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Review

Inhibition of glutamine metabolism as a therapeutic approach against pancreatic ductal adenocarcinoma

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Pancreatic ductal adenocarcinoma (PDAC) is a relatively rare tumor, however it is the seventh cancer related leading cause of death worldwide. Mean survival time after PDAC diagnosis is less than 1 year and the median survival of PDAC patients has hardly changed in the past 40 years. Until now, cytotoxic and/or targeted therapy produced disappointing results in the treatment of PDAC. Currently, surgical resection offers the only hope for survival, but it is suited for only 15% of PDAC patients. To complicate matters, the vast majority of PDAC patients relapse after surgery. Thus, there is a burning need to develop better therapeutic strategies for PDAC treatment. PDAC cells have adapted to survive and proliferate in a tumor microenvironment that is constitutively under deprivation of nutrients and oxygen, via mechanisms triggered by oncogenic *KRAS*. In this review, we highlight the metabolic alterations observed in PDAC, with a particular emphasis on past and ongoing strategies to develop inhibitors of KRAS effector signaling. This review provides an up to date information reported in the literature on the most relevant inhibitors of metabolism targets in PDAC. The review specifically provides an overall picture of the current state of the art with the aim of being thought provoking for plausible novel chemotherapeutic strategies of intervention. We anticipate that with our increased collective understanding of PDAC metabolic behavior, PDAC patients could hopefully benefit from these novel therapies.

Keywords

Pancreatic ductal adenocarcinoma; KRAS; glutamine metabolism; chemotherapeutics

1. Introduction

Pancreatic cancer (PC) is a relatively rare tumor (2% of all cancer cases), but it is the seventh leading cause of death from cancer worldwide [1, 2]. In 2018, PC ranked the 11th most common cancer in the world accounting for over 450,000 new cases and causing more than 430,000 deaths (4.5% of all deaths caused by cancer), 70% of which were in developing countries [1, 3]. PC falls into two main groups, based on the different types of cells found in the pancreas: (a) exocrine tumors, which account for 95% of all PCs, and (b) endocrine tumors, known as pancreatic neuroendocrine tumors or PancNETs. Overall, the most common type of PC, pancreatic ductal adenocarcinoma (PDAC), is an exocrine tumor and comprises about 90% of all malignant pancreatic neoplasms [1, 3]. PDAC has a very poor prognosis with mean survival time after first diagnosis less of than one year (only 24% of patients survive one year) and the 5-year survival rate is only 9% [2, 3, 4]. The poor prognosis is due to factors that render PDAC an aggressive cancer: late detection [5, 6], difficult anatomic location of the pancreas [7], metastatic spread when the primary tumor is too small to be detected [8], tumor interaction with stromal cells [9, 10], limited effectiveness of existing therapies [11] largely due to resistance to chemotherapy [12] and radiotherapy [13].

Due to the absence of symptoms at the first stages of the disease [14, 15], PDAC is not diagnosed until it has spread to distant locations [16]. When the tumor grows and presses nearby structures, the symptoms of PDAC become apparent [1]. The clinical manifestations of PDAC are nonspecific and include jaundice, unexplained weight loss, epigastric pain radiating to the back, nausea, onset of diabetes and, rarely, migratory thrombophlebitis [14, 15, 18]. When PDAC is suspected, medical imaging tests are used. The diagnoses use transabdominal ultrasound [19], in the introductory evaluation of the patient, along with computed tomography or magnetic resonance imaging [20]. Since a pathological analysis is required to establish a definitive diagnosis of PDAC, the majority of patients will undergo endoscopic ultrasound with fine needle aspiration biopsy [21]. Frequently, cases of PDAC are diagnosed in advanced stages. At the time of first diagnosis, 45% of the patients have metastases in distant sites, about 40% display a locally advanced tumor and only 15% have the disease at a stage that allows surgical removal [6]. Currently, pancreaticoduodenectomy is the only curative therapy for PDAC [22]. However, the majority of operated PDAC patients relapse, and their 5-year survival rate is less than 25% [2]. Complete surgical resection of localized PDAC followed by 6 months of adjuvant chemotherapy is the only recognized standard of care that improved patient survival, with a median overall survival up to 54.4 months [23]. The 5-year survival rate, in cases where it is not possible to operate the tumor (i.e. 85% of PDAC patients), is less than 3% [1].

Systemic cytotoxic treatments are the standard of care for most patients with PDAC [24]. In the US, PDAC patients with opera-

ble tumors are treated, in an adjuvant setting, with gemcitabine and chemoradiation based on 5-Fluorouracil (5FU). In the EU, gemcitabine monotherapy is the most common therapeutic option. However, almost all tumors display, or acquire resistance to these therapeutic regimens and follow their lethal progression [11]. Gemcitabine provided survival superiority over bolus 5-FU, and for more than a decade has been considered the standard treatment for metastatic PC. However, the median survival of patients with PDAC hardly changed in the last 40 years [25]. Gemcitabine-based combination regimens were evaluated subsequently for superiority over gemcitabine monotherapy in clinical trials. However, apart from erlotinib, a receptor tyrosine kinase inhibitor (TKI), which blocks epidermal growth factor receptor (EGFR), the addition of targeted agents to gemcitabine failed to produce any added benefit [26]. In 2011, the FOLFIRINOX regimen (oxaliplatin, irinotecan, leucovorin and 5-FU) also showed a comparative efficacy superior to that of gemcitabine monotherapy, but due to its severe toxicity, it is only suitable for young and fit patients [27, 28]. Nanoparticle albumin-bound-paclitaxel (Abraxane®, ABI 007 or nab-PTX) was approved in 2013 and is becoming, in combination with gemcitabine, the regimen of choice for the treatment of patients with advanced PC, especially in the USA [29]. The albumin nanoparticle formulation improves delivery to the tumor microenvironment (TME) and increases the drug load. FOLFIRINOX and nab-PTX are used in high-income countries, but the use of nab-PTX is not extensive in low- and mid-income countries. In the EU, nab-PTX is barely used since the National Health Systems usually do not finance it. The poly (ADP-ribose) polymerase (PARP) inhibitor olaparib remains the only molecularly matched therapy for PDAC treatment. However, olaparib is indicated in only ~4- 7% of PDAC patients, those who have a germline BRCA mutation [30]. In addition to the aforementioned therapies, there are other therapies that are under development [31]. These molecular targeted therapies include inhibition of growth factor receptors (EGFR, PDGFR, VGFR, IGF-1R), TKIs, complex liposome p53, programmed cell death protein 1 (PD-1), MEK1/2, mTOR blockade as well as PI3K and HER2-neu pathway inhibitors.

