and cell fate [51]. Main discoveries of GS as an essential metabolic enzyme in cancer are depicted at Fig. 3 [54–67]. In pioneer works at Souba laboratory, rats implanted subcutaneously with fibrosarcoma showed increased GS in liver [58], as well as in skeletal muscle and kidney [59] to support tumor avidity by Gln. GLUL overexpression was later detected also in liver cancer from human patients [60]. Because GS can synthesize Gln from Glu and thus allow cells to survive in Gln-depleted conditions, GS supports another anaplerotic flux for Gln, that feeds TCA cycle [68]. Significantly, Yang et al. (2016) induced tumor regression in an orthotopic mouse model for ovarian carcinoma by co-targeting GLUL in the mouse stromal cells (TME) and GLS in tumor cells, using GLS siRNA and GLUL siRNA, respectively, as well as a chitosan nanoparticle delivery system [69].

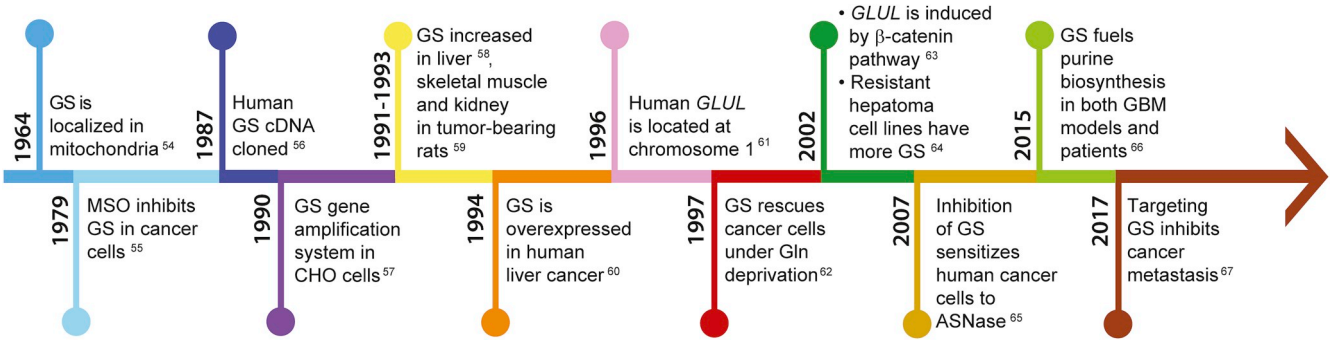

Fig. 3. Timeline of milestones for GS enzyme in cancer. Most relevant findings in scientific history that point out GLUL as a key enzyme in cancer growth are briefly depicted. ASNase, L-asparaginase; CHO, chinese hamster ovary; GBM, glioblastoma; Gln, glutamine; GLS, glutaminase isoenzyme; GLUL, γ-glutamyl:ammonia ligase gene; GS, glutamine synthetase; MSO, L-methionine sulfoximine.

3.2. Glutamine and glutamine synthetase

Some cancer cells can synthesize Gln *de novo* because of GS activity, but others import extracellular Gln, or use both metabolic capacities (GS and GLS) to produce and process Gln as a fuel for mitochondria [2]. Anyway, the absence of GS, associated to high GLS activity, makes tumor cells addicted to Gln, and this is usually linked with a more invasive, aggressive and resistant phenotype [51]. However, there are examples in which cancer cells express high levels of GS, such as papillary thyroid carcinoma [70], prostate neuroendocrine [71], prostate [72], breast [62,73] or liver cancers [74]. Cox et al. investigated zebrafish with hepatocyte-specific transgenic expression of mutated YAP (Yes-associated protein) that developed liver cancer as adults. In this model, GS increased Gln levels for nucleotide biosynthesis and the growth of liver cancer cells in a YAP-dependent manner [74]. GBM is another example where GS was upregulated in cancer [75]. These aggressive tumors have scarce GLS relative to surrounding brain tissue and show Gln independence [68]. GBM use Glc oxidation through pyruvate carboxylase (PC) during accelerated tumor growth [76]. These findings were confirmed by Tardito et al., who found that Gln-based anaplerosis was not essential for the proliferation of GBM. Experiments with both orthotopic GBM models and in patients demonstrated that GS produces Gln from TCA cycle-derived carbons. In fact, Gln required for the growth of GBM tumors is either synthesized by GS-positive glioma cells, or provided by surrounding astrocytes in a parasitic behavior [66]. Oligodendroglioma cells lack GS but are Gln-dependent [77]. As GBM, oligodendroglioma cells do not use Gln for anaplerosis. However, Gln starving induced a nutritional stress, blocking mTOR and Wnt/β-catenin pathways, that was rescued by artificial GLUL overexpression [77]. Notably, GLUL is a characterized downstream target in the Wnt/β-catenin signaling pathway *in vitro* and *in vivo* [63]. In addition, inhibitors of Gln transporters (ASCT2, SNAT2 and LAT2) elicited anti-proliferative effects on oligodendroglioma cells [77]. Different patterns (cancer heterogeneity) have been described in other tumor types [51]. Resistant leukemia cells depend more on glycolysis and TCA but less on GS [78]. This authors stated that reductions in GLUL expression and Gln dependence reflect an adaptation based on metabolic rewiring, that accompanies drug resistance in many cancer cells. Conversely, myeloma cells showed low expression of GLUL but high expression of Gln transporters, i.e.: ASCT2, that hint that these cells depend on extracellular Gln and use it for anaplerosis. In fact, stable ASCT2 silencing inhibited multiple myeloma growth in a murine xenograft model [79].