In the present review, we give a snapshot of the metabolic alterations observed in PDAC, with a particular emphasis on past and ongoing strategies to develop inhibitors of KRAS effector signaling. This work provides an up to date information reported in the literature on the inhibitors of PDAC metabolism targets. An extensive description of the compounds is avoided because of two main reasons. On the one hand, there are scarce studies of the scope of these inhibitors in PDAC; on the other hand, our aim is to provide an overall picture of the current treatment options with the aim of being thought provoking for development of plausible novel chemotherapeutic strategies of intervention.

2. Genetic alterations and metabolism in PDAC, potential breakthroughs

2.1 Genetic alterations in PDAC

Pancreatic intraepithelial neoplasms (PanINs) are the most common precursors of PDAC [32, 33]. *KRAS* gene alterations occur in 91% of PDACs, followed by *TP53* (61%), *CDKN2A* (44%) and *SMAD4* (40%) [34]. Other genes which may be mutated in PDACs, although their mutation frequency occurs in only a small fraction (2-17%), include *GATA6*, *ARID1A*, *RNF43*, *ATM*, *TGFBR2*, *MAP2K4*, *MLL3*, *PIK3CA*, *RBM10*, *ROBO2*, *SMARCA4*, *PBRM1*, *SLIT2*, *KDM6A*, *BRAF*, *BRCA2*, among others [35]. However, the role of these and other tumor promoting genes involved in the pathogenesis of PC remains to be elucidated. For instance, little is known about genomic and proteomic changes affecting myelocytomatosis (MYC) in PC. However, deregulation of c-MYC is common in PC [36]. Recent studies support the possibility that inactivation of MYC may be an effective therapeutic strategy for KRAS mutant tumors [37].

Currently, it is unknown why PDAC is associated exclusively with *KRAS* mutations. Genetic mutations indicate that PDAC cells are selected based on their competitive advantages when they encounter limitations in their hypovascular, fibrotic, hypoxic and nutrient deprived TME. The fibrotic layer around the tumor, which accounts for 90% of the tumor volume, creates a barrier to the supply and systemic penetration of drugs (poor drug delivery) and affects the vascularization of pancreatic tumor tissue. Thus, PDAC cells have adapted to survive and proliferate in a harsh TME being under attack by immune system cells, deprivation of nutrients and oxygen. With limited access to blood vessels, PDAC cells must rely on their ability to reprogram metabolic pathways to survive and proliferate [38, 39]. Although reprogrammed metabolism is a common feature of neoplasms, metabolic addictions vary among cancers and are determined mainly by their specific genetic mutations, tissue of origin or the TME [40]. PDAC cells show complex and heterogeneous reprogramming of glucose, amino acid and lipid metabolism. These features play an important role in disease evolution by inducing resistance to therapy [41]. In addition to changes in metabolism, PDAC cell survival and progression relies on enhancing nutrient acquisition through macropinocytosis and autophagy [42], and conducting metabolic crosstalk with other components within the TME [43].

As aforementioned, the oncogenic activation of *KRAS* occurs in the majority of PDAC cells. In fact, *KRAS* mutation is the initiating genetic event for PDAC [44]. The activation of *KRAS* originates most commonly from the mutation at the Gly12 residue, which prevents the interaction with GTPase activating proteins (GAPs) and keep KRAS constitutively bound to GTP, i.e. in its active form [45]. The aberrant downstream signaling pathways produce increased tumor cell proliferation, decreased apoptosis, and an invasive phenotype [46]. KRAS-GTP binds preferentially to at least 11 different downstream effector families with distinct catalytic functions. Therefore, it is not trivial to determine which effector pathways are the best to target [47]. Currently, there are no approved drugs that directly target mutated KRAS proteins. There are drugs that target KRAS indirectly by blocking proteins that interact with it, but they were ineffective against PDAC in clinical studies.

2.2 Metabolism in PDAC

One of the relevant metabolic changes occurs in the glutamine (Gln) pathway [48]. Gln is the most abundant amino acid in the plasma and can be synthesized endogenously but becomes essential in physiological or pathological conditions of high cell proliferation such as cancer. Gln is the most highly metabolized amino acid in PDAC tumors. Oncogenic *KRAS* has reprogrammed Gln uptake and metabolism to serve anabolic processes.

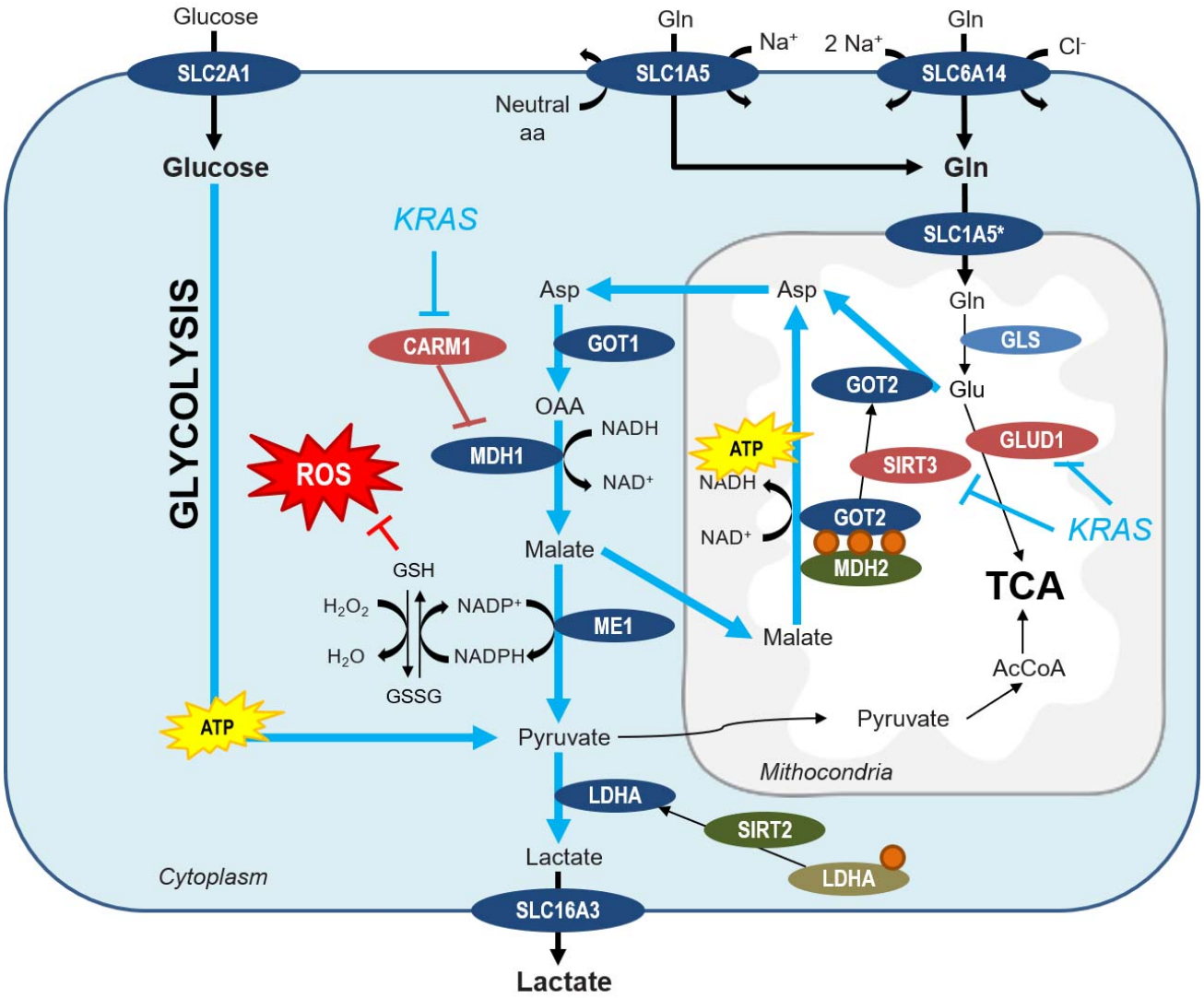

Figure 1. Glutamine (Gln) metabolism and redox homeostasis in PDAC cells. In PDAC, Gln enters the cell via the amino acid transporter SL6A14 and is converted by GLS to glutamate (Glu). *KRAS* inhibits GLUD1 expression and Glu becomes substrate of GOT2 leading to GSH production.

Gln enters cells through the amino acid transporters SLC6A14 ($ATB^{0,+}$), SLC6A19 (B^0AT1) and SLC1A5 (ASCT2; AlaSerCys Transporter 2). From the three transporters, elevated expression of SLC6A14 [49] or SLC1A5 [50] were observed in cancers from diverse origins, including PDAC, and is correlated with lower patient survival. Thus, both amino acid transporters play an important role in tumor cell growth, and represent promising pathological prognosis biomarkers for PDAC outcome. SLC6A14 can transport 18 of the 20 proteinogenic amino acids excluding the acidic amino acids glutamate and aspartate. In contrast, SLC1A5 exhibits functional asymmetry with an antiport mode of transport; some amino acids are transported only inwardly, whereas others are bidirectionally transported, allowing for regulation of amino acid balance in cells. In Gln "addicted" cells, SLC1A5 exchanges mainly Gln (the preferred natural substrate) with the release of Ser. Both transporters use Na^+ transmembrane gradients as the energy source to drive amino acid co-transport (Fig. 1).

When comparing the amino acid transporters, SLC6A14 is the only carrier that possesses all essential characteristics to promote tumor growth. The most relevant features include broad substrate selectivity (admits all essential amino acids as well as Gln), high concentrative capability due to coupling to three different energy sources (Na^+ gradient, Cl^- gradient, and membrane potential), as well as coupling to mTOR signaling [51]. SLC6A14 is the only amino acid transporter that generates an amino acid intracellular concentration gradient of more than 1,000-fold when compared to the extracellular milieu, making the transport practically unidirectional and directed towards the cytoplasm. More importantly, this Gln pathway is not used, extensively, by healthy cells [52].

Normal pancreas cells express SLC6A14 at much lower levels and their proliferation is not affected by blocking the transporter with α-methyl-tryptophan (α-MT). In contrast, in PDAC, SLC6A14 is clearly overexpressed (13- to 167-fold) and its pharmacological blockade with α-MT reduces the growth and prolifer-

ation of PDAC cells, in primary cultures and in xenografts of PC [52]. In SLC6A14-positive tumor cells, the inhibition of amino acid uptake induces cell death via four different mechanisms: (a) it halts the uptake of essential amino acids; (b) it targets the Gln addiction of PDAC cells; (c) it inhibits mTOR; and (d) it induces oxidative stress [51].

SLC1A5 plays a supportive role from the initial stages of PDAC formation. Tumor initiating cells have a mechanism for maximizing Gln uptake, which relies on CD9, a member of the tetraspanin family of proteins. PC cells show increased CD9 expression when compared to normal pancreatic tissues. High CD9 expression can initiate and sustain PDAC growth and correlates with poorer patient survival. CD9 augments Gln uptake by increasing the cell surface expression of SLC1A5, thereby enhancing PDAC growth and proliferation [53].

Once inside the cell, Gln is transported through the inner mitochondrial membrane before glutaminolysis can take place. However, data concerning the structure and function of the transport system are scarce [54]. Notably, a variant of SLC1A5 (SLC1A5*) induces metabolic reprogramming, ATP generation, glutathione synthesis, and gemcitabine resistance in PC cells [55]. Increased SLC1A5* expression was noted in PDAC and Kaplan-Meier survival analysis indicated a correlation with poor survival outcomes. SLC1A5* is an exclusive mitochondrial Gln transporter, while SLC1A5 localizes to the plasma membrane. Moreover, SLC1A5* is essential for PC growth.

Gln is transformed into glutamate (Glu) by glutaminase [56]. Humans have two glutaminase genes, *GLS* and *GLS2*. GLS has 3 isoforms, of which, isoform 3 is highly expressed in heart and pancreas [57]. GLS2 is highly expressed in liver and is moderately expressed in brain and pancreas. Whereas, GLS2 expression is significantly reduced in hepatocellular carcinomas [58]. All these glutaminases are known to be localized in mitochondria. Expression levels and enzymatic activity of GLS and GLS2 in different types of tumors are altered [59]. Therefore, the existing GLS or GLS2 could both be targeted in order to block tumor cell growth, taking into consideration that GLS is considerably overexpressed in PDAC cells and GLS2 is preferentially expressed in hypoxic PDAC cells [60].

In healthy cells, Glu enters the cycle of tricarboxylic acids (TCA), but oncogenic activation of *KRAS* in PDAC cells repurpose Glu through a distinct pathway in which mitochondrial glutamic-oxaloacetic transaminase 2 (GOT2) transforms Glu into aspartate (Asp), which is transported into the cytoplasm. Afterwards, successive reactions catalyzed by glutamic-oxaloacetic transaminase 1 (GOT1), malate dehydrogenase 1 (MDH1), and malic enzyme 1 (ME1), convert Asp to pyruvate and produce NADPH, maintaining the redox balance and ensuring cell proliferation (Fig. 1). Thus, *KRAS* holds an important role in Gln metabolic reprogramming in PDAC through the transcriptional upregulation of *GOT1* and the inhibition of glutamate dehydrogenase 1 (GLUD1) expression [61]. Relative to other cancer types, GLUD1 is not upregulated in PDAC [62]. Cancer cells depend on GLUD1 for the conversion of glutamate into α-ketoglutarate. However, PDAC cells rely on GOT1 and GOT2 to transform aspartate into pyruvate, which supports PDAC cell growth by maintaining the redox balance. Therefore, there must be a great deal of complex crosstalk among the metabolic processes of different energy sources that cooperatively regulate the malignant behavior of PDAC. The relevant druggable enzymes of this non-canonical Gln pathway, reprogrammed by the oncogenic *KRAS*, are GOT1, GOT2, MDH1 and ME1 [41, 63].