3.3. Glutamine synthetase expression as a therapeutic target against cancer

Remarkably, synergic antitumor capacity was achieved *in vitro* and *in vivo* by combination treatment with ASCT2 inhibition with benzylserine and the selective irreversible inhibitor of GS, L-methionine sulfoximine (MSO, Fig. 2). Ye et al. (2018) found a reinforced therapeutic efficacy of Gln-targeted treatment ASCT2-dependent in a gastric model [80]. Collectively, these results support the use of this combination therapy for treatment of patients whose gastric tumors express both ASCT2 and GLUL. Structural analog of Glu phosphinothricin and alendronate (Fig. 2), acting as GS inhibitors, have shown potential anticancer activity against lung cancer A-549 cell and breast cancer MCF-7 cell line [81]. Eisenberg and colleagues reviewed the GS inhibitors [82]. In SK-BR-3 breast cancer cells, it was demonstrated that knockdown of GLUL inhibited cell proliferation through inhibition of p38 mitogen-activated protein kinase (MAPK) and ERK1/2 signaling pathways [83]. In this research, the analysis of GLUL expression in clinical samples showed that GLUL expression was positively related with poor prognosis in breast cancer patients. In sarcoma cells, GS mediated proliferation of Gln-deprived cancer cells evoked nucleotide synthesis and mitochondrial bioenergetics [84]. Significantly, pharmacological (MSO) and shRNA-mediated inhibition of GLUL diminished xenograft tumor growth. Supporting results were found in ovarian cancer: knockdown of GLUL decreased the proliferation through inhibition of the p38-MAPK signaling pathway [85]. Besides, the analysis of GLUL expression in patient samples showed that high expression of GLUL was associated with poor prognosis in ovarian cancer patients. In ovarian cancer stem-like cells, GS triggered cell proliferation under ammonia stress, which enhanced tumor initiation *in vitro* and *in vivo* [86]. Mechanism was favored through hypoxia-inducible factor-1α (HIF-1α) stabilization by facilitating glycolysis and elevating Glc dependency.

Tumor-associated macrophages (TAMs) can act as metastasis promoters by creating an immune-suppressive environment, for which GLUL activity is critical [87]. GS deficiency in TAMs, induced either by GLUL silencing or MSO, led to lower T-cell suppression, reduction in angiogenesis, and tumor vessel normalization [87]. Conversely, when other types of ovarian cancer GS-overexpressing cells were subcutaneously injected into nude mice, tumor growth was fully inhibited [88]. Furthermore, in this model, the siRNA-mediated inhibition of the Gln transporter ASCT2 was more effective against tumor proliferation in the GS-deficient-ovarian cancer cells than in GLUL-expressing-ovarian cancer cells. Thus, downregulation of GLUL expression led to increased sensitivity to Gln depletion in these ovarian cancer cells.