GOT is a pyridoxal phosphate-dependent enzyme which exists in both cytoplasmic and inner-membrane mitochondrial forms, namely GOT1 and GOT2, respectively. In healthy cells, GOT plays a role in amino acid metabolism and the urea and tricarboxylic acid cycles [64]. In PDAC cells, GOT1 [65] and GOT2 [66] were found to be overexpressed. GOT1 is critical for connecting the mitochondria and cytosolic compartments in Gln anaplerosis, and hence, allows this metabolic process to complete [61]. The status of GOT1 in tumor tissue serves as an independent prognostic biomarker in PDAC [65]. Reduced NAD-dependent protein deacetylase sirtuin-3 (SIRT3) expression leads to an increase in GOT2 acetylation in PDAC cells. GOT2 acetylation at three lysine residues (K159, K185, and K404) augments the protein interaction between GOT2 and malate dehydrogenase 2 (MDH2), thereby stimulating the malate-aspartate shuttle stimulating and net transfer of cytosolic NADH into mitochondria to support ATP production [67].

Cytosolic MDH1 and mitochondrial MDH2 enzymes are overexpressed in PDAC patients. However, only high expression of MDH1 is associated with poor prognosis of the disease [66]. MDH1 is a cytoplasmic enzyme that exists as a mixture of monomers and dimers, where the homodimeric state is the catalytically active form. PDAC cells require MDH1 to maintain their cellular redox state by reprograming Gln metabolism, and MDH1 knockdown inhibits the viability of PDAC cells. Arginine 248 (R248) methylation of MDH1 by protein arginine methyltransferase 4 (PRMT4/CARM1) inhibited the enzyme through disrupting its homodimerization [68]. In clinical PDAC samples, MDH1 is overexpressed and hypomethylated. *KRAS* suppresses MDH1 methylation, contributing to Gln metabolism in PC [66].

In human cells, three isoforms of malic enzyme (ME) are known. ME1 localizes in the cytoplasm and it is important for NADPH production as well as keeping the redox balance in PDAC cells. In PC3 cells, ME1 depletion induced cellular senescence and suppressed tumor cell growth [69]. Thus, ME1 and GOT1 represent potential prognostic or sensitivity markers of radiotherapy [70]. Malic enzyme 2 (ME2) and malic enzyme 3 (ME3) are two redundant enzymes that reside in the mitochondria, where they help keep reactive oxygen species (ROS) levels under control. In PDAC, the homozygous deletion of *SMAD4* is often accompanied with homozygous deletion of *ME2* as well. A compensatory increase in *ME3* expression in *ME2*-null cell lines occurs [71]. In the absence of ME2, ME3 maintains indispensable NADPH synthesis in mitochondria. ME3 expression was higher in PC tissues of patients that had significantly shorter survival [72].

In contrast to other tumor types, PDAC cells do not rely extensively on glucose metabolism for energy demand and macromolecular biosynthesis. However, glycolysis is significantly higher than in normal cells. Furthermore, a high glycolysis phenotype in PDAC correlates with cancer metastasis [73]. *KRAS* reprogramming enhances glucose uptake and upregulates the primary glucose transporter SLC2A1 (also known as GLUT1), which corre-

lated with worse prognosis for PDAC [74]. Furthermore, SLC2A1 is indispensable for the preservation of PC stem cells [75]. Several rate-limiting glycolytic enzymes are also overexpressed in PDAC, including hexokinase 1 (HK1), HK2, phosphofructokinase 1 (PFK1) and lactate dehydrogenase A (LDHA). Additionally, the overexpression of NAD-dependent protein deacetylase sirtuin-2 (SIRT2) keeps LDHA deacetylated at K5 and retaining its enzymatic activity [76]. Clinical studies revealed that patients with a strong pyruvate kinase M2 (PKM2) and LDHA expression had significantly worse survival [77]. The dependence on glycolysis presents additional demands on mobilization and excretion of lactate to avert its intracellular accumulation and decreased cytosolic pH [78]. In PDAC cells, the transporter proteins SLC16A1 (also known as MCT1) and SLC16A3 (also known as MCT4) are overexpressed, with SLC16A3 playing a predominant role in this detoxification process and in the progression to metastasis [79].

2.3 A prominent role for biomarkers

In PDAC treatment, patients are treated with chemotherapeutic agents irrespective of tumor subtypes. However, diverse studies have shown the relationship between biomarkers and PDAC prognosis (Table 2). These proteins have great functional and prognostic importance for PDAC patients. The establishment of predictive biomarkers is essential for therapeutic decision-making and for treatment with targeted therapies. Routine cancer markers (like carbohydrate antigen 19-9 known as CA19-9) do not seem to be reliable in prediction and detection of early stage PDAC [80]. In practice, PDAC biomarkers are not established for diagnosis purposes. However, there is hope that emerging biomarkers may significantly have increased specificity and sensitivity in early PDAC detection. Liquid biopsy [81], proteomics [82], metabolomics [83], genomics [84], and miRNAs [85] appear most promising and might provide valuable biomarkers to improve selection of patients for optimal treatment regimens.

3. Chemotherapeutical approaches targeting PDAC metabolism

There is an unmet need for small molecule inhibitors of druggable metabolism targets in PDAC. Few inhibitors are available in the public domain (Fig. 2). As shown in Fig. 1, glutaminolysis and glycolysis meet at pyruvate. Significant progress was made in the discovery of molecules that act at various levels of the glycolytic pathway in tumor cells [79]. However, those compounds lay outside the scope of this review. Herein, we will give a brief overview of the inhibitors directed toward the proteins involved in *KRAS* reprogrammed Gln metabolism.

3.1 SLC6A14 inhibitors

The evaluation of tryptophan derivatives led to the identification of α-MT, which is not a transportable substrate, but is a weak inhibitor that blocks the transport function of SLC6A14 [87]. At present, no additional SLC6A14 inhibitors (iSLC6A14) are known. Recently, α- and γ-glutamyl tryptophan dipeptides [88], and naphthol-derived Betti bases [89] were proposed as iSLC6A14. Further studies are necessary in order to confirm these compounds as inhibitors of SLC6A14-mediated transport.

In addition to a plausible therapeutic target, SLC6A14 represents a strong candidate for the selective delivery of amino acid-based prodrugs to tumors [90, 91, 92].

3.2 SLC1A5 inhibitors

In contrast to SLC6A14, diverse small molecule compounds were discovered as pharmacological inhibitors of SLC1A5 (iSLSC1A5). Initial efforts consisted of the development of compounds derived from amino acids, the preferred natural substrates of the transporter. Thus, l-γ-glutamyl-*p*-nitroanilide (L-GPNA) was reported as one of the first synthetic iSLC1A5. Unfortunately, this compound showed very weak potency toward the transporter [93]. Subsequent research focused on obtaining new iSLC1A5 using amino acids (either l or d) as the main backbone, such as 2-substituted glutamylanilides (CHEMBL3576929) [94], phenylglycine derivatives (L-3,4diFPG and L-3OH,4FPG) [95], serine esters (CHEMBL3576945) [96], *O*-benzyl-serine (BnSer) and *S*-benzyl-cysteine (BnCys) [97], γ-(2-flurobenzyl)-proline (γ-FBP) [98], 4-aryl-prolines (CHEMBL4116473) [99], sulfonamides based on the 3-amino-alanine scaffold (12b) and sulfonic acid esters based on hydroxyproline (16b) [100], and 2,4-diaminobutanoic acid derivatives (CHEMBL3754498 and V-9302) [101, 102]. The most potent iSLC1A5 among all of these amino acid derivatives were CHEMBL3754498 and V-9302, which showed IC_{50} values in the low micromolar range (Table 3). All of these compounds blocked, with different potencies, SLC1A5-mediated amino acid uptake in live cells. In particular, V-9302 reduced cancer cell growth and proliferation, augmented cell death, and increased oxidative stress, both *in vitro* and *in vivo*. However, the study also showed that V-9302 efficacy is unrelated to SLC1A5 inhibition [103].

The rat but not the human SLC1A5 isoform contains two cysteine residues (Cys207 and Cys210) that form a CXXC metal binding motif. Mercurial compounds react with this site and inactivate the protein. This result was the rationale to design and synthesize iSLC1A5 containing a 1,2,3-dithiazole ring as the common core group. It was anticipated that the dithiazole group could covalently interact with the thiol group of C207/C210. The results provided the first inhibitors lacking an amino acid, which displayed potent activity at the low micromolar range (CHEMBL3753379) [104].

3.3 GLS inhibitors

One of the earliest GLS inhibitors (iGLS) is 6-diazo-5-oxo-l-norleucine (DON), who failed in clinical trials due to its low therapeutic index and no substantial activity in cancer patients [105]. However, the preclinical results of DON led to the search for new inhibitors. Allosteric iGLS 968 was shown to inhibit the growth of cancer cells, highlighting the potential of this enzyme as a druggable target [106]. Bis-2[5-phenylacetamido-1,2,4-thiadiazol-2-yl] ethylsulfide (BPTES) is another allosteric, time-dependent, and specific iGLS that also blocks tumor growth. Despite its remarkable selectivity, BPTES has poor solubility, which has limited its clinical development [107]. A recent study showed that nanoparticle encapsulation of BPTES (BPTES-NP) with dense PEG surface coatings provides an effective modality to deliver the inhibitor to pancreatic tumors while minimizing untoward toxicity [108]. However, hypoxic PDAC cells, which preferentially express GLS2, survived BPTES-NP monotherapy. The inability to target hypoxic PDAC cells with BPTES-NPs was overcome by treating the tumors with metformin. The promising results enhanced the

Table 1. Current approved drugs and drug combinations for PDAC treatment.

Drug	Cellular target	FDA	EMA
Gemcitabine (Gem)	Ribonucleotide Reductase (RRM); Deoxycytidine kinase (dCK); DNA replication chain termination	Locally advanced or metastatic and who have been treated with 5-FU	
Everolimus	FK506 binding protein-12 (FKBP-12); mTORC1	In adults with progressive neuroendocrine tumors that cannot be removed by surgery, are locally advanced, or have metastasized	Treatment of unresectable or metastatic, well or moderately differentiated neuroendocrine tumors of pancreatic origin in adults with progressive disease
Erlotinib	Epidermal growth factor receptor (EGFR)	In combination with Gem in patients whose disease cannot be removed by surgery, is locally advanced, or has metastasized	In combination with gemcitabine for the treatment of patients with metastatic pancreatic cancer
Olaparib	Poly (ADP-ribose) polymerases (PARP1, PARP2 and PARP3)	Maintenance therapy in adults with metastatic disease that has not progressed after first-line therapy with Pt chemotherapy and has certain germline mutations in the BRCA1 or BRCA2 genes	
Sunitinib	Platelet-derived growth factor receptors (PDGFRa and PDGFRb); Vascular endothelial growth factor receptors (VEGFR1, VEGFR2 and VEGFR3); Stem cell factor receptor (KIT); Fms-like tyrosine kinase-3 (FLT3); Colony stimulating factor receptor Type 1 (CSF-1R); The glial cell-line derived neurotrophic factor receptor (RET)	In patients with progressive neuroendocrine tumors that cannot be removed by surgery, are locally advanced, or have metastasized	
Irinotecan	DNA Topoisomerase I		In combination with (5-FU) and leucovorin, in adult patients who have progressed following Gem-based therapy
Paclitaxel	Microtubules		In combination with Gem for the first-line treatment of adults with metastatic PDAC
Nab-paclitaxel	Microtubules	In combination with Gem in patients with metastatic disease	
	Drug combinations		
FOLFIRINOX (leucovorin, 5-FU, irinotecan, oxaliplatin)		PC that has metastasized	
OFF (oxaliplatin, leucovorin, 5-FU)		PC that is advanced and has gotten worse after treatment with Gem	

search for new iGLS, most of which were based on the structure of BPTES [109, 110, 111]. As a result, the BPTES derivative telaglenastat (CB-839), a potent, selective, and orally bioavailable iGLS, has advanced to clinical trials [112]. Currently, 20 phase I/II clinical trials include CB-839 alone or in combination with other drugs [107]. However, none of these clinical trials includes PC patients. CHEMBL4080388 is a thiazolidine-2,4-dione that was optimized after a preliminary, high throughput screening against GLS of a library of 40,000 small molecule compounds [113]. In a virtual screen for more iGLS-like compounds conducted *in vivo* on 1,280 active drugs, ebselen, chelerythrine and (*R*)-apomorphine exhibited 10- to 1500-fold greater affinities than DON and BPTES [114]. Ebselen behaves as a mixed non-competitive inhibitor, while chelerythrine and (*R*)-apomorphine are competitive inhibitors.