SLC7A8 gene encodes L-type amino acid transporter 2 (LAT2), that is responsible for transporting neutral amino acids, including Gln, and functions as an oncogenic protein through Gln-dependent activation of mTOR pathway in pancreatic cancer [89]. LAT2 was upregulated, *in vitro* and *in vivo*, in gemcitabine-resistant pancreatic cancer cells, while artificial LAT2 overexpression decreased the level of GLS and increased that level of GS, as well as reduced gemcitabine sensitivity *in vitro* and *in vivo* [89]. Consequently, the LAT2-mTOR-GS pathway might be another interesting therapeutic target in pancreatic cancer. Notably, GLUL expression was upregulated in gefitinib-sensitive NSCLCs, but it was downregulated in gefitinib-resistant NSCLCs [90]. In this model, the increased Gln anabolism promoted by GLUL expression sensitized cancer cells to gefitinib by diminishing both ATP and GSH production, leading to cell death and lower invasive capacity [90]. Accordingly, GLUL knock out provoked gefitinib resistance [90]. In other study, Gln-addicted NSCLC cell lines were highly sensitive to treatment with tumor necrosis factor receptor associated protein 1 (TRAP1) inhibitor gamitrinib triphenylphosphonium (G-TPP, Fig. 2) [91]. G-TPP treatment released GS enzymatic activity in those Gln-addicted NSCLCs through phosphorylation of AMPK. Hence, this drug might be useful for therapeutic targeting of other Gln auxotrophic cancers.

Within the same tumor, stromal cells are a key element to provide Gln requirements [69]. Stromal cells, which show high GS to compensate for the low GS of tumor cells, include cancer associated fibroblasts, adipocytes, regulatory T cells and TAMs [51]. TAMs play key roles in angiogenesis, tumor cell invasion, and metastasis formation [87]. In fact, GS acts as mediator of the proangiogenic, immunosuppressive, and pro-metastatic function of macrophages, highlighting the possibility of GS targeting against cancer metastasis [67]. In addition, adipocytes in a leukemia microenvironment produced Gln by upregulating GS *in vitro*, *in vivo* and in human samples of ALL [92]. Adipocytes also secrete Gln by overexpressing GS to promote PDA cell proliferation. In return, PDA cancer cells down-regulate adipocyte GLS expression [93]. On the other hand, there exists an inter-cellular metabolic Gln symbiosis described in brain [7], kidney [94], liver [95], and breast [96]. Through the analysis of how different breast cancer cells respond to Gln deprivation, Kung et al. found a striking difference in the Gln requirement among different breast cancer cells, which tracks with the luminal *versus* basal type. In the luminal cells, GATA3 triggers expression of GLUL and contributes to Gln independence. High GS represses GLS (low expression) which would also help to maintain the cell-type specific phenotype. Basal-specific expression (high GLS, low GLUL) is supported by the absence of GATA3 and higher activities of c-Myc in the basal type cells. In addition, GLUL expression in tumor cells can vary depending on many different mechanisms, including

upregulation of thymine DNA glycosylase through c-Myc, which leads to demethylation of the GLUL gene promoter and its higher expression in subsets of cancer cell lines [97]. Moreover, GLUL was upregulated by Wnt pathway in c-Myc/E2F1 hepatocarcinogenesis [98]. GS was subject to feedback control by Gln, which promoted its post-translational acetylation and ubiquitination before its degradation in HCC cells [99]. On the other hand, phosphorylation and enhanced expression of liver GS was found in HCCs [100]. Besides, Gln, insulin and glucocorticoids regulated GLUL in HCCs and adipocytes [101]. Of note, DNA hypermethylation influenced dependency on Gln availability as exemplified by Gln auxotrophy in different cancer cell lines [102].

Bode and colleagues found that resistant and more aggressive hepatoma cell lines (HepG2, Hep3B, and Huh-7) had compensatory mechanisms for Gln supply, including higher GLUL expression, than the less differentiated hepatomas (SK-Hep, FOCUS, and PLC/PRF/5) [63]. Many studies have highlighted the critical role of GS and Gln for mTOR activation in HCC development [103]. So, several sets of HCCs are characterized by mutations of β-catenin and overexpression of GLUL [98,104,105].