Overall, the exact disease context where GLS inhibition will be most effective remains an area of active investigation. This opens a debate emphasizing the importance of defining the patient subpopulation likely to benefit from GLS inhibition. However, due to the widespread expression of GLS throughout the body, long-term and high-dose administration of an iGLS would not circumvent its likely toxicity. The results of the completed clinical trials conducted with CB-839 clearly suggest that combination therapy is a plausible option for developing future iGLS [107].

3.4 GOT inhibitors

While diverse studies report on inhibitors of GOT1 (iGOT1), the corresponding studies on GOT2 inhibition were limited to knockdown of *GOT2* [66]. The iGOT1 aminooxyacetic acid (AOA), while cytotoxic to triple negative breast cancer cells, showed acceptable toxicity profiles in small clinical trials of patients with tinnitus and Huntington's disease [115]. The prodrug approach demonstrated an effective strategy to improve the anti-proliferative potency of AOA *in vitro* and *in vivo* while reducing its untoward toxicity *in vivo* [116]. iGOT1-01 was discovered as iGOT1 during the screening of a large library

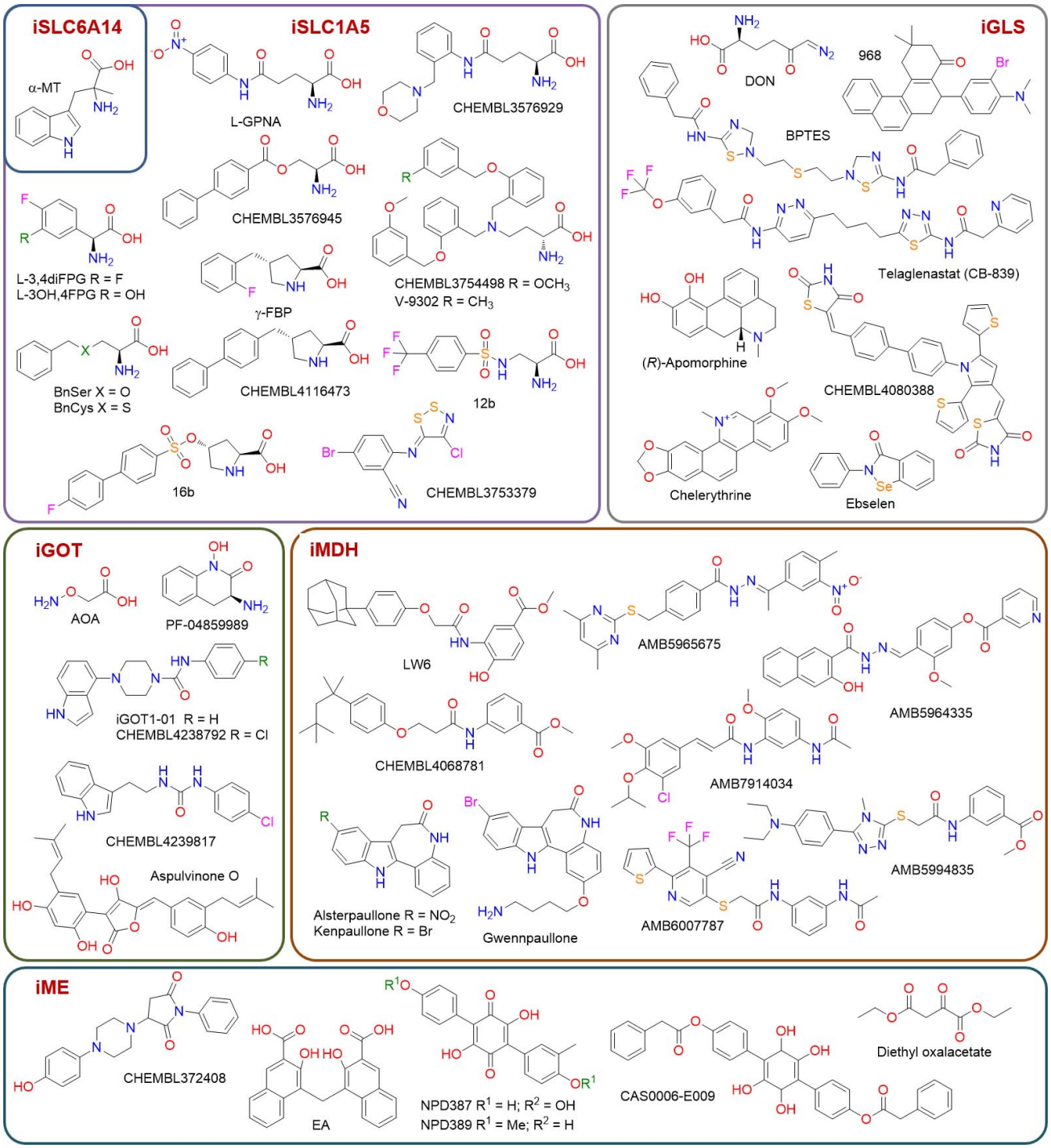

Figure 2. Small molecule inhibitors of druggable metabolism targets in PDAC.

of 800,000 small molecules [117]. Medicinal chemistry-based optimization of iGOT1-01 caused the identification of several analogs with an improvement in potency of at least 10-fold (e.g. CHEMBL4238792), along with the discovery of a tryptamine-based series (e.g. CHEMBL4239817) of iGOT1 [118]. PF-04859989, a known kynurenine aminotransferase II (KAT II) inhibitor, was developed to be applied in the treatment of several psychiatric and neurological disorders. Notably, it inhibited GOT1 in a time- and pyridoxal-5′-phosphate-dependent manner and showed selective growth inhibition of PDAC cell lines [119]. In the same study, PF-04859989 displayed lower inhibitory activity against GOT2. Aspulvinone O was identified from an in-house natural compound library as a new iGOT1 that significantly reduced proliferation of PDAC *in vitro* and *in vivo* [120].

3.5 MDH inhibitors

The role of MDH in cancer metabolism is not relevant at present. Inhibitors of MDH (iMDH) are scarce, although there is evidence of cancer-associated functions for MDH1 and MDH2. These findings motivated the search for iMDH. Thus, hypoxia-

Table 2. Biomarkers that correlate with poor prognosis in PDAC patients.