3.4. State of the art and perspectives

(R)-Etodolac (Fig. 2) lowered proliferation of HCC cells by diminishing GLUL expression [106]. Similarly, sorafenib (Fig. 2) decreased GS, through interfering Wnt/β-catenin signaling pathway in HepG2 cells and in HepG2 xenografts in mice, diminishing tumor volume and increasing median survival of sorafenib treated mice [107]. The antitumor enzyme L-asparaginase (ASNase) has been employed for many years in the treatment of ALL [92]. In that research, adipocytes protected leukemia cells from ASNase via Gln production through GS overexpression. Very recently it has been stated the possibility of anticancer combination therapy using two or more ASNases from different bacteria to achieve better therapeutic outcomes with lower side effects [108]. ASNase also increased GLUL expression and GS activity in several HCC lines [109]. This authors showed that ASNase had a significant antiproliferative effect in the β-catenin mutated HepG2 cell line through higher expression and activity of sodium-coupled neutral amino acid transporter 2 (SNAT2). Importantly, ASNase lowered the availability of extracellular Gln, and the GS inhibitor MSO prevented the intracellular synthesis of the amino acid. When simultaneously treated with ASNase and MSO, undetectable intracellular Gln and proliferative arrest and apoptosis was achieved in human osteosarcoma cells [65] and HepG2 cells [109]. The key role of β-catenin and GS for establishment and progression of HCC has been characterized *in vitro* [110] and *in vivo* [111]. Very recently, targeting β-catenin-GS-mTORC1 axis in HCC ameliorated cancer growth [112]. In this research, since no anti-β-catenin inhibitors are currently available in the clinic, mTOR inhibition with rapamycin in combination with MET-β-catenin inhibitor CG1 (sobetirome) were used. Successfully, this combined treatment synergistically reduced HCC burden. Strikingly, GS and mTOR use to be differently located at midlobular liver zone in HCC, instead of pericentral liver zone of normal hepatocytes, following a dysregulated mutated β-catenin profile in Wnt-driven liver cancer [113]. In patients suffering from HCC, after liver transplantation, immunohistochemical

Table 1
Glutaminase and glutamine synthetase specific inhibitors with anticancer properties.

Drug	Inhibition	Multi therapy	Cancer type	Ref.
DON	GLS/GLS2	ASNase	P388 and L1210 leukemia cells	[17]
Acivicin				
DON	GLS/GLS2	Lonidamine, orlistat	Colon	[21]
BPTES	GLS	Metformin	Pancreas	[2]
BPTES	GLS	*Gls* silencing	HCC, lymphoma	[20]
BPTES	GLS	5-FU	NSCLC	[26]
BPTES	GLS	Cisplatin	TNBC	[27]
BPTES	GLS	DBZ	T-ALL	[28]
BPTES	GLS	ARQ761	PDA	[43]
BPTES	GLS	Shikonin	Gastric cancer cells	[44]
BPTES	GLS	BSO	Breast/lung cancer cells	[117]
CB-839	GLS	TXNIP repression	PC3/DU145 prostate cancer cells	[22]
CB-839	GLS	AZD8055	TNBC	[23]
CB-839	GLS	MLN128	Lung SCC, head and neck SCC, osteosarcoma	[24]
CB-839	GLS	THZ1	NSCLC	[25]
CB-839	GLS	Phenformin/metformin	ESCC	[31]
CB-839	GLS	Etomoxir/BSO	PDA	[42]
CB-839	GLS	ARQ761, albendazole, MTX, MG-132	PDA	[43]
CB-839	GLS	BSO	Breast/lung cancer cells	[117]
968	GLS	DHA	HCC	[29]
968	GLS	Metformin, ribavirin, oxaliplatin	CRC	[30]
968	GLS	CQ	NSCLC	[31]
968	GLS	PP242	GBM	[32]
CPD-3B	GLS/GLUD	——	A-549 lung, HCT116 colon, H22 liver, U251 GBM, caki-1 renal cells	[33,34]
CPD-20	GLS	——	H22 liver/HCT116 colon	[35]
CPD-5	GLS/GLS2	Doxorubicin	MDA-MB-231 breast, AsPC-1 pancreas cells	[36]
3B	GLS	——	HCT116 colon cells	[37]
MSO	GS	Benzylserine	Gastric cancer cells	[79]
MSO	GS	——	Sarcoma cells	[80]
ALD/PPT	GS	——	A-549 lung, MCF-7 breast cancer cells	[83]
G-TPP	GS/AMPK	*Gls* silencing	NSCLC	[90]
Etodolac	GS/Wnt/β-catenin	——	HepG2/Hep3B liver cells	[105]
Sorafenib	GS/Wnt/β-catenin	——	HCC	[106]