Biomarker	Status	References
SLC6A14 ($ATB^{0,+}$)	Upregulation	[49]
SLC1A5 (ASCT2)	Upregulation	[52]
SLC1A5_var	Upregulation	[55]
GOTx/GLUD1 ratio	High	[62]
GOT2	Acetylation (3K)	[67]
MDH1	Hypomethylation	[68]
ME1	Upregulation	[72]
SLC2A1 (GLUT1)	Upregulation	[49] [74]
LDHA	Upregulation	[77]
SLC16A3 (MCT4)	Upregulation	[78]

inducible factor 1 (HIF-1) inhibitor LW6 is also a dual iMDH1/2 [121]. In PC cells, LW6 inhibited migration, proliferation and cell viability. These effects were enhanced synergistically when cells were treated with LW6 in combination with metformin [122]. LW6 served as basis for structure-activity relationship studies on a series of (aryloxyacetylamino)benzoic acids that led to the identification of novel iMDH [123]. In that study, the lead compound (CHEMBL4068781) competitively inhibited MDH1 and MDH2, and demonstrated significant *in vivo* antitumor efficacy in xenograft models using HCT116 cells. Affinity investigations revealed that paullones bind and inhibit MDH from various tissues. Subsequent studies showed that alsterpaullone, gwennpaullone and kenpaullone inhibited MDH1 and MDH2 in the low micromolar concentration range [124, 125]. Moreover, alsterpaullone induced apoptosis and inhibited proliferation via the p38MAPK signaling pathway [126]. The virtual screening of the compound library of Ambinter revealed 16 candidate molecules for further *in vitro* testing against MDH2. From this set, only 5 compounds were identified as iMDH2, with IC_{50} values in the range of 3.9-18.2 μM [127].

3.6 ME inhibitors

Among the three MEs, ME1 and ME2 were predominantly studied. However, the number of small molecules reported as inhibitors of ME (iME) is limited. A fragment-based virtual library design and virtual screening allowed synthesizing several compounds that were tested against ME1. The derivatives from this library combining the piperazine and 2,5-dioxopyrrolidine fragments have shown sub-micromolar inhibitory activity against ME1 (e.g. CHEMBL372408) [128]. The natural compound embonic acid (EA) inhibited ME2 and induced anti-proliferative effects in the non-small cell lung cancer H1299 cells [129]. A set of 12,683 natural products from the Chinese National Compound Library were tested against ME2 revealing 15 ME2 inhibitors with different structures [130]. From this set, compound NPD387 was the most potent inhibitor. Through structural modification, an even more potent iME2 was generated, NPD389, which is a fast-binding iME2 and acts as an uncompetitive inhibitor. The study of the effects of fumarate analogs on ME1 and ME2 led to the conclusion that diethyl oxaloacetate behaves as a weak allosteric iME2 [131]. The work paves the way to rational design of allosteric iME2.

Table 3. IC_{50} values of small molecule inhibitors of PDAC metabolism.

Target	Inhibitor	PIC_{50} (μM)	References
SLC6A14	α-MT	$\sim$ 250	[87]
SLC1A5	L-GPNA	1,200	[93] [101]
	CHEMBL3576929	312	[94]
	L-3OH,4FPG	133	[95]
	L-3,4diFPG	131	[95]
	γ-FBP	87	[98]
	CHEMBL3576945	30	[96]
	CHEMBL3754498	7.2	[101]
	ν-9302	9.6	[103]
	CHEMBL3753379	3.7	[104]
GLS	968	$\sim$ 3	[106]
	BPTES	3.3	[109] [110]
	CB-839	0.06	[110]
	CHEMBL4080388	0.05	[113]
	Ebselen	0.009	[114]
	Chelerythrine	0.03	[114]
	(R)-Apomorphine	0.6	[114]
GOT1	AOA	3-10	[116]
	iGOT1-01	85	[117] [118]
	CHEMBL4238792	8.2	[118]
	CHEMBL4239817	36	[118]
	Aspulvinone O	$\sim$ 0.3	[120]
GOT1/2	PF-04859989	8.0 / 55	[119]
MDH1/2	LW6	1.1 / 6.3	[121]
	CHEMBL4068781	1.07 / 1.06	[123]
	Alsterpaullone	2.2 / 6.2	[124]
	Gwennpaullone	3.3 / 22	[124]
	Kenpaullone	13 / 17	[124]
MDH2	AMB5965675	3.9	[127]
	AMB7914034	6	[127]
	AMB6007787	9.4	[127]
	AMB5964335	14.7	[127]
	AMB5994835	18.2	[127]
ME1	CHEMBL372408	0.15	[128]
ME2	EA	1.4	[129]
	NPD387	18.27	[130]
	NPD389	5.59	[130]
	CAS0006-E009	31.02	[130]
	Diethyl oxaloacetate	2,500	[131]

3.7 KRAS modulators

PROteolysis TArgeting Chimeras (PROTACs) are small molecules that selectively degrade target proteins by exploiting the intracellular ubiquitin-proteasome system (UPS) [132]. PROTACs have three connected chemical components: a ligand binding to a target protein, a ligand binding to E3 ubiquitin ligase, and a linker bridging these two ligands. Once the PROTAC-mediated target protein-E3 complex is formed, an E2 ubiquitin-conjugating enzyme transfers ubiquitin to lysine residues on the surface of the target protein. The recognition of polyubiquitination signal by UPS facilitates the degradation of the target protein [133]. In contrast to the stoichiometric occupancy-driven process of traditional inhibitors, PROTACs induce target protein degradation, in multiple rounds, at sub-stoichiometric levels. PROTACs allow degradation of previously "undruggable" proteins [133]. Even target proteins with low affinities with PROTACs can be effectively degraded if PROTACs can induce extensive protein-protein interac-

tions between target proteins and E3 ligases. PROTACs that trigger KRAS degradation would effectively shut down the alternative Gln pathway overexpressed in PDAC cells.

In the WO2019/19560A2 patent highlight report, there are six examples of PROTAC molecules that target KRAS [134]. In cells treated with 1 μM of the compound, two of these molecules triggered degradation of more than 50% of KRAS. These PROTACs were found to recruit either VHL or CRBN E3 ligases.

Recently, over 100 PROTACs targeting oncogenic KRAS^{G12C} were described [135]. The lead PROTAC successfully recruited the E3 ligase CRBN in cells, bound to KRAS^{G12C} *in vitro*, promoted CRBN/KRAS^{G12C} complex formation, and degraded GFP-KRAS^{G12C} in reporter cells in a CRBN-dependent manner. However, it failed to degrade endogenous KRAS^{G12C} in pancreatic and lung cancer cells. Although unsuccessful, this effort indicates the shortcomings that must be surpassed to achieve KRAS degradation in cancer cells.