ALD, alendronate; AMPK, adenosine monophosphate-activated protein kinase; ASNase, L-asparaginase; DBZ, dibenzazepine; BSO, L-buthionine-(S,R)-sulfoximine; CQ, chloroquine; CRC, colorectal cancer; DHA, dihydroartemisinin; DON, 6-diazo-5-oxo-L-norleucine; ESCC, esophageal squamous cell carcinoma; 5-FU, 5-fluorouracil; GBM, glioblastoma; *Gls*, glutaminase gene; GLS, glutaminase isoenzyme; GLS2, glutaminase 2 isoenzyme; GLUD, glutamate dehydrogenase; GS, glutamine synthetase; G-TPP, gamitrinib triphenylphosphonium; HCC, hepatocellular carcinoma; MTX, methotrexate; MSO, L-methionine sulfoximine; NSCLC, non-small-cell lung cancer; PDA, pancreatic ductal adenocarcinoma; PPT, phosphinothricin; SCC, squamous cell carcinoma; T-ALL, T-cell acute lymphoblastic leukemia; TNBC, triple negative breast cancer; TXNIP, thioredoxin-interacting protein.

marker GS showed a positive correlation with better survival ratios [114]. However, in another study, GS-positive staining of HCC tumors by itself, was not associated with any changes in intratumoral proliferation in mice neither in human patients [115].

4. Concluding remarks and future prospects

Different therapeutic targets and specific inhibitors dealing with metabolic dysregulation have been very recently described [116]. In Table 1 are depicted GLS and GS inhibitors described in this review, and their roles in corresponding cancer types. As stated by many authors (see this review and this special issue), inhibiting a single metabolic target is not sufficient to block cancer growth in preclinical trials. In Table 1 some multi therapy treatments which synergistically inhibit some types of cancer are outlined. Future prospects include diagnostic signature of GLS inhibition predicting *in vivo* efficacy profile of BPTES/CB-839 treatments. Recently, drug response in mesenchymal breast and lung tumors was envisioned, and confirmed in lung patient-derived xenograft models [117]. In this research, a co-dependency of GLS and the γ -glutamylcysteine synthetase (GCS) inhibitor BSO was established, suggesting that the control of redox balance is a fundamental role of GLS. On the other hand, for overcoming drug resistance by specific metabolic inhibition, double or triple combination therapies have been proposed [116]. Among key metabolic targets, Gln metabolism has been extensively tackled, as reported in last years [2,3,55,118].

Ralph DeBerardinis and colleagues confirmed that Gln is not only essential for feeding the biosynthesis of proteins, nucleotides and fatty acids, but also supports NADPH production and anaplerosis in tumor cells [119]. Although many cancer studies draw conclusions from a number of cell lines, large-scale metabolomic characterization of tumor samples will have direct implications for therapeutics targeting metabolism [102]. As found in most cancer models, a high ratio of GLS/GLUL expression in a tumor is indicative of extracellular Gln-dependent metabolism for cell proliferation and survival [120]. On the other hand, immunotherapy has a great potential to rewire the immune cycle through the generation of a bunch of alternative immune responses, and it has been established as another critical tool in the metabolic network of tumors to struggle against cancer [52]. Importantly, GLS controlled *in vitro* and *in vivo* for inflammatory effector T cell responses, enhancing mTORC1 and phosphatidylinositide 3-kinase (PI3K) signaling, to support glutaminolysis that integrates with glycolysis [121]. In a very recent research, GS and GLS have been characterized as basic targets in oral cancer [122]. Both metabolic enzymes are positively correlated with c-Myc overexpression, as well as with tumor growth and metastasis stage. Therefore, GLS and GS can be pointed out as essential metabolic enzymes to be targeted for cancer therapy. Anyhow, accurate patient stratification based on individual mutations and respective metabolic profiles is required to better plan the optimal combinations of targeted chemotherapies and immunotherapies needed for the success of personalized oncology [52,123].

CRediT authorship contribution statement

José M. Matés: Conceptualization, Funding acquisition, Project administration, Writing - original draft, Writing - review & editing. **José A. Campos-Sandoval:** Software, Visualization, Writing - review & editing. **Juan de los Santos-Jiménez:** Writing - original draft, Writing - review & editing. **Javier Márquez:** Funding acquisition, Project administration, Writing - review & editing.

Declaration of competing interest

The authors have no conflict of interest.

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