4. Drug shuttles

KRAS-transformed cells have developed key adaptations to generate metabolic substrates, namely autophagy [42], micropinocytosis [136] and macropinocytosis [137]. Autophagy cannot create a net increase in biomass since cells are degrading themselves. Alternatively, macropinocytosis provides amino acids as well as nutrients secreted by stromal cells through the non-specific bulk internalization of large portions from the extracellular fluid [138]. PDAC cells rely on macropinocytosis to meet their elevated metabolic demand. Lipids, glutamine, and in particular albumin have been actively scavenged by *KRAS*-transformed cells, including PDAC. Cultured PDAC cells can obtain enough amino acids to grow via protein scavenging of human serum albumin (HSA) as the sole amino acid source [139].

HSA possesses several characteristics that render this protein a strong candidate for the tumor targeted release of anticancer agents. To mention a few, HSA is the most abundant protein in plasma (comprising 50-60% of blood plasma proteins), has a very long half-life of about 19 days, evades renal clearance (molecular weight of 66.5 kDa), has multiple binding sites, and accumulates within the tumor interstitium due to the "enhanced permeation and retention (EPR) effect" [140]. Importantly, HSA increases the bioavailability and stability of systemically administered pharmaceuticals in biological fluids [141].

Unlike autophagy, where much of the machinery was identified, much less is known about the proteins that are critical for macropinocytosis. Caveolin-1 (Cav-1) is overexpressed and associated with poor prognosis in PC, and confers oncogenic properties including migration, invasion, and resistance to therapy [142]. Moreover, Cav-1 expression is important for intracellular transport of albumin [143].

Drug carriers based on nanoparticles (NPs) represent a promising tool for cancer therapy *via* precise and effective tumor-targeted drug delivery. Albumin based NPs are among the most capable nanocarriers for antitumor drugs since they are biodegradable, nontoxic and non-immunogenic. There are several ways of utilizing albumin properties to deliver drugs. The most common approach is the nab-technology, where albumin and hydrophobic drugs are processed together under high pressure to generate NPs with diameters of $>$ 100 nm, such as the use of nab-PTX [29]. In addition, nab-rapamycin (ABI-009; albumin-bound rapamycin NPs) is undergoing phase II clinical trials in patients with metastatic, unresectable, low or intermediate grade neuroendocrine tumors of the lung or gastroenteropancreatic system (NCT03670030; https://clinicaltrials.gov/).

An alternative to form albumin-based drug carriers is through binding polymers to albumin without causing any deleterious effects to the protein. The method takes advantage of the free thiol functionality on Cys34 of albumin for polymer conjugation and has a wider scope than nab-technology, allowing the formation of NPs with a much smaller size (10 nm) [144].

5. Conclusions and Future Perspectives

In the next decades, the incidence of PDAC will rise worldwide as a consequence of an increase in age. Predictions rank PDAC among the most common causes of cancer deaths in developed countries by 2030 (second in USA and third in the European Union) [1]. At present, PDAC remains one of the most lethal malignant neoplasms. The underlying reasons for the lack of improvement in the 5-year survival rate of treated PDAC patients are the formidable scientific and technical challenges posed by the previously mentioned late diagnosis, pathophysiological features, genetic alterations, *KRAS*-reprogrammed metabolism, scarce molecularly matched therapies as well as chemoresistance.

Currently, there are no biomarkers that can reliably allow for PDAC detection at an early stage of the disease [80]. Thus, early detection of PDAC remains a major challenge for a favorable outcome of the disease. Worldwide, several health-care organizations recommend a shift toward early detection [145]. There are challenges in early detection of PDAC such as low disease prevalence, which makes the screening of adult population unfeasible with the prevailing diagnostic methods because of the high rates of false-positive findings [146]. In general, biomarker levels quantified in cystic fluid or pancreatic juice appear closer to being ready for largescale biomarker validation trials than those measured in blood and will most likely be useful for high-risk patients [147]. Undoubtedly, advances in early detection demand improvements in chemotherapeutics to extend survival for PDAC patients.

The available drug treatments based on systemic anticancer drugs (Table 1) are minimally effective. Molecularly matched therapies showed that targeted treatments for patients with defined molecular alterations could be a possibility in PDAC [30]. Successful development of chemotherapeutics requires an in-depth understanding in disparate areas, which implies overcoming differences in the concepts, approaches, analysis and vocabulary. Understanding the core effects and features of PDAC requires cross-disciplinary approaches, using knowledge from medicinal chemistry, molecular pharmacology, genomics, materials science (drug delivery), toxicology, pathophysiology, and clinical trials, making the problem a truly cross-disciplinary challenge. The development of therapeutics for PDAC is a major hurdle and will remain a hot topic in the next decades. In the absence of biomarkers, identifying therapeutic targets relies more on serendipity. A number of drugs against PDAC are under intensive investigation in clinical trials [31]. However, Gln metabolism targets remain unexplored in PDAC patients despite CB-839 being studied against other neo-

plasms in 11 Phase II clinical trials (https://clinicaltrials.gov/). In this scenario, diverse studies have identified Gln metabolism biomarkers that correlate with worse prognosis in PDAC patients (Table 2). These biomarkers might help prioritize the group of PDAC patients for whom Gln metabolism inhibitors could be most beneficial.

PDAC cells show increased macropinocytosis to reuse extracellular proteins for tumor growth. This effect is closely related with autophagy [148]. This pathway, typical Gln transporters (i.e. SLC6A14 and SLC1A5), allows PDAC cells to maintain intracellular levels of Gln. Most importantly, Gln deprivation activates macropinocytosis-associated autophagy, while autophagy inhibition augments Gln uptake [149]. The implications of this compensatory response must be considered in the development of inhibitors of Gln metabolism in PDAC. Therefore, concomitant targeting of the Gln metabolism and macropinocytosis would provide an appropriate therapeutic rationale for PDAC. Also, of particular interest are the adaptive metabolic networks to Gln starvation, which allow PDAC cells to utilize available nutrients to sustain cell proliferation [150]. Thus, a combined metabolic inhibition could provide a more successful strategy to treat PDAC patients.

Finally, we should consider the central genes and their related pathways that have been shown to specifically upregulate Gln metabolism, such as *MYC* [151] and *p53* [152]. KRAS regulates MYC in PDAC and the stabilizing effect concurs with the phosphorylation of Ser 62 in the N-terminal domain of MYC [153]. Targeting MYC with microRNAs could be a viable therapeutic strategy for targeting KRAS-driven PDAC [154].

In this review, we have explored the current status and challenges ahead in the discovery and development of small molecule inhibitors of PDAC metabolism. We have emphasized the need for a multidisciplinary approach. In summary, small molecule therapeutics for PDAC treatment represent an excellent scientific problem and a challenging unmet clinical need.

Authors' Contributions

All authors wrote the manuscript, contributed to editorial changes in the manuscript, read and approved the final manuscript.

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Conflict of interest

The authors declare no competing interests.

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